ABSTRACT

DRAPER, DAVID W. The Role of Calcium and Phosphorylation in the Activation of $cPLA_2$ During TNF-induced Apoptosis (Under the direction of Scott M. Laster)

Tumor necrosis factor (TNF) is a pleotropic cytokine that mediates many inflammatory and innate immune responses. TNF also causes apoptosis in certain transformed cell lines and cells that are infected with certain viruses or intracellular bacteria. Cytosolic phospholipase A_2 ($cPLA_2$) is an inflammatory enzyme that mediates its activities, by specifically catalyzing the release of arachidonic acid leading to the generation of eicosanoids. The activity of $cPLA_2$ is necessary during TNF-induced apoptosis and the goal of this study was to identify signals that mediate the activation of $cPLA_2$ during cell death. Intracellular calcium and phosphorylation are well documented to activate $cPLA_2$ under many inflammatory conditions. Therefore, I examined the ability of these signals to regulate $cPLA_2$ during TNF-induced apoptosis. I first examined calcium levels during the TNF-induced apoptosis of C3HA fibroblasts and determined that an influx of extracellular calcium occurs early during cell death. This influx, as well as the release [^{3}H]arachidonic acid, was blocked by verapamil indicating that the calcium response is necessary for the activation of $cPLA_2$ during this process. To analyze the effects that phosphorylation has during TNF-induced apoptosis, $cPLA_2$ proteins, containing serine phosphorylation site mutations, were stably overexpressed in WM793 melanoma cells. Although PMA was able to enhance the release of [^{3}H]arachidonic acid from cells that overexpressed $cPLA_2$, the treatment of the same cells with TNF and cycloheximide had no effect. However, subsequent experiments using PMA

demonstrated novel roles for the phosphorylation of Ser-437 and –727. One was an activation role for Ser-437 as its phosphorylation enhanced [^{3}H]arachidonic acid release. The other was an inhibitory role for the phosphorylation of Ser-727 as its mutation suppressed the release in response to PMA. In conclusion, though the activation of $cPLA_2$, by calcium responses, during apoptotic and non-apoptotic systems is consistent, the regulation of $cPLA_2$ by phosphorylation may involve both positive and negative regulatory signals.

THE ROLE OF CALCIUM AND PHOSPHORYLATION IN THE ACTIVATION OF $cPLA_2$ DURING TNF-INDUCED APOPTOSIS

By,
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DEDICATION

I dedicate this degree to my parents, Richard and Rebecca Draper. Their support and guidance helped me through difficulties time and time again and I would not be where I am today without their strong encouragement. I am truly blessed to have had them throughout my life. Thank you for always being there!

BIOGRAPHY

David William Draper was born in October of 1976 in Glen Ridge, NJ, to Richard and Rebecca Draper. David was raised in North Caldwell, NJ, a suburb of New York City, where he attended West Essex Regional High School. During David's young life and high school years, he participated in many extracurricular activities. His mother was an avid tennis fan and began teaching Dave when he was young. Dave made the high school varsity tennis team for three years and played both singles and doubles. He also developed a love for musical instruments at an early age. He learned to play several but particularly excelled with the clarinet, which he played in both high school symphonic and marching bands as well as several NJ Regional bands. Dave and his father were also active participants in the Boy Scouts of America. Dave designed and directed a project to completely renovate the clubhouse at the North Caldwell Walker's Skating Pond as a requirement for his Eagle Scout Award, which he received at age 16. Dave graduated from West Essex in the Spring of 1994 and moved to Raleigh, NC to pursue a Bachelor of Science in Animal Science at North Carolina State University. Dave had aspirations to become veterinarian, but his interests changed along the way and Dave became fascinated with the immune system after taking a course in general microbiology. Dave received a Bachelor of Science in Animal Science in May of 1998 and shortly after, began working as a technician for Dr. Scott Laster at North Carolina State University. This experience taught David the basics of molecular and cellular research experimentation and strengthened his enthusiasm to study the human immune system. Dr. Laster saw a potential for a career microbiology/immunology in David and

encouraged him to pursue a doctor of philosophy degree. David enrolled in the doctorate program in the Department of Microbiology at North Carolina State University in July of 1999 where he began training under the direction of Dr. Scott Laster.

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Literature Review

The Biology of TNF

Tumor necrosis factor alpha (TNF) is a member of a superfamily of trimeric cytokines (1) that is primarily produced by activated macrophages but is also synthesized within T-cells, NK cells, keratinocytes, adipocytes, and other non-lymphoid cells. TNF plays an important role in many cellular activities that include immune cell activation and migration during the innate immune response as well as cellular proliferation and differentiation (2). In addition, the apoptotic effects of TNF have been linked to antiviral immunity (3-5). However, like many other inflammatory mediators, the dysregulation of TNF signaling processes can have adverse affects. For example, the effects of TNF have been linked to the disease progression of many infectious disorders including cerebral malaria (6) and chronic hepatitis (7). In addition, TNF-induced apoptosis is thought to mediate tissue damage associated with several autoimmune diseases such as type I diabetes (8) and multiple sclerosis (9). Currently, recombinant TNF receptors and anti-TNF antibodies, which inhibit the signaling through TNF receptors, are successfully being used to reduce the tissue degeneration that occurs within individuals that suffer from rheumatoid arthritis and Crohn's disease (10, 11). However, because TNF mediates several other responses that are required during innate immunity, this type of therapy may certainly have drawbacks. The goal or our research is to define the TNF-induced signaling mechanisms that mediate disease progression in an effort to provide the groundwork that will lead to the production of better pharmaceuticals to treat these disorders.

TNF and Apoptosis

TNF is probably most well known for its ability to induce apoptosis, which is a process that plays a crucial role during the development of multicellular organisms as well as the maintenance of cellular homeostasis and immune regulation. This cell death process differs from necrotic cell death as it occurs in and organized and step-wise manner. During necrosis, cellular swelling occurs followed by the lysis of the plasma membrane resulting in the release of the cellular contents (12). Typically, an inflammatory response ensues. In contrast, apoptosis is characterized by several well documented morphological and biochemical changes within the cell that are not detected during necrotic cell death. These include caspase activation, cellular membrane blebbing, mitochondrial inactivation, microfilament depolymerization, chromatin condensation and DNA fragmentation, as well as the degradation of intracellular proteins such as enzymes involved in DNA repair (13, 14). Finally, the cell is broken into "apoptotic bodies" surrounded by an intact plasma membrane, which are engulfed by phagocytic cells.

The cytotoxic effects of TNF were originally characterized by Carswell *et al.* (15) who determined that TNF could induce the death of certain tumor derived cell lines. The ability of TNF to induce apoptosis has since been extended to several normal cell types as well (16). Though most cells are normally resistant to the lytic effects of TNF (17), many can be rendered sensitive by infection with different types of organisms. These include viruses such adenovirus, herpes virus, hepatitis B virus, and HIV (4, 18-21), as well as intracellular bacteria such as *Salmonella typhimurium* and *Listeria Monocytogenes* (22). Sensitization therefore allows for the selective removal of infected cells suggesting an important role for TNF in combating infection.

The mechanism by which infection induces susceptibility is thought to be attributed to the inhibition of resistance gene product expression (23). This is supported as inhibitors of transcription and translation (ITT), such as actinomycin D (24) and cycloheximide (25) respectively also induce susceptibility to TNF in several resistant cell lines. The resistance to TNF-induced cell death also depends upon the activity of the transcription factor NF-κB since studies have shown that cells lacking the expression of NF-κB inducible genes display enhanced susceptibility to TNF (26, 27). Taken together, this evidence demonstrates that TNF induces two independent pathways. One is a pro-apoptotic pathway, which functions independently of protein synthesis while the other is an anti-apoptotic pathway that requires *de novo* mRNA and protein expression.

TNF Receptor Signaling

TNF mediates its diverse activities by binding to two high affinity receptors named TNFR1 (p55) and TNFR2 (p75) (28). Signaling through these receptors can be induced by both the membrane bound form of TNF, as well as the soluble form that is released by the TNF-a converting enzyme (TACE) (29-31). The TNF receptors themselves have no intrinsic signaling capacity, and instead mediate the effects of TNF by recruiting a wide variety of adapter proteins, most of which can associate with either receptor (32). Signaling through both receptors has been shown to induce apoptosis in some cases (33). However, TNFR2 signaling is commonly associated with cellular proliferation (34). Since in vitro analysis has demonstrated that that the two receptors mediate distinct signaling pathways (35), and because the exact role that TNFR2 has

during the induction of apoptosis is controversial, this review will focus on the signaling through TNFR1.

The active TNF cytokine exists as a homotrimer consisting of 157 amino acid subunits (36). Likewise, upon binding, receptor trimerization occurs that is facilitated by the extracellular pre-ligand-binding assembly domain (PLAD) (37). To induce signal transduction, the trimerized receptor must initially recruit several adapter proteins to form a multi-component signaling complex (32). A major signaling component of this complex is a protein known as the TNF receptor-associated death domain (TRADD) that interacts directly with the trimerized TNFR1. TRADD then serves to recruit at least three additional signaling proteins to the receptor complex. These are Fas-associated death domain (FADD), receptor-interacting protein (RIP), and TNF-R-associated factor 2 (TRAF2). The resulting complex induces downstream apoptotic signaling events mediated by caspase activation as well as antiapoptotic gene expression through activation of the NF-?B transcription factor.

Caspases are a family of cysteine-dependent proteases that cleave substrates at the carboxyl side of aspartate residues. These proteases are the driving force of the apoptotic process as inhibition of these proteins, through mutation or by use of specific inhibitors, can completely block apoptosis (38). To date, there are 14 caspases that have been identified in humans and more than half of them have been shown to play a role during apoptosis. The activation of the caspase signaling cascade is mediated through TRADD and FADD that recruit the "initiator" caspase-8 (FLICE) to the TNFR1 signaling complex resulting in the formation of the death-inducing signaling complex (DISC) (1). Caspase-8 is subsequently activated, presumably by autoproteolysis, and released to the

cytosol where it activates downstream effector or "executioner" caspases. These include caspase-3, 6, and 7, which share significant sequence homology. Though all three have been demonstrated to potentate apoptosis, many believe that caspase-3 is most critical as its activities cannot be compensated by the other enzymes (39). In addition, the inhibition of caspase-3, in many systems, prevents the hallmarks of apoptosis including membrane blebbing and DNA fragmentation (40, 41). Caspase-3 is activated by two different mechanisms during apoptosis. One is mitochondria-dependent and occurs through direct activation by the caspase-9 subunit of the multimeric protein complex known as the apoptosome (42). In addition to caspase-9, this complex consists of cytochrome c, which is released from the mitochondria, as well as APAF-1, which recruit procaspase-9 for processing and activation (43). This mechanism is initiated by caspase-8 that cleaves Bid, a pro-apoptotic Bcl-2 protein, into its truncated form (tBid) that then translocates to the mitochondria to release cytochrome c (44). Alternatively, caspase-3 can be activated in a mitochondria-independent pathway. This mechanism is also initiated by caspase-8 but bypasses the mitochondria under circumstances where sufficient caspase-8 molecules are recruited to the DISC to directly activate caspase-3 by proteolysis (45). Ultimately, the activated caspase-3, and caspases-6 and 7, target and either activate or degrade many intracellular substrates resulting in cell death (38).

The NF-?B transcription factor typically exists as a heterodimer consisting of NF-?B1 and RelA and is sequestered in an inactive cytosolic state the by the Inhibitor of ?B protein (I?B). The activation of NF-?B is responsible for many of the TNF's activities including its roles during inflammation and immunity. In addition, NF-?B activation controls cell death and survival during TNF receptor signaling through apoptotic

resistance gene expression (46). A few mechanisms have been proposed as to how NF-?B activation occurs. These are mediated by the recruitment of signaling molecules to RIP and TRAF2 (47-49) and it is accepted that all mechanisms result in the recruitment of the IKK signaling complex. This complex, consisting of two catalytic subunits (IKKa and IKKß) and a regulatory subunit (IKK?) (50), is then activated through dual phosphorylation within the activation loop of IKKß (51). The activated IKK complex subsequently activates NF-?B by phosphorylating the Inhibitor of ?B protein (I?B) and induces its degradation by the 26S proteosome (52). This reaction then releases NF-?B, allowing it to translocate to the nucleus to induce transcription of TNF-dependent genes.

NF-?B has been demonstrated to be a repressor of apoptosis in both mice and humans (26, 53). NF-?B-inducible proteins shown to be involved in this process include the inhibitor of apoptosis proteins (IAP) cIAP1, cIAP2, and XIAP, as well as FLIP, MnSOD, Bcl-2 proteins, and TRAF1. The IAP's and FLIP (FLICE-inhibitory protein) inhibit TNF-induced apoptosis by disrupting the caspase signaling cascade. FLIP is transcribed as two alternatively spliced messages $FLIP_S$ and $FLIP_L$. The proteins these code for contain two death effector domains (DED) similar to that of FADD. Also, $FLIP_L$ contains a caspase-like domain similar to procaspase-8 but lacks the catalytic serine required for protease activity (54) Both FLIP species can interact with procaspace-8 and FADD, which inhibits the interaction of these proteins with each other, and therefore block the processing and activation of procaspase-8 (54). The IAP's have tissue specific expression patterns but have been demonstrated to block caspase-3 and caspase-7 activity and abrogate TNF-induced apoptosis (55). In addition, ectopic expression of cIAP1 and cIAP2 has been shown to inhibit caspase-8 activation and TNF-mediated

apoptosis in a fibrosarcoma cell line (56). MnSOD is a mitochondrial enzyme that dismutates superoxide anions, which are a byproduct of normal respiration. MnSOD expression in induced by TNF and is essential for resistance to TNF-mediated cytotoxicity (57). Most likely, MnSOD expression provides resistance by preventing the formation of reactive oxygen species, in response to TNF, which can cause cytotoxicity by damaging multiple cellular components. Antiapoptotic Bcl-2 family proteins, such as A1 and Bcl-x_L, are upregulated by TNF (58, 59). These proteins have been shown to inhibit apoptosis by localizing to the mitochondria and preventing the release of cytochrome c thereby inhibiting procaspase-9 activation (58, 60). Bcl-x_L has also been shown to directly inhibit apoptosome formation by directly interacting with cytochrome c (61). TRAF1 is also induced by NF-?B in response to TNF and has an antiapoptotic role during apoptosis (62). However, the exact mechanism of its activity is unclear. One possibility is that TRAF1 expression strengthens the affinity of TRAF2 for antiapoptotic proteins at the TNF receptor complex. This was observed in the case of the cIAP's as well as a TRAF2 interacting protein A20 (63, 64).

Phospholipase A_2 Enzymes and cPLA$_2$

Phospholipase A_2 enzymes catalyze the hydrolysis of the ester bond at the *sn*-2 position of membrane phospholipids to release free fatty acid, a reaction that has been demonstrated to play a role in many biological processes (65). Many phospholipase A_2 enzymes have been identified, which are organized into at least thirteen groups containing isoforms that are characterized as low molecular weight secretory enzymes (sPLA$_2$), calcium independent enzymes (iPLA$_2$), or cytosolic enzymes (cPLA$_2$).

Cytosolic Phospholipase A_2 ($cPLA_2$) is a member of the Group IV subfamily of phospholipase A_2 enzymes that also contains the $cPLA_2\beta$, and $cPLA_2\gamma$ isoforms. These enzymes share sequence homology as well as a catalytic domain containing the lipase consensus sequence (GXSGS). $cPLA_2$a and $cPLA_2\beta$ both contain an N-terminal calcium dependent lipid binding domain (CalB or C2) that regulates enzyme activity by controlling the intracellular localization of the enzyme in a calcium-dependent manner. On the other hand, $cPLA_2\gamma$ lacks the C2 domain suggesting that its activity is regulated in a calcium-independent manner. In contrast to $cPLA_2$a, the β and γ isoforms have only recently been identified and their physiological roles are not well characterized (66). Therefore, this review focuses on the regulation $cPLA_2$a (herein referred to simply as $cPLA_2$) and its roles during physiological and inflammatory processes.

$cPLA_2$ Identification and Properties

$cPLA_2$ was initially discovered in different cell types including renal mesangial cells, macrophages, and U937 monocytes (67-69). Sequencing immediately revealed that the enzyme at hand was distinct from the known secreted PLA_2 enzymes (70, 71). First, the cytosolic phospholipase A_2 enzyme shared no sequence homology with the $sPLA_2$'s (71). In addition, $cPLA_2$ was active at physiological concentrations of intracellular calcium (nM), unlike the $sPLA_2$'s that require μM concentrations (72). In addition, $cPLA_2$ showed a preference for arachidonic acid at the *sn*-2 position, whereas the $sPLA_2$'s do not have specificity for particular fatty acids (67).

Sequence analysis of the $cPLA_2$ cDNA, as well as the recent elucidation of the $cPLA_2$ protein structure by X-ray crystallography (73), has provided insight into how the

enzyme's activity is regulated. Unlike the other groups within the PLA_2 family, $cPLA_2$ consists of two domains. The N-terminal portion of the enzyme contains a calcium dependent lipid binding domain (CalB or C2) that consists of 8 anti-parallel ß sheets (74). It was identified due to its sequence similarities with other with other calcium dependent membrane-binding proteins that contain similar motifs (70). The role of this domain is to drive the translocation of the enzyme to membranes in the presence of intracellular calcium. These mechanisms will be discussed in greater detail in a subsequent section. The C-terminal portion of $cPLA_2$ is responsible for catalytic activity. This catalytic domain shares some similarities with other lipases that are classified as a/ß hydrolases (75) such as a catalytic serine residue (Ser-228) as well as a consensus lipase motif (76). However, one structural component within the catalytic domain differentiates $cPLA_2$ from classical a/ß hydrolases, as well as all other members of the PLA_2 family. This is the presence of a "cap" that is composed of two sets of anti-parallel ß sheets and four a helices (73). The cap normally conceals the hydrophobic core, which contains the active site, and is connected to the rest of the catalytic domain by two stretches of amino acids that are proposed to be highly mobile. Analysis of the $cPLA_2$ crystal structure suggests that upon membrane association, the cap is opened, exposing the catalytic site to arachidonic acid. In addition, three of the five serine phosphorylation sites, contained within $cPLA_2$, are located within the cap suggesting a role for phosphorylation in the structural rearrangement of this region during $cPLA_2$ activation.

cPLA$_2$ Effector Functions

cPLA$_2$ is unique among other phospholipase A$_2$ enzymes in that it is highly selective for the hydrolysis of arachidonic acid. This reaction has been determined to be the rate-limiting step in the production of inflammatory mediators as arachidonic acid is the precursor of bioactive eicosaniods, which include prostaglandins, thomboxanes, and leukotrienes (77). Arachidonic acid has also been demonstrated to mediate its effects directly as a second messenger. *In vitro* analysis has demonstrated that arachidonic acid can induce mitochondrial permeability transition, which may result in cell death (78). In addition, arachidonic acid also activates NADPH oxidase (79), an enzyme that mediates the production of superoxide anion and hydrogen peroxide during the respiratory burst in phagocytic cells (80). Finally, as cPLA$_2$ is ubiquitously expressed, its involvement in the production of eicosanoids suggests a key regulatory role for the enzyme during inflammatory processes throughout the body.

While the use of primary cells and cell lines, as well as specific inhibitors has demonstrated the importance of cPLA$_2$ for the synthesis of inflammatory mediators, the recent generation of cPLA$_2$ knockout mice has provided great insight into other physiological processes that involve cPLA$_2$. The cPLA$_2$ knockout mice, generated independently by Bonvertre, as well as Shimizu, develop without any gross abnormalities (81, 82). Female mice, however, display fertility defects both in terms of copulation (82) and parturition (81). On a cellular level, the agonist-induced production of specific prostaglandins and leukotrienes is significantly reduced (81, 82). These studies also demonstrated that cPLA$_2$ contributes to tissue injury within the brain and lung associated with ischemia and adult respiratory distress syndrome (ARDS) respectively (83), the type

I allergic response (anaphylaxis) following challenge with allergen (81), as well as intestinal tumorigenesis (84). Taken together, these studies in $cPLA_2$ knockout mice demonstrate non-redundant roles for $cPLA_2$, as its activities cannot be compensated by other PLA_2 enzymes.

Finally, the activity of $cPLA_2$ has been linked to the ability of TNF to induce apoptosis (to be discussed in detail in a later section). TNF-induced signaling pathways result in the upregulation of $cPLA_2$ gene expression as well as its activity within cells (85, 86). Moreover, several studies have demonstrated that the activity of $cPLA_2$ is essential for cell death (87-92). The cytotoxic effects of TNF have been attributed to the tissue damage that is associated with several types of disease, thus making $cPLA_2$ an attractive target for pharmacological prevention of disease progression.

Regulation of $cPLA_2$ by Intracellular Calcium

It is well documented that the activity of $cPLA_2$ is enhanced by two post-translational regulatory signals. The first activation signal is an increase in the levels of intracellular free calcium $[Ca^{2+}]_i$. The second signal is the phosphorylation of $cPLA_2$ on serine residues (to be discussed in detail in the next section). $cPLA_2$ is generally confined to the cytosol in resting cells (93). Thus, to release arachidonic acid, the enzyme must be activated and induced to translocate to membranes where the fatty acid is located (68). The role that intracellular calcium plays during this process has been studied extensively in many cell types responding to different stimuli. These include primary cell cultures, such as macrophages and platelets responding to physiological agonists such as zymosan and thrombin respectively (94, 95). Other studies utilized calcium ionophores such as ionomycin or A23187, which artificially induce a rapid increase in $[Ca^{2+}]_i$ (96, 97).

Translocation is mediated by the C2 domain, which is both necessary and sufficient to induce translocation of $cPLA_2$ to membranes in the presence of calcium (74). This has been demonstrated both in vitro, using artificial phosphatidylcholine vesicles (98), as well as within intact cells (99). The C2 domain modulates $cPLA_2$ activity in a catalysis independent manner (100) by coordinating the association of two calcium ions onto calcium binding residues (Asp-43 and -93) contained within the calcium binding cleft (101). Mutation of one or both of these residues has been shown to impair the translocation as well as activity of $cPLA_2$ after stimulation (102). Typically, $cPLA_2$ translocates to the nuclear envelope and endoplasmic reticulum in response to calcium fluxes (97, 103). The extent to which the enzyme translocates has been shown to depend on how high $[Ca^{2+}]_i$ is elevated (96) as levels in excess of 210 nM are required for complete translocation to the perinuclear region. It has also been reported that the elevated calcium levels must be sustained in order to maintain the perinuclear localization and enhanced enzyme activity (104). The C2 domain also contributes to the interaction of $cPLA_2$ with membranes. This is accomplished by several hydrophobic residues contained within the calcium binding loops of the C2 domain that penetrate the hydrophobic core of lipid membranes (102). Finally, agonists that do not mobilize calcium, such as okadaic acid, also require a functional C2 domain to induce translocation (101), supporting the evidence that suggests basal levels of $[Ca^{2+}]_i$ are required for $cPLA_2$ to associate with membranes (94).

Regulation of $cPLA_2$ by Phosphorylation

Many groups have demonstrated that serine phosphorylation regulates $cPLA_2$ activity in a wide variety of systems (105-109). Early studies using CHO cells that overexpressed $cPLA_2$ demonstrated that serine phosphorylation accompanied $cPLA_2$ activation (110). This observation was observed in response to many agonists that induce arachidonic acid release, including PMA, which is a strong activator of protein kinase C (PKC). The *in vitro* activity of $cPLA_2$, within lysates made from these cultures, was sensitive to phosphatase treatment. In addition, a functional Ser-505 phosphorylation site was required for enhanced $cPLA_2$ activity. This was suggestive of a role for PKC-dependent phosphorylation in the activation of $cPLA_2$ as Ser-505 is contained within the MAPK consensus sequence for phosphorylation (Pro-Leu-Ser505-Pro) (111). These observations strongly suggest that phosphorylation regulates $cPLA_2$ activity through the activation of MAP kinase family members such as ERK1/ERK2, p38 (SAPK), and JNK.

However, in contrast to the well defined role for calcium and the CalB domain, the role that MAPK family members play, in the activation of $cPLA_2$, is less clear. Studies have demonstrated that $cPLA_2$ requires PKC and MAPK-dependent phosphorylation to induce enzyme activity in different systems (97, 112, 113). However, other studies have shown that the phosphorylation of $cPLA_2$ occurs in a PKC/MAPK-independent pathway (95). In addition, though zymosan treated macrophages and thrombin treated platelets display p38 activation (94, 95), the p38-dependent phosphorylation of $cPLA_2$ is only required for enzyme activity in macrophages (107). These observations suggest that the involvement of MAP kinases is cell type and agonist

specific. Further investigations must be performed to clarify the role that these kinases play in that activation of $cPLA_2$.

Trypic digests of $cPLA_2$, derived from insect cells infected with recombinant baculovirus to express $cPLA_2$, have shown that four major phosphorylation sites within the enzyme are serine resides 437, 454, 505, and 727 (114). These sites have since been demonstrated to exist in mammalian cells as well (114, 115). Recently, a fifth serine phosphorylation site (Ser-515) has been identified in smooth muscle cells (116). The identification of these serine phosphorylation sites has facilitated the effort to determine the impact that phosphorylation has on $cPLA_2$ activity. Since, it has been demonstrated that the *in vitro* phosphorylation of Ser-505 results in an increase in $cPLA_2$ catalytic activity (117). The phosphorylation of Ser-727 has been shown to occur within intact cells in response to several agonists (113-115). However, as determined by mutational analysis, a functional role for Ser-727 phosphorylation has only been demonstrated in some systems, (101). In addition, Ser-515 phosphorylation has been attributed to the CaM kinase II-dependent activation of $cPLA_2$ (116). Finally, it has been recently shown, by *in vitro* enzyme analysis that Ser-505 phosphorylation directly contributes to $cPLA_2$ translocation, enhanced catalytic activity, as well as a strong membrane affinity (118). Taken together, these observations suggest that multiple signaling pathways that result in the phosphorylation of $cPLA_2$ may regulate its activity through a variety of different mechanisms.

cPLA$_2$ and TNF-Induced Apoptosis

The first strong evidence suggesting a necessary role for PLA$_2$ activity during TNF-induced apoptosis was presented by Hollenbach *et al* (119). In this study, TNF induced PLA$_2$ activity within C3HA fibroblasts several hours before the onset of cell death. In addition, both PLA$_2$ activity and cell death could be inhibited by dexamethasone suggesting that the enzyme activity was a cause and not an effect of cytolysis (119). Hayakawa *et al*. later generated a clonal cell line (C12), derived from L929 fibroblasts, that was resistant to the cytotoxic effects of TNF (120). They later determined that the resistance was due to a defect in cPLA$_2$ production and by overexpressing ectopic cPLA$_2$, sensitivity could be restored suggesting that cPLA$_2$ is the necessary PLA$_2$ enzyme required for cell death (92). Our findings using C3HA as a model support these observations as TNF-induced apoptosis, as well as the release of arachidonic acid that accompanies cells death, was blocked by antisense expression and specific cPLA$_2$ inhibitors (87). We have also demonstrated that, like the L929 derived C12 cell line, the resistance of WM793 melanoma cells to TNF is due to low levels of endogenous cPLA$_2$ expression. Like Hayakawa's observations, when cPLA$_2$ is overexpressed within WM793, both cell death and arachidonic acid release is enhanced following treatment with TNF and CHI (88).

Though TNF-induced apoptosis requires cPLA$_2$ activity, the exact mechanism by which the enzyme mediates cell death is still largely unknown. One proposed mechanism is that cPLA$_2$ activates sphingomylenases to induce cell death through ceramide production. Neutral sphinogomylenases hydrolyze sphingomyelin within membranes to produce ceramide, which is an apoptosis inducer (121). The TNF-induced apoptosis of

L929 fibroblasts coincides with a large intracellular accumulation of ceramide. And as the C12 cell line is defective in $cPLA_2$ production and are resistant to TNF, they also do not effectively produce ceramide during TNF-induced apoptosis. Stable expression of $cPLA_2$ within these cells was not only sufficient to restore neutral sphingomyelinase activity and ceramide production, but it also enhanced apoptosis following treatment with TNF (122).

We and others have also demonstrated that arachidonic acid can induce apoptosis directly (78, 123), possibly through the induction of mitochondrial permeability transition and the release of cytochrome c (78). Observations by Reid *et al*. support this theory. In their studies, TA1-R6 adipocytes failed to release arachidonic acid or undergo apoptosis following treatment with TNF (124). Interestingly, no defect was found in $cPLA_2$ expression or activity. Instead, the failure to respond to TNF was due to a defect in arachidonic acid synthesis and the addition of exogenous arachidonic acid to media was able to enhance susceptibility and arachidonic acid release upon treatment with TNF. However, in a study by Suffys *et al*, exogenous arachidonic acid supplementation failed to counteract the resistance to TNF that was induced by various inhibitors (125). Indeed, a direct role for arachidonic acid during apoptosis may be cell type specific. On the other hand, $cPLA_2$ activity may mediate TNF-induced apoptosis in an arachidonic acid-independent manner. Though $cPLA_2$ typically translocates to the perinuclear region following activation, the intranuclear localization of $cPLA_2$ has been demonstrated in some studies (126-128). Though the role that $cPLA_2$ plays within the nucleus is not yet clear, Sheridan *et al.* demonstrated that it associates with a nuclear protein (PLIP) (128). In addition, the formation of this complex is required for apoptosis that is induced by

serum starvation. In light of these findings, $cPLA_2$ may mediate TNF-induced apoptosis by interacting with one or more nuclear signaling proteins. Taken together, the activity may mediate apoptosis through different mechanisms. Further investigations must be performed to clarify the role that $cPLA_2$ plays during apoptosis.

This research focused on understanding the signals that regulate $cPLA_2$ activity during TNF-induced apoptosis. An emphasis was placed on intracellular calcium and phosphorylation as suggestive evidence has implicated a role for both these signals during TNF-induced cell death (88, 129). Studies have demonstrated that TNF induces the phosphorylation of $cPLA_2$, which may be mediated by members of the MAP kinase family including Erk1/2, p38, or the c-Jun N-terminal kinase (JNK) (108, 130, 131). Our earlier work has shown that within C3HA fibroblasts sensitized by cycloheximide, sustained phosphorylation of $cPLA_2$ is necessary for arachidonic acid release and cell death in response to TNF (129). As the phosphorylation of specific serine residues within $cPLA_2$ has been shown to directly enhance the activity of $cPLA_2$, these observations suggest that the TNF-induced phosphorylation of $cPLA_2$ mediates cell death. However, the specific sites that are phosphorylated and whether they directly contribute to $cPLA_2$ activity is unknown. Our studies have also demonstrated that rapid phosphorylation of $cPLA_2$ occurs within minutes, which precedes the release of arachidonic acid by 2 to 3 hours suggesting a secondary signal is likely to be required for complete activation of $cPLA_2$. A large body of evidence has accumulated that supports a role for Ca^{2+} in different types of cell death including thymocytes stimulated with glucocorticoids, self-reactive T-cells, targets of cytotoxic lymphocytes, as well as T-cells exposed to toxic metals such as cadmium (132-135). Presently, the identification of a

role for Ca^{2+} during TNF induced cell death is a matter if some controversy. Some studies have demonstrated that intracellular Ca^{2+} levels remain unchanged in response to TNF (136, 137) while others propose that a sustained increase in Ca^{2+} is necessary for apoptosis (138). Our research supports the latter. We found that the calcium channel blocker, Verapamil, inhibits the release of arachidonic acid from stimulated cells and abrogates cell death suggesting that an influx of extracellular Ca^{2+} may act together with one or more kinases to fully activate $cPLA_2$ during TNF-induced apoptosis (129). Taken together, though these observations provide suggestive evidence that phosphorylation and intracellular calcium are important, whether or not these signals directly activate $cPLA_2$ during TNF-induced apoptosis has yet to be determined. The studies, described herein, were conducted to answer these questions.

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2

Calcium and its Role in the Activation of $cPLA_2$ During TNF-Induced Apoptosis

ABSTRACT

In this report, a role for calcium as a second messenger in the apoptotic activation of $cPLA_2$ was investigated. A murine fibroblast cell line (C3HA) was used as a model that was induced to undergo apoptosis by a combination of TNF and cycloheximide (CHI). Using fura-2 Ca^{2+} imaging, we found strong evidence for a calcium response after 1 h of treatment. The response was strongest in the perinuclear region where mean levels rose 83% (144 ± 14 nM in untreated cells vs. 264 nM ± 39 in treated) while cells with morphological evidence of apoptosis displayed the highest levels of calcium (250-1000 nM). Verapamil blocked this response indicating an extracellular source for the calcium. Elevated levels of intracellular calcium were also detected in C3HA cells that were rendered sensitive to TNF by infection with a mutant human adenovirus (*dl309*). In addition, verapamil prevented the release of [^{3}H]arachidonic acid from C3HA cells induced to undergo apoptosis by the chemotherapeutic agents vinblastine, melphalan, and cis-platinum. Together, these data suggest that calcium is important for $cPLA_2$ activation by diverse apoptotic stimuli.

INTRODUCTION

TNF is a 17-kDa inflammatory cytokine that mediates a broad range of inflammatory responses (Reviewed in 1). This report focuses on the ability of TNF to induce apoptosis. Though most normal and transformed cells are resistant to lysis, TNF can cause the death of certain tumor-derived cell lines (2). Susceptibility to TNF can also be induced by infection with certain viruses (3-7), intracellular bacteria (8), or treatment with inhibitors of transcription or translation (9, 10). In resistant cells, TNF activates NFκB that induces the expression of resistance gene products (11, 12) and sensitivity arises when this process is inhibited (11). The TNF-induced death of sensitized, normal cells has been implicated in a number of pathologies, including; HIV Dementia (13), type I diabetes (14), and Hepatitis (15).

Phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of acyl groups, at the *sn*-2 position, from membrane glycerophospholipids. These enzymes are organized into groups within three families known as cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$), and Ca^{2+} independent PLA_2 ($iPLA_2$) (16). Of the three isozymes within the $cPLA_2$ family, cytosolic phospholipase A_2-alpha, referred to herein as $cPLA_2$, is most understood. This 85 kDa enzyme, is unique among other PLA_2 enzymes as it is highly selective for arachidonic acid at the *sn*-2 position (17, 18) and its activity is tightly regulated by phosphorylation (18-21), and/or intracellular calcium (22, 23). The activity of $cPLA_2$ plays several roles during inflammation because arachidonic acid is the intermediate in the production of eicosanoids such as leukotrienes and prostaglandins (24, 25). $cPLA_2$ has also been shown to be necessary for TNF to induce apoptosis. We have

reported previously that both adenovirus (26) and metabolic inhibitors (27) sensitize human and murine cells to TNF in a $cPLA_2$-dependent fashion. $cPLA_2$ is also required for TNF-induced apoptosis of macrophages infected with *Mycobacterium tuberculosis* (28), and a variety of other tumor-derived cell lines (29-31). The role $cPLA_2$ and arachidonic acid play in apoptosis is, however, controversial. One study suggests that arachidonic acid activates one or more sphingomyelinases that in turn induce apoptosis through ceramide production (32). More recently, arachidonic acid was found to act directly on the mitochondria to induce the permeability transition leading to cytochrome c release and cell death (33).

The regulation of $cPLA_2$ by phosphorylation and intracellular calcium has been well documented (18, 19, 21-23). Phosphorylation of $cPLA_2$ results from the activation of agonist-induced MAPK signaling cascades while Ca^{2+} binds the N-terminal C2 domain, allowing for the translocation and efficient membrane binding of $cPLA_2$. Our lab has been interested in identifying the signals necessary for activation of $cPLA_2$ during TNF-induced apoptosis. Our previous data has shown that phosphorylation of $cPLA_2$ is likely to be important since sustained phosphorylation of $cPLA_2$ leading to arachidonic acid release and cell death occurred only in sensitized cells (34). We also postulated a second signal since the phosphorylation of $cPLA_2$ preceded arachidonic acid release. Since verapamil blocked arachidonic acid release and cell death, we proposed that calcium functioned as a second signal for $cPLA_2$ activation during TNF-induced apoptosis.

In this study, we sought to obtain direct evidence of a calcium response during TNF-induced apoptosis. As our model, we used a murine fibroblastic cell line (C3HA) in

which the morphological and biochemical apoptotic markers are well characterized during TNF-induced apoptosis. Our results show that a sustained rise in the intracellular free calcium concentration ($[Ca^{2+}]_i$) does indeed occur that is necessary for $cPLA_2$ activation. Calcium was also linked to the release of arachidonic acid from cells triggered to undergo apoptosis by various chemotherapeutic agents, suggesting that calcium is important to many $cPLA_2$-dependent, apoptotic responses.

MATERIALS AND METHODS

Cell culture and reagents

C3HA is a 3T3-like murine fibroblast cell line kindly provided by L. Gooding, (Emory University, Atlanta, GA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37°C in 8% CO_2. Media and reagents, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Cycloheximide and verapamil were purchased from Calbiochem (La Jolla, CA). Fura-2 acetoxymethyl ester (fura-2 AM) was obtained from Molecular Probes (Eugene, OR). ^{3}[H]arachidonic acid ([5,6,8,9,11,12,14,15-^{3}H(N)] was purchased from PerkinElmer Life Sciences, Inc. (Boston MA). Protein assay kits were purchased from Pierce (Rockford, IL).

Virus and infections

dl309 is an adenovirus mutant that is derived from an Ad5 virus and lacks the right-hand region of the E3 transcription unit, including the 10.4K, 14.5K, and 14.7K protein genes. C3HA fibroblasts were plated overnight, washed, and then incubated with 20 pfu/cell for 2 h in serum-free medium. The cells were then returned to medium with serum and incubated for an additional 20 h prior Ca^{2+} analysis. The *dl309* was kindly provided by L. Gooding (Emory University, Atlanta, GA).

^{51}Cr-Release Assay

100 mm^2 plates, containing 2 X 10^6 cells, were labeled overnight with 100 µCi of $Na_2{}^{51}CrO_4$ (^{51}Cr). The cells were harvested by trypsinization and seeded into 96-well flat-bottom tissue culture plates at a concentration of 1 X 10^4 cells/well. After 16 h of treatment with TNF and CHI, 100 µl of the supernatants were removed and counted with an autogamma counter (Packard, Downers Grove, IL). The maximum release of ^{51}Cr was determined by adding 100 µl of 1 N HCl to labeled, but untreated cells. The % specific ^{51}Cr release (mean ± SEM) was calculated as follows: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. All experiments were performed in triplicate.

[^{3}H]Arachidonic acid release assays

1 X 10^5 cells were plated into 12-well flat-bottom tissue culture plates (Fisher Scientific, Pittsburgh, PA) and labeled overnight with 0.1 µCi/ml ^{3}[H]arachidonic acid. The following morning, the cells were washed 2X with Hank's balanced salt solution (HBSS), allowed to recover for an additional 2 h, and washed again prior to treatment. Cells were treated with TNF (20 ng/ml) and CHI (25 µg/ml), vinblastine (1 µM), melphalan (200µg/ml), or cis-platinum (30 µM) with or without the presence of verapamil (10 µM) in a total of 600 µl of media. At indicated time points after treatment, 300 µl aliquots of media were removed from the wells and centrifuged to remove debris. 200 µl of the supernatant was removed for scintillation counting (Beckman model LS 5801, Fullerton, CA) and total [^{3}H]arachidonic acid release was calculated by multiplying by a factor of 3. Each point was performed in triplicate and maximum radiolabel

incorporation was determined by lysing untreated controls with 0.01% SDS and counting the total volume.

Fura-2 AM loading and $[Ca^{2+}]_i$ measurements

Cells were plated on chambered coverglass slides (Nalge Nunc International, Naperville IL) and incubated overnight. For 1 h treatments, the C3HA cultures were washed with HBSS and loaded with 4 µM fura-2 AM in serum-free DMEM for 1 h at 37°C in the dark. The dye was then removed and the cells were washed two more times prior to stimulation with the appropriate reagents. In experiments where verapamil was used, 10 µM verapamil was added during dye loading as well as throughout the duration of the experiment. The slide was then mounted on a Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany) and cells were viewed through a "Fluar" 40x/ 1.3 numerical aperture, oil immersion objective lens (Zeiss). For 3 h treatments, cells were washed and then treated with TNF and CHI for 3 h. Cells were loaded with fura-2 AM during the last hour of treatment (35).

Dual-excitation wavelengths of 340 and 380 nm stimulated indicator loaded cells and emitted light was monitored at 510 nm. Fluorescence and differential interference contrast (DIC) images were captured by a CCD camera (Princeton Instruments, Trenton NJ) and processed using Metafluor imaging software (Universal Imaging Corporation, West Chester PA). After background fluorescence was subtracted, user defined regions generated within Metamorph imaging software (Universal Imaging Corporation, Great Britain) were drawn to enclose an entire cell or the indicated cellular compartment within the images generated by each wavelength. The ratio of the 340 nm to 380 nm average

pixel intensities were converted to $[Ca^{2+}]_i$ using a calibration curve (Molecular Probes, Eugene OR) as described by Grynkiewicz (36) after adjustments for the *in situ* versus *in vitro* fura-2 dissociation constant were made (37).

Statistical analysis

Statistical analysis was performed using the Student's t-test (standard two-tailed *t* procedure). Values of $p < 0.05$, < 0.01, or < 0.001 were considered the thresholds for defining statistical significance where indicated.

RESULTS

Verapamil abrogates [^{3}H]arachidonic acid release during apoptotic responses.

C3HA cells are murine, 3T3-like fibroblasts that are normally resistant to TNF but can be rendered sensitive by treatment with inhibitors of transcription or translation such as CHI (38), or by infection with adenovirus deletion mutants that lack the E3 14.7K resistance gene (26). The TNF-induced lysis of these cells is accompanied by the release of arachidonic acid (39) and earlier experiments have determined, by expressing antisense oligonucleotides specific for $cPLA_2$, that the TNF-induced arachidonic acid release and cytolytic effects in this system are dependent upon the activity of $cPLA_2$ (26, 27). As reported previously, Figure 1 shows that [^{3}H]arachidonic acid is released from C3HA cells that have been sensitized by CHI (25 µg/ml) and treated with TNF (20 ng/ml) for 6 h, as an approximate 100% increase in radiolabel release is observed above control values (Figure 1).

Verapamil is a calcium channel antagonist that blocks the influx of calcium into cells by interacting with L-type calcium channels in the plasma membrane (40), and is commonly used to study the regulation of $cPLA_2$ by calcium. To determine if a correlation could be made between a calcium influx and $cPLA_2$ activation during TNF-induced apoptosis, the ability of verapamil to inhibit the TNF-induced [^{3}H]arachidonic acid release from C3HA was examined. As shown in Figure 1, verapamil suppressed the [^{3}H]arachidonic acid release from CHI-sensitized C3HA cells approximately by 50%, suggesting that calcium is important during this response.

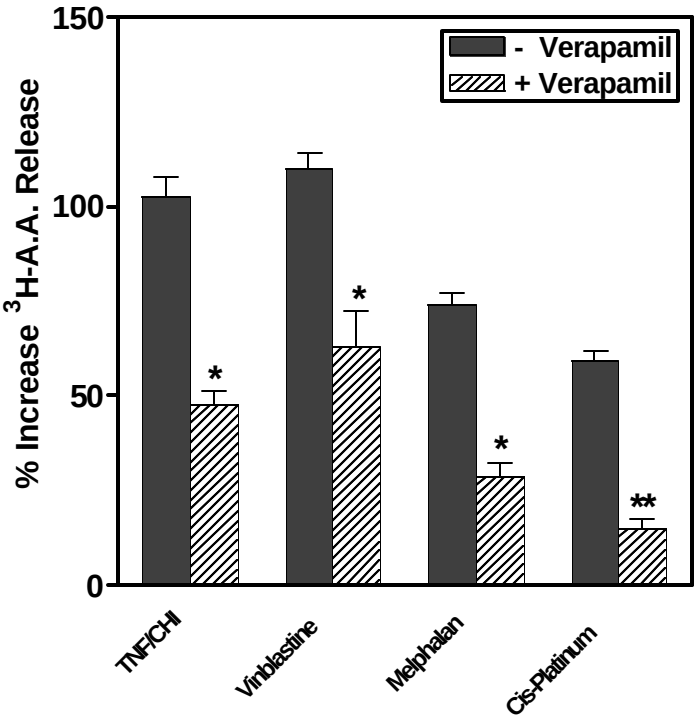


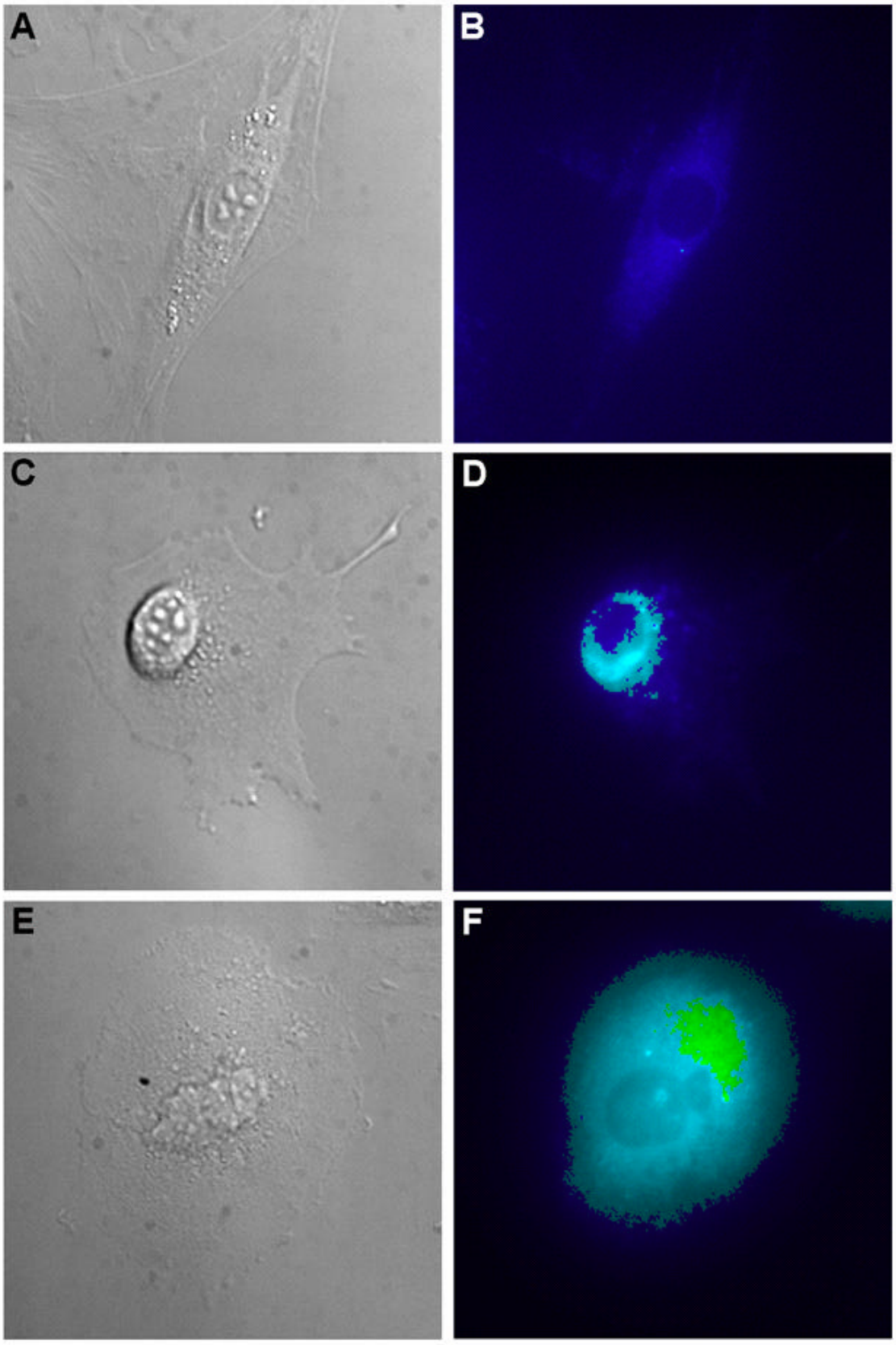
Figure 1: Verapamil abrogates agonist-induced [^{3}H]arachidonic acid release from C3HA fibroblasts. C3HA cells, labeled overnight with [^{3}H]arachidonic acid, were treated with 20 ng/ml TNF, 25 µg/ml CHI, 1 uM vinblastine , 200 µg/ml melphalan, 30 µM cis-platinum and 10 µM verapamil where indicated. [^{3}H]arachidonic acid release was measured after a 6 hour treatment with TNF in combination with CHI, vinblastine, and melphalan; and after a 24 hour treatment with cis-platinum. The experiment shown was performed in triplicate and is representative of 3 repeats. The results are presented as the % increase (mean ± SEM) over the spontaneous release from untreated cells. *, $p < 0.05$; **, $p < 0.01$.

Finally, to determine if a calcium influx is associated with $cPLA_2$ activation during other apoptotic responses, we tested the effects of verapamil on the release of [^{3}H]arachidonic acid induced by vinblastine, melphalan, and cis-platinum; chemotherapeutic agents and known inducers of apoptosis (41-43). In preliminary experiments, concentrations of 1 µM vinblastine, 200 µg/ml melphalan, and 30 µM cis-platinum were found to readily induce cell death (not shown) as has been reported (41-43). We also found that each compound triggered a marked increase in the release of [^{3}H]arachidonic acid above control values following a 6 h treatment with either vinblastine or melphalan, or a 24 h treatment with cis-platinum (Figure 1). Similar to its effects on the TNF-induced response, verapamil significantly abrogated the [^{3}H]arachidonic acid release induced by these chemotherapeutic agents by 50 – 75%. Taken together, these data provide suggestive evidence of a role for calcium in the activation of $cPLA_2$ during multiple apoptotic responses.

Intracellular Ca^{2+} levels increase during early stages of TNF-induced apoptosis.

To determine if a calcium response occurs during the TNF-induced death of C3HA fibroblasts, cell cultures were treated with TNF and CHI. We selected a 1 hour time point that is early in the apoptotic response of C3HA, preceding large-scale release of arachidonic acid into the culture media (39). To analyze changes in $[Ca^{2+}]_i$, the fluorescent Ca^{2+} indicator, fura-2 AM was used. A typical DIC image of an untreated cell is shown in Figure 2*A* while Figure 2*B* shows the ratiometric image of the same cell in pseudocolor. The dark blue color indicates $[Ca^{2+}]_i$ between 79 and 154 nM. Following 1 hour of treatment with TNF and CHI, though most cells had not begun the

Figure 2: The TNF-induced calcium response at early stages of apoptosis. C3HA fibroblasts were loaded with fura-2 AM, left untreated (*A* and *B*) or treated with TNF (20 ng/ml) and CHI 25 (µg/ml) for 1 hour (*C*-*F*). The panels on the left side are DIC images. The panels on the right are ratio images of the same cells displaying relative $[Ca^{2+}]_i$. Levels are depicted as dark blue (low), light blue (intermediate), and green (high). Each image is representative of cells from 3 separate experiments.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apoptotic process, a subpopulation of cells within the culture that had could be identified by well-characterized changes in morphology. It was also evident that $[Ca^{2+}]_i$ had risen within these cells (Figure 2*C* – 2*F*). Figure 2C shows a cell displaying an elevated and pronounced nucleus, which is one of the earliest discernable changes in the morphology of C3HA cells as they undergo apoptosis (44). Cells of this type contained enhanced $[Ca^{2+}]_i$, depicted by the light blue color (250 to 300 nM), that localized to the perinuclear region (Figure 2*D*). Cells further along in the apoptotic process, i.e., those with small blebs on their surfaces yet still attached to the substrate (Figure 2*E*) contained the highest levels of $[Ca^{2+}]_i$ (green color) that ranged from 350 to 900 nM (Figure 2*F*). In addition, $[Ca^{2+}]_i$ was elevated in both cytosolic and nuclear compartments within these cells.

Elevated $[Ca^{2+}]_i$ is sustained through the late stages of apoptosis.

To observe the latter stages of apoptosis, longer incubations with TNF and CHI were required. However, we found that the leakage rate of fura-2 AM was too high and levels sufficient for calcium measurements were not retained beyond two hours (1 hour of labeling plus 1 hour of treatment). Therefore, the protocol was adjusted and cell cultures were treated with TNF and CHI for a total of 3 hours, with the addition of Fura-2 AM during the last hour of treatment. Representative images from these experiments are shown in Figure 3. Figure 3*A* shows a cell in a later stage of the apoptotic process, which has undergone considerable cytoplasmic shrinkage and lost most of its adhesiveness. This cell also contains elevated levels of $[Ca^{2+}]_i$ (Figure 3*B*). Finally, Figure 3*C* shows a cell in the end stages of apoptosis that was completely overcome with large membrane blebs and was no longer attached to the substrate. Again, levels of $[Ca^{2+}]_i$ are clearly

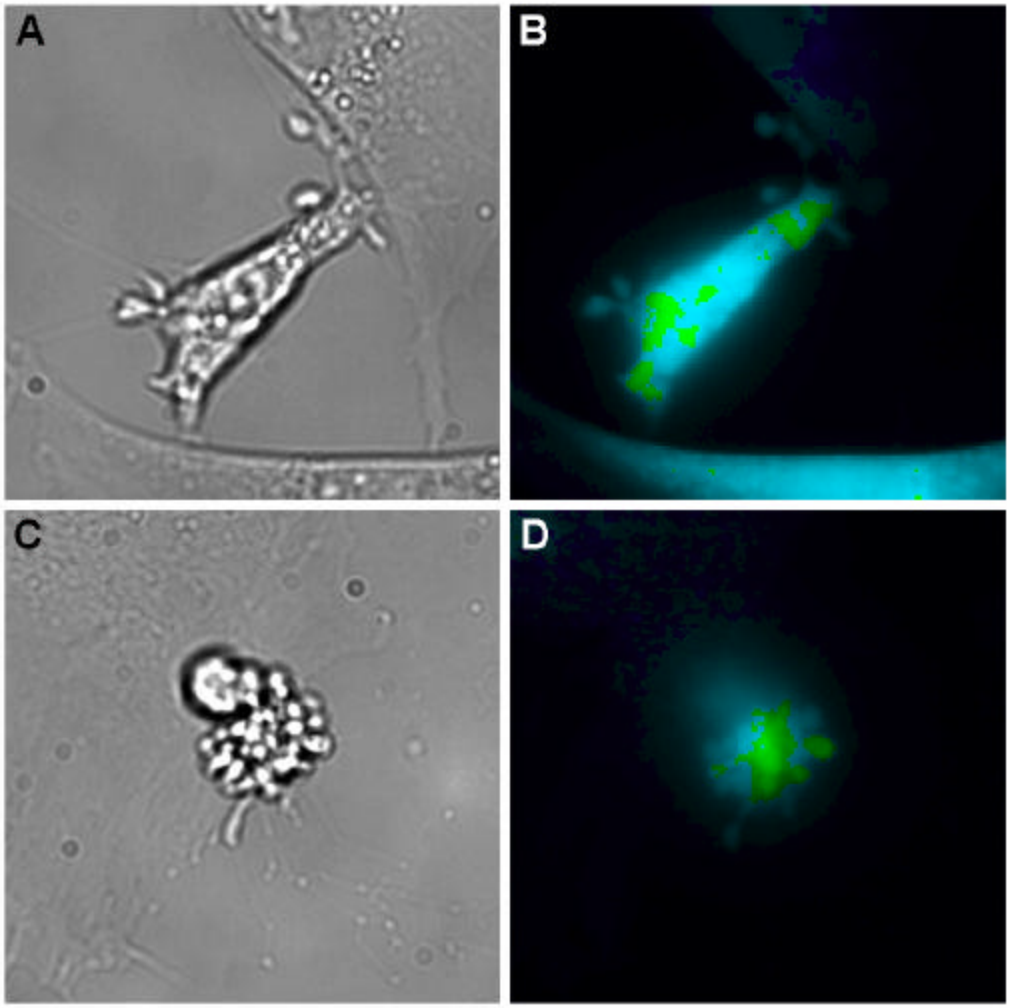

Figure 3: The TNF-induced calcium response at late stages of apoptosis. C3HA fibroblasts were treated with TNF (20 ng/ml) and CHI 25 (µg/ml) for 3 hours and loaded with fura-2 AM as described in the Materials and Methods. (*A* and *C)* DIC images. (B and D) Ratio images displaying relative $[Ca^{2+}]_i$ that are depicted as dark blue (low), light blue (intermediate), and green (high). All images are representative of 3 separate experiments.

elevated (Figure 3*D*). Overall, 21 cells with characteristic apoptotic morphology were examined and all of these displayed enhanced $[Ca^{2+}]_i$ with one exception. Since this particular cell was at the end stages of apoptosis, it is likely that calcium and fura-2 AM was released from the cell due to the rupture of the plasma membrane during secondary necrosis.

Verapamil inhibits the Ca^{2+} influx associated with TNF-induced apoptosis.

If the source of Ca^{2+} for the response noted above is extracellular, calcium channel antagonists should block the elevations. To test this hypothesis C3HA cells were treated with TNF and/or CHI for 1 hour, with or without verapamil, and $[Ca^{2+}]_i$ was measured by ratio imaging. A total of 24 cells, from 3 separate experiments were selected at random and $[Ca^{2+}]_i$ was determined for the whole cell as well as subcellular regions that correlated with the peripheral cytoplasm, perinuclear region, and nucleus. The results of these experiments are shown in Figure 4 and means levels of $[Ca^{2+}]_i$ are summarized in Table I. In untreated C3HA cultures, whole cell $[Ca^{2+}]_i$ was found to be 101 ± 6 nM (mean ± SEM) (Table I). $[Ca^{2+}]_i$ was lowest in the peripheral cytosol and highest in the perinuclear region (Figure 4*A*). Treatment with either TNF or CHI alone (Figure 4B and 4C) was not sufficient to induce the calcium response seen in Figure 2. In fact, treatment with these compounds resulted in a reduction in the mean whole cell levels of $[Ca^{2+}]_i$. In contrast, significant elevations in $[Ca^{2+}]_i$ were seen following treatment with TNF and CHI with mean whole cell levels rising 57% to 159 ± 17 nM (Figure 4*D* and Table I). The increase in $[Ca^{2+}]_i$ was especially pronounced in the perinuclear region where mean levels rose 87% to 264 ± 39 nM., and as high as 900 nM in certain cells. Verapamil itself caused a small decrease in $[Ca^{2+}]_i$ with mean whole cell

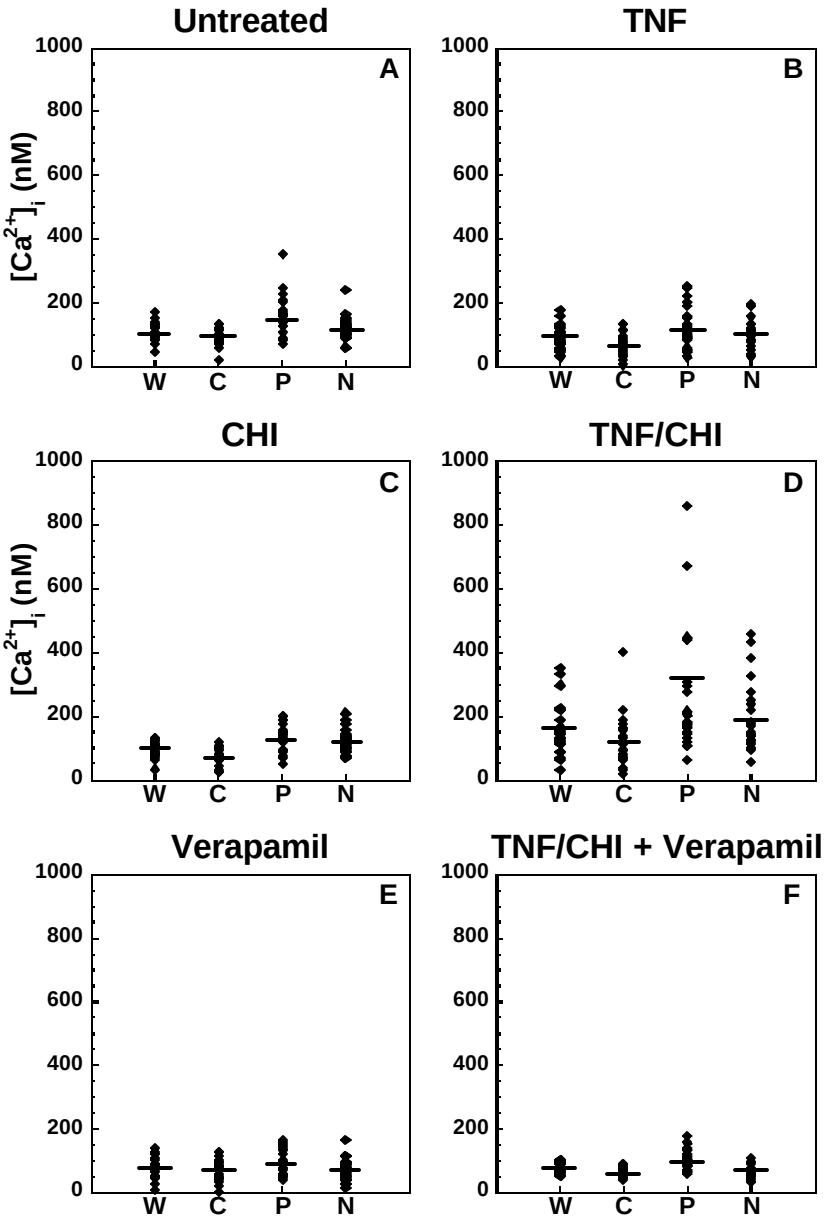

Figure 4: The effect of verapamil on the TNF-induced calcium response. C3HA fibroblasts were loaded with fura-2 AM and left untreated (A), or treated with 20 ng/ml TNF (B), 25 µg/ml CHI (C), or both (D) for 1 hour with (E) or without verapamil pretreatment (F). $[Ca^{2+}]_i$ measurements were taken from areas corresponding to the whole cell (W), cytosolic (C), perinuclear (P), and nuclear (N) regions of individual cells. Each data point represents $[Ca^{2+}]_i$ within a subcellular region of an individual cell (n = 24 from 3 experiments) and mean values are indicated by a bar. Statistical analysis of mean $[Ca^{2+}]_i$ differences between treated and untreated cells is presented in Table 1.

Table I. *Mean $[Ca^{2+}]_i$ within whole cells and subcellular compartments following treatment.*

	$[Ca^{2+}]_i$ within region (nM)			
Treatment	Whole Cell	Cytoplasmic	Perinuclear	Nuclear
Untreated	101 ± 6	88 ± 5	144 ± 14	108 ± 8
TNF	87 ± 8	68 ± 7	118 ± 13	107 ± 9
CHI	97 ± 5	73 ± 5	130 ± 9	120 ± 8
TNF/CHI	159 ± 17**	120 ± 16	264 ± 39**	193 ± 23**
Verapamil	81 ± 6	73 ± 7	95 ± 8**	72 ± 7**
TNF/CHI + Verapamil	78 ± 4**	66 ± 3	100 ± 6**	67 ± 4**

$[Ca^{2+}]_i$ was measured within whole cells, and subcellular regions within C3HA fibroblasts. Cells were pre-treated with or without verapamil following a 1 h treatment with TNF, CHI, or both as described in the *Materials and Methods.* Data is expressed as the mean ± SEM of 24 cells that were taken from 3 separate experiments. **, $p < 0.01$ (compared with untreated cells).

values dropping by 20% to 81 ± 6 nM (Figure 4*E* and Table I). When verapamil-treated cells were stimulated with TNF and CHI (Figure 4*F* and Table I), a similar reduction in $[Ca^{2+}]_i$ was observed (23%) and the calcium response seen in Figure 4*D* was blocked completely. These results, in conjunction with the observations that verapamil prevents cell death and the release of arachidonic acid, strongly suggest that the influx of extracellular Ca^{2+} is necessary for $cPLA_2$ activation and cell death in C3HA fibroblasts treated with TNF. In addition, these experiments show that elevated levels of $[Ca^{2+}]_i$ can be detected in the whole population, prior to the appearance of the apoptotic morphology, since at 1 hr apoptotic cells are extremely rare in culture. Finally, as shown in Table I, increased $[Ca^{2+}]_i$ was not seen with individual TNF or CHI treatments suggesting that the TNF-induced rise in calcium is normally blocked by the action of constitutive or TNF-induced gene products.

Adenovirus infection elevates $[Ca^{2+}]_i$ within C3HA cells.

C3HA fibroblasts that are infected with adenovirus deletion mutants lacking the E3 14.7K resistance gene also become sensitive to TNF and release [^{3}H]arachidonic acid during apoptosis. Previously we had found, however, that verapamil did not block this effect (34) suggesting that calcium may not be important for $cPLA_2$ activation during TNF-induced apoptosis of adenovirus-infected cells. Alternatively, we postulated that the adenovirus infection itself raises $[Ca^{2+}]_i$, making subsequent treatment with verapamil ineffective. To test this hypothesis, C3HA cells were infected with adenovirus *dl309* and $[Ca^{2+}]_i$ within single cells was determined after 24 h. The results from these experiments are presented in Figure 5 and demonstrate that $[Ca^{2+}]_i$ was elevated, within infected cells,

in all cellular compartments that were analyzed. Mean whole cell levels were elevated by 238% to 352 ± 28 nM and the increase was most pronounced in the perinuclear region where levels in some cells exceeded 900 nM. Taken together, these observations implicate a role for calcium in the activation of $cPLA_2$ during the TNF-induced death of C3HA that are sensitized by *dl309* infection.

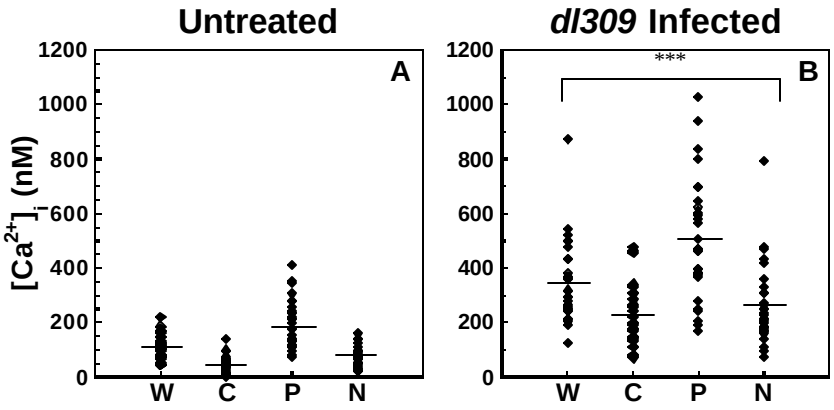

Figure 5: The effect of dl309 infection on $[Ca^{2+}]_i$ within C3HA. C3HA fibroblasts were either left uninfected or infected with *dl309* and incubated for 20 hours. Uninfected cells (*A*) and infected cells (*B*) were then loaded with fura-2 AM as described in the *Materials and Methods* and calcium measurements were taken from areas corresponding to the cytosolic (C), perinuclear (P), nuclear (N), and whole cell (W) regions of individual cells. Each data point represents $[Ca^{2+}]_i$ within a subcellular region of an individual cell (n = 30 from 3 experiments) and mean values are indicated by a bar. ***, $p < 0.01$ (compared with uninfected cells).

DISCUSSION

In this report, a role for calcium in the activation of $cPLA_2$ during TNF-induced apoptosis was examined. These experiments were performed using a murine fibroblast cell line that was rendered sensitive to TNF by CHI because this system is representative of in vivo situations where toxins or microorganisms cause normally resistant cells to become sensitive to TNF. We found that a calcium increase, resulting from an influx of extracellular calcium, occurred early in the apoptotic process. A role for calcium was also implicated in the TNF-induced apoptotic death of adenovirus-infected cells and for apoptosis induced by chemotherapeutic compounds. Together, these results suggest that calcium is important for many apoptotic processes that involve $cPLA_2$.

Calcium first gained attention as a possible second messenger during apoptosis when it was discovered that the death of glucocorticoid-treated thymocytes was dependent on an influx of extracellular calcium (45). It is now clear that, in thymocytes and mature T cells, a calcium response occurs following a number of apoptotic stimuli including gamma-irradiation (46), TCR and Fas ligation (47, 48), toxic metals (49), as well as the removal of specific growth factors from culture media (50). Calcium is believed to activate a number of calcium binding proteins during apoptosis (51-54) as well as calcium-dependent endonucleases that mediate DNA fragmentation (55-58).

In contrast to the well-established role for calcium during T cell apoptosis, little is known about the involvement of calcium during TNF-induced apoptosis. Two studies, in L929 (59) and BT-20 (35) cell lines (which are spontaneously susceptible to TNF) demonstrated that cellular calcium levels were elevated after treatment. The increase in

calcium was gradual and spread to the peripheral cytosol, but initially localized in and around the nucleus where levels remained most pronounced. In addition, apoptosis was mediated by an influx of extracellular calcium because the cell death was abrogated by verapamil or by chelating calcium from incubation media. In contrast, other investigations have failed to find evidence for a calcium response during TNF-induced apoptosis. Calcium levels were unmodified within TNF treated in U937 monocytes (51), as well as KYM-1 and HeLa cells that overexpress the p75 TNF receptor (60).

The results of our experiments were similar to the findings of Bellomo *et al*. (35), and Kong *et al.* (59) with BT-20 and L929 cell lines. We found that a large increase in $[Ca^{2+}]_i$ accompanied the death of C3HA cells treated with TNF and CHI, which was present in cells within all stages of apoptosis. The response appeared within the first hour after treatment, before the vast majority of cells displayed any morphological indications of apoptosis. At 1 h, a few cells showing early signs of apoptosis were observed within the culture that all had elevated levels of $[Ca^{2+}]_i$. Examination of the apoptotic process revealed that $[Ca^{2+}]_i$ rose even higher within cells that began forming membrane blebs yet remained spread and attached to the substrate. We also observed that levels of $[Ca^{2+}]_i$ remained elevated within cells during the late stages of apoptosis. Finally, we noted that this response only occurred following treatment with TNF and CHI, not when either compound was used independently. The role of CHI in these experiments is to inhibit the expression of TNF-induced resistance gene products. The results of these experiments suggest that one of these gene products acts by preventing TNF-induced increases in $[Ca^{2+}]_i$.

The major thrust of our investigations was to define calcium's role in the activation of $cPLA_2$ during TNF-induced apoptosis. Culver, CA and Laster, SM recently addressed calcium's role in the intracellular translocation of $cPLA_2$ during TNF-induced apoptosis as calcium responses influence $cPLA_2$ localization in several other systems (61-63). Moderate levels of intracellular calcium (100-200 nM) have been shown to cause translocation of $cPLA_2$ to the golgi apparatus, while higher levels of calcium (>200 nM) caused movement to the endoplasmic reticulum and perinuclear region (61). Culver and Laster performed immunofluorescence microscopy, using a monoclonal antibody against $cPLA_2$, and revealed an intranuclear staining pattern occurred within C3HA following a 3 h treatment with TNF and CHI. However, pretreating the cells with verapamil blocked this effect suggesting that the calcium response reported here is necessary for the intranuclear localization of $cPLA_2$ during apoptosis. The intranuclear localization of $cPLA_2$ has only been documented in a few other studies (64-66) but several possible reasons for the nuclear translocation of $cPLA_2$ can be envisioned. For instance, arachidonic acid can be a substantial component of nuclear membranes (67, 68), which may be the source of the arachidonic acid released by $cPLA_2$ during apoptosis. Alternatively, arachidonic acid may be released from cytosolic membranes, and nuclear $cPLA_2$ is playing an as yet uncharacterized role in apoptosis. In a recent study of apoptosis induced by serum-starvation, $cPLA_2$ was found to interact with a nuclear protein (PLIP) (65), a splice variant of the transcriptional regulator, Tip60. It is possible, therefore, that nuclear $cPLA_2$ functions during apoptosis by interacting with one or more nuclear proteins. Finally, the nuclear $cPLA_2$ we have observed may not be critical to

apoptosis and results merely from the prolonged, high levels of calcium in apoptotic cells.

An interesting observation emerged from our earlier experiments with verapamil (34). While verapamil was shown to be effective at preventing cell death and the release of arachidonic acid from C3HA cells sensitized by CHI, we were unable to demonstrate suppression of either parameter when these cells were rendered sensitive by adenovirus infection. It is possible, therefore, that the pathway activated by TNF in infected cells is calcium independent. Alternatively, we hypothesized that the infection with adenovirus itself, elevated levels of intracellular calcium. Thus, verapamil would be ineffective when added with TNF since calcium levels had all ready raised. We tested this hypothesis by infecting C3HA fibroblasts with the adenovirus mutant *dl309*, and indeed, calcium levels were significantly higher after infection. These results suggest that the TNF-induced death of adenovirus-infected cells is indeed calcium-dependent and provide a possible explanation for the failure of verapamil to inhibit this response. Increased levels of intracellular calcium following adenovirus infection have not been reported previously and the mechanism by which adenovirus causes calcium levels to rise is not known.

Our results place the TNF-induced calcium response, which begins following 1 h of treatment, at an early time point during the $cPLA_2$ activation process as this precedes the bulk of arachidonic acid release from the culture (39). This suggests that the response is an early event during the apoptosis of C3HA. Our earlier studies support this hypothesis as we revealed that characteristic morphological and biochemical changes that occur during apoptosis do not begin until several hours after treatment. These markers

include membrane permeablization, mitochondrial membrane depolarization, and PARP cleavage. Recently, additional tests were conducted by Culver and Laster (personal communication) to place the calcium response in context with the initiation and effector phases of apoptosis. The effects of TNF and CHI on PS externalization, which is an early and common event among apoptotic processes, was analyzed (69). TNF has been documented to induce PS externalization within 2 h of treatment in an extracellular calcium dependent manner (70). Similarly, Culver and Laster revealed that PS exposure on C3HA occurred as early as one hour post treatment, the same time that increased $[Ca^{2+}]_i$ can be observed suggesting that the calcium response begins early during apoptosis. The second test performed was an analysis of procaspase processing during the apoptosis of C3HA. Caspases are generally organized in two groups known as initiator caspases and effector caspases (71). Effector caspases are synthesized as inactive procaspases. The procaspases are subsequently activated, during apoptosis, by proteolysis and the removal of a pro-peptide. This event occurs downstream in the apoptotic signaling cascade and results in the cleavage of many cellular substrates. The cleavage of procaspases-3 and-7, which are known to be processed during TNF-induced apoptosis (72), was analyzed. The results demonstrated that both of these downstream procaspases become processed during the apoptosis of C3HA after 4 h of treatment with TNF and CHI, several hours after the calcium response initiates. Together, these results suggest that the TNF-induced calcium response, which occurs at the same time as the onset of PS exposure, is an early event during apoptosis preceding many downstream markers of apoptosis.

Finally, we investigated whether calcium is important for $cPLA_2$ activation during apoptosis induced by chemotherapeutic compounds. Substantial arachidonic acid release from C3HA cells was observed following treatment with vinblastine, melphalan, and cis-platinum, and in each case, treatment with verapamil abrogated this response. In light of these results, it is likely that calcium is important for many apoptotic responses that require the activity of $cPLA_2$.

In summary, these studies suggest that calcium is important for the activation of $cPLA_2$ during TNF-induced apoptosis. These results may explain why verapamil has a therapeutic effect on the pathology associated with autoimmune disorders such as experimental allergic neuritis (73), cyclosporine nephrotoxicity (74), and hepatitis (75), all of which may involve TNF (75-77). Further research into the effects of inhibitors, that block calcium mobilization, will determine whether these compounds may be generally useful for reducing tissue damage that is associated with TNF.

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3

The Regulation of $cPLA_2$ by Phosphorylation

ABSTRACT

In this report, we investigated a role for serine phosphorylation in the activation of cytosolic phospholipase A_2 ($cPLA_2$) during TNF-induced apoptosis. As a model, we used WM793 melanoma derived cell lines that stably overexpressed recombinant human $cPLA_2$ with or without a mutation at one of serine residues 437, 454, 505, or 727. The effect of each mutation on TNF-induced $cPLA_2$ activation and apoptosis was analyzed. TNF and CHI failed to induce $cPLA_2$ activation within these lines as WT, which expressed unaltered $cPLA_2$, did not display any enhanced [^{3}H]arachidonic acid release following treatment. However, the treatment of these lines with phorbol ester, an activator of PKC and MAPK signaling cascades, implicated novel roles for serine phosphorylation in the activation of $cPLA_2$. PMA induced the activation of $cPLA_2$ within 30 minutes following treatment and a significant increase in [^{3}H]arachidonic acid release was observed throughout 2 hours of treatment. The substitution of Ser-505 and -437, with alanine, blocked the PMA-induced activation of $cPLA_2$. In contrast, the substitution of Ser-727 enhanced [^{3}H]arachidonic acid release as a 3.75 fold increase in [^{3}H]arachidonic acid release was observed over WT after 30 minutes of treatment. Together these results demonstrate that dual phosphorylation on Ser-437 and -505 enhances the activation of $cPLA_2$ by MAPK signaling cascades while Ser-727 phosphorylation is inhibitory.

INTRODUCTION

Cytosolic Phospholipase A_2 ($cPLA_2$) is a member of the PLA_2 family of enzymes, which are responsible for the hydrolysis of the *sn*-2 ester of membrane phospholipids. Though the PLA_2 family consists of many enzymes, only $cPLA_2$ is highly selective for arachidonic acid at the *sn*-2 position making it a key inflammatory enzyme (1). Upon hydrolysis, arachidonic acid is metabolized into potent inflammatory mediators known as eicosanoids (2). The other product of the arachidonic acid hydrolysis is lysophospholipid, a precursor to platelet activating factor that also has been demonstrated to play an important role in inflammation (3). The activity of $cPLA_2$ and the release of arachidonic acid is also necessary during tumor necrosis factor (TNF)-induced apoptosis (4-9). The apoptotic activity of TNF has been implicated in several autoimmune diseases such as rheumatoid arthritis, type I diabetes, Crohn's disease, as well as Hepatitis. Thus, the regulation of $cPLA_2$ is an attractive target for pharmaceuticals designed to better treat the pathology associated with these disorders.

TNF is a 17-kDa inflammatory cytokine that mediates a broad range of inflammatory responses including apoptosis (10). Though $cPLA_2$ mediates the TNF-induced apoptotic response, the signals required for the activation of $cPLA_2$ and cell death are less clear. It is well documented that both intracellular calcium and serine phosphorylation play and important role during the activation of $cPLA_2$. The role of calcium is to induce the translocation of $cPLA_2$ to the nuclear envelope and endoplasmic reticulum in response to stimulation (11, 12). This is mediated by the N-terminal portion of the enzyme that consists of a calcium binding domain (CalB or C2), which is both

necessary and sufficient to induce translocation of $cPLA_2$ to membranes in the presence of calcium (13). Though the role of phosphorylation in the activation of $cPLA_2$ is still controversial, MAP kinases, such as ERK1/2, p38, and JNK, are thought to be largely involved (14-16). Evidence for this was initially presented by Lin *et al.* who showed that serine phosphorylation accompanied $cPLA_2$ activation (17). These experiments were performed in CHO cells treated with different agonists including PMA, a strong activator of MAPK signaling pathways via protein kinase C (PKC) (18). The *in vitro* activity of $cPLA_2$, within lysates made from these cultures, was sensitive to phosphatase treatment. In addition, the phosphorylation of Ser-505, which is contained within the MAPK consensus sequence for phosphorylation, was required for enhanced $cPLA_2$ activity suggesting a role for PKC-dependent phosphorylation in the activation of $cPLA_2$ (19). However, other studies have shown that the phosphorylation of $cPLA_2$ occurs in a PKC/MAPK-independent pathway (20). In addition, p38 is activated within macrophages and platelets, treated with zymosan and thrombin respectively, resulting in the phosphorylation of $cPLA_2$ (20, 21). However, p38 inhibitors, which block $cPLA_2$ phosphorylation in both cell types, only inhibit $cPLA_2$ activity in the macrophages (16). Taken together, these observations suggest that the involvement of MAP kinases is cell type and agonist specific.

Several groups, including our own, have shown that TNF induces both the phosphorylation and activation of $cPLA_2$ (22-24). Our earlier report demonstrated that a sustained phosphorylation of $cPLA_2$ accompanies enhanced enzyme activity in C3HA cells induced to undergo apoptosis with TNF and cycloheximide (24). Interestingly, neither CHI nor TNF is sufficient to induce sustained phosphorylation or activate $cPLA_2$

suggesting an essential role for phosphorylation. However, the residues within $cPLA_2$ that serve as phosphorylation sites have yet to be identified.

Trypic digests of $cPLA_2$, derived from insect cells that express $cPLA_2$, have shown that four major phosphorylation sites within $cPLA_2$ are serine resides 437, 454, 505, and 727 (12). In this study, we used site-directed mutagenesis to investigate the role that these sites play in the activation of $cPLA_2$ during TNF-induced apoptosis. Mutant $cPLA_2$ constructs, containing serine residues substituted with alanines, were stably overexpressed in the melanoma derived WM793 cell line. Unexpectedly, TNF and CHI failed to activate $cPLA_2$ in these transfectants as determined by [^{3}H]arachidonic acid release. However, PMA treatment of WT did induce [^{3}H]arachidonic acid release, demonstrating that the overexpressed $cPLA_2$ protein was indeed functional. Subsequently, we identified novel roles for Ser-437 and -727 in the activation and down-regulation of $cPLA_2$ activity respectively, during PMA-induced $cPLA_2$ activation.

MATERIALS AND METHODS

Supplies and reagents

Media and reagents, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA). Bovine pituitary extract was purchased from Biosource International (Carmarill, CA). Standard chemicals and supplies were purchased from Fisher Scientific (Suwanee, GA). Cycloheximide was purchased from Calbiochem (La Jolla, CA). QuickChange Site-Directed Mutagenesis Kits were purchased from Stratagene (La Jolla, CA). The pHygEGFP vector was purchased from Clontech (Palo Alto, CA). FuGENE 6 transfection reagent was purchased from Roche (Indianapolis, IN). Hygromycin B was purchased from Calbiochem (La Jolla, CA). Protein assay and SuperSignal chemiluminescence kits were purchased from Pierce (Rockford, IL). Radiolabled compounds were purchased from PerkinElmer Life Sciences, Inc. (Boston MA).

Cell culture

C3HA is a 3T3-like murine fibroblast cell line kindly provided by L. Gooding, (Emory University, Atlanta, GA). Cells were maintained at 37°C in 8% CO_2 and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma D-7777) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. WM793, which is a melanoma derived cell line, was kindly provided by M. Herlyn at the Wistar Institute, Philadelphia, PA. WM793 was maintained at 37°C with 5% CO_2 and cultured in MEL

media (4 parts MCDB 152, 1 part L-15, with 2 mM $CaCl_2$, 0.8 mg/ml bovine pituitary extract, and insulin (5 µg/ml) and 2 mM L-glutamine). Untransfected WM793 melanoma cells were cultured in MEL media supplemented with 2% FBS. Stable WM793 transfectants were cultured in MEL media supplemented with 10% FBS and 200 µg/ml Hygromycin B.

^{51}Cr-release assay

100 mm^2 plates, containing 2 X 10^6 cells, were labeled overnight with 100 µCi of $Na_2{}^{51}CrO_4$ (^{51}Cr). The cells were harvested by trypsinization and seeded into 96-well flat-bottom tissue culture plates at a concentration of 10^4 cells/well. After 16 h of treatment with TNF and CHI, 100 µl of the supernatants were removed and counted with an autogamma counter (Packard, Downers Grove, IL). The maximum release of ^{51}Cr was determined by adding 100 µl of 1 N HCl to labeled, but untreated cells. The % increase (mean ± SEM) in ^{51}Cr release from treated cell over that of untreated cell was calculated as follows: [(experimental release – spontaneous release)/spontaneous release] X 100. The % specific ^{51}Cr release (mean ± SEM) was calculated as follows: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. All experiments were performed in triplicate.

[^{3}H]Arachidonic acid release assays

1 X 10^5 cells were plated into 12-well flat-bottom tissue culture plates (Fisher Scientific, Pittsburgh, PA) and labeled overnight with 0.1 µCi/ml ^{3}[H]arachidonic acid. Cells were washed twice with HBSS to remove unincorporated label and then allowed to recover in

DMEM for 2 h. The cells were washed two additional times prior to treatment with the appropriate chemicals in a final volume of 600 µl. At indicated time points after treatment, 300 µl aliquots of media were removed from the wells and centrifuged to remove debris. 200 µl of the supernatant was removed for scintillation counting (Beckman model LS 5801, Fullerton, CA) and total [^{3}H]arachidonic acid release was calculated by multiplying by a factor of 3. Each point was performed in triplicate and maximum radiolabel incorporation was determined by lysing untreated controls with 0.01% SDS and counting the total volume.

Site-directed mutagenesis

cDNA coding for human cPLA$_2$ contained within the pMT-2 vector (Accession number M72393) (25), was provided by Jim Clark at the Genetics Institute (Cambridge, MA). The pCI mammalian expression vector was purchased from Promega (Madison, WI). cPLA$_2$ cDNA was excised from pMT-2 and subcloned into the *Sal*1 cloning site of pCI (5). The QuickChange Site-Directed Mutagenesis Kit was used to generate constructs to express mutant cPLA$_2$ enzymes with alanine substitutions for serine residues 437, 454, 505, and 727. Mutagenic PCR primers, designed to generate single or double base substitutions, were synthesized by IDT, Inc. (Coralville, IA) and were as follows: 5'-GAGTAATGATAGCTCGGACGCTGATGATGAATCACACGAACCC-3', 5'-GGGTTCGTGTGATTCATCATCAGCGTCCGAGCTATCATTACTC-3' (SA437); 5'-GGCACTGGAAATGAAGATGCTGGAGCTGACTATCAAAGTG-3', 5'-CACTTTGATAGTCAGCTCCAGCATCTTCATTTTCAGTGCC-3' (SA454); 5'-CTCAATACATCTTATCCACTGGCTCCTTTGAGTGACTTTGCC-3', 5'-

GGCAAAGTCACTCAAAGGAGCCAGTGGATAAGATGTATTGAG-3' (SA505); 5'-GACAGAATCCATCTCGTTGCGCTGTTTCCCTTAGTAATG-3', 5'-CATTACTAAGGGAAACAGCGCAACGAGATGGATTCTGTC-3' (SA727). PCR reactions were carried out according to Stratagene's recommendations and the base substitutions were confirmed by sequence analysis (Iowa State DNA Sequencing and Synthesis Facility, Ames IA).

Generation of stable transfectants

Stable cell lines expressing recombinant human $cPLA_2$, or mutants, were generated by co-transfecting WM793 melanoma cells with $cPLA_2$ constructs and pHygEGFP. Transfections were performed with FuGENE 6 Transfection Reagent according to the manufacture's instructions. Briefly, 2.6 X 10^5 WM793 melanoma cells were seeded into 35 mm^2 plates. Following 24 hours of incubation, media was replaced with 2 mL MEL media prior to transfection. A 100 µl transfection solution in Minimal Essential Medium Eagle (MEME) without, serum, was made by combining equal volumes of a DNA solution (2.5 µg Plasmid DNA and 200 ng pHyg EGFP) with the FuGENE 6 solution (containing 4 µL FuGENE 6). The transfection solution was incubated at room temperature for 20 minutes and then added to the WM793 cells. 24 hours later, the media was replaced with selection media (MEL media containing 200 µg/ml Hygromycin B). Selection was performed for several days until individual colonies could be isolated. The clonal cell lines were then trypsinized and transferred to 24-well plates for culturing using Pyrex Brand Cloning Cylinders (Fisher).

Western blot analysis

60 mm^2 plates containing near confluent cell cultures were scraped, resuspended in lysis buffer (50 mM Hepes pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5% Sodium dodecyl sulfate, 200 nM Sodium Orthovanadate, and freshly added protease inhibitors), and transferred to 1.5 mL centrifuge tube. Crude protein extracts were centrifuged at 14,000 X g to concentrate genomic DNA at the bottom of the tube for removal. Total protein concentrations were determined using the BCA protein assay kit (Pierce) as described by the manufacture's instructions. Equal amounts of protein from each sample were loaded in 8% Tris-glycine gels and subjected to SDS-PAGE using the NOVEX system (San Diego CA). After transfer to nitrocellulose and blocking, protein was probed with a rabbit polyclonal anti-$cPLA_2$ antiserum at a 1/1000 dilution (provided by J. Clark, Genetics Institute, Cambridge MA). A 2° horseradish peroxidase-coupled goat α-rabbit antibody was purchased from Sigma and bands were visualized using the SuperSignal chemiluminescence kit.

Statistical analysis

Statistical analysis was performed using the Student's t-test (standard two-sample *t* procedure). The significance threshold was set to $p < 0.05$ to define statistical significance where indicated.

RESULTS

The TNF-induced [^{3}H]arachidonic acid and ^{51}Cr release from WM793 and C3HA.

To determine if WM793 melanoma cells are an appropriate model for mutant $cPLA_2$ expression and analysis, their ability to undergo cell death and release [^{3}H]arachidonic acid was analyzed following treatment with TNF and CHI. C3HA fibroblasts were also analyzed for comparison as C3HA display high levels of $cPLA_2$-dependent cytotoxicity and [^{3}H]arachidonic acid release during TNF-induced apoptosis (5).

^{51}Cr release assays were performed to analyze the TNF-induced cytolysis of WM793 and C3HA. Cell cultures were labeled overnight with ^{51}Cr, sensitized with CHI, and then treated with different concentrations of TNF. The results from this experiment are presented in Figure 6. In C3HA, TNF induced cell death in a dose dependent manner with a 125% increase in radiolabel release upon treatment with the high dose of 20 ng/ml TNF treatment (Figure 6*A*). In WM793, TNF induced a slight dose dependent increase with TNF. However, the levels were significantly reduced when compared to C3HA with only a 16% increase upon treatment with 20 ng/ml TNF. To determine the % lysis of the cultures, the % specific ^{51}Cr release was measured (Figure 6*B*). Again, a dose dependent increase was observed from CHI sensitized C3HA cells with a 91% release at 20 ng/ml TNF, indicating a near complete lysis of the culture. In contrast, WM793 was highly resistant to TNF-induced cell death. Although a slight dose dependent increase in the % release is observed from WM793, only 2.4% of the total radiolabel was released following treatment with 20 ng/ml TNF.

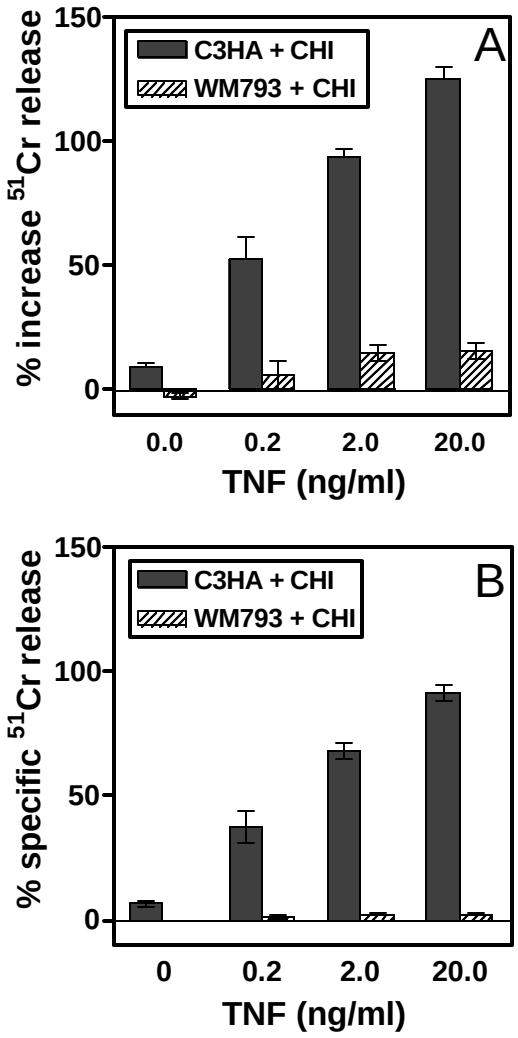


Figure 6: The effects of TNF and CHI on ^{51}Cr release from C3HA and WM793 cell lines. C3HA and WM793 cells, labeled with ^{51}Cr, were treated with TNF (0.2, 2.0, or 20 ng/ml) in the presence of CHI (10 μg/ml). The % increase in ^{51}Cr release (*A*) and the % specific ^{51}Cr release (*B*) was measured after 16 hours. The experiment shown was performed in triplicate and is representative of several repeats.

The levels [^{3}H]arachidonic acid that are released from C3HA and WM793 cultures treated with TNF and CHI, have been shown to directly correlate with the percent cell death within the culture (5). This experiment was repeated as a control and C3HA and WM793 cell cultures were labeled overnight with [^{3}H]arachidonic acid and treated with TNF, CHI, or both for 6 hours. The results from this experiment are presented in Figure 7. In C3HA, CHI alone induced a 10% increase in [^{3}H]arachidonic acid release above that of untreated cells, while TNF induced a 46% increase. When combined, TNF and CHI synergized to induce a 132% increase in the release of [^{3}H]arachidonic acid, correlating with the extensive cytolysis within the culture. In contrast, WM793 cells displayed only a 37% increase in radiolabel release, which is consistent with their resistance to TNF-induced cell death. The response was mostly due to the effects of CHI and the increase was not significantly different than the sum of those induced by TNF and CHI independently ($P < 0.05$).

To examine the levels of endogenous $cPLA_2$ that is expressed in C3HA and WM793 cell lines, western blot analysis was performed. Protein extracts from these cell lines were probed with a rabbit polyclonal antiserum raised against full length recombinant human $cPLA_2$ (7905). The immunoblot from this experiment is presented in Figure 8. Lane 1 contains proteins that were extracted from C3HA fibroblasts and shows the expression of the full length $cPLA_2$ protein that is estimated to have a molecular weight of ~110 kDa (26). Lane 2 contains protein from WM793 melanoma cells and also shows that the full length $cPLA_2$ is also expressed within these cells albeit at lower levels when compared to C3HA. As previously published, these experiments indicates that the resistance of WM793 to TNF correlates with reduced levels of endogenous $cPLA_2$

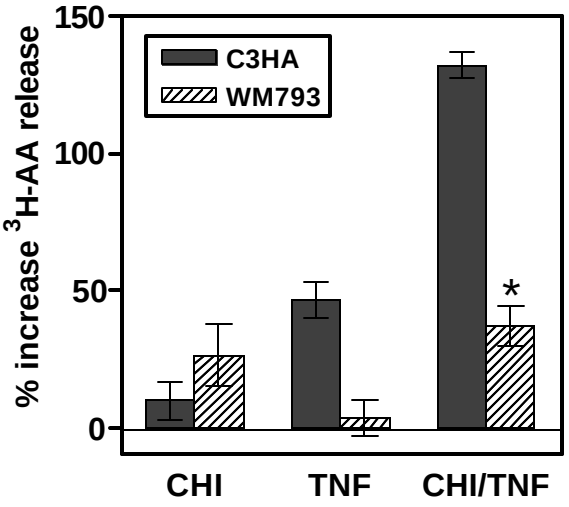


Figure 7: The effects of TNF and CHI on [^{3}H]arachidonic acid release from C3HA and WM793 cell lines. Cell cultures were labeled overnight with [3H]arachidonic acid and treated with TNF (20 ng/ml), CHI (25 μg/ml), or both. [^{3}H]arachidonic acid release was measured at indicated time points as described in the *Materials and Methods*. The results are presented as the % increase (mean ± SEM) over the spontaneous release from untreated cells. The experiment shown is representative of at least 3 repeats. *, $P < 0.05$ (compared to C3HA).

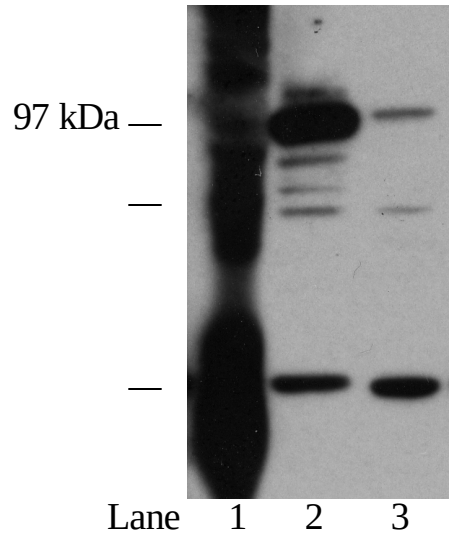


Figure 8: The endogenous expression of $cPLA_2$ in C3HA and WM793 cell lines. C3HA and WM793 cell cultures were grown near to confluency. Total cellular protein was extracted from these cultures and subjected to anti-$cPLA_2$ western blot analysis as described in the *Materials and Methods*. (Lane 1) Protein marker. (Lane 2) Protein extracted from C3HA. (Lane 3) Protein extracted from WM793. The immunoblot shown is representative.

protein as well as low levels of [^{3}H]arachidonic acid release upon stimulation with TNF and CHI. Earlier studies have demonstrated that TNF-induced apoptosis is dependant upon the activity of $cPLA_2$ (5, 9). In addition, the over-expression of wild type $cPLA_2$ within WM793 enhances the ability of TNF to induce apoptosis (5). Therefore, WM793 was selected as a model to determine the roles that $cPLA_2$ phosphorylation sites play during TNF-induced apoptosis.

The effects of TNF and CHI on [^{3}H]arachidonic acid release from stable WM793 transfectants overexpressing wild type or mutant $cPLA_2$.

To determine whether the phosphorylation of Ser-437, -454, -505, or –727 contributes to the activation of $cPLA_2$ during TNF-induced apoptosis, we performed site-directed mutagenesis. cDNA coding for $cPLA_2$ was altered to replace each phosphorylation site with an alanine residue and each construct, as well as the unaltered $cPLA_2$ cDNA, was transfected and stably expressed in WM793 melanoma cells. Clones were selected based on similar overexpression levels, as determined by western analysis, and are herein referred as WT, SA437, SA454, SA505, and SA727 (Figure 9). To determine the effects that each mutation has on $cPLA_2$ activation, cell cultures were labeled with [^{3}H]arachidonic acid and treated with TNF and CHI for 8 hours. The results from this experiment are presented in Figure 10. In contrast to a previous study involving WM793 melanoma cells transiently overexpressing $cPLA_2$, TNF and CHI reproducibly failed to induce enhanced [^{3}H]arachidonic acid release from WT over pCI (vector control) or parental WM793 cells. Therefore, it was impossible to draw any conclusions regarding the impact that phosphorylation of the serine residues has on

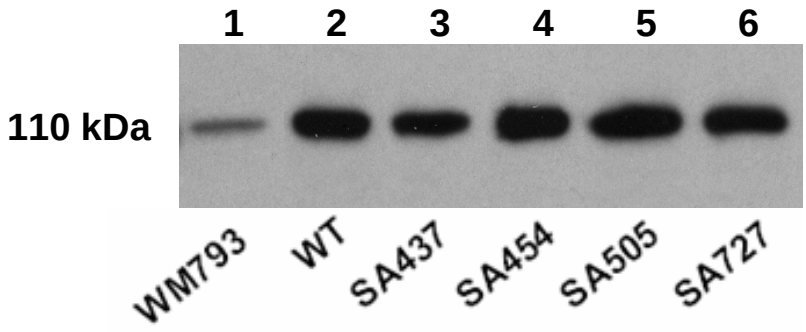


Figure 9: Overexpression levels of $cPLA_2$ constructs in stably transfected clonal cell lines. Cell cultures were grown near to confluency. Total cellular protein was extracted and subjected to anti-$cPLA_2$ western blot analysis as described in the *Materials and Methods*.

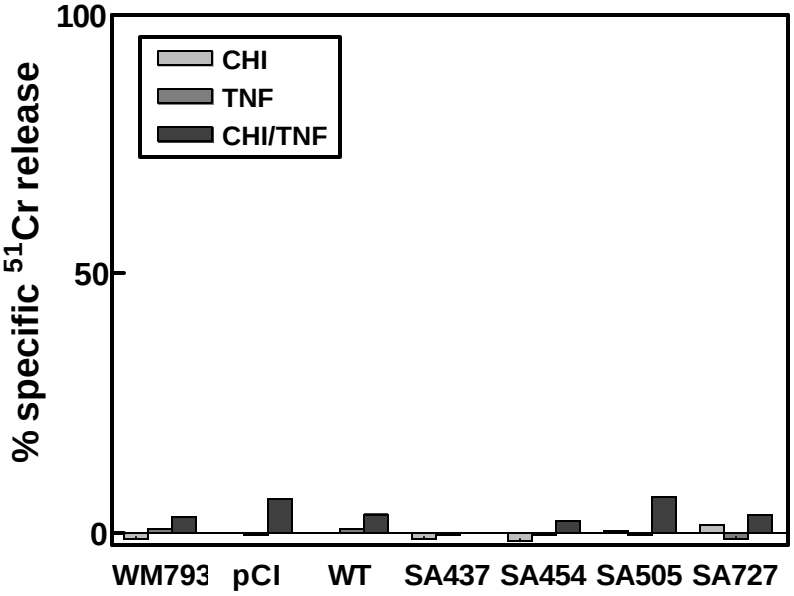


Figure 10: The effects of TNF and CHI on [^{3}H]arachidonic acid release from WM793 melanoma cells stably transfected with cPLA$_2$ constructs. Stable cell lines were labeled with [^{3}H]arachidonic acid overnight and then treated with TNF (20 ng/ml), CHI (25 µg/ml) or both. [^{3}H]arachidonic acid release was measured after 16 hours. The experiment shown is representative of several repeats.

$cPLA_2$ activity. Therefore, additional control experiments were performed in order to confirm that the method of $cPLA_2$ overexpression that was used resulted in synthesis of a functional enzyme.

PMA stimulation activates $cPLA_2$ that is stably overexpressed in WM793

Phorbol esters, such as PMA, are commonly used to study the regulation of $cPLA_2$ by phosphorylation (12, 17, 21, 27). It is well documented that PMA activates PKC and enhances $cPLA_2$ activity through the MAPK/ERK pathway (14, 18). Upon treatment of cells with PMA, this signaling cascade leads to the phosphorylation of $cPLA_2$ on ser-505 resulting in enhanced [^{3}H]arachidonic acid release (19). If the overexpressed $cPLA_2$ in WT is functional, treatment of WT cultures with PMA should also result in enhanced arachidonic acid release. To test this, [^{3}H]arachidonic acid release from parental WM793, pCI, and WT was measured following treatment with PMA and the results from this experiment are presented in Figure 11. In contrast to the effects that TNF and CHI had, PMA induced enhanced [^{3}H]arachidonic acid release from WT cells over WM793 and pCI. A dose dependent increase was observed when WT was treated with PMA that was significantly higher than the increase observed from WM793 and pCI. The higher concentrations of PMA (1 and 10 ng/ml) more optimally induced [^{3}H]arachidonic acid release. It was also observed that the cells transfected with the empty pCI expression vector displayed suppressed PMA-induced [^{3}H]arachidonic acid release compared to parental WM793 cells. These results clearly demonstrate that the $cPLA_2$ enzyme that is overexpressed in WT is functional and the expression method that was used should confer the ability to study the overexpressed $cPLA_2$ mutants.

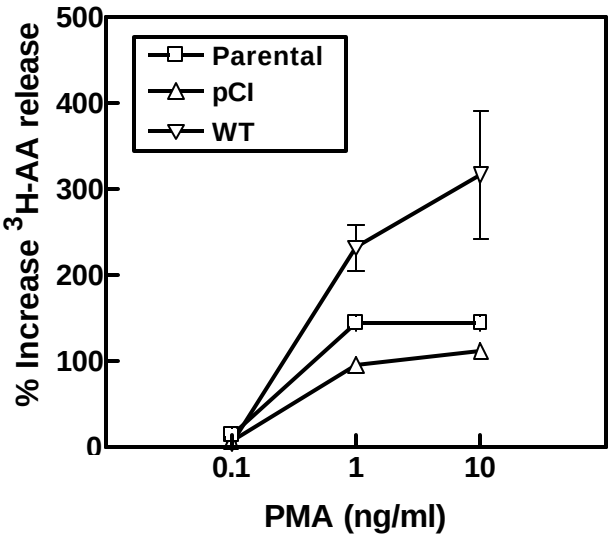


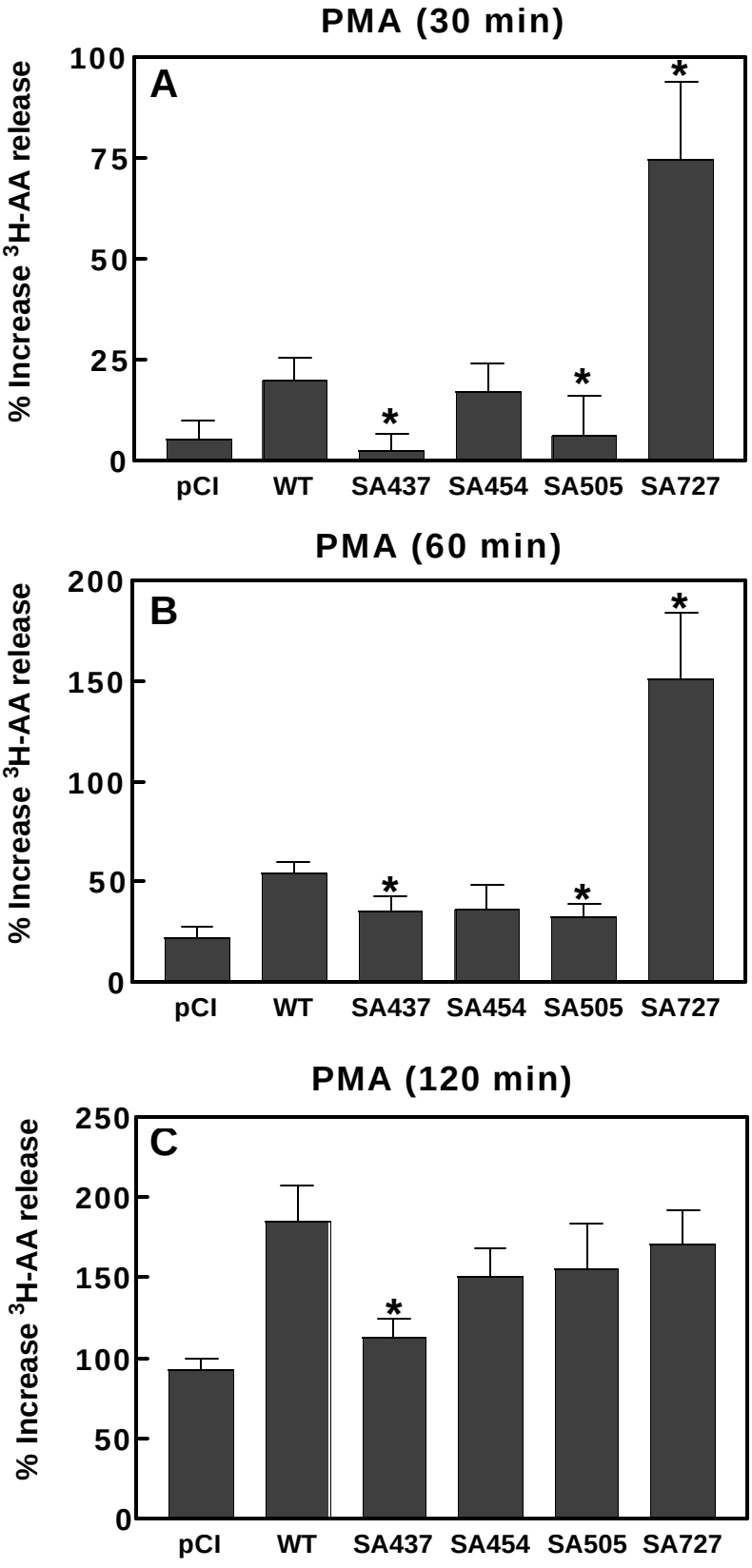
Figure 11: The effects of PMA on WM793 œlls that overexpress $cPLA_2$. The effects stable $cPLA_2$ overexpression on PMA-induced [^{3}H]arachidonic acid release. Parental and WM793 clones stably transfected with pCI or $cPLA_2$ were labeled with [^{3}H]arachidonic acid overnight. [^{3}H]arachidonic acid release was measured after 2 hours of treatment with 0.1, 1, or 10 ng/ml PMA). The experiment shown is representative of at least 3 repeats.

Ser-437 and ser-727 regulate the activation of $cPLA_2$ by PMA.

Though the phosphorylation of Ser-505 is known to contribute to the PMA-induced activation of $cPLA_2$ (17), no roles for other serine phosphorylation sites have yet been identified. Therefore, we analyzed the effects that the serine substitutions at positions 437, 454, and 727 had on [^{3}H]arachidonic acid release from the transfectant cell lines following PMA treatment. The responses are presented as the % increase in [^{3}H]arachidonic acid release above the spontaneous release from untreated. Several experiments were performed on each cell line (n = 6-13) which were pooled and the results from these experiments are presented in Figure 12**.**

Following 30 minutes of treatment with PMA (Figure 12*A*), WT displayed a 4 fold increase in the levels of [^{3}H]arachidonic acid release was observed from WT over pCI indicating the activation of $cPLA_2$. The increase was maintained through 60 and 120 minutes and the differences were significant at all times examined. As expected, radiolabel release from SA505 was significantly lower at 30 minutes when compared to WT, supporting the evidence implicating an important role for Ser-505 phosphorylation. The response from SA437 was also significantly lower at this time point suggesting a role for Ser-437 phosphorylation during $cPLA_2$ activation. Abrogated responses were also observed from SA505 and SA437 at 60 minutes post treatment (Figure 12*B*) but only from SA437 at 120 minutes (Figure 12*C*). Surprisingly, the substitution of Ser-727, in the SA727 cell line, resulted in significantly higher levels of [^{3}H]arachidonic acid release following treatment with PMA compared to WT. Following 30 minutes, SA727 displayed a 3.75 fold increase in mean levels over those observed from WT (Figure 12*A*). A significant increase was also observed at 60 minutes post treatment (Figure 12*B*) but

Figure 12: The kinetics of [^{3}H]arachidonic acid release from WM793 clones overexpressing wild-type and mutant cPLA$_2$ proteins. Stable WM793 transfectants were labeled overnight with [^{3}H]arachidonic acid and treated with PMA (10 ng/ml). [^{3}H]arachidonic acid was measured at 30 minutes (*A*), 60 minutes (*B*), and 120 minutes (*C*). Several experiments using each clone were combined (n=6-13) and the results are presented as the % increase (mean ± SEM) over the spontaneous release from untreated cells. * indicates a significant difference compared to WT, $P < 0.05$.


PMA (30 min)
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% Increase ^{3}H-AA release
100
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SA727
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like SA505, the response from SA727 at 120 minutes closely resembled that of WT (Figure 12*C*).

Taken together, the results from these experiments suggest that the phosphorylation of both Ser-505 and Ser-437 mediates the PMA-induced activation of $cPLA_2$. They also indicate an inhibitory role for the phosphorylation of Ser-727 in this system. Interestingly, in contrast to SA505, the reduced response from SA437 remains significant throughout 120 minutes of PMA treatment suggesting that the mutation of Ser-505 only partially inhibits the activation of $cPLA_2$. Ser-437, on the other hand, appears to completely block $cPLA_2$ activation suggesting a critical role for this phosphorylation site in the activation of the enzyme. In addition, the elevated response from SA727 was only observed at 30 and 60 minutes following treatment suggesting that Ser-727 phosphorylation delays the activation of $cPLA_2$ by PMA.

DISCUSSION

In this study, the roles of serine phosphorylation sites within $cPLA_2$ during enzyme activation were examined. Treatment of the WM793 line stably overexpressing the wild-type $cPLA_2$ (WT) failed to release [^{3}H]arachidonic acid in response to TNF and CHI treatment making the study of the mutants $cPLA_2$ proteins, in this system, a moot issue. However, treatment of WT with PMA demonstrated that the overexpressed $cPLA_2$ enzyme was indeed functional. The subsequent investigation into the roles that these phosphorylation sites play, during PMA-induced $cPLA_2$ activation, yielded interesting findings. The substitution of Ser-505 with alanine (SA505) abrogated the activation of $cPLA_2$. The disruption of the Ser-437 phosphorylation site blocked enhanced ^{3}H]arachidonic acid release at all time points suggesting an important role for the phosphorylation of this residue. Finally, overexpression of $cPLA_2$ containing a mutation at Ser-727 enhanced ^{3}H]arachidonic acid release suggesting that phosphorylation of Ser-727 has an inhibitory effect on $cPLA_2$ activation in response to PMA. As PMA is a strong activator of PKC, these results indicate that $cPLA_2$ phosphorylation sites, other than Ser-505, contribute to the regulation of $cPLA_2$ induced by PKC-dependent MAPK signaling cascades.

Stable cell lines that overexpressed the various $cPLA_2$ constructs were generated because initial transient transfection experiments yielded inconsistent results. Enhanced [^{3}H]arachidonic acid release was observed from transient transfectants following treatment with TNF/CHI or PMA but only in ~10% of the experiments. It is possible that the ~40% transfection efficiency that was obtained with Lipofectamine 2000 (determined by transient GFP-tagged protein expression; not shown) was not sufficient to produce

optimal conditions to study $cPLA_2$ overexpression. Therefore, the stable transfectants were generated to obtain stable cells lines that overexpressed equal levels of protein.

The reason that WT, which stably expresses $cPLA_2$, also failed to respond to treatment with TNF and CHI was not identified. One possible explanation is that the activation of the enzyme is blocked by an unidentified regulatory mechanism as a protective measure. As the $cPLA_2$ enzyme is a highly inflammatory molecule and has been shown to mediate apoptotic processes, the deactivation of $cPLA_2$ in WT that are responding to TNF in may be triggered to promote the viability of the cell line. An investigation of this hypothesis would be beyond the scope of this study and was not conducted.

Lin *et al.* was the first to provide direct evidence that phosphorylation of $cPLA_2$ enhances the activity of the enzyme (19). In that study, treatment of CHO cells, that expressed $cPLA_2$, with different agonists (including PMA) resulted in the phosphorylation on serine resides. The enhanced activity that coincided with phosphorylation was sensitive to phosphatase treatment suggesting a critical role for serine phosphorylation. A later study demonstrated that one target of phosphorylation, which is necessary for activity, is Ser-505 (17). Ser-505 contains the consensus sequence for MAP kinase phosphorylation (28), which has been shown to be phosphorylated both *in vitro* and *in vivo* (17, 29). Since, the phosphorylation of Ser-505, as well as other serine residues, has been shown to be induced by different agonists in many systems (12, 27, 30, 31). Though the phosphorylation of $cPLA_2$ is largely thought to depend upon MAP kinase dependent signaling pathways, the serine residues that are phosphorylated have been shown to be cell type and agonist specific. And because evidence exists that

suggests that MAPK-independent pathways in response to these agonists also contribute to $cPLA_2$ phosphorylation and activity, it is obvious that the regulation of the enzyme by phosphorylation is very complex (20). Presently, Ser-727 is the only serine residue, other that Ser-505, whose phosphorylation has been directly demonstrated to contribute to the activation of $cPLA_2$ (27). Clearly, the investigation into the roles of other serine residues is warranted. The results of this study implicate a novel role for the phosphorylation of serine residues 437 and 727 during the PMA-induced activation of $cPLA_2$. These were found to mediate both the activation and inhibition of the enzyme respectively.

The results from this study are in agreement with earlier reports that demonstrated Ser-505 phosphorylation mediates arachidonic release from phorbol ester treated cells. However, the Ser-505 mutation did not completely block the ability of PMA to activate $cPLA_2$. The inhibitory effect that this mutation had was largest at 30 minutes and decreased at later time points suggesting that activation had occurred independently of Ser-505 phosphorylation. PMA is a potent activator of PKC and the responses to PMA treatment are commonly measured within 10 minutes of treatment as the activation of MAPK and phosphorylation of Ser-505 is rapid. However, MAP kinase activation is only one consequence of PKC activity as many cellular substrates for PKC exist (32). Therefore, the longer treatment periods used in this study may have resulted in the activation PKC mediated signaling systems, independent of MAP kinase activity that activated $cPLA_2$. One possibility is that intracellular calcium concentrations become elevated after prolonged exposure to PMA. Though the PMA-induced activation of $cPLA_2$ has not been attributed to calcium level modifications, phorbol esters have been shown to activate L-type calcium channels in the plasma membrane in a PKC-dependent

manner (33, 34). If this phenomenon occurs in WM793 following prolonged treatment with PMA, the enhanced calcium levels could certainly activate $cPLA_2$ by inducing membrane translocation.

Unlike SA505, the response of SA437 was similar to that of pCI at all time points examined suggesting that PMA completely fails to activate $cPLA_2$ when Ser-437 is not phosphorylated. Treatment of cells with phorbol esters does not increase Ser-437 phosphorylation, which is probably why a role for this site has not been previously investigated. However, studies have shown that Ser-437 is constitutively and highly phosphorylated within untreated macrophages, and insect cells that overexpress $cPLA_2$ (12). Furthermore, when the insect cells were labeled with ^{32}P, phosphopeptide analysis demonstrated that Ser-437 is phosphorylated to a higher degree than any other site examined. Therefore, it is possible that the constitutive phosphorylation of $cPLA_2$ is necessary for the PMA-induced arachidonic acid release from cells. In addition, because the suppressive effects of the Ser-505 mutation may be overcome at later time points by other signaling cascades, the observation that the Ser-437 mutation blocks enhanced [^{3}H]arachidonic acid release throughout 2 hours suggests that Ser-437 is required for $cPLA_2$ activity in many systems.

In contrast to the suppressive effects that the Ser-437 and Ser-505 mutations had, the mutation of Ser-727 enhanced the [^{3}H]arachidonic acid release from cells treated with PMA. This suggests an inhibitory role for the phosphorylation of Ser-727. The effect of this mutation was only evident at 30 and 60 minutes after treatment. Therefore it is likely that Ser-727 phosphorylation modulates the PMA-induced [^{3}H]arachidonic acid release by increasing the kinetics of $cPLA_2$ activation. Phosphorylation that results in the

inhibition, rather than activation, of activity is less common but does occur in some enzymes including glycogen synthase kinase-3 (35), as well as members of the Src kinase family (36). In addition, evidence for a phosphorylation-dependent mechanism that inhibits $cPLA_2$ activity has been previously documented (37). Murthy *et al.* demonstrated that the activation of cAMP and cGMP-dependent protein kinases, in smooth muscle cells, results in the phosphorylation of $cPLA_2$ and a decrease in $cPLA_2$ activity. Though the site of phosphorylation was not identified, phorbol esters have been demonstrated to activate cAMP-dependent protein kinase suggesting that Ser-727 may be the target (38).

The inhibitory role of Ser-727 phosphorylation during PMA signaling, however, is difficult to justify because it is at odds with some earlier observations that linked the phosphorylation of Ser-727 with $cPLA_2$ activation. De Carvolho *et al.* initially demonstrated that the treatment, of sf9 insect cells overexpressing $cPLA_2$, with okadaic acid resulted in Ser-727 phosphorylation and an increase in [^{3}H]arachidonic acid release (12). It was later demonstrated that the substitution of ser-727 had no effect on the okadaic acid-induced [^{3}H]arachidonic acid release indicating that the phosphorylation of this site does not have a functional role in this system (39). The treatment of platelets with thrombin has also been shown to result in the phosphorylation of both Ser-505 and 727, as well as an increase in arachidonic acid release (40). However, no mutational analysis was performed and therefore, the arachidonic acid release may be mediated solely by Ser-505 phosphorylation. Another study also refutes the hypothesis that phosphorylation of Ser-727 plays an inhibitory role. Hefner *et al.* demonstrated that arachidonic acid release was suppressed from PMA-treated COS cells overexpressing

$cPLA_2$ with the Ser-727 mutation (27). In addition, this study demonstrated that the MAPK-interacting kinase 1 (MNK1) was capable of phosphorylating Ser-727 in a p38-dependent manner. These results provide stronger evidence to support a role for Ser-727 phosphorylation that is involved in $cPLA_2$ activation. However, although p38 has been shown to contribute to the PMA-induced [^{3}H]arachidonic acid release, it does so in a phosphorylation independent manner (41). Further investigations must be performed to clarify the how this phosphorylation site contributes to $cPLA_2$ activity.

Taken together, the results from this study indicate that the activation of $cPLA_2$, by phorbol esters, is not solely regulated by the phosphorylation of Ser-505. We propose that novel regulatory mechanisms exist that also modulate $cPLA_2$ activity. These are mediated by constitutive phosphorylation of Ser-437 that is necessary for arachidonic acid release. In addition, PMA-induced phosphorylation of Ser-727 controls arachidonic acid release by inhibiting over-activation of the enzyme. Through the production of eicosanoids, $cPLA_2$ plays an important role during inflammatory responses such as type I allergy (42). The identification of these, and other novel regulatory mechanisms, will provide a foundation for the development of better pharmaceuticals that target the signaling pathways that mediate these responses.

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4

SUMMARY

SUMMARY

The goal of these studies was to identify a role for intracellular calcium and serine phosphorylation in the activation of $cPLA_2$ during TNF-induced apoptosis. We focused on intracellular calcium and phosphorylation on serine residues because these signals have been shown to activate $cPLA_2$ in other systems. Our results demonstrated that a rise in intracellular calcium levels occurs early during apoptosis of C3HA fibroblasts that have been sensitized to TNF by cycloheximide, and this is necessary for arachidonic acid release. In addition, calcium-dependent $cPLA_2$ activation was consistent during apoptosis induced by various chemotherapeutic agents. To study serine phosphorylation, we generated cell lines that stably overexpress $cPLA_2$ protein containing mutations at specific serine phosphorylation sites. Unexpectedly, the overexpressed enzymes, including the wild-type $cPLA_2$, were unresponsive to stimulation with TNF. They did however respond to PMA stimulation and our results yielded two interesting findings. First, the cell line, that contained $cPLA_2$ with a mutation at ser-437, was hypo-responsive to PMA treatment. Second, the cell line that over-expressed $cPLA_2$ with a mutation at ser-727 was hyper-responsive. Taken together, our results suggest that calcium responses are a general requirement for the activation of $cPLA_2$ during many apoptotic processes and that $cPLA_2$ activity can be both positively and negatively regulated by serine phosphorylation.

To directly measure changes in intracellular calcium levels during apoptosis, we used the fluorescent indicator fura-2 AM. Elevated calcium levels, within apoptotic C3HA fibroblasts, were evident as early as 1 hour after treatment with TNF and CHI and were highest in the perinuclear region. The calcium response was also inhibited by the

calcium channel blocker, verapamil, suggesting that the source of calcium was the extracellular milieu. We also found that the apoptosis-inducing chemotherapeutic agents, melphalan, cis-platinum, and vinblastine readily induced the death of C3HA. Given that verapamil inhibited arachidonic acid release to the same extent, regardless of the inducer, a rise in intracellular calcium appears to be required for $cPLA_2$ activation during multiple apoptotic responses. Fluorescence experiments, performed by Culver and Laster demonstrated a near complete nuclear staining pattern of $cPLA_2$ by 3 hours of treatment with TNF and CHI. The nuclear localization was also sensitive to verapamil treatment suggesting that the role of calcium, in the activation of $cPLA_2$, was to induce the nuclear translocation. Finally, infection with *dl309*, an adenovirus that also sensitizes C3HA to TNF, induced a similar calcium response suggesting that TNF-induced apoptosis of virally infected cells also has a calcium requirement to activate $cPLA_2$.

Our experiments do not rule out the possibility that other PLA_2 enzymes contribute to arachidonic acid release following treatment with chemotherapeutic agents. Inhibitors are available that specifically block the activity of different PLA_2 enzymes and would be useful in clarifying this issue. In addition we did not confirm that the cell death induced by the chemotherapeutic agents was apoptosis. To address this, a variety of apoptotic markers can be analyzed including cytoplasmic membrane blebbing, pro-apoptotic caspase activation, phosphatidylserine exposure, as well as mitochondrial depolarization. To complete these studies, intracellular calcium levels and $cPLA_2$ localization should be analyzed following treatment with the chemotherapeutics. As a calcium response appears to be required for $cPLA_2$ activation during multiple apoptotic

responses, it is possible that $cPLA_2$ also translocates into the nucleus during these responses as well and future experiments are to be performed to this hypothesis.

Another goal is to identify the role of nuclear $cPLA_2$ during apoptosis. The nuclear membrane contains substantial amounts of arachidonic acid and therefore, one possibility is that arachidonic acid released from the nuclear membrane is required for apoptosis. Future experiments will be designed to identify which membrane(s) is the source of arachidonic acid liberation. Another possibility is that $cPLA_2$ interacts with one or more nuclear proteins to induce apoptosis. A variety of procedures can be performed to address this including co-immunoprecipitation for apoptotic nuclei or yeast two-hybrid analysis. Nevertheless, if this is the case, a cellular expression system can be designed to determine if the interactions are necessary for apoptosis. Indeed, to demonstrate that the interaction of $cPLA_2$ and nuclear protein(s) is a common requirement for cell death in other apoptotic systems would be exciting. This would also prove that PLA_2 enzymes have other functions in cellular signaling in addition to fatty acid liberation.

The roles that serine phosphorylation sites, other than ser-505, have during PMA-induced $cPLA_2$ activation have not been identified. To analyze the function of these residues in response to PMA, we generated WM793 melanoma-derived cell lines that stably overexpressed $cPLA_2$ constructs. The respective $cPLA_2$ proteins contained a mutation at different serine phosphorylation sites. Our results suggest that the phosphorylation of both ser-437 and ser-727 regulate the activity of $cPLA_2$. These observations are exiting for two reasons. First, no role has yet been identified for ser-437 in any system. We observed that the levels of [^{3}H]arachidonic acid release from the cell line expressing $cPLA_2$, mutated at ser-437, were significantly lower than those from the

cell line expressing the unaltered enzyme. This was consistent throughout 2 hours after PMA treatment suggesting that the phosphorylation of this residue is critical for enzyme activity. Second, our results implicate the phosphorylation of ser-727 in negatively regulating $cPLA_2$. This observation suggests a novel discovery because evidence for the down-regulation of $cPLA_2$ activity by phosphorylation has been proposed but the mechanism by which the inhibition is mediated has not been identified.

Cellular and biochemical tests can be performed to determine the mechanism in which ser-437 and ser-727 regulate $cPLA_2$ activity. However, additional clonal cell lines that express $cPLA_2$, with mutations at these residues, should first be tested for PMA-induced [^{3}H]arachidonic acid release. Although our experiments were repeated several times, we tested only one cell line for each mutation. To regulate $cPLA_2$ activity, the phosphorylation of these sites most likely modulates the catalytic activity of the enzyme or affects the intracellular localization of $cPLA_2$. A variety of experiments can be designed to distinguish between these possibilities. *In vitro* enzyme assays can be performed to determine the effect of the mutations on the intrinsic catalytic activity of $cPLA_2$. Membrane binding kinetics of the enzymes can also be measured to determine if the mutations affect the membrane association rate of $cPLA_2$ following stimulation. Finally, immunofluorescence and membrane fractionations can determine if the subcellular localization of $cPLA_2$ is affected by mutations after PMA-treatment.

The reason why TNF and CHI failed to activate the overexpressed $cPLA_2$ in the WM793-derived cell line was not explored. One possibility is that $cPLA_2$ inhibitory gene(s) became upregulated upon treatment with TNF in these cells. The inhibitory protein(s) could block arachidonic acid release by several mechanisms including

sequestering $cPLA_2$ away from substrate, inhibiting its catalytic activity, or by inducing its degradation by proteolysis. Nevertheless, we concluded that stable overexpression of $cPLA_2$ in WM793 was not an appropriate system to study the role of serine phosphorylation during TNF-induced apoptosis. In the future, we can possibly overcome this problem by selecting a different cell line to overexpress $cPLA_2$. Ideally, we could develop a knock out cell line that completely lacks $cPLA_2$ expression.

In summary, the results from these studies provide insight into the mechanisms that regulate the activity of $cPLA_2$. $cPLA_2$ specifically releases arachidonic acid from membrane phospholipids. Arachidonic acid is the precursor to eicosanoid synthesis, which makes $cPLA_2$ a key regulatory molecule during inflammation. The activity is also required during TNF induced apoptosis, a programmed cell death mechanism that has been linked to tissue damage during several autoimmune diseases. Understanding the molecular basis for the activation of $cPLA_2$ during this process will lead to the production of pharmaceuticals that have potential to lessen damage associated with disease.

4

APPENDICES

Appendix A: Protocol for Site-Directed Mutagenesis of $cPLA_2$ cDNA

Introduction

To substitute the $cPLA_2$ serine residues 437, 454, 505, and 727 with alanine, we used the QuickChange Site-Directed Mutagenesis Kit (Stratagene) as it has previously been used to successfully generate $cPLA_2$ mutations at Ser-505 and Ser-727 (1). Following mutagenesis, the plasmid DNA was propagated in *E. coli.,* purified, and then successful mutagenesis was confirmed by sequence analysis.

Materials

The QuickChange Site-Directed Mutagenesis Kit, which contains the *Pfu Turbo* DNA polymerase and XL1-Blue supercompetent cells, was purchased from Stratagene (La Jolla, CA). The CloneChecker plasmid detection system was purchased from Invitrogen (San Diego, CA). All standard chemicals and supplies were purchased from Fisher (Suwanee, GA).

Results

Site-directed mutagenesis

The cDNA coding for human $cPLA_2$ (Accession number M72393, Clark, *et al.* 1991) was obtained from Jim Clark (Genetics Institute, Cambridge MA) contained within the pMT-2 vector. Christina Voelkel-Johnson later excised the $cPLA_2$ gene and subcloned it into the *Sal*1 cloning site of the pCI mammalian expression vector (Promega, Madison WI) generating the target plasmid we used for site-directed mutagenesis (2).

Two primers designed to generate each mutation, which corresponded to the positive and negative strand of the plasmid (primer #1 and primer #2 respectively), were synthesized by IDT, Inc. (Coralville, IA) and the sequences are listed in Table 2. 50 ng of plasmid DNA was added to a freshly made reaction mixture (5 µl 10X reaction buffer, 125 ng primer #1, 125 ng primer #2, 1 µl dNTP mixture, 2.5 Units *Turbo* DNA polymerase), which was brought to a final volume of 50 µl in water. A variation of manufacturer's instructions for PCR was used as follows: denaturation 95 ?C for 5 minutes, followed by 12 cycles at 95 ?C for 1 minute, 55 ?C for 1 minute, and a final extension at 68 ?C for 16 minutes. Each sample was then treated with 10 Units *Dpn* I, a restriction enzyme specific for methylated and hemi-methylated DNA with a target sequence of 5'-Gm6ATC-3'. This step is necessary to eliminate parental plasmid DNA, thus ensuring that remaining plasmid contains the mutation(s).

Table 2: Sequences of the oligonucleotide primers used to generate mutant $cPLA_2$ constructs.

Primer	**Sequence (5' to 3')**
SA437 #1:	GAGTAATGATAGCTCGGACGCTGATGATGAATCACACGAACCC
SA437 #2:	GGGTTCGTGTGATTCATCATCAGCGTCCGAGCTATCATTACTC
SA454 #1:	GGCACTGGAAATGAAGATGCTGGAGCTGACTATCAAAGTG
SA454 #2:	CACTTTGATAGTCAGCTCCAGCATCTTCATTTTCAGTGCC
SA505 #1:	CTCAATACATCTTATCCACTGGCTCCTTTGAGTGACTTTGCC
SA505 #2:	GGCAAAGTCACTCAAAGGAGCCAGTGGATAAGATGTATTGAG
SA727 #1:	GACAGAATCCATCTCGTTGCGCTGTTTCCCTTAGTAATG
SA727 #2:	CATTACTAAGGGAAACAGCGCAACGAGATGGATTCTGTC

Transformation into *E. coli*.

10 µl of *Dpn* I treated plasmid DNA was transferred to a pre-chilled Falcon 2059 polypropylene tube that contained 50 µl XL1-Blue supercompetent cells (transformation mixture). The rest of the procedure was carried out as follows: Chill the transformation mixture on ice for 30 minutes, heat pulse at 42 ?C for 45 seconds, Add 0.5 mL NZY+ broth (10 g casein hydrolysate, 5 g yeast extract, 5 g sodium chloride per liter, supplemented with 12.5 mM magnesium chloride, 12.5 mM magnesium sulfate, and 20 mM glucose) and shake at 37 ?C for 1 hour (250 rpm). The transformation cultures were then plated on LB nutrient agar plates and incubated overnight at 30 ?C. Colonies were selected and screened using the CloneChecker system according to the manufacture's instructions. Mutations were confirmed by sequence analysis (Iowa State DNA Sequencing and Synthesis Facility; Ames, IA).

Appendix B: Protocols for Transient and Stable Expression of $cPLA_2$ in WM793

Introduction

The generation of WM793 melanoma cells that transiently and stably overexpressed wild-type and mutant $cPLA_2$ enzymes required a great deal of optimization. This appendix was added to provide a detailed description of the protocols used for future reference. Described herein, are the results that were obtained using various transfection reagents to overexpress $cPLA_2$ under different conditions. Also described is the protocol used to generate stable WM793 cells.

Materials and Methods

Materials and cell culture

Lipofectin and Lipofectamine 2000 transfection reagents were purchased from Invitrogen (San Diego, CA). The FuGENE 6 transfection reagent was purchased from Roche (Indianapolis, IN). The pHygEGFP vector was purchased from Clontech (Palo Alto, CA). FuGENE 6 transfection reagent was purchased from Roche (Indianapolis, IN). Hygromycin B was purchased from Calbiochem (La Jolla, CA). Mini and MidiPrep plasmid preparation kits were purchased from Qiagen (Valencia, CA). Standard chemicals and supplies were purchased from Fisher Scientific (Suwanee, GA). All media was purchased from Sigma (St. Louis, MO). The C3HA fibroblast cell line was kindly provided by L. Gooding, (Emory University, Atlanta, GA) and cultured in DMEM (Sigma D-7777) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The WM793 Melanoma cell line was generously provided by M. Herlyn (Winstar Institute, Philadelphia PA). Untransfected cells were maintained at 37°C with 5% CO_2 and

cultured in MEL media (4 parts MCDB 152, 1 part L-15, with 2 mM $CaCl_2$, 50 µg/ml pituitary extract, and 5 µg/ml insulin and 2 mM L-glutamine). Stable WM793 transfectants were cultured in MEL media supplemented with 10% FBS and 200 µg/ml Hygromycin B.

Western blot analysis

See Materials and Methods in Chapter 2.

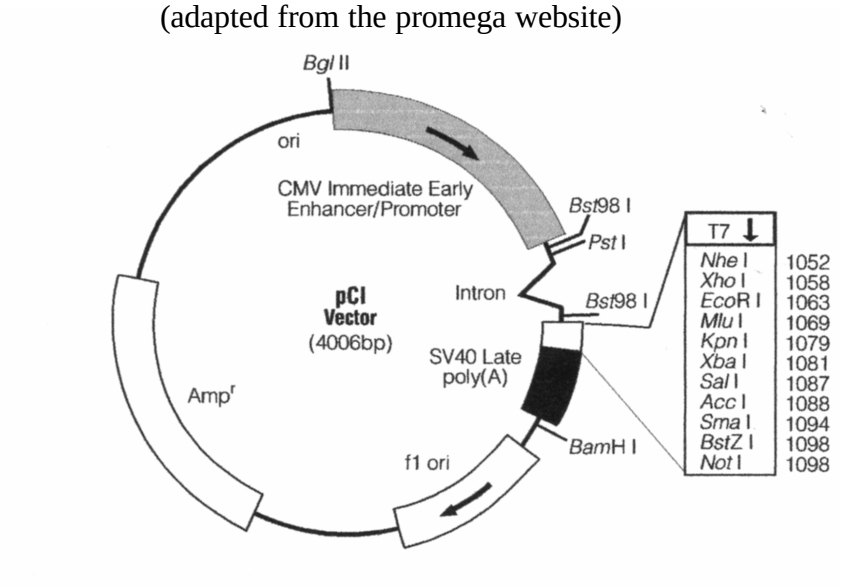
Results

The pCI expression vector and $cPLA_2$ construct preparation.

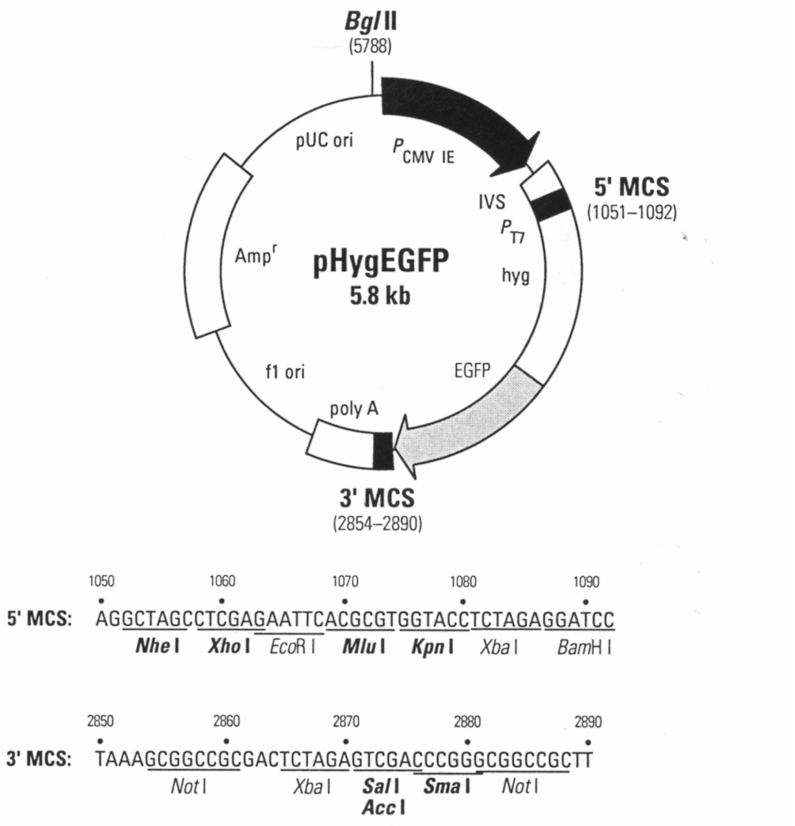
The site-directed mutagenesis experiments targeted $cPLA_2$ cDNA that was cloned into the pCI mammalian expression vector (Promega Madison, WI). This vector contains a multiple cloning site that is under the control of the human cytomegalovirus (CMV) promoter for expression of target genes within mammalian cells. The vector also contains the ampicillin resistance gene in order to propagate and select the plasmid in *E. coli*. An illustration of the vector map is provided in Figure 13. Prior to transfections, wild-type and a mutant $cPLA_2$ constructs were extracted from 25 mL XL1-Blue *E. coli.* using the Qiagen Midi plasmid preparation kit. The plasmid DNA was subjected to a final phenol/chloroform extraction, to remove any remaining protein contaminants, and resuspended in sterile H_20 for transfection.

Figure 13: Vector Maps of pCI and pHygEGFP.

pCI mammalian expression vector and multiple cloning site
(adapted from the promega website)



pHygEGFP vector and multiple cloning site
(adapted from the Clonetech website)



The optimization of transient transfections using the Lipofectin, Lipofectamine 2000, and FuGENE 6 transfection reagents.

We tested three different transfection reagents for efficiency and toxicity. Two were cationic liposome-mediated transfection reagents named Lipofectin and Lipofectamine 2000. The third was a multi-component lipid-based transfection reagent called FuGENE 6. The transfection protocols, for cells within 35 mm^2 plates, were as follows: Unless otherwise indicated, 2.6 X 10^5 WM793 melanoma cell were seeded in 35 mm^2 tissue culture plates one day prior to transfection, which resulted in a cell density of ~80% confluency, and 1 µg of plasmid DNA was used for transfection. For Lipofectin: the reagent and DNA solutions were each made separately in 0.5 mL centrifuge tubes, to a final volume of 100 µl in serum free Minimal Essential Medium Eagle media, and incubated for 35 minutes at room temperature. These solutions were combined, incubated for an additional 12 minutes at room temperature, and then added to the WM793 melanoma cells with 2 mL freshly added MEL growth media. For Lipofectamine 2000, the reagent and DNA solutions were made as described above. However, the two solutions were incubated for 5 minutes prior to combination. Following combination, the Reagent/DNA solution was incubated for 20 minutes before it was added for cells that had 2 mL freshly added growth media. For FuGENE 6, the solutions were also made separately but brought to a final volume of 50 µl for each. The solutions were incubated for 5 minutes prior to combination, and then for an additional 10 minutes. The combined mixture was added to cells that had 750 µl fresh growth media. For all three reagents, the transfection process was allowed to proceed for 5 hours

before the transfection media was replaced with fresh growth media. Western blot analysis was performed after 42 hours.

A comparison of Lipofectin, Lipofectamine 2000, and FuGENE 6 is presented in Figure 14. Different Reagent/DNA ratios, as well as cell densities were tested for optimization (variations of the manufacture's instructions). As shown in Figure 14*A*, transfections using 15 µl Lipofectin failed to result in any noticeable $cPLA_2$ overexpression when compared to untransfected WM793. In contrast, 7.5 µl Lipofectamine 2000 mediated high levels of $cPLA_2$ expression that were similar when combined with either 2 or 4 µg plasmid DNA. We also tested the transfection efficiency of Lipofectamine 2000 when a higher cell density (3.2×10^5 cells/$35mm^2$ plate) was used (Figure 14*B).* High levels of $cPLA_2$ were detected when 2 µl of Lipofectamine 2000 was used in combination with 1 µg plasmid DNA. However, a considerable loss in the levels of overexpression was observed when only 1 µl was used. 5 µl FuGENE 6 with 2 µg DNA resulted in the highest level of $cPLA_2$ overexpression (Figure 14*B).* 15 µl Lipofectin and 7.5 µl Lipofectamine 2000 resulted in considerable cytotoxicity. The lower concentration of Lipofectamine 2000 (2 µl/transfection) resulted in a reduced but still noticeable cytotoxic effects. The transfection with FuGENE 6 resulted in no noticeable cytotoxic effect on WM793 (not shown). In light of these experiments, we selected to use the Lipofectamine 2000 transfection reagent for the transient transfection experiments. The decision was based on overexpression levels and cytotoxic effects induced by each reagent as well as cost. The transient transfection experiments were also performed using 2 µl reagent and 1 µg DNA with a cell density of $2.6X10^5$ cells/35 mm^2

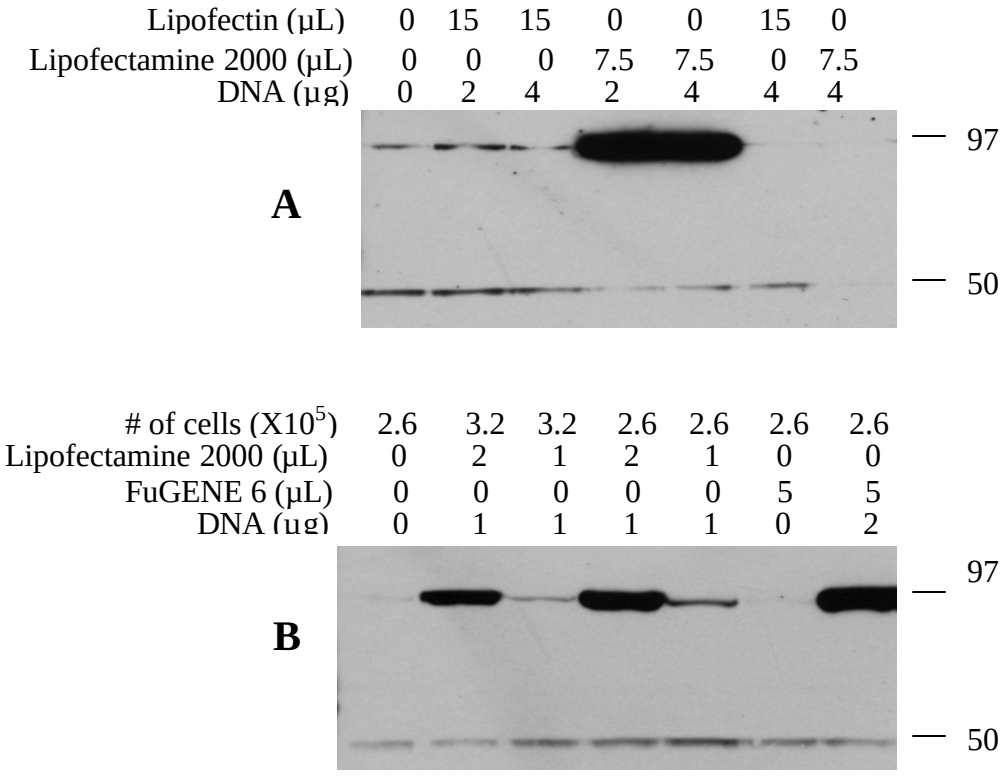


Figure 14: Comparison of Lipofectin, Lipofectamine 2000, and FuGENE transfection reagents. $2.6X10^5$ (unless otherwise indicated) WM793 cells were seeded in 35 mm^2 tissue culture plates and incubated overnight. (*A*) Lipofectin and Lipofectamine 2000 were used at the indicated concentrations to transfect either 2 or 4 µg $cPLA_2$ cDNA. (*B*) Lipofectamine 2000 or FuGENE 6 was used at indicated concentrations to transfect 1 µg $cPLA_2$ cDNA into WM793 at indicated cell densities. $cPLA_2$ protein was detected using the 7905 polyclonal anti-serum.

plate, as these conditions resulted in the highest levels of $cPLA_2$ overexpression with the least amount of cytotoxity.

The generation of stable WM793 cell lines overexpressing wild-type and mutant $cPLA_2$ constructs.

Stable WM793 cell lines were generated to obtain cell lines that contained equal levels of $cPLA_2$ construct expression within each cell. The FuGENE 6 transfection reagent was used according to a variation of the protocol described above. For the purpose of selection, the pHygEGFP vector was purchased, which contains the hygromycin B resistance gene under the control of the CMV promoter (Figure 13). This vector was co-transfected into WM793 with pCI, containing a $cPLA_2$ construct, at a ratio of 200 ng/2.5 µg. 48 hours after transfection, the media was replaced with fresh MEL media containing 200 µg/ml hygromycin B. The selection media was replaced every 3 days and resistant colonies appeared after 2 weeks of selection. When the colonies reached ~100 cells in size, they were enclosed within a cloning cylinder, harvested in 50 µl trypsin, and then transferred to a 24 well tissue culture plates.

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