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In Situ Regulation of Cytosolic Phospolipase A_2

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ABSTRACT

The 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) is an agonist-responsive effector for intracellular signal transduction through the arachidonate cascade. *In vitro* studies have demonstrated that this enzyme is regulated by sub-micromolar calcium and is specific for arachidonate as the *sn*-2 fatty acyl group of phospholipid substrates. However, very little data is available regarding *in situ* mechanisms which govern the activity of $cPLA_2$. The primarily objective of these studies was to develop an *in situ* system for the study of $cPLA_2$, and investigate mobilization of arachidonate during signal transduction events .

Dimethylsulfoxide differentiation of the human lymphoma cell line, U937, induced an enhanced capacity to mobilize arachidonate in response to the calcium ionophore A23187. The arachidonate mobilizing activity in differentiated cells was consistent with characteristics reported for $cPLA_2$ *in vitro*. Although undifferentiated U937 cells have exceptionally high quantities of $cPLA_2$, A23187-stimulated arachidonate mobilization was low, and not specific for arachidonate. Thus, differentiation of U937 induced $cPLA_2$ regulatory elements that mediate arachidonate mobilization.

Differentiation induced significant changes in the capacitative pathway of intracellular calcium elevation. Both the size of intracellular calcium stores, as well as the characteristics of calcium influx channels were altered with differentiation. Agonist-

stimulated arachidonate mobilization was coupled to these differentiation-induced alterations. $cPLA_2$ activity was initiated upon agonist-stimulated depletion of intracellular calcium stores, and continued until maximum elevations of intracellular free calcium were attained. The data suggest that $cPLA_2$ may be coupled to the generation of a calcium influx factor, which serves as a communication link between intracellular calcium stores and store-operated calcium influx channels. Consistent with this hypothesis, exogenous free arachidonate activated calcium influx in differentiated U937, consistent with activation of store-operated capacitative calcium influx channels.

Based on the data obtained in this study, a model for agonist-stimulated $cPLA_2$ activity is presented. This model suggests a novel role for $cPLA_2$. Apart from the well known role in initiation of the arachidonate cascade, $cPLA_2$ may be part of an intracellular effector system which regulates agonist-stimulated influx of extracellular calcium during activation of the capacitative pathway.

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CHAPTER ONE

INTRODUCTION

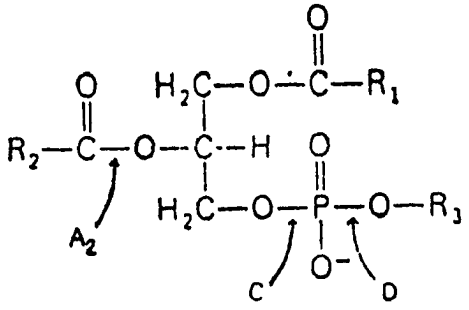
Arachidonic acid is a 20 carbon fatty acid, containing 4 double bonds. In resting mammalian cells, arachidonate is primarily found in its storage form, esterified to the *sn*-2 position of membrane phospholipids (1). Agonist stimulation by a diversity of compounds including thrombin (2, 3), bradykinin (4), cytokines (5), growth factors (6), vasopressin (7), platelet activating factor (8), and the non-hormonal calcium ionophore A23187 (1, 4) result in liberation of free arachidonic acid. Arachidonate is mobilized from phospholipid stores by either direct or indirect pathways (Figure 1A). The indirect pathways involve phospholipid hydrolysis by phospholipases C (PLC) and D (PLD). Hydrolysis by PLD results in the formation of phosphatidic acid, which can be further hydrolyzed by the combined actions of phosphatidate phosphohydrolase and diglyceride lipase, yielding free arachidonic acid. Additionally, the combined actions PLC and diglyceride lipase can also yield free arachidonate. However, these two pathways are considered to provide only a minor contribution to the total agonist-stimulated arachidonate release (9). The primary mechanism for generation of free arachidonate is *via* the action of phospholipase A_2 (PLA_2), which hydrolyzes the ester

FIGURE 1

A. PHOSPHOLIPASES INVOLVED IN RELEASE OF FREE ARACHIDONATE

B. THE ARACHIDONATE CASCADE

A.



B.

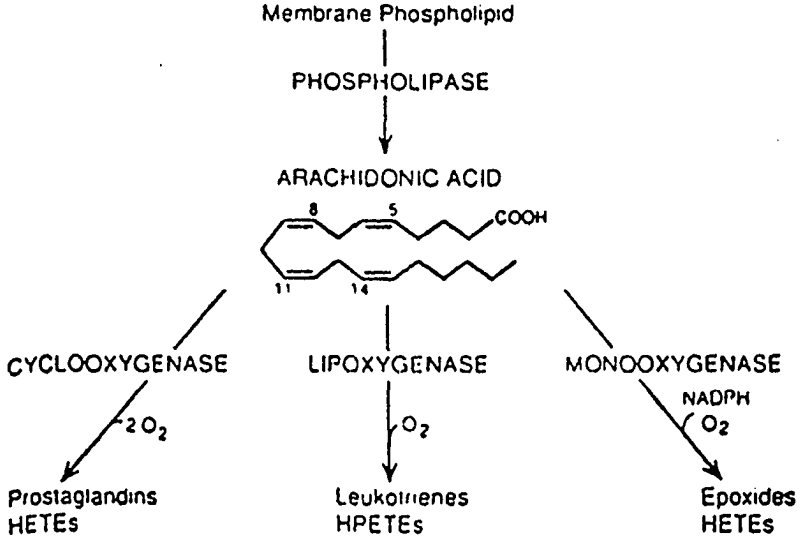


FIGURE 1

linkage of the fatty acid at the C2 position of a phospholipid molecule.

Once released, arachidonic acid is a key intermediate in a diversity of signal transduction pathways. Free arachidonate can be metabolized *via* three basic oxygenation pathways which comprise what is known as the arachidonate cascade (Figure 1B). Metabolism by the cyclooxygenase pathway results in the formation of prostaglandins, thromboxanes, and prostacyclin. The lipoxygenase pathway catalyzes the formation of leukotrienes, and hydroperoxy- and hydroxy-eicosatetraenoic acids (HPETES & HETES). Finally, cytochrome P450 epoxygenases catalyze the NADPH-dependent formation of epoxyeicosatrienoic acids (EETS). Taken together, these 20-carbon oxygenated metabolites of arachidonate are collectively known as "eicosanoids". Eicosanoids are potent lipid mediators, which differentially regulate a wide range of responses including cell growth, vascular and bronchial smooth muscle tone, platelet aggregation, tissue injury, allergy, and the inflammatory response (8, 10, 11). It has also been suggested that arachidonate itself may be directly involved in signal transduction via the regulation of intracellular free calcium ($[Ca^{2+}]_i$) levels (12-14), mediation of growth and differentiation (15, 16), and regulation of low molecular weight GTPase activating proteins (17-19).

Regardless of the particular metabolic pathway taken by released arachidonate, the first and rate limiting step in the synthesis of these lipid mediators is the release of free arachidonate from membrane phospholipids, primarily by the action of PLA_2. As a first committed step to a biochemical pathway, it is expected that the action of PLA_2 is under stringent regulatory control. A comprehensive knowledge of these controlling elements will provide more effective treatment for pathological conditions related to

activation of the arachidonate cascade.

Two forms of PLA_2 have been implicated in the inflammatory response in mammalian tissues, each with distinctly different characteristics. A 14 kDa secretory PLA_2 ($sPLA_2$) has been isolated from extracellular fluids at inflammatory sites (20, 21) and has been associated with extracellular propagation of the inflammatory response (22, 23). $sPLA_2$ appears to contain a signal sequence, homologous to those of other secretory proteins, which directs secretion to the extracellular matrix in response to inflammatory stimuli (20, 25). Distinguishing properties of this enzyme include a lack of preference for sn-2 fatty acyl group and a millimolar calcium requirement (22). Although $sPLA_2$ has long been considered the agonist responsive phospholipase involved in regulation of intracellular signal transduction through the arachidonate cascade, the characteristics of this enzyme suggest that this may not be so. Physiological intracellular calcium transients are in the sub-micro to micromolar range (6). The millimolar calcium requirement of $sPLA_2$ implies that this enzyme may not function as an intracellular effector system for receptor mediated events. The lack of preference for fatty acyl substrate lends further support to this hypothesis.

An 85 kDa cytosolic PLA_2 ($cPLA_2$), has recently been identified in many cell types, including platelets (24), mast cells (5), pulmonary endothelium (25), adrenal chromaffin cells (26), U937 monocytic leukemia cells (27-29), and macrophages (30, 31). This enzyme has no sequence homology with the 14 kDa $sPLA_2$ (32). Unlike $sPLA_2$, $cPLA_2$ requires sub-micro to micromolar calcium for activation; which is within the range of $[Ca^{2+}]_i$ concentrations. In further contrast to $sPLA_2$, $cPLA_2$ exhibits a preference for sn-2 arachidonyl-containing phospholipid substrates (22, 31).

A direct coupling between $cPLA_2$ mediated arachidonate release and hormonal stimulation has been demonstrated in Chinese hamster ovary cells overexpressing transfected U937 $cPLA_2$, but not in those overexpressing transfected $sPLA_2$ (33). Furthermore, platelets that were depleted of $sPLA_2$ by stimulation with platelet activating factor, were still capable of releasing thromboxane in response to a thrombin stimulus (34). This suggests that release of precursor arachidonate may not be mediated by $sPLA_2$. These observations support the hypothesis that $cPLA_2$ is an agonist responsive effector for signal transduction *via* the arachidonate cascade.

Current knowledge of $cPLA_2$ suggests that it is regulated by two primary mechanisms: phosphorylation and calcium. The calcium requirements of $cPLA_2$ have been determined *in vitro* using the purified U937 cell enzyme (28, 29, 35), as well as in subcellular fractionation studies (28, 36). $cPLA_2$ was found to translocate to membrane vesicle substrates *in vitro* at calcium concentrations above 150-300 nM. In fractionation studies, $cPLA_2$ was found in the cytosolic fraction of cells which were disrupted in calcium-free medium. However if cells were disrupted in medium with a calcium concentration above 250-300 nM, the enzyme was found in the membrane fraction (28, 36). Although $cPLA_2$ does not require calcium for catalysis (37, 38), calcium is required for association of the enzyme with membrane substrates (39). Sequence analysis of $cPLA_2$ has identified an amino acid domain homologous to the calcium dependent lipid binding domain of protein kinase C (PKC), GTPase activating protein (GAP), and PLC, which is believed to participate in the calcium dependent translocation process (32).

Presumably, activation of $cPLA_2$ requires translocation of the enzyme to

membrane substrates in response to an increase in $[Ca^{2+}]_i$. However, much of the data on calcium dependent activation of $cPLA_2$ has been obtained from *in vitro* experiments with purified enzyme. Direct correlations between physiological intracellular free calcium levels and *in situ* mobilization of arachidonate remain unclear. Although release of arachidonate has been observed following receptor mediated activation of various G protein signal transduction pathways which elevate $[Ca^{2+}]_i$ (13, 33, 40-43), we know very little about the effector mechanisms which couple receptor mediated events to translocation and activation of $cPLA_2$.

In addition to regulation by calcium, the activity of $cPLA_2$ is further enhanced by serine\threonine phosphorylation in response to phorbol 12-myristate 13- acetate (PMA), thrombin, and ATP (33), as well as other agonists (30, 44). *In vitro*, phosphorylation results in increased release of arachidonate primarily due to an increase in the V_{max} of the enzyme, while substrate affinity and calcium requirements remain unaltered (45). $cPLA_2$ has been reported to contain multiple phosphorylation sites, and the extent of phosphorylation may vary with the type of stimulus (33, 46, 47). Additionally, $cPLA_2$ contains potential recognition sites for protein kinase C (PKC), protein kinase A (PKA), casein kinase II, and mitogen activated protein kinase (MAP kinase) (48). Various effector proteins have been identified *in vitro* as potential catalysts for phosphorylation of $cPLA_2$. PMA has been shown to potentiate arachidonate release in U937 and other cells (28, 33, 36, 49). Since PMA activates PKC (50, 51), and PKC is capable of phosphorylating $cPLA_2$ *in vitro* (33, 47), it is possible that PKC may regulate *in situ* phosphorylation of $cPLA_2$. However treatment of cells with the PKC inhibitors staurosporine and sphingosine increased arachidonate

mobilization in some cell lines (52, 53), while effects were inhibitory in others (33, 54). These results suggest potential involvement of PKC isoforms, which may be differentially inhibited (50).

Alternatively, other lines of evidence suggest regulation of effector proteins for $cPLA_2$ phosphorylation *via* a kinase cascade, involving both PKC and non-PKC mediated events. In murine macrophages, zymosan-stimulated eicosanoid formation was inhibited by the tyrosine kinase inhibitors genistein and tyrphostin (55). This suggests that inhibition of tyrosine phosphorylation may inhibit agonist-stimulated arachidonate release in some cells. Furthermore, treatment of macrophages and monocytes with the tyrosine phosphatase inhibitors orthovanadate (56) and okadaic acid (30) increased agonist stimulated arachidonate release. Although $cPLA_2$ is not phosphorylated on tyrosine directly, it can be phosphorylated *in vitro* by MAP kinase (46, 57) which is in turn activated by both tyrosine and threonine phosphorylation (58, 59). In U937 cells, PMA treatment resulted in activation of both MAP kinase and a MAP kinase activator (58). Thus PMA may potentiate arachidonate mobilization by activation of a tyrosine kinase upstream of effector proteins which phosphorylate $cPLA_2$ directly, such as MAP kinase. Furthermore, the calcium ionophore A23187, which stimulates arachidonate release in many cells (60) also increases tyrosine phosphorylation. Thus, in addition to the direct effects of calcium on the translocation of $cPLA_2$, calcium may also affect upstream tyrosine kinases in a phosphorylation cascade which ultimately ends in phosphorylation of $cPLA_2$.

Although phosphorylaton may increase the specific activity of $cPLA_2$, an elevation in $[Ca^{2+}]_i$ appears to be essential for initiation of hydrolysis. In the neutrophil

(49), macrophage (30), and other cells (26), it has been reported that PMA alone is capable of activating arachidonate release, suggesting a role for phosphorylation alone in activation of $cPLA_2$. However it is unclear whether a calcium transient was also generated under the conditions used in these experiments, as neutrophils and macrophages are often activated during experimental manipulation (47, 61). In vasopressin-stimulated platelets (62), chemokine-stimulated monocytes (63), LPS-primed neutophils (64), and interleulkin-1-stimulated rat mesangial cells (35), phosphorylation of $cPLA_2$ was an ineffective stimulus for arachidonate release, unless combined with a calcium signal. Thus, although phosphorylation further increases the *in vitro* activity of $cPLA_2$, the actual intracellular effects of phosphorylation remain to be elucidated.

It has been suggested that a basal level of phosphorylated enzyme may exist in various cells, the activity of which is initiated by a calcium signal (47, 65). Additional phosphorylation might further increase enzymatic activity. However, *in vitro* experiments have demonstrated that the unphosphorylated enzyme is both active and responsive to calcium (33, 22). Alternatively, dual regulation of $cPLA_2$ by both calcium and phosphorylation may occur. Whereas calcium transients initiate catalysis by activation of $cPLA_2$-substrate association, the phosphorylation state of the enzyme regulates V_{max}. Thus a calcium transient may initiate translocation and activation of the $cPLA_2$ enzyme, the activity of which is further enhanced by phosphorylation.

In summary, *in vitro* studies have shown that $cPLA_2$ is regulated by both calcium, and phosphorylation. However, *in situ* regulation of arachidonate mobilization by $cPLA_2$ remains unclear. Although many $cPLA_2$ activating ligands have

been identified, (9, 22, 33) we know very little about the effector mechanisms which transduce receptor mediated stimuli into activation of $cPLA_2$. This study is designed to elucidate *in situ* mechanisms by which $cPLA_2$ is activated in U937 cells. The initial experiments presented in Chapter 3 were designed to identify a model cellular system for the study of $cPLA_2$. These experiments bypass receptor mediated stimulation, and focus on direct activation of $cPLA_2$ in response to A23187-stimulated increases in intracellular free calcium. Arachidonate release in this system was characterized with respect to stimulation by both calcium and PMA. Results from these initial experiments provoked further investigation into intracellular calcium dynamics, and the coupling of these dynamics to $cPLA_2$ activation, presented in Chapters 4 and 5. Chapter 6 examines intracellular calcium and $cPLA_2$ activity during actual receptor mediated events. Finally, a model for $cPLA_2$ regulation in response to agonist stimulation is presented in Chapter 7.

CHAPTER TWO

METHODOLOGY

2.1 Materials

U937 histiocytic lymphoma cells (66) were obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium was purchased from Sigma (St. Louis, MO). Fetal calf serum was obtained from Hyclone (Logan, UT). Dulbecco's phosphate buffered saline (PBS), HEPES buffer, and glutamine, were purchased from GIBCO (Grand Island, NY). Fatty acid free bovine serum albumin was from ICN Immuno Biologicals (Lisle, IL). Calcium ionophore A23187, SKF96365, staurosporine, and thapsigargin were from Calbiochem (San Diego, Ca). Dimethylsulfoxide (DMSO), phorbol myristate acetate (PMA) and nordihydroguaretic acid (NDGA), were from Sigma (St. Louis, MO). Stock solutions of A23187, PMA, and thapsigargin were prepared in DMSO and stored at -20°C. For arachidonate release assays, working solutions were prepared daily in PBS/2.2 mM glucose containing 2.5 μM albumin. [^{3}H]arachidonate (100 Ci/mmol) and [^{3}H]oleate (10 Ci/mmol) obtained from New England Nuclear (Boston, MA). Fura-2 AM was obtained from Molecular Probes (Eugene, OR). Thin layer chromatography plates were purchased from Supelco (Bellafonte, PA). Free fatty acids, phospholipids, and acylglycerol standards were from Sigma (St. Louis, MO) Prostaglandin B oligomer (PGBx) and HPLC standards were from Cayman Biochemical (Ann Arbor, MI). Econazole was obtained from R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). All other reagents were from Sigma (St. Louis, MO).

2.2 Cell Culture

U937 cells were grown in suspension culture using RPMI medium supplemented with 2 mM glutamine, 10 mM HEPES, and 10% heat inactivated fetal calf serum. Cultures were maintained in a 37°C humidified incubator, with 5% CO_2. Stock cultures were maintained at a cell density of less than 1 x 10^6 /ml. All experiments utilized cells between generations 4 and 26 in our laboratory.

For differentiation of U937 cells, cultures were seeded at an initial density of 0.2 x 10^6/ml, and were differentiated in growth medium supplemented with 1.5% DMSO (v/v) for 96 hours. Cell density was maintained at or below 0.8 x 10^6 cells/ml during the differentiation process by addition of fresh growth medium supplemented with 1.5% DMSO. Cell viability was monitored by trypan blue exclusion, and was greater than 99% during the differentiation time course.

Differentiation was monitored by growth cessation (as determined by cell count) and development of the capacity to reduce nitro-blue tetrazolium (NBT) (67). Reduction of NBT is characteristically used for assessment of the macrophage phenotype (68). Briefly, 2 x 10^6 cells were centrifuged and resuspended in 1.0 ml Dulbecco's phosphate buffered saline (PBS). One ml of 0.2% NBT (w/v) solution and 0.16 μM PMA were added, and the cells were incubated at 37°C for 30 minutes in a shaking water bath. After incubation, a smear of the cell suspension was prepared on a glass slide. Cells capable of reducing NBT contained purple-black formozan granules intracellularly. Results were tabulated by microscopic evaluation, as a percentage of cells reducing NBT. A minimum of 150 cells were counted. In undifferentiated U937 cell cultures 8-10% of the cells

reduced NBT. After the 96 hour differentiation period, >90% of the cells reduced NBT.

2.3 Radiolabeling

Differentiated or undifferentiated cells were radiolabeled by addition of [^{3}H]arachidonate (0.1 μCi/10^6 cells) or [^{3}H]oleate (0.2 μCi/10^6 cells) to the growth medium 24 hours prior to assay. 82 -85 % of the radiolabel was incorporated intracellularly during this period. After the labeling period, cells were washed once with PBS supplemented with 2.2 mM glucose and 2.5 μM albumin.

To determine the amount of radioactivity incorporated into phospholipids, aliquots of labeled cells were extracted according to the method of Bligh & Dyer (69). Extracts were thin layer chromatographed in the solvent system petroleum ether/diethyl ether/glacial acetic acid (82:18:1). Phospholipids, mono-, di-, and triacylglycerols, and free fatty acids were identified by co-chromatography of authentic standards. Radioactivity in the various lipid classes was quantitated using a Bioscan (Washington, DC) radioactivity scanner. Results confirmed that 96-99% of the radiolabel was present in phospholipid. Thin layer chromatography using the solvent system chloroform/methanol/glacial acetic acid/water (50:30:8:3) confirmed that [^{3}H]arachidonate was similarly distributed within the phospholipid classes of differentiated and undifferentiated cells.

2.4 Stimulation of fatty acid mobilization

[^{3}H]Arachidonate labeled U937 cells were resuspended in

PBS/glucose/albumin (Section 2.3). Aliquots containing 1 x 10^6 cells, were pre-incubated for 10 minutes at 37°C in 0.8 ml PBS/glucose/albumin with or without PMA (10 nM). Where indicated, soluble diglycerides, EGTA, or inhibitors (NDGA, PGBx, econazole, SKF96365, Ni^{2+}) were added during this initial pre-incubation period. PBS/glucose/albumin containing A23187, thapsigargin, fatty acids, or receptor-mediated agonists were then added in a volume of 0.2 ml, and the cells were incubated for an additional time period (2 - 10 minutes as indicated in Figure legends). The final assay volume was 1.0 ml in all cases. Controls consisted of cells treated with PBS ± PMA (and/or inhibitors); but without further stimulation. Reactions were stopped by rapid centrifugation in a microfuge at 1600 x g for two minutes. Aliquots of the supernatants were removed and radioactivity was quantitated by liquid scintillation. Release of radiolabel was expressed as a percentage of the total incorporated cellular radioactivity.

Release of phospholipid into the medium can indicate cell damage during the incubation period. To monitor this, incubation supernatants from selected experiments were extracted according to the method of Bligh & Dyer (69). Aliquots of the lipid extracts were chromatographed against authentic lipid standards in the solvent system petroleum ether/diethyl ether/glacial acetic acid (82:18:1). Results confirmed that the agonist-stimulated release of radiolabel was in the free fatty acid form (>95%).

Cell viability after the incubation period was monitored by trypan blue exclusion. Cell viability was greater than 98% under all incubation conditions, except A23187 doses in excess of 5 μM. Long term survival rates were assessed by washing agonist treated cells twice in PBS/glucose/albumin, resuspending the cells in growth

medium, and determining cell number and trypan blue viability 1 hour post treatment.

For experiments in which arachidonate release was measured in Ca^{2+} free medium, or in the presence of extracellular strontium, radiolabeled cells were washed twice in Ca^{2+} free PBS, and resuspended in the same. All reagents used were also prepared in Ca^{2+} free PBS. Strontium or calcium (1.1 mM) was added just prior to the initial ten minute pre-incubation.

2.5 Time course of differentiation

For these experiments, cells were differentiated with 1.5% DMSO as described above. At 12 hour intervals, replicate flasks were assayed for agonist-stimulated arachidonate mobilization. For all time points assayed, [^{3}H]arachidonate was added to the culture medium 24 hours prior to assay, except for the 12 hour time point, which was radiolabeled 12 hours prior to agonist stimulation. The zero time point utilized radiolabeled cells at a density of 0.2 x 10^6/ml (initial differentiation density) that had not been treated with DMSO. Cell viability, growth, and NBT reduction were assessed at each time point.

2.6 Time course of A23187-stimulated arachidonate release

Cells were radiolabeled and stimulated as described above. Reactions were stopped by addition of methanol at various time intervals after thapsigargin, ATP, or fMLP stimulation. Controls consisted of radiolabeled cells incubated for similar time intervals, without agonist stimulation. After addition of methanol, phosphatidylcholine

(10 μg in 2 μl) and cold arachidonate (5μg in 2 μl) were added as carrier to minimize loss of lipids during extraction. The combined cells and incubation medium were extracted, concentrated under nitrogen, and stored at -20°C. Extracts were chromatographed using the solvent system petroleum ether/diethyl ether/glacial acetic acid (82:18:1). Phospholipids; mono-, di-, and triacylglycerols, and free arachidonate, were identified by co-chromatography of authentic lipid standards. Results were quantitated using a Bioscan radioactivity scanner. No changes in radioactivity associated with mono or diglyceride fractions were observed during the time course of thapsigargin stimulation.

Comparison of arachidonate release measured using methanol to stop agonist-stimulated reactions, with those obtained by stopping the reaction with centrifugation, confirmed that both methods produced similar results for incubation times $\geq$ two minutes. However, arachidonate release at earlier time points could not be accurately measured using the centrifugation method.

Released arachidonate can be re-incorporated into cellular triglycerides (9, 70) following agonist stimulation. Re-incorporation can therefore result in an underestimation of actual agonist-stimulated arachidonate release. Thus, re-incorporation was measured by determining radiolabel present in the triglyceride fraction. Re-incorporation of arachidonate, as evidenced by increased radioactivity in the triacylglycerol fraction, was minimal (less than 1.0% of radiolabeled cellular lipids) from 0 to 3 minutes post thapsigargin.

2.7 Analysis of P450 arachidonate metabolites: thin layer chromatography.

For analysis of cytochrome P450 arachidonate metabolites, 2 x 10^6 radiolabeled

cells were pre-incubated for ten minutes at 37^{0}C in 1.6 ml PBS/glucose/albumin, with or without PMA (10 nM). PBS/glucose/albumin containing 1 μM thapsigargin was then added in a volume of 0.4 ml. The final incubation volume was 2.0 ml. An increased cell number is utilized for these experiments to assure adequate detection of small amounts of arachidonate metabolites. Reactions were stopped by addition of methanol, and were extracted and concentrated under nitrogen as previously described. Extracts were thin layer chromatographed using the solvent system ethyl acetate/iso-octane/acetic acid/water (110:50:20:100) (71). The solvent system was prepared in a separatory funnel, mixed well, and allowed to stand until phases separated (approx. 5 minutes). The organic phase was used for thin layer chromatography. Prostaglandins, leukotrienes, free arachidonate, and P450 arachidonate metabolites were identified by co-chromatography of authentic lipid standards. Results were quantitated using a Bioscan radioactivity scanner. Further confirmation of the identity of arachidonate metabolites was obtained using indomethacin to inhibit the cyclooxygenase pathway as described in Section 5.2.3. Any metabolites formed that were not inhibited by indomethacin would be either cytochrome P450 or lipoxygenase metabolites.

2.8 Analysis of P450 arachidonate metabolites: HPLC

For HPLC analysis of agonist-stimulated arachidonate metabolites, radiolabeled cells were incubated in a final volume of 2.0 ml, (± PMA, ± indomethacin) as described in section 2.7. Reactions were stopped by rapid centrifugation. The resultant supernatants were removed and acidified to pH 4.0 with 20 μl 10% formic acid, and extracted twice with two volumes of ethyl acetate. Extracts were concentrated under nitrogen, and

stored at -20°C. For HPLC, extracts were dried under nitrogen and resuspended in 0.5 ml of methanol/water (70/30). Arachidonate metabolites were separated by HPLC (Beckman Instruments Inc., Fullerton, CA) on a reversed phase C-18 column (Beckman ODS, 4.6 mm x 250 mm) with a flow rate of 1 ml/minute. After a five minute elution of the column with methanol/water/phosphoric acid (550/450/2 pH 5.7) the methanol composition was increased from 55 to 100% over 35 minutes. Lipoxygenase metabolites were detected by in line UV absorption at 270 nm (235 nm for 5-HETE). For detection of prostaglandins, P450 arachidonate metabolites, lipoxygenase metabolites, and free arachidonate, column effluent fractions were collected at 1 minute intervals. Radioactivity in the fractions was determined by liquid scintillation. Radiolabeled arachidonate metabolites were identified by their reported retention times (72).

2.9 Intracellular Free Calcium

Differentiated or undifferentiated cells were centrifuged and resuspended in PBS/albumin/glucose at a density of 2 x 10^6 cells/ml. Cells were loaded with Fura-2/AM (5 μM) at 37°C for 1 hour. After loading, cells were then centrifuged to remove unincorporated Fura-2/AM, and resuspended in PBS/glucose/albumin with or without calcium as indicated. Aliquots of cell suspension (500 μl) were added to the spectrofluorometer cuvette, maintained at 37°C with stirring, and measurements were initiated. Cells were stimulated with DMSO solutions (2.5-5 μl) of A23187 or thapsigargin. Addition of this volume of DMSO to Fura-2 loaded cells did not affect $[Ca^{2+}]_i$. Although A23187 is reported to have intrinsic fluorescence, A23187 ($\leq$ 2 μM) did not contribute to background fluorescence under the conditions utilized in these experiments.

$[Ca^{2+}]_i$ was measured in a SPEX ARCM spectrofluorometer. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz (73):

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

Briefly, autofluorescence was determined using aliquots of unloaded cells, and was subtracted from all measurements. The Fura-2 signal was calibrated in loaded cells by addition of 0.1% (w/v) digitonin (to obtain the fluorescence with maximum calcium at 340 and 380 nm;), followed by 10 mM EGTA (to obtain the fluorescence with minimum calcium at 340 and 380). R_{max} represents the ratio of Fura-2 fluorescence at 340/380 nm in medium containing saturating concentrations of calcium (+ digitonin). R_{min} represents the ratio of Fura-2 fluorescence at 340/380 nm in calcium free medium (+EGTA). S_{f2} and S_{b2} represent the fluorescence ratios of Fura-2 obtained at 380 nm in the absence and presence of saturating levels of calcium, respectively. For a given sample, computer driven measurements were made every 0.1 second, and were averaged to obtain a data point at 1 second intervals. For each data point the fluorescence values obtained following excitation at 340 nm were divided by those obtained at 380 nm giving a corrected ratio (R). $[Ca^{2+}]_i$ was then calculated by a computer program designed for the spectrofluorometer, using the equation given above. The K_d for the Fura-2/calcium complex was assumed to be 224 nM at 37°C. The final result was an experimental trace of $[Ca^{2+}]_i$ versus time.

For PMA pre-incubations, aliquots of Fura-2 loaded cells were incubated with 10 nM PMA for 10 minutes at 37°C. Where indicated, EGTA (1 mM), NDGA (20-40 μM), PGBx (10-50 μg/ml), econazole (2μM), and SKF96365 (1 μM) were added 2 minutes prior to thapsigargin stimulation.

For experiments in which strontium was used as a surrogate for calcium, cells were loaded with Fura-2 as described above. After Fura-2 loading, cells were washed twice in calcium free PBS, and resuspended in the same. Sr^{2+} or Ca^{2+} (1 mM, 2.5 μl) were added to cells immediately prior to addition of thapsigargin. Intracellular Sr^{2+} was quantitated as described above, using a dissociation constant of 510 nM for the Fura-2/Sr^{2+} complex (74-76).

To examine the effects of free fatty acids (arachidonic, linoleic, oleic) on $[Ca^{2+}]_i$, cells were loaded as described above, washed, and resuspended in PBS/glucose with or without albumin as indicated in figures. Stock solutions of fatty acids were prepared in ethanol. Working dilutions of the free fatty acids were prepared daily in PBS by sonication of an aliquot of the concentrated stock. Concentrations of ethanol in experimental measurements did not exceed 0.1%.

To examine receptor-mediated events, ATP and the chemotactic peptide N-formyl-methionyl-L-leucyl-L-phenylalanine (fMLP), were added to 0.5 ml of cell suspension in the cuvette using a volume of 2.5 - 5.0 μl. $[Ca^{2+}]_i$ was measured as described above.

2.10 Statistics

Data points, except where indicated in figure legends, represent the mean ±

standard error of the mean (SE) for a minimum of 3 experiments performed in duplicate.

For most data points, the actual number of individual experiments averaged to obtain the mean exceeds 3, and this is indicated in figure legends. Statistical significance was determined by Student's T test. A probability of 0.05 or less was considered significant.

CHAPTER 3

EFFECTS OF DMSO-INDUCED DIFFERENTIATION ON ARACHIDONATE MOBILIZATION IN THE HUMAN HISTIOCYTIC LYMPHOMA CELL LINE U937: RESPONSIVENESS TO SUB-MICROMOLAR CALCIUM IONOPHORE A23187 AND PHORBOL ESTERS.

3.1 Background

Although $cPLA_2$ has been extensively characterized *in vitro* (22), very little data is available regarding *in situ* regulation. Initial studies were designed to establish a model cellular system in which $cPLA_2$ could be effectively studied. The human histiocytic lymphoma cell line U937, is a rich source for $cPLA_2$ and has been a standard cell line for purification of the human enzyme (27-29, 77, 78). We therefore chose to investigate arachidonate release in U937 cells, as a potential model system for studying regulation of $cPLA_2$.

U937 is a transformed cell line of the myeloid lineage, considered to represent a pre-macrophage. The development of U937 cells has been arrested late in hematopoetic development, after the commitment to neutrophil or macrophage has been made (66,

68). Differentiation of U937 with DMSO and other agents induces morphological and functional maturation into a macrophage-like cell. Differentiated cells display growth inhibition, alterations in surface glycoproteins, reduction of NBT, development of the chemotactic response, and phagocytosis (68). However, reports indicate that the intrinsically high levels of $cPLA_2$ in U937 increase only slightly with differentiation (32, 77-80).

In order to identify U937 as a model cellular system for *in situ* study of $cPLA_2$, one must first demonstrate arachidonate mobilization in response to agonist stimulation. Secondly, arachidonate mobilization should be consistent with the characteristics reported for $cPLA_2$ *in vitro* (responsiveness to low calcium and specificity for arachidonate). The results presented in this Chapter represent experiments designed to define such a system in U937. These initial experiments utilized the calcium ionophore A23187 as an agonist for $cPLA_2$ activation. We reasoned that use of A23187 to directly elevate $[Ca^{2+}]_i$, would activate $cPLA_2$ independent of any complications related to receptor-mediated events. Unexpectedly, our observations indicate that, although undifferentiated U937 cells contain considerable amounts of functional $cPLA_2$, arachidonate release is only weakly stimulated by the calcium ionophore A23187. Dimethylsulfoxide (DMSO) differentiation of U937 dramatically enhances A23187 stimulatable arachidonate mobilization. Furthermore, the characteristics of this response are consistent with those reported for $cPLA_2$ *in vitro*. Our observations suggest that differentiation may induce $cPLA_2$ regulatory elements which are inactive in undifferentiated cells.

3.2 Results

3.2.1 Effects of calcium ionophore A23187 on arachidonate release in differentiated and undifferentiated U937 cells.

Our initial investigations examined the calcium ionophore A23187-stimulated release of [^{3}H]arachidonate from undifferentiated U937 cells. A23187 transports calcium across biological membranes, down its concentration gradient (81). Exposure to A23187 thereby results in an elevation of $[Ca^{2+}]_i$, independent of plasma membrane-receptor mediated events. Since $cPLA_2$ is activated by elevations in $[Ca^{2+}]_i$, we reasoned that A23187 should activate arachidonate mobilization in undifferentiated U937. However, in contrast to the high levels of extractable enzyme in U937 cells, A23187 stimulatable arachidonate mobilization in undifferentiated cells was low and only weakly dose-dependent on A23187 (Figure 2). A23187 stimulated release of incorporated [^{3}H]arachidonate was only 1 - 2 % above basal (unstimulated) release of radiolabel at A23187 concentrations below 2.5 μM.

DMSO differentiation of U937 cells induced a dramatic alteration in A23187-stimulated arachidonate release (Figure 2). Differentiated cells exhibited a 2-4 fold increase in release of arachidonate in response to A23187 as compared to undifferentiated cells. Differentiated cells also exhibited an increased sensitivity to concentrations of A23187 in the sub-micromolar range. Furthermore, the dose-response curve for differentiated cells was biphasic, with an initial rapid increase over the A23187 concentration range 0.05-0.3 μM. This initial increase was followed by a

FIGURE 2.

EFFECTS OF DMSO-INDUCED DIFFERENTIATION ON A23187-STIMULATED [^{3}H]ARACHIDONATE RELEASE IN U937 CELLS.

U937 cells were cultured and differentiated with DMSO as described in Methods section 2.2. Cells were labeled with [^{3}H]arachidonate during the last 24 hours as described in 2.3. Radiolabeled cells were washed and resuspended in PBS/glucose/albumin. Arachidonate release was stimulated with calcium ionophore A23187 as described in Methods section 2.4. Results represent mean $\pm$ SE of 5 separate experiments performed in duplicate.

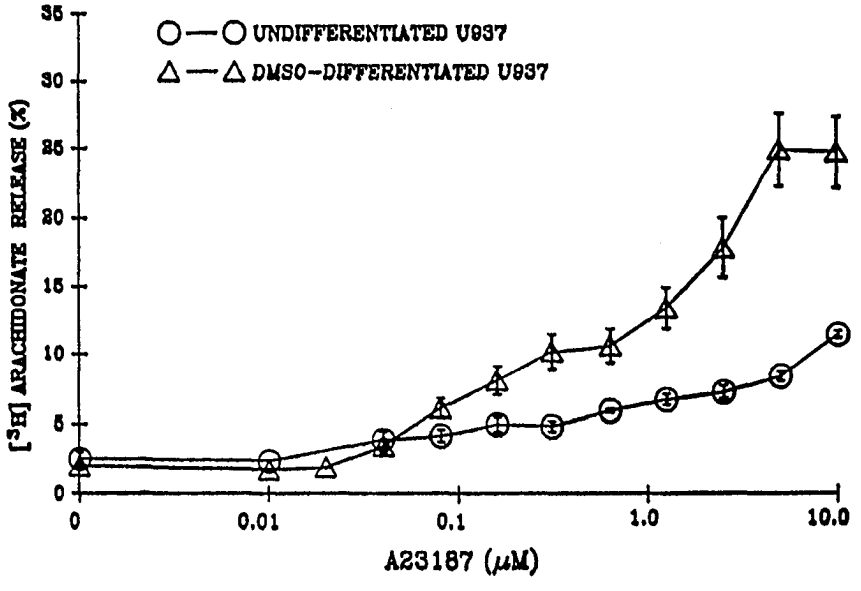


FIGURE 2

plateau, during which arachidonate release remained at the same level of activity (0.3-0.8 μM A23187). A second increase occurred over the concentration range 1.25 - 10 μM; during which release of [^{3}H]arachidonate approached 15%.

Cell viability at all A23187 concentrations tested was greater than 99%, except at 10 μM. A 3-4 % initial loss of viability was observed at this A23187 dose. However, the 1 hour survival rate of cells exposed to 10 μM ionophore, washed, and returned to incubation medium was only 60-70%. Furthermore, considerable amounts of radiolabeled phospholipid were released during incubations with 5-10 μM A23187 (30-40% of the released radioactivity was phospholipid at 10 μM A23187, data not shown). Thus, the response to A23187 concentrations of 5 μM and above are markedly different from that observed with A23187 concentrations less than 2.5 μM, where >95% of the released radioactivity was free arachidonate (data not shown). The increased release of phospholipid, combined with the decrease in viability observed, may indicate cytotoxic effects of A23187 at concentrations above 5 μM. Similar effects have been reported in other cell lines (82, 83). Subsequent experiments utilized ionophore concentrations of 2.5 μM or less.

3.2.2 Effects of PMA on A23187-stimulated arachidonate release

PMA has been shown to enhance agonist-stimulated arachidonate mobilization in several cell types (33, 47). This synergism may be due, in part, to PMA induced activation of protein kinase C (PKC). Activation of PKC results in phosphorylation of $cPLA_2$, thereby enhancing enzymatic activity (47, 53, 57). We therefore examined the effects of PMA on A23187-stimulated arachidonate mobilization in U937 cells.

Differentiated and undifferentiated U937 cells were incubated with PMA concentrations ranging from 1 to 100 nM and for incubation times from 10 minutes to 3 hours. No significant stimulation of arachidonate mobilization was observed in either differentiated or undifferentiated U937 cells exposed to PMA alone (results not shown). This suggests that activation of PKC without a subsequent calcium signal (such as that provided by A23187) is an insufficient stimulus for arachidonate mobilization in U937 cells.

In undifferentiated cells, a ten minute pre-incubation with PMA (10 nM) followed by A23187 challenge had no significant effect on A23187-stimulated arachidonate mobilization (Figure 3). Pre-incubation with higher concentrations of PMA (25-100 nM) were without additional effects. However, in differentiated cells, the priming effect of PMA on A23187 stimulated arachidonate release was quite pronounced (Figure 4). The PMA + A23187 dose-response retained the biphasic characteristics observed with A23187 alone, and PMA pre-incubation further potentiated this response by 4 to 6 fold. The most dramatic increases in arachidonate mobilization were evident at sub-micromolar to micromolar concentrations of A23187 (0.04 - 1.25 μM). For example, PMA + A23187 (0.04 μM) resulted in a 14 % release of incorporated label, while 0.04 μM A23187 alone resulted in only a 3% release. The inactive isomer of PMA, 4 α phorbol myristate acetate, was without effect in either differentiated of undifferentiated cells (data not shown).

FIGURE 3.

EFFECTS OF PMA ON A23187-STIMULATED [^{3}H]ARACHIDONATE MOBILIZATION IN UNDIFFERENTIATED U937 CELLS.

Undifferentiated cells were cultured and radiolabeled as described in Methods (2.2-2.3). Radiolabeled cells were resuspended in PBS/glucose/albumin and pre-incubated with 10 nM PMA for ten minutes prior to A23187 challenge, as described in section 2.4. Results represent mean $\pm$ SE of 3 separate experiments performed in duplicate.

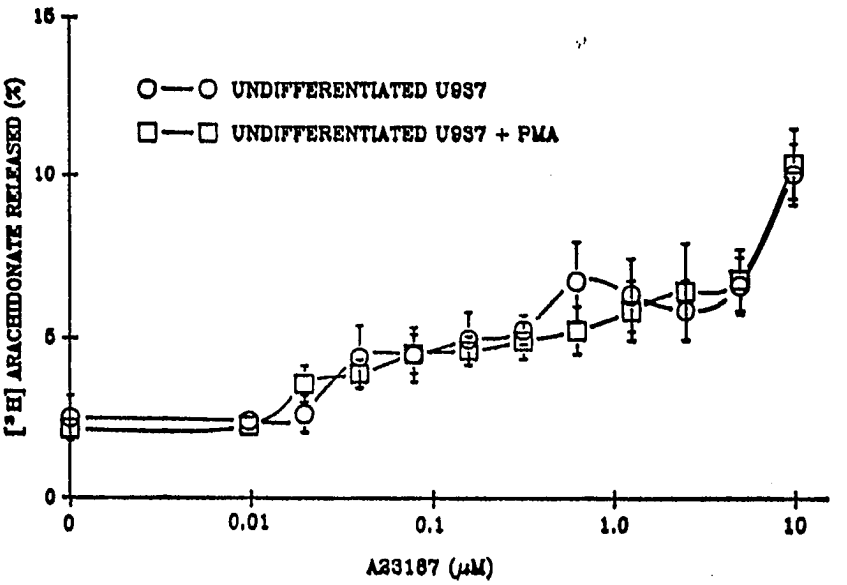


FIGURE 3

FIGURE 4.

EFFECTS OF PMA ON A23187-STIMULATED [^{3}H]ARACHIDONATE MOBILIZATION IN DIFFERENTIATED U937 CELLS.

Differentiated cells were prepared and radiolabeled as described in Methods (2.2-2.3). Radiolabeled cells were resuspended in PBS/glucose/albumin and pre-incubated with 10 nM PMA for ten minutes prior to A23187 challenge, as described in section 2.4. Results represent mean $\pm$ SE of 5 separate experiments performed in duplicate.

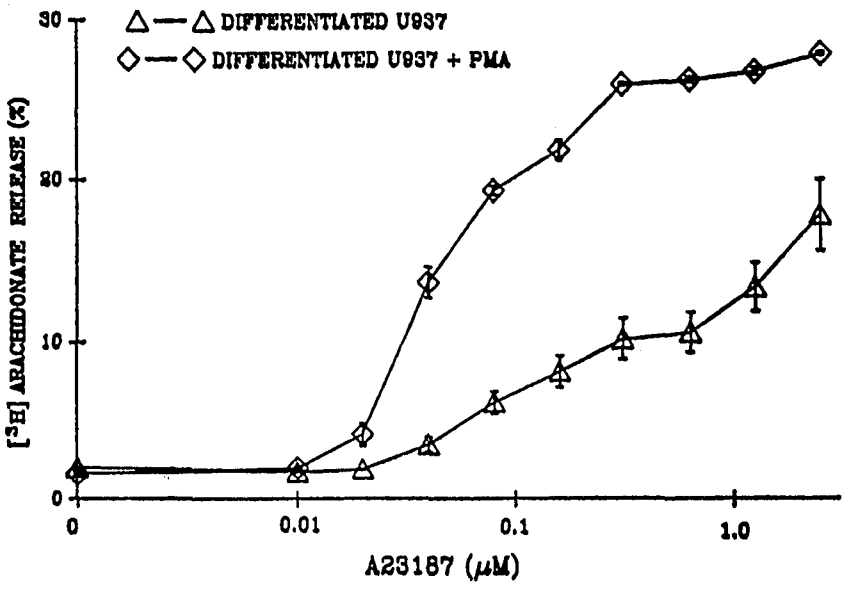


FIGURE 4

3.2.3 Specificity of fatty acid release

$cPLA_2$ purified from U937 as well as other cell types exhibits a specificity for phospholipids containing arachidonate in the *sn*-2 position (22). It was therefore of interest to examine the specificity of A23187-stimulated arachidonate release in differentiated U937 cells. For these experiments, differentiated and undifferentiated cells were radiolabeled with [^{3}H]oleate or [^{3}H]arachidonate for 24 hours. Cells were pre-incubated with PMA and subsequently challenged with increasing concentrations of A23187. In undifferentiated cells, basal (unstimulated) release of both oleate and arachidonate were low (3-4% of incorporated radiolabel, Figure 5). Pre-incubation of undifferentiated cells with PMA, followed by A23187 challenge, did not significantly increase A23187-stimulated release of either fatty acid (Figure 5). Thus, A23187-stimulated fatty acid release in undifferentiated cells was not specific for arachidonate.

In differentiated cells, basal (unstimulated) release of oleate was low (2-3%) and only slightly higher than that of arachidonate. Concentrations of A23187 up to 1.25 μM did not stimulate release of oleate above basal levels (Figure 6). Consistent with activation of $cPLA_2$, the PMA + A23187 stimulated release of free fatty acids in differentiated U937 cells is specific for arachidonate.

3.2.4 Effects of PMA and soluble diglycerides on A23187-stimulated arachidonate release

Diacylglycerols are physiological activators of PKC (50). In the neutrophil, soluble diacylglycerol analogs such as 1,2-dioctanoylglycerol (diC8) and

FIGURE 5.

SPECIFICITY OF PMA + A23187 STIMULATED [^{3}H]ARACHIDONATE RELEASE IN UNDIFFERENTIATED U937.

Cells were cultured and radiolabeled with either [^{3}H]arachidonate or [^{3}H]oleate as described in Methods (2.3). Labeled cells were resuspended in PBS/albumin/glucose and pre-incubated with 10 nM PMA for 10 minutes, followed by A23187 challenge (Methods 2.4). Results shown represent mean $\pm$ SE of 3 separate experiments performed in duplicate.

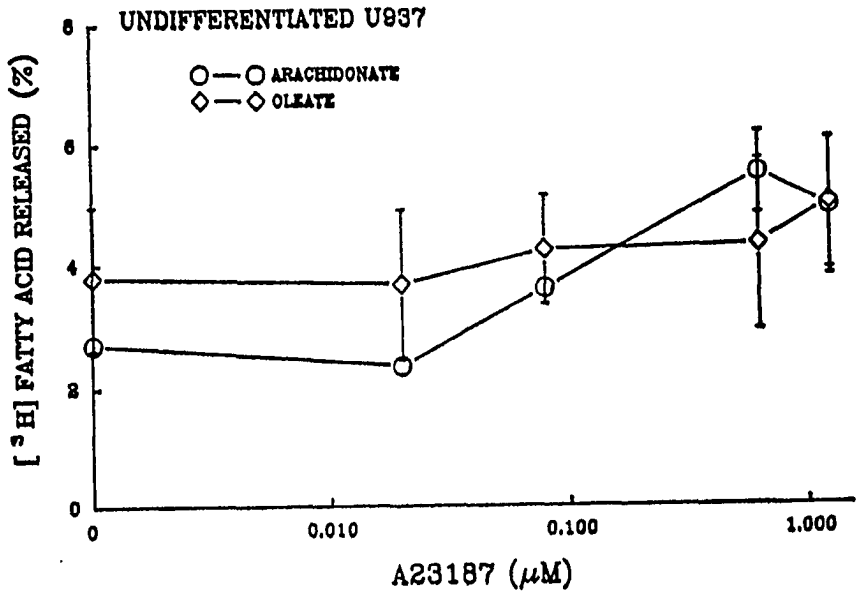


FIGURE 5

FIGURE 6.

SPECIFICITY OF PMA + A23187 STIMULATED [^{3}H]ARACHIDONATE RELEASE IN DIFFERENTIATED U937.

Cells were differentiated and radiolabeled with either [^{3}H]arachidonate or [^{3}H]oleate as described in Methods (2.3). Labeled cells were resuspended in PBS/albumin/glucose and pre-incubated with 10 nM PMA for 10 minutes, followed by A23187 challenge (Methods 2.4). Results shown represent mean $\pm$ SE of 3 separate experiments performed in duplicate. Error bars are too small to print.

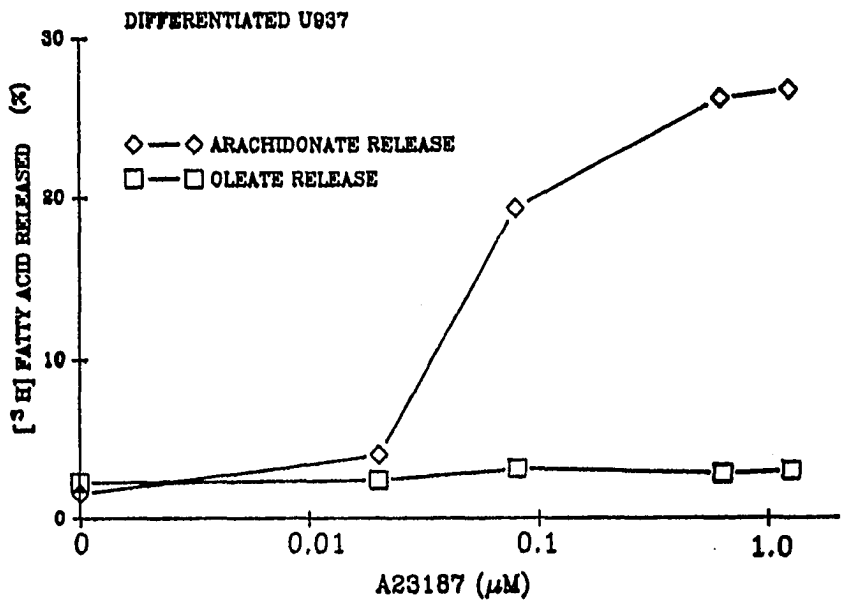


FIGURE 6

oleoylacetylglycerol (OAG) are equipotent with PMA in enhancement of A23187-stimulated arachidonate release (49). Therefore, we investigated the effects of these soluble diglycerides on arachidonate mobilization in differentiated U937 cells. Unlike results observed in the neutrophil, micromolar concentrations of the soluble diglycerides produced only a minor (10-20%) increase in A23187 stimulated arachidonate release in differentiated U937 cells (Figure 7A). In contrast, PMA potentiation of A23187-stimulated arachidonate release was dose-dependent, and maximal effects were observed at a relatively low (10 nM) concentration (Figure 7B). Thus, neither soluble diglyceride was as effective as PMA in potentiation of A23187-stimulated arachidonate mobilization in differentiated U937. It is possible that U937 cells lack the PKC-independent pathway by which soluble diglycerides prime arachidonate release in the neutrophil (84). However, diacylglycerols can be hydrolyzed by various esterases to yield glycerol and free fatty acids (51). Differentiated U937 contains high levels of non-specific esterases (68) which may decrease the efficacy of diacylglycerols in these cells.

3.2.5 A23187-stimulated arachidonate release during the time course of differentiation.

Our observations indicated that DMSO-differentiation resulted in a marked enhancement in both A23187 and PMA+A23187-stimulated [^{3}H]arachidonate mobilization. We therefore sought to examine A23187 stimulated arachidonate mobilization during the temporal development of monocytic differentiation.

The normal doubling time for U937 cells in our laboratory is approximately 24 hours. As can be seen from Figure 8A, DMSO treatment significantly decreased this

FIGURE 7.

EFFECTS OF PMA AND SOLUBLE DIGLYCERIDES ON A23187 STIMULATED [^{3}H]ARACHIDONATE RELEASE IN DIFFERENTIATED U937 CELLS.

In figure 7A, differentiated cells were pre-incubated for 10 minutes in PBS/albumin/glucose containing either OAG or diC8, at various concentrations as indicated in the figure. A23187 (1.25μM) was then added and the cells were incubated an additional 10 minutes (Methods 2.4). Results represent mean ± SD of 3 separate experiments. In figure 7B, differentiated cells were pre-incubated with increasing concentration of PMA for 10 minutes, followed by A23187 (1.25 μM) challenge as described above.

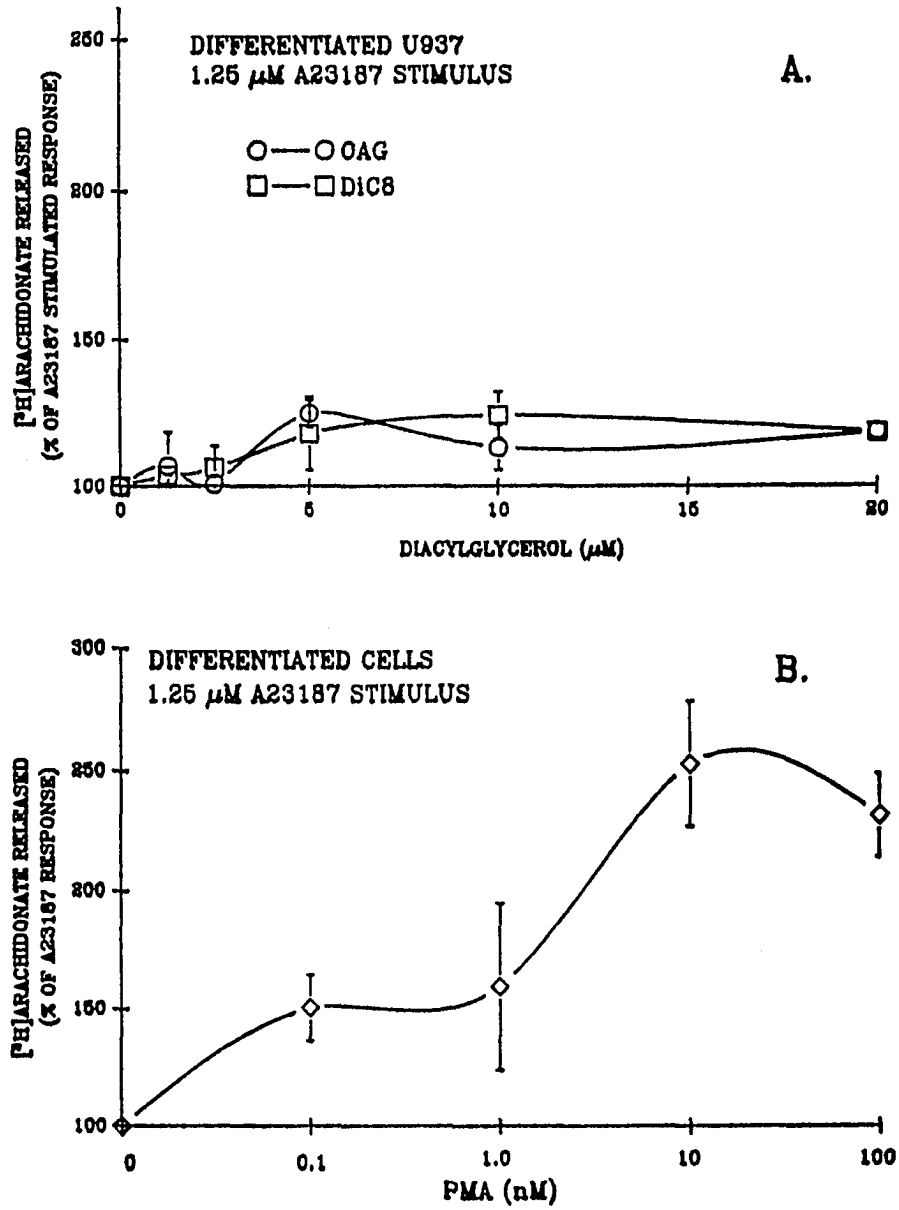


FIGURE 7

FIGURE 8.

TIME COURSE OF DMSO DIFFERENTIATION: CELL GROWTH AND NBT REDUCTION.

Cells were differentiated at an initial density of 0.2 x 10^6 cells/ml as described in Methods 2.5. At 12 hour intervals aliquots of the cultures were removed and cell density (Figure 8A) and NBT reduction (Figure 8B) were determined as described in Methods 2.2. Results shown are representative of 3 separate experiments.

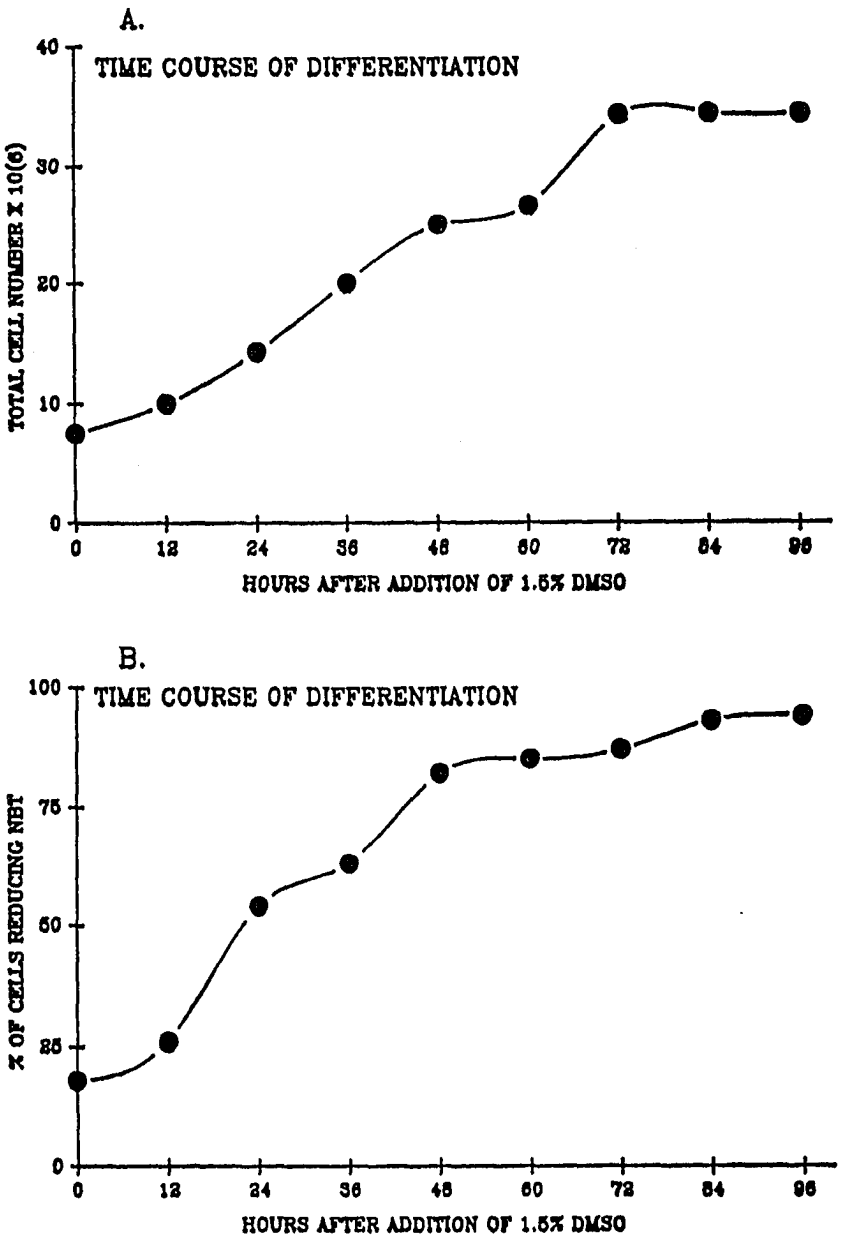


FIGURE 8

growth rate. However, complete growth inhibition did not occur until 72 hours post DMSO. Cell viability at all time points analyzed was greater than 99%.

The capacity to reduce and phagocytose NBT was used as a marker for monocytic differentiation of U937 cells (67, 68). During the first 48 hours of differentiation, the percentage of cells reducing NBT increased in a linear manner, with 80% of the cells reducing NBT by this time (Figure 8B). Maximum development of NBT reduction did not occur until 72 - 84 hours post DMSO, at which point 90% of the cells were positive for NBT reduction. Thus, the development of the capacity to reduce NBT correlates with the onset of growth inhibition in differentiating U937 cells.

During the course of DMSO-induced differentiation, development of the A23187 response occurred at two distinct phases (Figure 9). Enhanced arachidonate release in response to 1.25 μM A23187 challenge was observed within 12 hours of DMSO exposure. Arachidonate release increased linearly until 36 hours post DMSO, and remained at this elevated level through 84 hours post DMSO. Between 84 and 96 hours post DMSO, a second sharp increase in A23187-stimulated arachidonate mobilization was observed. This enhanced level of responsiveness was maintained through 144 hours post DMSO (data not shown). Therefore, the onset of maximal arachidonate mobilization in response to A23187 challenge correlates with both onset of growth inhibition and development of the capacity to reduce NBT.

In contrast, the ability of PMA to potentiate the A23187 response developed early in the differentiation time course (Figure 9). Maximal PMA effects were attained by 36 hours after initial DMSO treatment. PMA primed the 1.25 μM A23187 stimulated arachidonate release by 2-3 fold during this early phase, and maintained a

FIGURE 9.

TIME COURSE OF DMSO DIFFERENTIATION OF U937 CELLS: ARACHIDONATE MOBILIZATION.

U937 cells were differentiated and radiolabeled with [^{3}H]arachidonate as described in Methods 2.5. At 12 hour intervals after addition of DMSO, cells were evaluated for A23187 (1.25 μM) or PMA + A23187-stimulatable arachidonate mobilization, as described in Methods 2.4. Results shown are representative of 3 separate experiments.

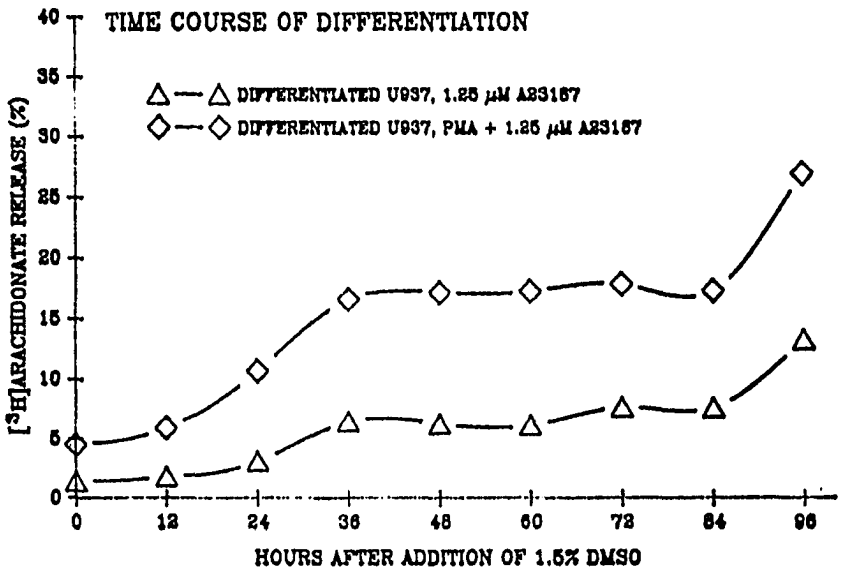


FIGURE 9

steady 1.5-2 fold enhancement throughout the time course of differentiation. Therefore, the second phase of increased arachidonate release observed at 96 hours appears to be primarily due to increases in the A23187 response rather than an increase in PMA potentiation.

3.3 DISCUSSION

Quite unexpectedly, our results indicate that undifferentiated U937 cells are only weakly stimulated to release arachidonate in response to the calcium ionophore A23187. Furthermore, A23187-stimulated release of fatty acids in undifferentiated U937 cells is not specific for arachidonate (as compared to oleate). In contrast to our *in situ* observations, $cPLA_2$ activity in high speed supernatants from undifferentiated whole cell homogenates is quite high (27, 28, 29). Recent reports indicate that only modest (less than 10-20%) increases in $cPLA_2$ protein are observed with DMSO differentiation (32, 77-80). Furthermore, purified enzyme from undifferentiated U937 cells as well as $cPLA_2$ activity in U937 cell cytosol, are activated *in vitro* by physiological (150-600 nM) calcium (29), indicating that the enzyme is indeed functional and responsive to calcium in undifferentiated cells.

In contrast, DMSO-differentiation of U937 cells results in significant enhancement of A23187 stimulatable arachidonate release. This implies that $cPLA_2$ regulatory elements may be lacking or non-functional in undifferentiated cells. The enhanced capacity to mobilize arachidonate observed in differentiated cells may be due to induction of these regulatory elements.

The characteristics of the A23187 stimulatable mobilization of arachidonate in differentiated U937 cells are consistent with those observed for *in vitro* $cPLA_2$ (22); the enzyme is activated by low levels of calcium and demonstrates a marked preference for arachidonate over oleate. *In vitro* activation of purified $cPLA_2$ (29) and translocation of the enzyme to membrane fractions of cell homogenates (27, 28, 44) is reported to occur at calcium concentrations in the micro to sub-micromolar range. Preliminary calcium measurements indicated that A23187 doses of 0.01-1 μM do, indeed, correlate with intracellular free calcium concentrations within the sub-micro to micromolar range (data presented in Chapter 4). Our results indicate that arachidonate release observed at these low ionophore concentrations appears to be consistent with the physiological intracellular calcium elevations required for signal transduction and $cPLA_2$ activation. In fact, measurement of arachidonate release under conditions of sub-micromolar calcium may tend to rule out a major contribution to arachidonate release from $sPLA_2$ in U937 cells.

In addition to regulation by calcium, the activity of $cPLA_2$ is further enhanced by serine/threonine phosphorylation in response to PMA, thrombin, ATP, and other agonists (31, 33, 42). Pre-incubation of differentiated U937 cells with PMA, followed by A23187 challenge, resulted in a synergistic potentiation of arachidonate mobilization. This "priming" effect of PMA on fatty acid mobilization exhibited a preference for arachidonate over oleate. The most dramatic PMA-induced effects (4-6 fold) were evident in differentiated cells stimulated with low doses of A23187. These observations suggest that PKC is involved in regulation of arachidonate mobilization in U937 cells.

However the precise role of PKC is, at present, unclear. $cPLA_2$ is phosphorylated and activated by a MAP kinase *in vitro* (46, 57). In U937 cells, PMA activated both a MAP kinase and a MAP kinase activator (58). Thus, PMA may potentiate arachidonate mobilization in U937 cells by indirect activation of kinases which lie upstream of effector proteins such as MAP, which phosphorylate $cPLA_2$ directly. However, as PMA alone was without effect in either differentiated or undifferentiated cells, either mode (direct or indirect) of PMA-induced $cPLA_2$ phosphorylation may be insufficient to stimulate arachidonate mobilization in differentiated U937 cells unless combined with an elevation of $[Ca^{2+}]_i$, which brings $cPLA_2$ in contact with its membrane substrate.

Since undifferentiated U937 cells contain substantial $cPLA_2$ activity, our results suggest that the dramatic increases in arachidonate mobilization observed in differentiated cells may be due to induction of $cPLA_2$ regulatory elements. Two such $cPLA_2$ regulatory elements are calcium, which regulates translocation; and phosphorylation, which further enhances enzymatic activity. Although $cPLA_2$ does not require calcium for catalysis (37, 38), it does require calcium for translocation to membrane phospholipid substrates. Thus, differentiation-induced changes in the capacity of intracellular calcium pools or extracellular calcium influx pathways could subsequently effect translocation of $cPLA_2$ and mobilization of arachidonate.

The present results suggest that differentiation may also induce changes in levels of PKC isoforms, or PKC substrates, which in turn regulate activity of $cPLA_2$. PMA pre-incubation does not enhance arachidonate release in undifferentiated cells, suggesting that the necessary phosphorylation mechanism may be lacking. The ability of

PMA to potentiate A23187-stimulated arachidonate mobilization developed during the first 36 hours of differentiation, and remained at this level through 96 hours. In contrast, the dramatic increase in arachidonate release observed after 96 hours differentiation appears to be primarily related to increased A23187-responsiveness. In fact, the early (36 hr) increase in responsiveness to PMA preincubation may also be due to development of the ability to increase $[Ca^{2+}]_i$, thus translocating PKC as well as $cPLA_2$.

In summary, we have demonstrated that DMSO-differentiation of U937 cells induces an enhanced capacity to mobilize arachidonate in response to calcium ionophore A23187 stimulation. The arachidonate mobilizing activity in differentiated U937 cells is consistent with observed characteristics reported for $cPLA_2$ from both differentiated and undifferentiated cells *in vitro*. However, in intact cells, this activity is only weakly detectable prior to DMSO-differentiation. Although both increased calcium and phosphorylation regulate the activity of $cPLA_2$ in vitro, our results suggest that a calcium transient is a necessary prerequisite for *in situ* arachidonate mobilization. A differentiation-induced alteration in intracellular free calcium dynamics may regulate differentiation-induced mobilization of arachidonate in response to A23187 and PMA. Further studies, presented in Chapter 4, were designed to dissect the role of intracellular calcium in mobilization of arachidonate in U937 cells.

CHAPTER 4

ARACHIDONATE MOBILIZATION IS COUPLED TO DEPLETION OF INTRACELLULAR CALCIUM STORES AND INFLUX OF EXTRACELLULAR CALCIUM IN DIFFERENTIATED U937 CELLS.

4.1 Background

U937 cells contain high quantities of $cPLA_2$, and are a standard source for isolation of functional enzyme (27-29). Nonetheless, we have found that undifferentiated U937 cells release little free arachidonate in response to stimulation with A23187. The results presented in Chapter 3 identify an A23187-stimulatable arachidonate mobilizing activity in differentiated U937 cells, consistent with characteristics reported for $cPLA_2$ *in vitro* (sensitivity to low calcium and a preference for arachidonyl-containing phospholipid substrates). Given that undifferentiated cells contain functional $cPLA_2$, the levels of which increase only slightly with differentiation (77-80), we reasoned that the increased A23187 responsiveness observed in differentiated cells must be due to induction of $cPLA_2$ regulatory elements.

Two possible regulatory elements for $cPLA_2$ are intracellular free calcium ($[Ca^{2+}]_i$) levels and phosphorylation. In intact cells, $cPLA_2$ is phosphorylated in response to PMA, zymosan, and purinergic agonists (31, 33, 61). Although phosphorylation further increases the *in vitro* activity of $cPLA_2$, an elevation in $[Ca^{2+}]_i$ appears to be essential in activation of $cPLA_2$ *in situ* (35, 62-64). Our previous work

has shown that PMA alone, which causes phosphorylation of $cPLA_2$ (22, 33, 31) is an insufficient stimulus for arachidonate mobilization in DMSO differentiated U937. Therefore, we reasoned that differentiation-induced alterations in $[Ca^{2+}]_i$ dynamics could control arachidonate mobilization in U937 cells.

In non-electrically excitable cells such as U937, a principal mechanism for elevation of $[Ca^{2+}]_i$ is through the "capacitative" or "second messenger operated" calcium influx pathway described by Putney (85, 86). Activation of this pathway is initiated by receptor-ligand binding, involving either G-protein or tyrosine-kinase linked receptors (87). Receptor-ligand binding is then transduced into activation of phosphoinositide specific phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the formation of the second messengers diacylglycerol (DAG) and inositol 1, 4, 5 trisphosphate (IP_3) (87, 88). According to the capacitative model of Putney (85), IP_3 binds to receptors on intracellular calcium stores, signaling release of calcium from these stores. An initial, rapid elevation of $[Ca^{2+}]_i$ is produced. Depletion of store calcium results in the generation of a diffusible messenger or calcium influx factor (CIF), which directs the opening of "store operated channels" (SOC) or "second messenger operated" (SMOC) calcium channels in the plasma membrane (85, 86, 89). Influx of extracellular calcium through these channels and into the cytoplasmic compartment results in a sustained phase of elevated $[Ca^{2+}]_i$. Therefore, the capacitative signal is biphasic, consisting of two components. The first is an elevation of $[Ca^{2+}]_i$ due to intracellular store depletion. This is followed by a second phase consisting of influx of extracellular calcium, which is regulated by the filling state of intracellular stores.

The precise location of intracellular calcium stores is presently unclear. Subcellular fractionation studies revealed that the location of IP_3-responsive organelles did not correlate with markers for the plasma membrane, endoplasmic reticulum, mitochondria, golgi, or other known organelles (90). In bovine endothelial cells, the IP_3 receptor was immunolocalized to granular-like structures in the perinuclear region of the cells (91). Lew (92) reported that the IP_3 responsive organelles in the neutrophil appeared to be of a unique type, termed the "calciosome", which is considered to be a modified portion of the endoplasmic reticular network (93).

The precise nature of the CIF is also unknown. There are two basic hypotheses regarding the nature of the intracellular signal that results in store-controlled calcium influx. The first involves release of a diffusible second messenger upon depletion of intracellular calcium stores, which results in the opening of plasma membrane calcium influx channels (SOC). The store-controlled generation of a diffusible messenger has recently been demonstrated, and is thought to involve a phosphorylation/dephosphorylation reaction (89, 94). Alternatively, the CIF may represent a more a physical connection between calcium stores and calcium influx channels, in which empty stores induce some form of conformational change which is transmitted to plasma membrane channels by a direct coupling (86). Recent studies implicate involvement of a low molecular weight guanine nucleotide binding protein that may direct transport of vesicles containing calcium influx channels to the plasma membrane (95, 86).

The capacitative calcium influx pathway has been identified in U937, as well as other cell types (87, 96). Several $cPLA_2$ activating ligands, such as thrombin (42), ATP

(13, 33), prostaglandins (41, 43), PDGF (40), and histamine (97) also activate the capacitative pathway. We therefore chose to investigate the role of capacitative calcium entry as a differentiation-induced regulatory mechanism for $cPLA_2$ in U937.

The studies described in the previous chapter utilized the calcium ionophore A23187 to elevate $[Ca^{2+}]_i$ independent of receptor-mediated events. However, A23187 elevates $[Ca^{2+}]_i$ by both transport of extracellular calcium across the plasma membrane into the cytosol and by transport of calcium from intracellular stores into the cytoplasmic compartment (98). This dual action of A23187 makes it difficult, if not impossible, to separate elevations in $[Ca^{2+}]_i$ due to influx calcium from those generated by depletion of intracellular stores. Therefore, many of the experiments in this chapter make use of the pharmacological agent thapsigargin (99). This terpene compound selectively inhibits the Ca^{2+}-ATPase located on IP_3 sensitive intracellular stores. The Ca^{2+}-ATPase of the plasma membrane is not affected. In the absence of agonist stimulation, there is a small but continuous leakage of calcium from the stores to the cytoplasm. The Ca^{2+}-ATPase pumps calcium back into the stores, maintaining their filled state (88). By inhibition of the Ca^{2+}-ATPase, thapsigargin treatment results in depletion of intracellular stores and activation of the capacitative pathway, independent of plasma membrane receptor-mediated events (99).

In the present chapter, we demonstrate that differentiated U937 cells attained higher elevations of $[Ca^{2+}]_i$ in response to both thapsigargin and A23187, consistent with enhancement of capacitative calcium entry. Differentiation induced alterations in the size of intracellular calcium stores, and the characteristics of plasma membrane calcium influx channels. Mobilization of arachidonate in differentiated cells was coupled

to activation of the capacitative pathway. An initial arachidonate mobilizing activity was identified during depletion of intracellular calcium stores, prior to capacitative calcium influx. Results suggest that this initial release of arachidonate may be involved in the generation of a CIF that regulates the opening of plasma membrane calcium influx channels. A second phase of arachidonate mobilization was coupled to influx of extracellular calcium, and resulted in maximal levels of arachidonate release. PMA pre-incubation was without effect on either arachidonate release or $[Ca^{2+}]_i$ elevations resulting from depletion of intracellular calcium stores. However, PMA pre-incubation potentiated influx of extracellular calcium and arachidonate release coupled to this phase of the capacitative pathway. Based on these results, we propose a model for regulation of $cPLA_2$-mediated arachidonate release in differentiated U937.

4.2 Results

4.2.1 Effects of A23187 on intracellular free calcium elevations in differentiated and undifferentiated U937 cells.

Elevations in $[Ca^{2+}]_i$ stimulated by increasing concentrations of A23187 were measured in both differentiated and undifferentiated cells (Figure 10). These results confirm that A23187 concentrations of ≤ 1 μM do, if fact, produce $[Ca^{2+}]_i$ levels within the sub-micro to micromolar range within which $cPLA_2$ in known to translocate *in vitro.*

The results in Figure 10 also indicate that differentiated U937 cells attained significantly higher elevations of $[Ca^{2+}]_i$ in response to A23187 than undifferentiated cells. PMA pre-incubation further increased A23187-stimulated elevations of $[Ca^{2+}]_i$ in

FIGURE 10

EFFECTS OF DMSO DIFFERENTIATION ON A23187-STIMULATED ELEVATION OF $[CA^{2+}]_I$.

Undifferentiated or differentiated U937 cells were loaded with Fura-2 as described in Methods 2.9. Cells were stimulated with A23187 at the concentrations indicated and $[Ca^{2+}]_i$ was determined as described (Methods 2.9). For PMA pre-incubation, aliquots of Fura-2 loaded cells were incubated with 10 nM PMA for 10 minutes prior to measurement of $[Ca^{2+}]_i$. Results represent the mean $\pm$ SE of the maximum elevations of $[Ca^{2+}]_i$ attained in response to the indicated concentration of A23187 (n=8-10)

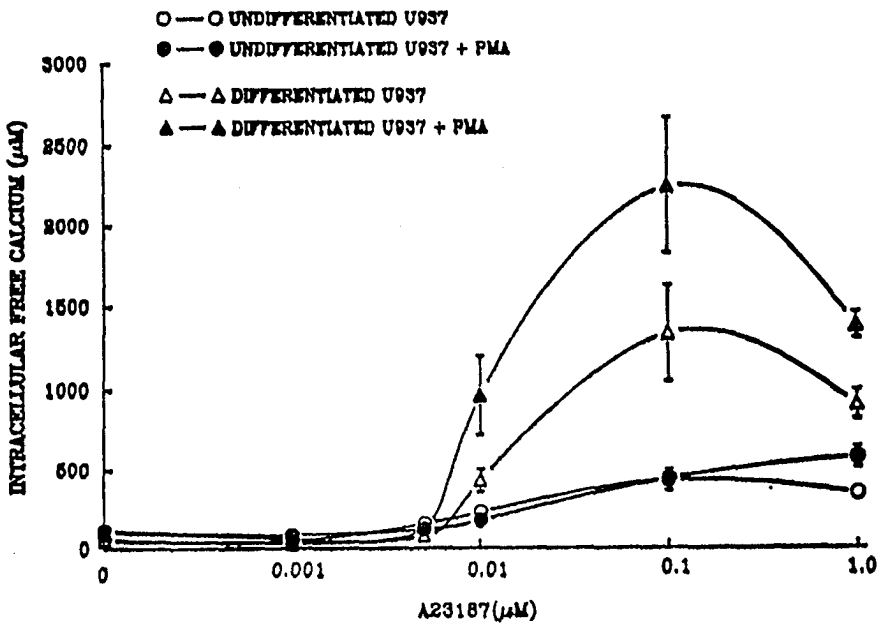


FIGURE 10

differentiated cells by 50 - 100% over the concentration range of 0.01 - 1.0 μM A23187. PMA was without effect in undifferentiated cells. The effects of PMA on A23187-stimulated elevations in $[Ca^{2+}]_i$ were essentially abolished (>95%) by staurosporine (1 μM), suggestive of PKC mediated events (data not shown).

4.2.2 Effects of depletion of intracellular calcium stores on $[Ca^{2+}]_i$ elevations in differentiated and undifferentiated U937 cells.

A23187 elevates $[Ca^{2+}]_i$ by direct transport of calcium across the plasma membrane, as well as across the membranes of intracellular stores, into the cytoplasmic compartment (81). It has been suggested that low doses of ionophore ($\leq$ 1 μM) elevate $[Ca^{2+}]_i$ primarily by depletion of intracellular stores and activation of capacitative calcium influx (100). We therefore sought to examine whether the A23187-stimulated elevations in $[Ca^{2+}]_i$ observed in differentiated U937 were due to increased activation of capacitative calcium entry. For these experiments, cells were challenged with thapsigargin, which results in intracellular calcium store depletion and activation of the capacitative calcium influx pathway (99). Undifferentiated cells responded to 1 μM thapsigargin with a weak elevation of $[Ca^{2+}]_i$, from a basal level of 55 nM to 216 nM (Figure 11). In contrast, differentiated cells exhibited a dramatic 14 to 18 fold increase over basal levels of $[Ca^{2+}]_i$ in response to thapsigargin (0.01-1.0 μM). Thus, the capacitative pathway was, indeed, enhanced by DMSO-differentiation.

A 10 minute pre-incubation of differentiated or undifferentiated cells with 10 nM PMA essentially doubled basal levels of $[Ca^{2+}]_i$ (Figure 11); from 55 to 114 nM in undifferentiated cells, and from 48 to 106 nM in differentiated cells. PMA pre-

FIGURE 11

EFFECTS OF DMSO-DIFFERENTIATION ON THAPSIGARGIN-STIMULATED ELEVATIONS OF $[CA^{2+}]_I$ IN U937 CELLS.

Cells were prepared and $[Ca^{2+}]_i$ was measured as described in Figure 10. The indicated concentrations of thapsigargin were added in a volume of 2.5 µl DMSO. Results represent the mean ± SE of the maximum elevations of $[Ca^{2+}]$ attained in response to the indicated concentration of thapsigargin (n=6-8).

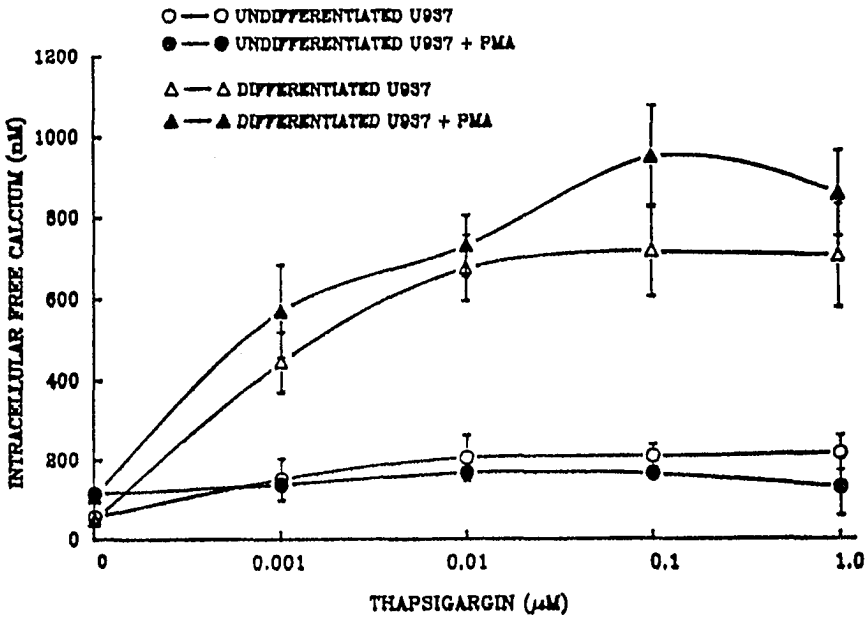


FIGURE 11

incubation weakly inhibited thapsigargin-stimulated elevations of $[Ca^{2+}]_i$ in undifferentiated cells at only at higher thapsigargin concentrations (0.1-1.0 μM). In contrast, PMA pre-incubation potentiated thapsigargin (0.1-1.0 μM)-stimulated elevations of $[Ca^{2+}]_i$ in differentiated cells, but not as dramatically as the response seen with PMA + A23187 (Figure 10).

Although the PMA effect on maximum elevations of $[Ca^{2+}]_i$ was weak, PMA dramatically increased the rate of $[Ca^{2+}]_i$ elevation (Figure 12). Without PMA pre-incubation, differentiated U937 cells attained maximal $[Ca^{2+}]_i$ levels within 4-5 minutes of thapsigargin addition. There was a lag period of approximately 2 minutes after thapsigargin addition, during which elevation of $[Ca^{2+}]_i$ was minimal. This lag period is not unusual for thapsigargin (99). Once the Ca^{2+}-ATPase is inhibited, the intracellular stores are depleted by slow leakage. Thus, the lag period represents the time required for significant store depletion to occur, and capacitative calcium influx to begin (illustrated in Figure 12 by the rapid increase in $[Ca^{2+}]_i$ which begins at approximately 190-200 seconds). However, when differentiated U937 cells were pre-incubated with PMA, the lag period was reduced by 30 - 45 seconds. The effects of PMA were inhibited (>90%) by 1 μM staurosporine (data not shown).

4.2.3 Differentiated and undifferentiated U937 cells have dissimilar capacitative calcium entry pathways.

Further experiments were conducted to dissect the respective roles of the two phases of the $[Ca^{2+}]_i$ elevation associated with capacitative influx, and examine differentiation-induced alterations. The relative size of intracellular free calcium stores in

FIGURE 12

EFFECTS OF PMA ON THAPSIGARGIN-STIMULATED ELEVATIONS OF $[CA^{2+}]_I$ IN DIFFERENTIATED U937 CELLS

Cells were prepared as described in Figure 10. After Fura-2 loading, differentiated cells were incubated with 10 nM PMA for 10 minutes. After pre-incubation, cells were stimulated with thapsigargin (1 μM) and $[Ca^{2+}]_i$ was measured as described in Methods 2.9. The tracing shown is representative of at least 10 similar measurements performed on different days.

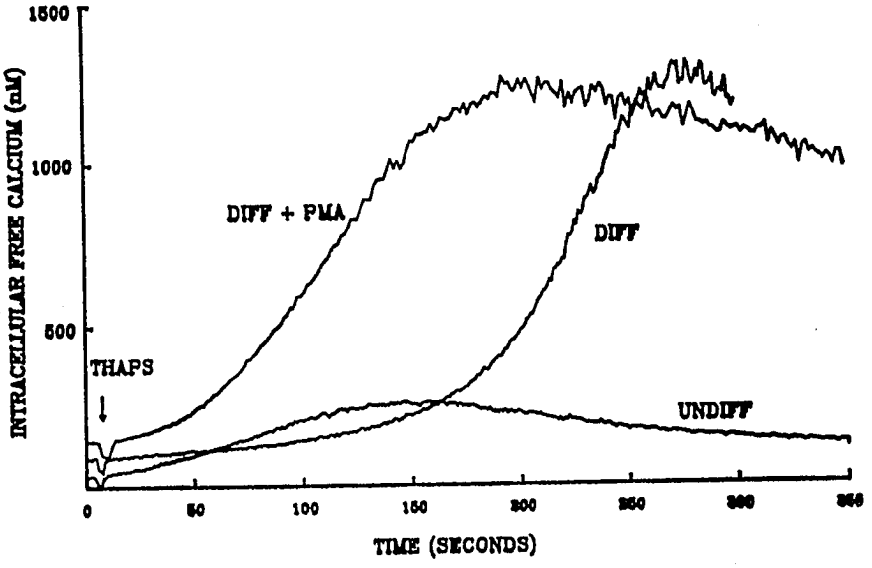


FIGURE 12

differentiated and undifferentiated cells was determined by thapsigargin stimulation in Ca^{2+}-free medium. Under these conditions, any observed increases in $[Ca^{2+}]_i$ could only be generated from emptying of the intracellular stores. Incubation of differentiated cells in calcium free medium inhibited 70-85% of the thapsigargin-stimulated elevations of $[Ca^{2+}]_i$ (Figure 13) However differentiated cells did exhibit a small but significant increase in $[Ca^{2+}]_i$ in response to 1 μM thapsigargin (approximately 2 -2.5 fold over basal levels). In contrast, when undifferentiated cells were incubated in calcium-free medium, 95-98% of the response to 1 μM thapsigargin was inhibited. Similar results were observed when cells were incubated in medium containing Ca^{2+}, and extracellular calcium was chelated by addition of 1.1 mM EGTA (Figure 14). These results indicate that either the intracellular calcium stores, or the filling state of the stores, is altered upon differentiation.

Additional experiments were conducted to characterize capacitative influx of extracellular calcium in differentiated and undifferentiated cells. As the maximal thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ are higher in differentiated cells, we reasoned that differentiation might simply induce more capacitative channels. Quite unexpectedly, the results shown in Figure 14 indicate that the characteristics of the calcium influx channels appear to be altered with differentiation. Econazole (2 μM), an inhibitor of capacitance calcium influx in many cell types (98), did not inhibit thapsigargin stimulated elevations in $[Ca^{2+}]_i$ in undifferentiated cells. In contrast, 2 μM econazole inhibited 44-49% of the thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ in differentiated cells. A second inhibitor of capacitance calcium entry channels, SKF96365 (101) also failed to inhibit thapsigargin induced calcium influx in

FIGURE 13

EFFECTS OF DIFFERENTIATION ON THE SIZE OF INTRACELLULAR CALCIUM STORES.

Differentiated and undifferentiated cells were loaded with Fura-2 as described in Methods 2.9. After loading, cells were washed three times with Ca^{2+}-free PBS, and resuspended in the same buffer. Thapsigargin (1 μM) was added as indicated by the arrow and $[Ca^{2+}]_i$ was measured as described. The tracings shown are representative of at least 5 similar experiments performed on different days.

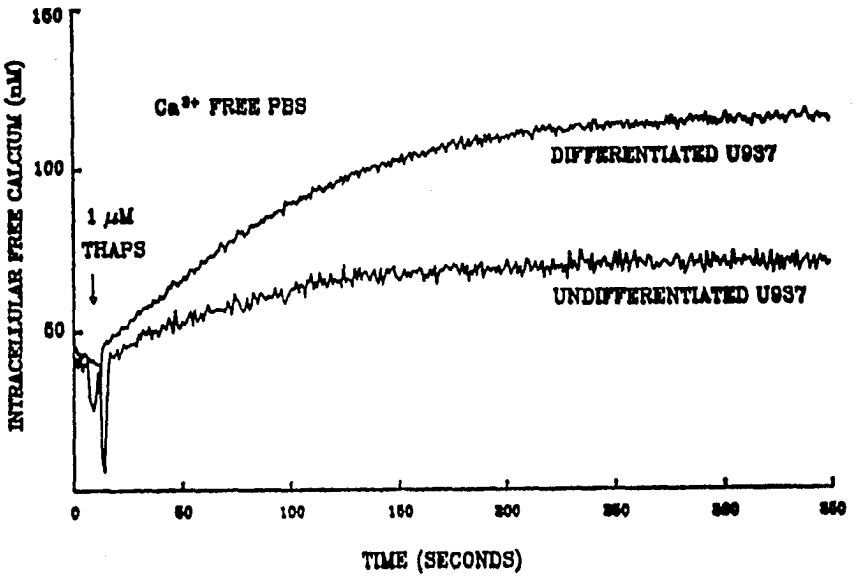


FIGURE 13

FIGURE 14

EFFECTS OF VARIOUS INHIBITORS OF CAPACITATIVE CALCIUM INFLUX ON THAPSIGARGIN-STIMULATED ELEVATIONS OF $[CA^{2+}]_I$ IN UNDIFFERENTIATED (PANEL A) AND DIFFERENTIATED (PANEL B) U937 CELLS.

Differentiated (filled bars) or undifferentiated cells (open bars) were loaded with Fura-2 as described in Methods 2.9. For Ca^{2+} free experiments, cells were prepared as described in Fig 13. Aliquots (0.5 ml) containing 10^6 cells were added to the spectrofluorometer cuvette and measurement of $[Ca^{2+}]_i$ was initiated. EGTA (1 mM), Econazole (2μM) , Ni^{2+}(5 mM), and SKF96365 (1 μM) were added in volumes of 2.5 μl. 1 μM thapsigargin was then added, and $[Ca^{2+}]_i$ was measured for an additional 10 minutes. The maximum elevation in $[Ca^{2+}]_i$ in response to thapsigargin was determined for each treatment. Results were normalized to the maximum $[Ca^{2+}]_i$ level achieved by 1μM thapsigargin (215 ±45 nM in undifferentiated cells; 705 ± 127 nM in differentiated cells) and represent the percentage of the 1 μM thapsigargin response. Data shown represents the mean ± SE of 3-5 separate experiments.

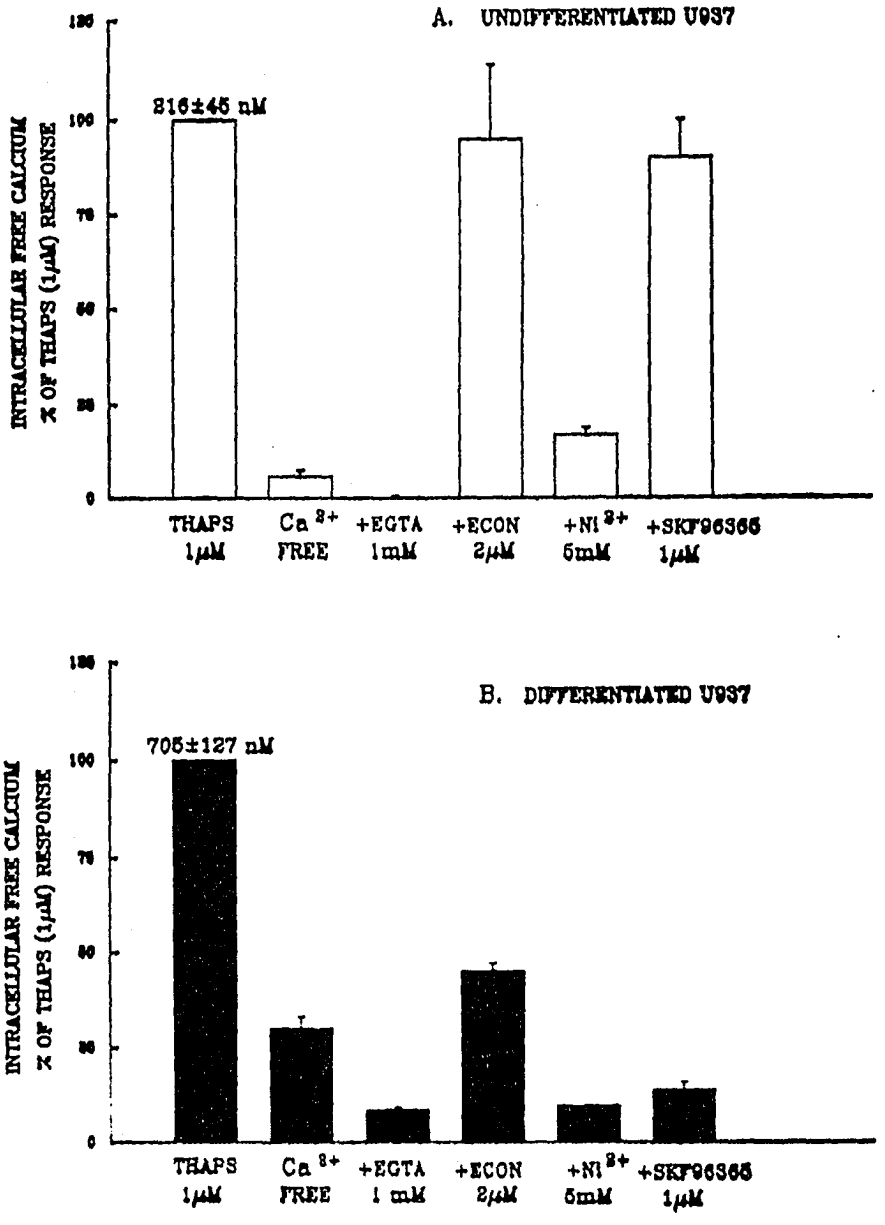


FIGURE 14

undifferentiated cells. However 1 μM SKF96365 resulted in an 80% inhibition of thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ in differentiated U937 cells. Nickel (5mM), which also blocks calcium influx channels (88), was also a somewhat more effective inhibitor of calcium influx in differentiated cells. Taken together, these results suggest that thapsigargin-induced capacitative calcium influx occurs through dissimilar channels or is under altered regulatory control in differentiated and undifferentiated U937.

4.2.4 Differentiation induced changes in the capacitative calcium entry pathway are consistent with differentiation induced changes in arachidonate release.

If the activation of $cPLA_2$ and subsequent arachidonate release are, indeed, regulated by development of a specific differentiation-induced capacitative calcium entry pathway in U937, then stimulation of differentiated U937 with thapsigargin should also activate arachidonate release. Figure 15 demonstrates that thapsigargin produced a dose dependent increase in arachidonate release in differentiated U937 cells. PMA pre-incubation potentiated thapsigargin-stimulated arachidonate release 2-3 fold, similar to the response seen using A23187 as stimulus (Figure 4, Chapter 3). Quite strikingly, thapsigargin or PMA + thapsigargin were completely ineffective at stimulating even a slight arachidonate release in undifferentiated cells. Thus, differentiation induced arachidonate mobilization appears to be coupled to the differentiation-induced development of a capacitance calcium influx pathway.

This coupling is particularly evident when A23187-stimulated effects on $[Ca^{2+}]_i$ and arachidonate release are compared to thapsigargin-stimulated effects. A23187 (0.1 μM) produced a $[Ca^{2+}]_i$ elevation of 2200 nM in differentiated U937 (Figure 16B).

FIGURE 15

EFFECTS OF THAPSIGARGIN ON ARACHIDONATE MOBILIZATION IN DIFFERENTIATED AND UNDIFFERENTIATED U937 CELLS.

U937 cells were differentiated and radiolabeled as described in Sections 2.2-2.3. Arachidonate mobilization in response to thapsigargin stimulation was measured as described in Section 2.4. Release of radiolabeled arachidonate is expressed as a percentage of the total cellular incorporated radioactivity. Data points represent mean ± SE of 3-4 separate experiments performed in duplicate.

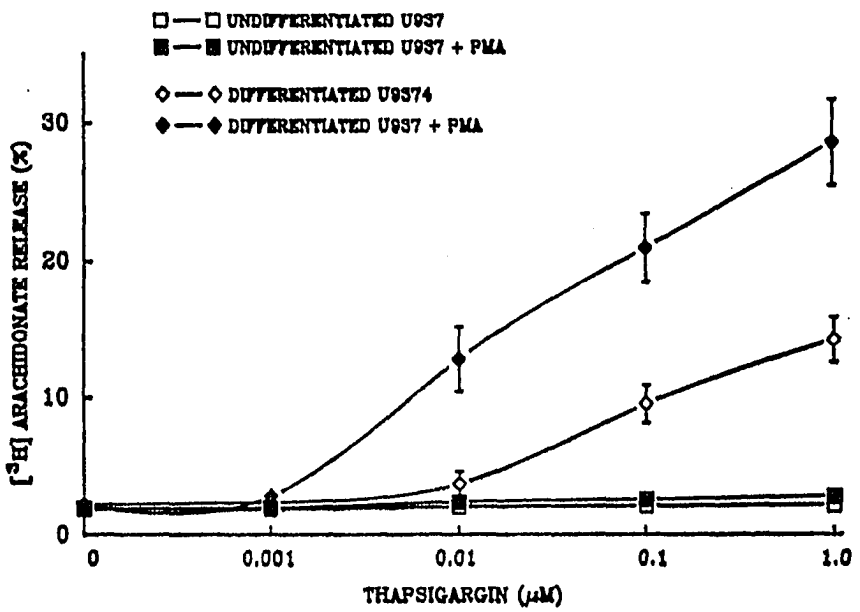


FIGURE 15

FIGURE 16

COMPARISON OF THAPSIGARGIN AND A23187-STIMULATED ELEVATIONS IN $[Ca^{2+}]_i$ AND ARACHIDONATE MOBILIZATION IN DIFFERENTIATED U937.

Figure 16A is a composite of Figures 4 and 15, and compares A23187-stimulated arachidonate mobilization to thapsigargin-stimulated arachidonate mobilization. Figure 16B is a composite of Figures 10 and 11, and represents a comparison of A23187 and thapsigargin-stimulated elevations in $[Ca^{2+}]_i$

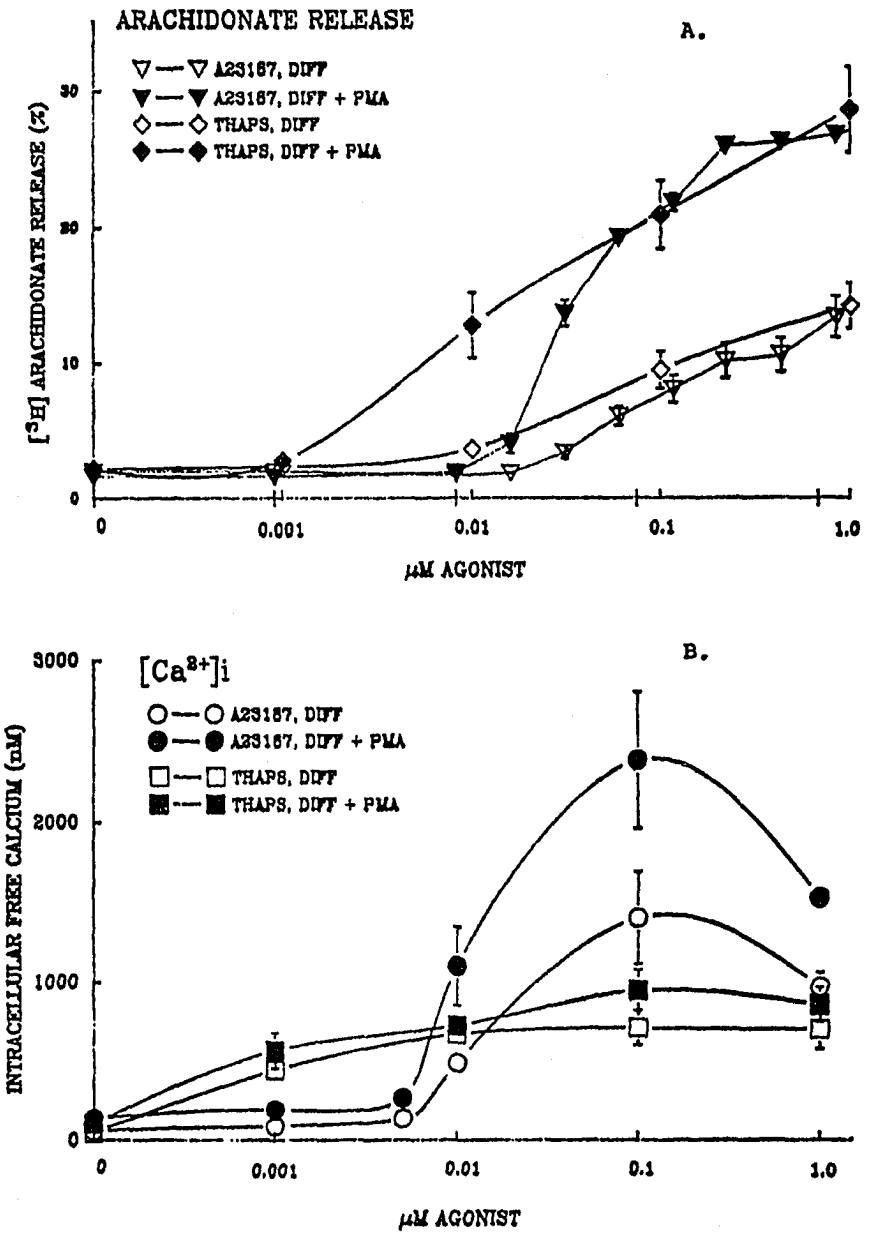


FIGURE 16

Thapsigargin (0.1 μM) produced a $[Ca^{2+}]_i$ elevation of only 1000 nM (Figure `16B). If $cPLA_2$ were solely dependent on $[Ca^{2+}]_i$, one would expect that A23187 would also produce greater arachidonate release than thapsigargin. However at concentrations of 0.1-1 μM, thapsigargin stimulation resulted in a 7-12 % (respectively) release of total incorporated arachidonate (Figure 16A). A23187 (0.1-1.0 μM) produced the same levels of arachidonate release. During A23187 stimulation, a portion of the elevated intracellular calcium is due to direct transport of calcium across the plasma membrane by A23187. Likewise, a portion of the A23187 effect is due to depletion of intracellular calcium stores and activation of capacitative influx. These results suggest that only the latter mechanism of $[Ca^{2+}]_i$ elevation, (depletion of stores followed by capacitative calcium entry) is coupled to activation of arachidonate release. Elevation of $[Ca^{2+}]_i$ by means other than the capacitative pathway, such as direct ionophore mediated transport of extracellular calcium across the plasma membrane, does not appear to activate arachidonate release in these cells.

To further support the role of extracellular calcium influx through store operated calcium channels in arachidonate mobilization, Sr^{2+} was utilized as a surrogate ion for calcium. In many biochemical processes, calcium can be replaced by Sr^{2+} without compromise of biological function (74-76). In the absence of extracellular calcium, Sr^{2+} is transported through store operated calcium influx channels in response to a stimulus (76). As cells contain little or no intracellular Sr^{2+}, changes in Fura 2 fluorescence in the presence of extracellular Sr^{2+} (and absence of extracellular calcium) would indicate influx of extracellular cation. The results shown in Figure 17A demonstrate that thapsigargin stimulated influx of Sr^{2+} from the extracellular medium in both

FIGURE 17

EFFECTS OF EXTRACELLULAR Sr^{2+} ON THAPSIGARGIN-STIMULATED ELEVATIONS OF INTRACELLULAR DIVALENT CATION AND ARACHIDONATE MOBILIZATION IN DIFFERENTIATED AND UNDIFFERENTIATED U937 CELLS.

For measurement of intracellular free Sr^{2+} (Figure 17A), cells were loaded with Fura-2 as described in Methods 2.9. After loading, cells were resuspended in Ca^{2+}-free PBS as described in Figure 13. Aliquots of cell suspension containing 10^6 loaded cells were added to the spectrofluorometer cuvette and measurements were initiated. Sr^{2+} (1 mM) was added in a volume of 2.5 µl PBS/albumin/glucose, followed by thapsigargin (1 µM). Intracellular free Sr^{2+} was calculated as described in Methods 2.9. Results represent the mean ± SE of three separate experiments.

For measurement of thapsigargin stimulated arachidonate release in the presence of extracellular Sr^{2+}, (Figure 17B), cells were radiolabeled as described in Methods 2.3. Following labeling, cells were washed twice in Ca^{2+}-free PBS and resuspended in the same. Sr^{2+} (1 mM) was added prior to thapsigargin challenge. Thapsigargin stimulation and quantitation of released radiolabel were as described in Section 2.4. Results represent the mean ± SE of three separate experiments performed in duplicate.

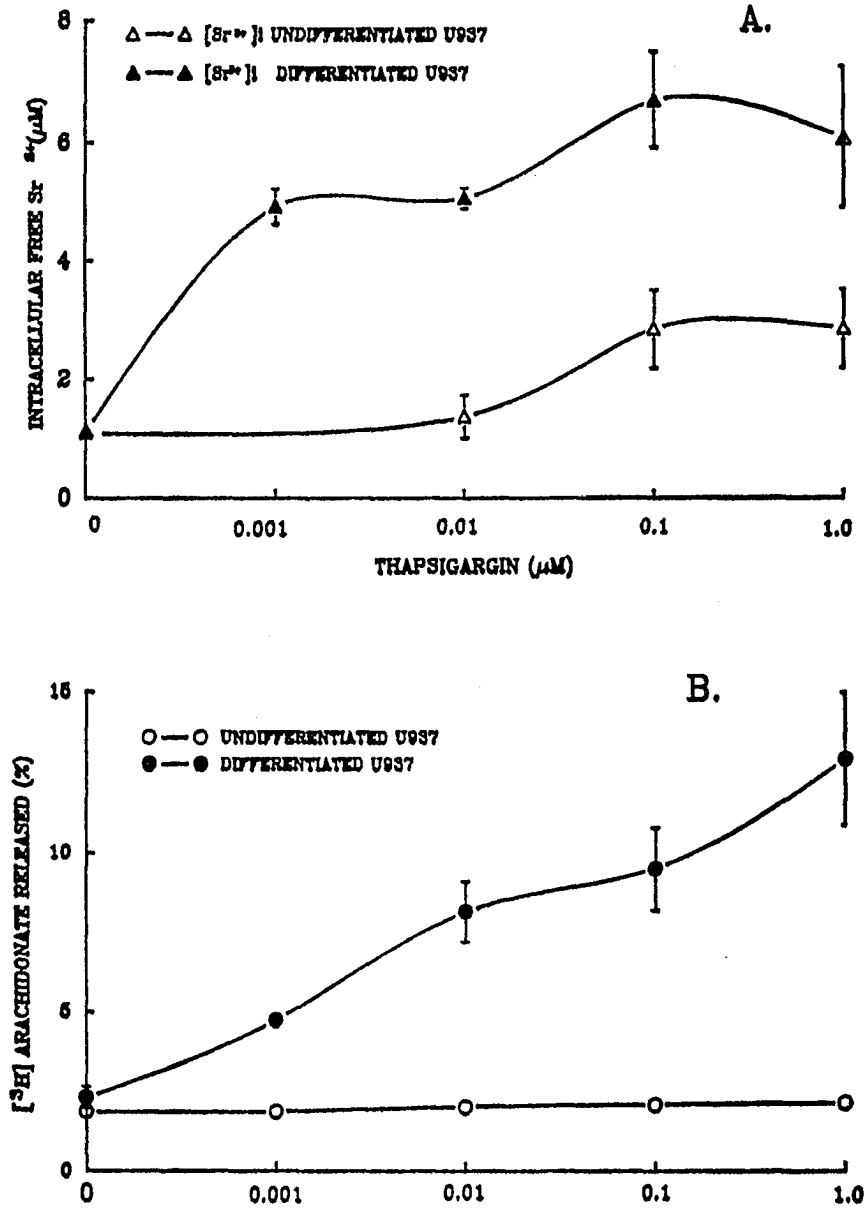


FIGURE 17

differentiated and undifferentiated cells. Consistent with effects observed with calcium, differentiated cells achieved significantly higher elevations of intracellular free Sr^{2+} than did undifferentiated cells. Furthermore, this elevation of intracellular free Sr^{2+} was coupled to arachidonate release in differentiated, but not undifferentiated cells (Figure 17B). Thapsigargin-stimulated arachidonate mobilization in the presence of extracellular Sr^{2+} was similar to that observed with Ca^{2+} as extracellular divalent cation (Figure 15). This suggests that arachidonate release in differentiated U937 is coupled to divalent cation influx through a differentiation-induced capacitative channel.

4.2.5 Arachidonate release is coupled to elevations in $[Ca^{2+}]_i$ generated from store depletion, as well as through store operated capacitative influx channels.

If arachidonate release is coupled solely to capacitative calcium influx in differentiated U937 cells, then inhibition of capacitative influx should have similar effects on arachidonate mobilization. Differentiated U937 cells were stimulated with thapsigargin in the presence of various inhibitors of capacitance calcium entry as shown in Figure 18. Stimulation of differentiated cells in medium containing calcium chelated with EGTA, or in calcium free medium, resulted in inhibition of 70-85% of thapsigargin induced arachidonate release. Thus, arachidonate release is primarily coupled to influx of extracellular calcium. However, one cannot ignore the fact that significant thapsigargin-stimulatable arachidonate release (15-20% of that observed in response to 1 μM thapsigargin in calcium-containing medium) remains, even after removal of all extracellular calcium. This suggests that a portion of the total arachidonate release observed may be due to $[Ca^{2+}]_i$ elevations resulting directly from store depletion. In the

FIGURE 18

EFFECTS OF INHIBITION OF CAPACITATIVE CALCIUM INFLUX ON THAPSIGARGIN-STIMULATED ARACHIDONATE RELEASE IN DIFFERENTIATED U937.

Cells were radiolabeled and stimulated ± PMA as described in Section 2.4. PBS/albumin/glucose solutions of EGTA (1mM), Ni^{2+} (5mM), SKF96365 (1 μM), and econazole (2 μM) were added during the pre-incubation period. For Ca^{2+}-free experiments, cells were radiolabeled and washed twice in Ca^{2+}-free PBS and resuspended in the same. Results were normalized to the arachidonate release stimulated by 1 μM thapsigargin in Ca^{2+} containing medium (14.19 + 1.67 % of total incorporated radiolabel), and represent mean ± SE for 3-5 separate experiments performed in duplicate

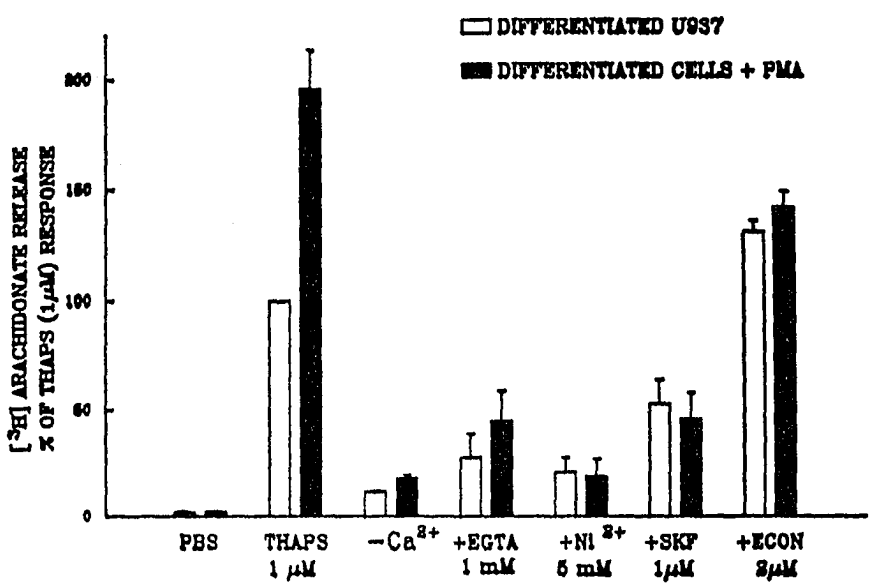


FIGURE 18

absence of extracellular calcium, PMA potentiation of thapsigargin-induced arachidonate release was weak. Thus, PMA potentiation of arachidonate release appears to be primarily dependent on that arachidonate release associated with influx of extracellular calcium (Figure 18).

Nickel (5mM) which physically blocks calcium entry though plasma membrane channels, also blocked arachidonate release, but not as effectively as it blocked elevations in $[Ca^{2+}]_i$ (compare Figures 18 & 14). Similar results were observed with SKF96365, which inhibited only 50% of the thapsigargin-stimulated arachidonate release (Figure 18) at concentrations that blocked 75-80% of the thapsigargin-stimulated elevation of $[Ca^{2+}]_i$ (Figure 14). Both these inhibitors act at the level of the store-operated channel. These results suggest an initial release of arachidonate occurs proximal to the opening of plasma membrane calcium influx channels. This is further supported by experiments in which econazole was used as an inhibitor. Econazole, at the same concentration that produced 50% inhibition of capacitance calcium influx, resulted in a 10-20% increase in arachidonate mobilization. It has been reported that econazole blocks capacitative calcium entry by inhibition of a cytochrome P450 dependent step integral to the opening of plasma membrane calcium channels (98). This suggests that initiation of arachidonate release may lie upstream of P450-mediated capacitance calcium influx, and may be controlled by calcium mobilization from the stores themselves.

PMA potentiation of thapsigargin-simulated arachidonate release was effectively blocked by inhibitors of calcium influx. This suggests that PKC may potentiate arachidonate release at the level of the calcium influx channel.

4.2.6 The time course of thapsigargin-stimulated arachidonate release correlates with the time course of thapsigargin-stimulated elevations in $[Ca^{2+}]_i$.

Arachidonate release appears to be coupled to both phases of capacitative calcium entry. Therefore, thapsigargin-stimulated release of arachidonate should follow a time course similar to thapsigargin-stimulated elevations of $[Ca^{2+}]_i$. Initially, $[Ca^{2+}]_i$ rose slowly in response to thapsigargin stimulation (Figure 19A). This slow increase continued until approximately 150 seconds, at which time $[Ca^{2+}]_i$ reached 110-125 nM. This was consistent with thapsigargin-induced elevations in $[Ca^{2+}]_i$ due to depletion of stores alone (Figure 13). This phase was followed by a rapid increase in $[Ca^{2+}]_i$ consistent with capacitative influx (Figure 19A). Maximal elevations of $[Ca^{2+}]_i$ were achieved by 300 seconds post thapsigargin. The thapsigargin stimulated time course of arachidonate release is shown in Figure 19B. The most striking finding is that arachidonate release is enhanced 2 fold over basal levels within 30 seconds of addition of thapsigargin, as intracellular calcium stores are undergoing depletion. A linear release of arachidonate continued through 300 seconds post-thapsigargin, at which time arachidonate release was maximal. Stimulation for longer time periods (up to 10 minutes) did not result in further increases in arachidonate release (data not shown). Thus, arachidonate mobilization occurs as the intracellular calcium stores are being depleted, and continues until maximal elevations of $[Ca^{2+}]_i$ are attained.

PMA pre-incubation raised initial basal $[Ca^{2+}]_i$ levels (Figure 19A, zero time point). The enhanced elevations of $[Ca^{2+}]_i$ observed in PMA + thapsigargin treated cells appear to be additive to that of thapsigargin induced elevations of $[Ca^{2+}]_i$ for the first 150 seconds of stimulation. Thus, PMA pre-incubation does not

FIGURE 19

TIME COURSE OF THAPSIGARGIN-STIMULATED ELEVATIONS OF $[CA^{2+}]_I$ AND ARACHIDONATE RELEASE IN DIFFERENTIATED U937 CELLS.

In panel A, $[Ca^{2+}]_i$ was measured in Fura 2 loaded cells ± PMA pre-incubation as described in Methods 2.9. In panel B, arachidonate mobilization was stimulated as described in Methods 2.6. Reactions were stopped at the indicated time intervals after thapsigargin stimulation, by addition of methanol. Cells plus incubation media were extracted and chromatographed in petroleum ether/diethyl ether/glacial acetic acid (82:18:1) as described in Methods 2.6. Released arachidonate was quantitated using a Bioscan radioactivity scanner. All results represent the mean ± SE of 3 experiments performed in duplicate

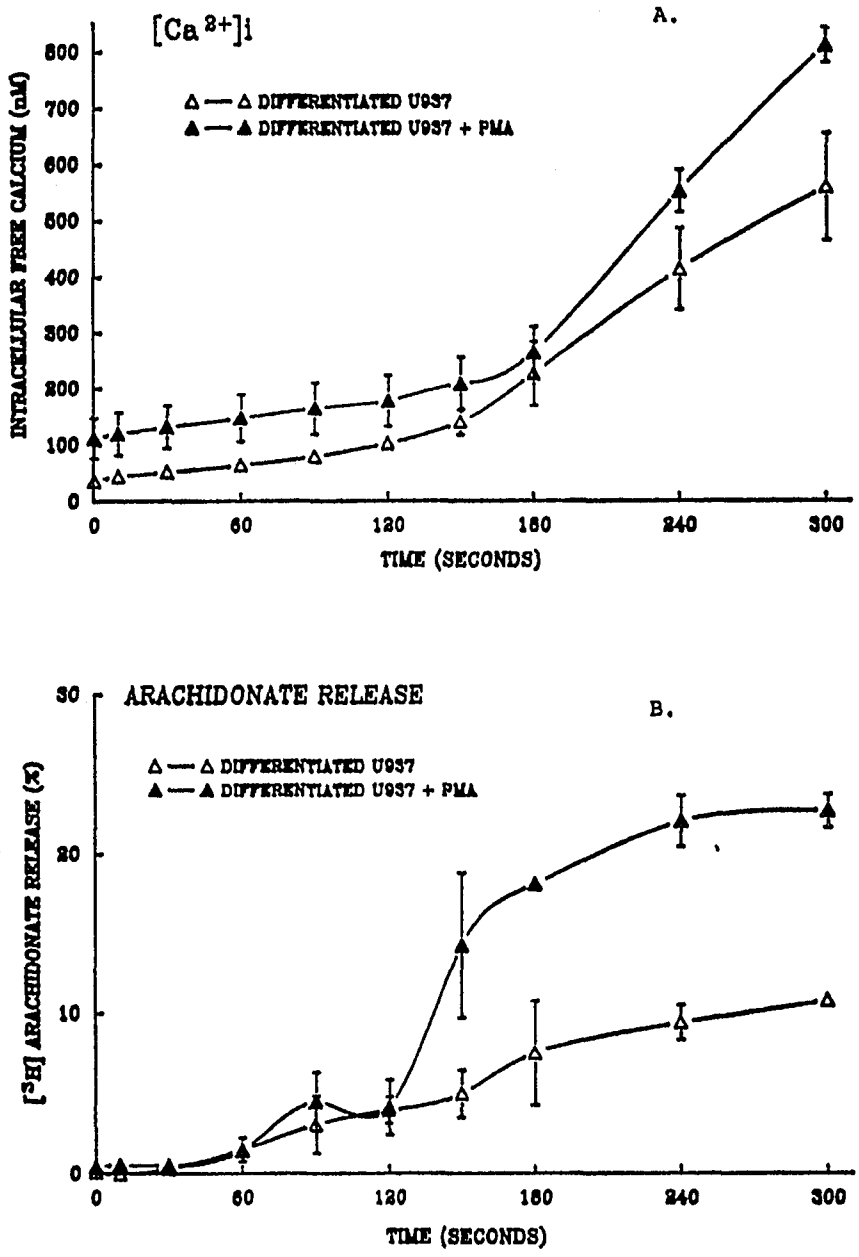


FIGURE 19

seem to directly effect thapsigargin-induced intracellular calcium store depletion. PMA did, however, significantly potentiate the sustained phase of capacitative calcium influx. Similar effects were observed with the time course of PMA + thapsigargin stimulated arachidonate release (Figure 19B). Arachidonate mobilization coupled to intracellular calcium store depletion was not affected by PMA pretreatment. However, arachidonate release coupled to influx of extracellular calcium was significantly potentiated by PMA. This suggests differential regulation of $cPLA_2$, dependent on either the source of intracellular calcium, or on an actual threshold level of $[Ca^{2+}]_i$ above which PMA potentiates arachidonate release.

Further evidence for this differential regulation is presented in Figure 20. A plot of arachidonate release as a function of the change in $[Ca^{2+}]_i$ demonstrates that in the absence of PMA pretreatment, the relationship is approximately linear during store depletion and calcium influx (open circles). Thus, release of arachidonate correlates well with $[Ca^{2+}]_i$ elevations. In PMA pre-treated cells, thapsigargin-stimulated arachidonate release is linear with respect to $[Ca^{2+}]_i$ elevation up to a change in $[Ca^{2+}]_i$ of approximately 75 nM. This change in $[Ca^{2+}]_i$ is approximately equivalent to that attained by depletion of intracellular calcium stores alone. Thus, it appears that PMA potentiation of arachidonate release is observed during calcium influx, downstream of store depletion.

FIGURE 20

THAPSIGARGIN-STIMULATED ARACHIDONATE RELEASE AS A FUNCTION OF $[Ca^{2+}]_i$.

Data for thapsigargin-stimulated arachidonate release presented in Figure 19 is graphed as a function of change in $[Ca^{2+}]_i$

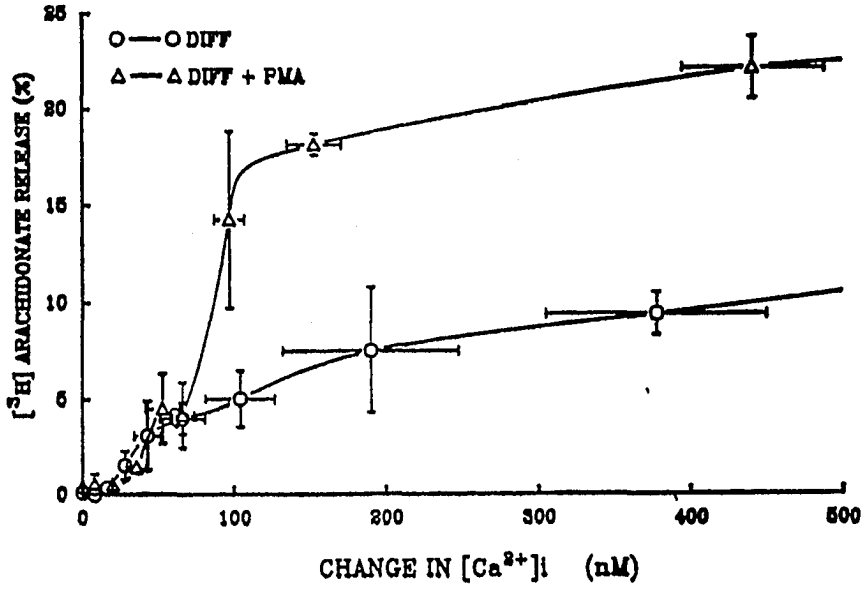


FIGURE 20

4.2.6 Thapsigargin stimulated effects on $[Ca^{2+}]_i$ elevation and arachidonate mobilization are blocked by PLA_2 inhibitors

The previous results suggest that arachidonate release is coupled to both phases of the capacitative calcium entry pathway; store depletion and influx of extracellular calcium. Furthermore, arachidonate release preceded opening of capacitative calcium entry channels. We therefore sought to examine whether inhibition of arachidonate release could inhibit capacitance calcium influx. For these experiments, differentiated U937 cells were pre-incubated with either NDGA or PGBx. PGBx is an oligomer of prostaglandin B, which inhibits $cPLA_2$ (and $sPLA_2$) *in vitro*, and inhibits arachidonate release in U937 and other cells (49). PGBx inhibited thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ as well thapsigargin-stimulated mobilization of arachidonate.

NDGA is a non-metabolizable substrate analog which inhibits 5-lipoxygenase and P450 pathways of the arachidonate cascade (72). U937 cells lack a 5-lipoxygenase (77). Therefore, the expected effects of NDGA in U937 would be primarily on a P450 system involved in the metabolism of free arachidonate. Like PGBx, NDGA also inhibited thapsigargin induced elevations in $[Ca^{2+}]_i$ (Figure 21). However, NDGA also inhibited thapsigargin-stimulated arachidonate mobilization. This indicates that although NDGA may inhibit cytochrome P450-mediated arachidonate metabolism in some cells, it can also act as a $cPLA_2$ inhibitor in differentiated U937. Taken together, these results suggests that released arachidonate and/or a metabolite thereof, may participate in regulation of capacitative calcium influx.

FIGURE 21

EFFECTS OF PGBx AND NDGA ON THAPSIGARGIN-STIMULATED ELEVATIONS OF $[Ca^{2+}]_i$ AND ARACHIDONATE MOBILIZATION IN DIFFERENTIATED U937 CELLS.

For arachidonate release (filled bars) cells were prepared, radiolabeled and stimulated as described in Methods 2.4. PGBx and NDGA were added during the pre-incubation period, as described. Results were normalized to the arachidonate release stimulated by 1 μM thapsigargin. For measurement of $[Ca^{2+}]_i$, cells were loaded with Fura-2 and calcium was measured as described in Methods 2.9. NDGA or PGBx were added 3 minutes prior to addition of thapsigargin, and $[Ca^{2+}]_i$ was monitored for an additional 10 minutes. Results are normalized to the maximum $[Ca^{2+}]_i$ elevation obtained in response to 1 μM thapsigargin. Result represent mean ± SE of at least 3 separate experiments.

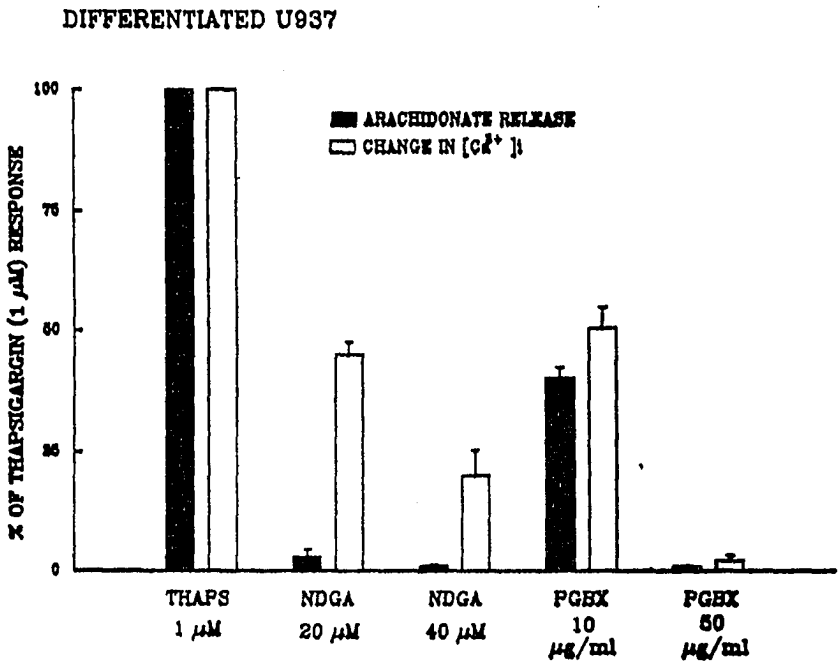


FIGURE 21

4.3 DISCUSSION

In Chapter 3, we demonstrated that differentiation of U937 cells induced significant A23187-stimulatable arachidonate release, not present in undifferentiated cells. The present chapter demonstrates that this enhanced capacity to release arachidonate in response to a calcium signal is coupled to differentiation-induced development of a capacitative calcium influx pathway. Furthermore, our data suggests that release of arachidonate may contribute to the activation of this calcium influx pathway. Based on the results presented here, a model for $cPLA_2$ regulation is proposed in Figure 22. According to this model, differentiation induces alterations in both the size of thapsigargin-stimulatable intracellular calcium stores, and the characteristics of store or second messenger operated calcium influx channels (SOC/SMOC). Stimulation of differentiated U937 cells with thapsigargin results in an initial release of calcium from intracellular stores, causing a localized increase in calcium near the membrane of intracellular stores. Thus, $cPLA_2$ translocates to the membranes of intracellular stores and initiates mobilization of arachidonate. Released arachidonate and/or a metabolite thereof may participate in generation of a second messenger or CIF, which regulates opening of SOC/SMOC in the plasma membrane. Influx of extracellular calcium through SOC results in a localized calcium increase at the plasma membrane, thereby recruiting more $cPLA_2$ to this subcellular location.

Several lines of evidence support $cPLA_2$ as the enzyme mediating thapsigargin-stimulated release of arachidonate. First, as the 14 kDa secretory PLA_2 requires millimolar calcium for catalysis, it does not appear likely that it is active under the sub-micromolar levels of $[Ca^{2+}]_i$ we observe in these experiments. Second, strontium was

FIGURE 22

REGULATION OF $cPLA_2$ IN DIFFERENTIATED U937 CELLS.

Thapsigargin (THAPS) inhibits the calcium pump located on the endoplasmic reticulum (ER) or possibly nuclear (NUC) membrane. Calcium then leaks into the cytoplasm through a channel, possibly the IP_3 receptor/channel. The local high concentration of Ca^{2+} generated near this channel causes translocation and activation of $cPLA_2$ (ACT $cPLA_2$), to the calcium store membrane, generating free arachidonic acid (AA). AA could then be converted, via cytochrome P450, to a calcium influx factor (CIF) which could then activate the store or second messenger operated calcium influx channel (SOC/SMOC) located in the plasma membrane, allowing entry of calcium from the extracellular space into the cytoplasm. Alternatively, CIF may not be an arachidonate metabolite itself, but could be generated by activation of a low molecular weight GTPase by free arachidonic acid. The local high concentration of Ca^{2+} near the plasma membrane calcium channel would then translocate and activate $cPLA_2$ (ACT-$cPLA_2$) to promote mobilization of AA at the plasma membrane

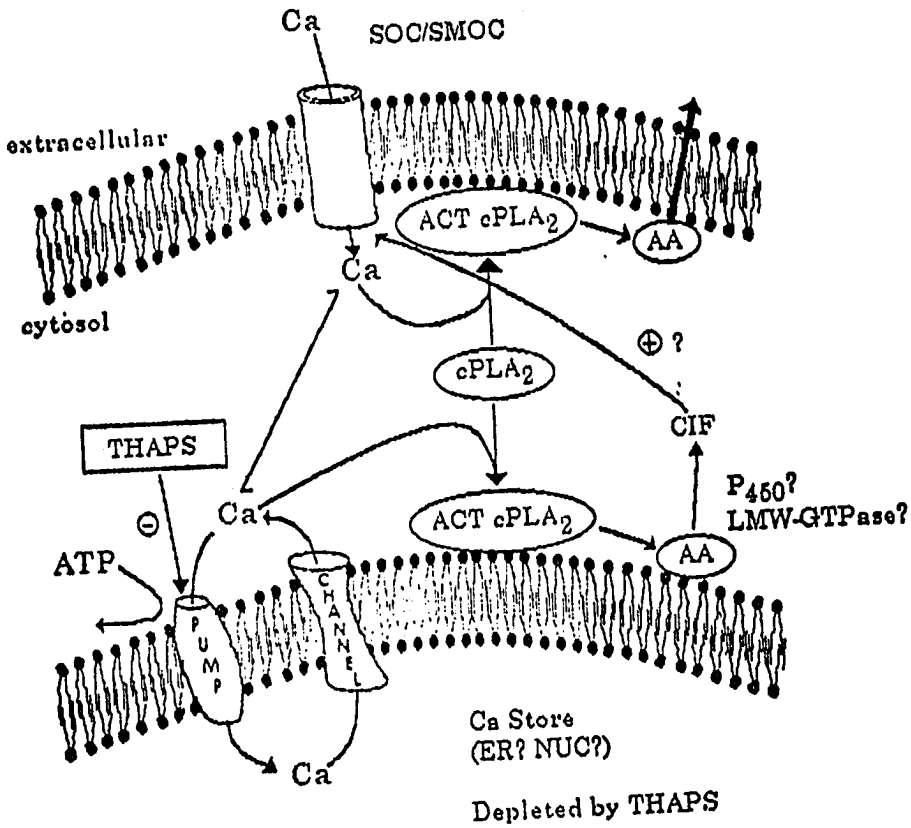


FIGURE 22

capable of supporting arachidonate release in response to thapsigargin stimulation, to the same extent as that observed with calcium as extracellular divalent cation. *In vitro*, $sPLA_2$ has an absolute requirement for calcium, and cannot utilize strontium for catalysis (102). However, $cPLA_2$ is active in the presence of strontium (39). Third, results in Chapter 3 have previously demonstrated that arachidonate release in differentiated U937 cells exhibits a preference for arachidonate, consistent with the characteristics of $cPLA_2$ activity. These results strongly suggest that arachidonate mobilization in response to thapsigargin stimulation is mediated by $cPLA_2$.

Free arachidonate can also be generated by the combined actions of phospholipases C or D, and diglyceride lipase. However, thapsigargin treatment does not result in result in the generation of IP_3, and therefore does not appear to activate PLC (14, 99). Furthermore, we do not observe any changes in the arachidonate labeling of the mono or diglyceride lipid fractions during the time course of thapsigargin stimulation (data not shown). Taken together, these results preclude a major role for PLC in thapsigargin-stimulated arachidonate mobilization in U937. Arachidonate mobilization was blocked by PGBx, an inhibitor of $cPLA_2$. Phospholipase D is not inhibited by PGBx (49), suggesting that PLD does not contribute to thapsigargin-stimulated arachidonate release.

Figure 22 suggests that differentiation induces alterations in two primary components of capacitative calcium influx, the first of which is the intracellular calcium store. Our results indicate that differentiated cells have increased thapsigargin-stimulatable intracellular calcium stores, as compared to undifferentiated cells (Figure 13). This could indicate that the physical size of intracellular calcium stores increases

with differentiation. Alternatively, myeloid leukemia cells lines such as U937 are reported to contain high intrinsic levels of IP_3, which decrease upon differentiation (103). Thus, the stores themselves may not necessarily be much larger, but may simply be "more full".

The second component of capacitative calcium influx is the store or second messenger operated calcium influx channel (SOC/SMOC). Figures 11 and 14 suggest that capacitative calcium influx channels are either dissimilar or under altered regulatory control in differentiated and undifferentiated U937. Undifferentiated cells do appear to exhibit some elevations in $[Ca^{2+}]_i$ in response to thapsigargin stimulation in calcium-free medium. Thus, the capacitative pathway does appear to function (although it is weak) in these cells. However, the small elevation in $[Ca^{2+}]_i$ observed could also be due to influx of trace amounts of calcium remaining in the medium. Nevertheless, thapsigargin-stimulated elevations $[Ca^{2+}]_i$ in undifferentiated cells is weak and is not coupled to mobilization of arachidonate. Differentiated cells however, respond to thapsigargin with dramatically enhanced levels of capacitative calcium influx.

Inhibitors of capacitative calcium influx channels, econazole and SKF96365, failed to block thapsigargin stimulated elevations of $[Ca^{2+}]_i$ in undifferentiated cells. Both these compounds were quite effective at inhibition of capacitative calcium influx in differentiated cells. It has been suggested that econazole, at the concentrations used in these experiments, blocks capacitative calcium entry by inhibition of a cytochrome P450 dependent step in the opening of store controlled capacitative channels (98). Lack of inhibition of thapsigargin-stimulated $[Ca^{2+}]_i$ elevations by econazole suggests that this P450-mediated pathway may not contribute to calcium influx in undifferentiated cells.

SKF96365 blocks capacitative calcium influx by physical association with calcium influx channels, inhibiting passage of the Ca^{2+} ion. The ineffectiveness of SKF96365 in undifferentiated cells suggests that the channel activated in these cells has a different physical conformation. Furthermore, PMA pre-incubation had contrasting effects on thapsigargin-stimulated influx in differentiated and undifferentiated U937 (Figure 11). In undifferentiated cells, PMA pre-incubation had a slight inhibitory effect on thapsigargin-induced elevations of $[Ca^{2+}]_i$. In contrast, PMA potentiated thapsigargin-stimulated $[Ca^{2+}]_i$ elevations in differentiated cells. Staurosporine (1 μM) blunted these effects, which implies regulation via PKC. Thus, PKC activation had contrasting effects on capacitative calcium influx in differentiated and undifferentiated U937. This suggests the existence of either different PKC isoforms in differentiated and undifferentiated cells, or different calcium influx channels. Taken together with results from inhibitor experiments, this suggests that differentiation does not merely induce an increased number of capacitative calcium influx channels. Rather, it may induce either a dissimilar channel, or a structural alteration of an existing channel, thereby enhancing capacitative influx.

$cPLA_2$ activity appears to be coupled to both phases of the capacitative pathway, store depletion and influx of extracellular calcium. Our observations indicate that significant thapsigargin-stimulated arachidonate release occurs even in the absence of extracellular calcium. This is consistent with observations in thrombin stimulated platelets, in which arachidonate release was increased 2-3 fold over basal levels even in the absence of extracellular calcium (45). Furthermore, inhibition of calcium entry by agents which block capacitative influx at the level of the calcium channel did not

completely block arachidonate release. This suggests that elevations of $[Ca^{2+}]_i$ resulting from store depletion can, indeed, result in mobilization of arachidonate. The time course of thapsigargin-stimulated arachidonate release further supports this point. Arachidonate release begins within 30 seconds of addition of thapsigargin. The rapid increase in $[Ca^{2+}]_i$, indicative of capacitative calcium influx, does not begin until approximately 150 seconds after thapsigargin addition (Figures 19A & 19B). Taken together, these results indicate that initial release of arachidonate appears to occur as intracellular calcium stores are undergoing depletion.

We suggest that the initial thapsigargin-stimulated arachidonate mobilizing activity occurs at the membrane of the intracellular stores. In the absence of extracellular calcium, the maximum elevation of $[Ca^{2+}]_i$ attainable by depletion of the stores with 1 μM thapsigargin was approximately 120 nM. This concentration of $[Ca^{2+}]_i$ is well below the reported threshold calcium concentration of 250 nM, at which $cPLA_2$ appears to translocate to membrane substrates *in vitro* (22). However, Fura 2 is likely to be evenly distributed throughout the cytoplasm (88). Thus, measurements of $[Ca^{2+}]_i$ represent an average calcium concentration throughout the cytoplasmic compartment. The actual concentration of free Ca^{2+} immediately surrounding the stores could possibly be much higher (88, 104). Thus, $cPLA_2$ would be more likely to associate with membranes in the area of a localized increase in free calcium. In Swiss 3T3 fibroblasts, chelation of intracellular free calcium with BAPTA or EGTA-AM failed to inhibit $cPLA_2$ mediated arachidonate release, suggesting that localized increases in near-membrane calcium could control $cPLA_2$ activity (40). Thus, during the initial phase of thapsigargin-stimulated arachidonate release, a localized increase of calcium at

the store membrane may result in $cPLA_2$ translocation to this membrane.

The fate of arachidonate generated during intracellular calcium store depletion is at present, unclear. It has been suggested that arachidonate and/or its metabolites may be associated with activation of capacitative calcium entry channels (12-14). As demonstrated in time course experiments, as well as in experiments utilizing inhibitors of capacitance entry channels, an initial phase of arachidonate release precedes influx of extracellular calcium. Of particular interest is the effect of econazole on arachidonate release. In differentiated U937, econazole effectively blocked capacitative calcium influx at its reported IC_{50} (100). However, the same concentrations of econazole stimulated release of arachidonate. Free arachidonate can be metabolized by several different pathways, one of which is a P450 mediated process resulting in the production of HETES and epoxide derivatives. If a P450 arachidonate metabolite were involved in activation of capacitative influx, then inhibition of P450 by econazole would block production of this metabolite and inhibit calcium influx. $cPLA_2$-mediated release of arachidonate in response to intracellular calcium store depletion might be unaffected. Thus, the observed effects of econazole on $[Ca^{2+}]_i$ and arachidonate release suggest activation of capacitative influx by a P450 arachidonate metabolite. Recent evidence suggests that a non-cyclooxygenase metabolite of arachidonate regulated agonist-induced capacitative calcium influx in thyroid FRTL-5 cells (13). Additionally, exogenous arachidonate activated calcium influx in several cell lines (12, 14). A GTP dependent step in the capacitative calcium entry pathway has recently been identified (86, 95), which is dependent on hydrolysis of GTP to GDP. Arachidonate has been reported to participate in regulation of GTPase activating proteins (17-19, 105). Thus,

there is evidence that arachidonate and/or its metabolites can participate in activation of capacitative calcium entry at several stages.

The second phase of the capacitative calcium influx pathway consists of the influx of extracellular calcium through store controlled plasma membrane channels. Our results suggest that arachidonate mobilization is also coupled to influx of extracellular calcium. Removal of extracellular calcium from the medium, inhibited 70-85% of thapsigargin-stimulated arachidonate release (Figures 13 & 18). Inhibition of capacitative calcium influx channels by nickel and SKF96365 produced similar effects. As depicted in the model in Figure 22, influx of calcium through plasma membrane channels would cause an increase in $[Ca^{2+}]_i$, with local elevated levels at the plasma membrane (104). This local increase in calcium could then recruit $cPLA_2$ to the plasma membrane. As these calcium concentrations are possibly even higher than those seen due to store depletion, arachidonate release would be even more rapid, as more $cPLA_2$ is recruited to this subcellular location.

PMA pre-incubation potentiated thapsigargin-stimulated calcium influx, as well as arachidonate release. However, PMA potentiation of arachidonate mobilization was only observed during the calcium influx phase of the thapsigargin response (Figs 19 & 20). PMA did not effect arachidonate release associated with $[Ca^{2+}]_i$ elevation due to store depletion. PMA effects were inhibited by staurosporine, suggestive of PKC mediated events. PMA induces phosphorylation of $cPLA_2$ *in situ*, which enhanced *in vitro* activity of the partially purified enzyme (33, 61). Thus, PMA may enhance arachidonate release *via* PKC mediated phosphorylation $cPLA_2$ itself. Additionally, our observations indicate that PMA may enhance capacitative calcium influx itself, resulting

in increased $[Ca^{2+}]_i$ for $cPLA_2$ translocation. This elevation in $[Ca^{2+}]_i$ alone may account for the increased arachidonate mobilization observed in PMA pre-incubated cells. Thus, PKC may have a dual role in regulation of arachidonate release. It may have effects on calcium influx, as well as on $cPLA_2$ activity directly. Alternatively, there may be some threshold $[Ca^{2+}]_i$, above which PMA effects on arachidonate mobilization are observed. Regardless of the specific mechanism involved, PMA potentiation of thapsigargin-stimulated arachidonate release only occurred during capacitative influx, suggesting that PKC mediated events lie downstream of calcium store depletion and initial release of arachidonate.

The proposed model studies suggests that the subcellular localization of $cPLA_2$ is regulated by localized increases in $[Ca^{2+}]_i$. Thus, $cPLA_2$ may be regulated by calcium dependent "compartmentalization". Actual physical compartmentalization of enzymatic activity, as evidenced by confinement of various enzymes to specific membrane bound organelles, provides discrete areas dedicated to specific enzymatic processes. In the case of $cPLA_2$, compartmentalization may be dependent on the free calcium concentration of the membrane microenvironment. This may assist in recruiting $cPLA_2$ to specific subcellular locations, thereby directing subsequent metabolism of arachidonate. For example, localization at the intracellular store membrane may favor metabolism by a P450 dependent pathway resulting in opening of capacitative calcium entry pathways. Likewise, localization of the enzyme to the nuclear membrane in conjunction with 5-lipoxygenase activating protein (106) may favor leukotriene production.

In summary, the results presented in this chapter demonstrate that DMSO

differentiation of U937 cells induces an enhanced capacitative calcium influx pathway. In differentiated cells, $cPLA_2$ is closely coupled to both phases of capacitative calcium influx. As intracellular calcium stores are depleted, mobilization of calcium from these stores activates arachidonate release. Evidence suggests that arachidonate liberated during calcium store depletion may participate in the store controlled opening of capacitative calcium influx channels. Since the experiments presented in this chapter do not discriminate between released arachidonate and its metabolites, it is presently unclear whether a P450 arachidonate metabolite is involved in capacitative calcium entry. Further experiments aimed at clarification of this issue are presented in Chapter 5

Additionally, arachidonate release is also coupled to capacitative calcium influx. This phase of arachidonate release is potentiated by PMA. This biphasic coupling of arachidonate release to capacitance calcium influx may be related to calcium dependent translocation of $cPLA_2$ to localized increases in intracellular free calcium.

The experiments presented thus far focus on stimulation of capacitative calcium influx and mobilization of arachidonate independent of receptor-mediated events. This approach simplifies the study of effector systems downstream receptor-ligand binding. However, in order to accurately define a model for $cPLA_2$ activation, the events described in our model should also occur in response to actual receptor mediated events. Such a study is the focus of Chapter 6.

CHAPTER 5

EXOGENOUS FREE ARACHIDONIC ACID STIMULATES INFLUX OF EXTRACELLULAR CALCIUM AND RELEASE OF ENDOGENOUS ARACHIDONATE IN DIFFERENTIATED U937 CELLS.

5.1 Background

During stimulation of differentiated U937 cells with thapsigargin, an initial phase of $cPLA_2$ activity was coupled to depletion of intracellular calcium stores, preceding the opening of capacitative calcium influx channels in the plasma membrane (Section 4.2.5). This data suggests that this initial $cPLA_2$ activity may participate in the formation of a calcium influx factor (CIF). CIF may be a discrete molecule, such as the diffusible second messenger described by Randriamampita (89). Alternatively, CIF may represent the assembly of a molecular complex involving the activity of low molecular weight GTPases (86, 95). Whatever its nature, the CIF serves as a communication link between intracellular calcium stores and store operated calcium influx channels in the plasma membrane (SOC), thereby regulating influx of extracellular calcium.

Free fatty acids can non-specifically effect ion fluxes and membrane permeability several cell systems (14, 107-109). In contrast to these non-specific effects, specific effects of arachidonate on calcium influx have been identified in pancreatic islets (110),

neural tissue (15), adrenal chromaffin cells (12), and thyroid cells (13). In differentiated U937 cells, inhibition of $cPLA_2$ with PGBx blocks capacitative calcium influx (Section 4.2.6), supporting a role for free arachidonate (or an arachidonate metabolite) in generation of a CIF.

Experiments with econazole and other imidazole antimycotics suggest that a metabolite of a cytochrome P450 system may be involved in the generation of a CIF (98, 111). The precise P450 system involved is presently unknown. Arachidonic acid can be metabolized by cytochromes of the P450-4A series, resulting in the formation of arachidonic acid epoxide derivatives. Therefore, a P450 metabolite of arachidonic acid could be critical to the formation of the CIF. Econazole blocked thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ in differentiated U937, yet potentiated release of arachidonate (4.2.6). If a cytochrome P450 metabolite of arachidonate were involved in generation of CIF, one might expect these results. Inhibition of cytochrome P450 would block P450-mediated arachidonate metabolism and subsequent generation of a CIF. Influx of extracellular calcium would then be inhibited. However, arachidonate release coupled to depletion of intracellular calcium stores might remain unaffected.

The evidence implicating a P450 metabolite in generation of a CIF is based solely on the use of imidazole antimycotics (econazole, miconazole, and ketoconazole). Recent reports suggest that antimycotics such as econazole, may inhibit capacitative calcium influx by physical blockage of calcium channels, similar to SKF96365 (112). Both SKF96365 and econazole do have quite similar structures (n-substituted imidazole). Thus, although econazole is reported to act through inhibition of cytochrome P450, the precise mode of action of this drug in the capacitative pathway is

presently unclear.

If arachidonate and/or a metabolite thereof participates in the generation of CIF, it follows that:

1.) Exogenous application of arachidonate (and/or a metabolite) should stimulate influx of extracellular calcium

2.) Exogenous application of arachidonate (and/or a metabolite) should activate arachidonate release, as $cPLA_2$ activity is also coupled to influx of extracellular calcium (Chapter 4).

3.) Arachidonate and/or a metabolite, should be detected in response to thapsigargin-stimulation.

Experiments designed to investigate these parameters are presented in this chapter.

5.2 Results

5.2.1 Effects of free arachidonate on $[Ca^{2+}]_i$.

If arachidonate and/or a metabolite is involved in the formation of a CIF, then exogenous free arachidonate should provoke an elevation in $[Ca^{2+}]_i$. However this type of experiment poses several technical problems. Experiments utilizing U937 cells are generally performed in PBS containing 2.5 μM fatty acid free albumin, the presence of which serves two main functions. First, albumin coats the cell, and enhances viability in PBS. Second, since albumin has multiple binding sites for free fatty acids, it serves as a "trap" in assays for arachidonate mobilization, by minimizing re-incorporation of [^{3}H]arachidonate released in response to agonist stimulation. When exogenous arachidonate is given to cells suspended in PBS/glucose/albumin, a portion of the free

fatty acid is immediately bound to albumin, and is therefore less readily available to the cell. To eliminate these difficulties, initial experiments on $[Ca^{2+}]_i$ elevations were performed on cells resuspended in albumin-free PBS/glucose immediately prior to assay.

Arachidonate (100 μM) had no effect on $[Ca^{2+}]_i$ in undifferentiated cells (data not shown). However 100 μM arachidonate elevated $[Ca^{2+}]_i$ in differentiated cells 4 fold over basal levels (from 38 to 173 nM, Figure 23). The response to exogenous arachidonate was composed of an initial rapid elevation in $[Ca^{2+}]_i$ (first 5 seconds of response in figure 23) followed by a sustained phase, and was dose-dependent over the range 10-100 μM (data not shown). Linoleate (100 μM) also induced an elevation in $[Ca^{2+}]_i$, from a basal level of 38, to approximately 79 nM (Figure 23). However, the response to linoleate was much weaker, and lacked the initial phase of rapid elevation seen with arachidonate. Oleate (100 μM) was without effect. Viability after all incubations with free fatty acids was > 98%.

In the presence of albumin, the response to 100 μM arachidonate in differentiated cells was reduced by approximately 40-50% (Figure 24). Additionally, the dose-response for arachidonate was dramatically blunted (Figure 25). Arachidonate (50 μM) produced only a weak elevation in $[Ca^{2+}]_i$, (0.5 fold over basal levels), while 25 μM arachidonate was without effect. These results are not surprising, considering that albumin reduces the effective concentration of arachidonate available to the cell. Thus, concentrations of $\geq$ 100 μM may be the minimal concentration of arachidonate required to saturate albumin binding sites and allow availability of free fatty acid for stimulation. To promote viability and maintain consistency with arachidonate mobilization assays, all

FIGURE 23

EFFECTS OF EXOGENOUS FATTY ACIDS ON $[Ca^{2+}]_i$ IN DIFFERENTIATED U937 CELLS

Differentiated U937 cells were loaded with Fura-2 as described in Methods 2.9. Following loading, cells were washed twice in albumin-free PBS and resuspended in the same buffer. Cells were stimulated with 2.5-5 µl aliquots of the indicated fatty acid and [Ca2+]i was measured as described in Section 2.9. Results shown are representative of 3-4 similar experiments performed on different days.

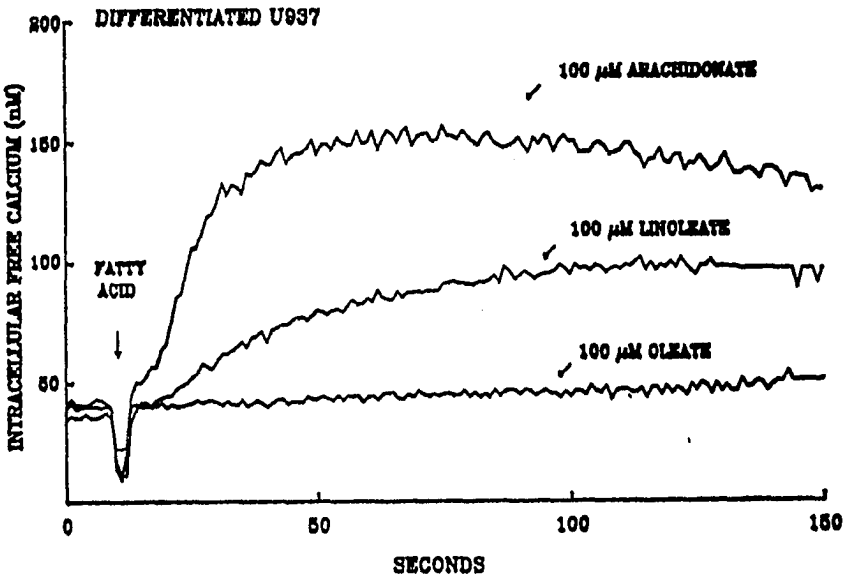


FIGURE 23

FIGURE 24

THE EFFECTS OF ALBUMIN OF EXOGENOUS ARACHIDONATE-STIMULATED INCREASES IN $[Ca^{2+}]_i$.

Differentiated U937 cells were loaded with Fura-2 as described in Figure 23. After loading, cells were washed and resuspended in PBS with or without albumin (2.5 μM), as described in Section 5.2.1. Arachidonate (100 μM) was added as indicated by the arrow. Results shown are representative of 3 separate experiments.

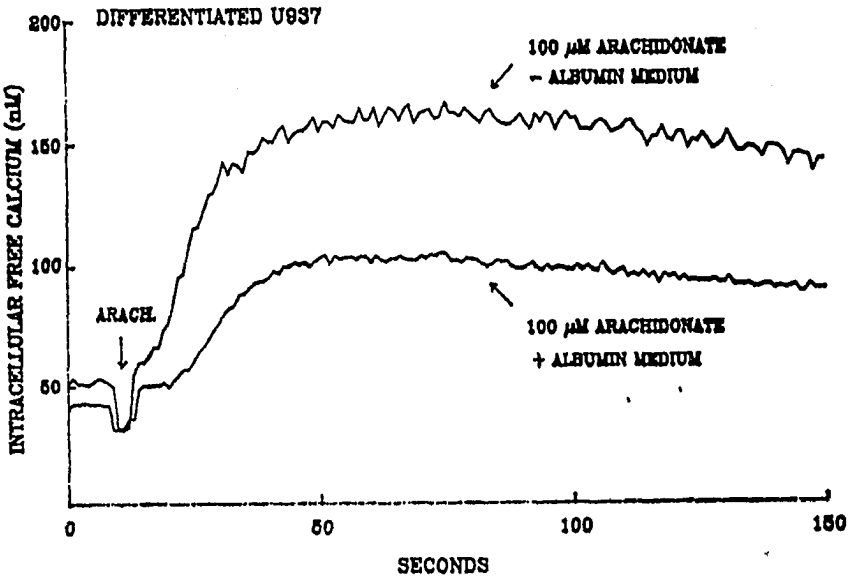


FIGURE 24

FIGURE 25

EFFECTS OF EXOGENOUS ARACHIDONATE ON $[Ca^{2+}]_i$: DOSE RESPONSE.

Differentiated cells were loaded with Fura-2 and prepared as described in Methods 2.9. Exogenous arachidonate stimulated elevations in $[Ca^{2+}]_i$ were determined in albumin containing medium as described in Figure 24. Results are representative of 3-4 separate experiments.

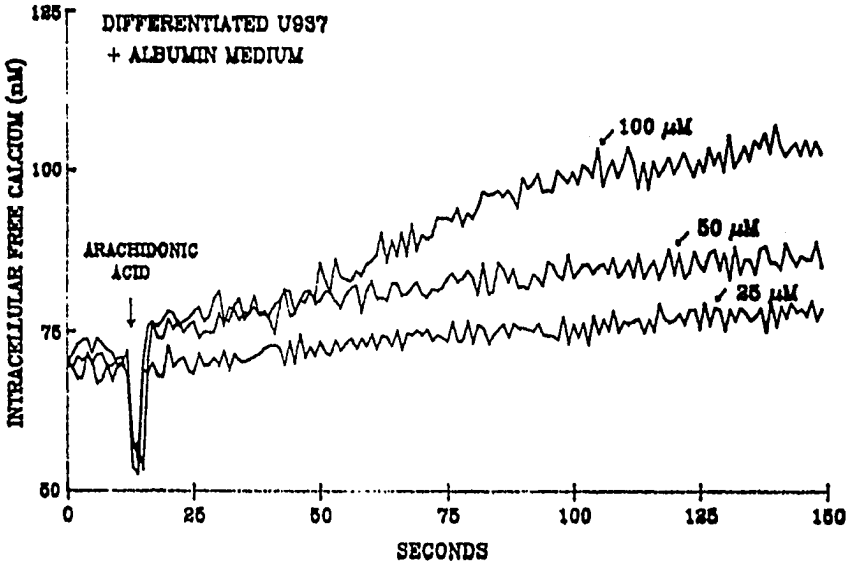


FIGURE 25

further experiments were performed in albumin (2.5 μM) containing medium, using a fatty acid concentration of 100 μM. We reasoned that under these conditions, sufficient free fatty acid would be present, unbound to albumin, to allow adequate stimulation of cells.

To determine the requirements for extracellular calcium, differentiated cells were suspended in albumin containing medium. EGTA (1.1 mM, 2.5 μl) was added to cells in the spectrofluorometer cuvette, followed by 100 μM arachidonate (2.5 μl). Chelation of extracellular calcium with EGTA essentially abolished the effects of 100 μM arachidonate (Fig. 26). This indicates that the elevations of $[Ca^{2+}]_i$ observed in response to stimulation with exogenous arachidonate are due to influx of extracellular calcium.

Pre-treatment of differentiated U937 with NDGA, an inhibitor of cytochrome P450-mediated arachidonate metabolism, had no effect on $[Ca^{2+}]_i$ elevation stimulated by 100 μM arachidonate (Fig 26B). As NDGA inhibits cytochrome P450-medaited arachidonate metabolism, these results suggest that metabolism of arachidonate by this pathway is not required for stimulation of influx of extracellular calcium by exogenous arachidonate.

Pre-treatment of differentiated cells with PMA enhanced thapsigargin-stimulated influx of extracellular calcium (Chapter 4). It was therefore of interest to determine whether PMA pre-incubation could enhance the effects of exogenous arachidonate on elevations in $[Ca^{2+}]_i$. Results shown in Figure 27 demonstrate that a 10 minute pre-incubation of differentiated cells with 10 nM PMA potentiated exogenous arachidonate-stimulated elevations in $[Ca^{2+}]_i$ by 2 fold.

FIGURE 26

A. EFFECTS OF EXOGENOUS ARACHIDONATE ON ELEVATIONS IN $[Ca^{2+}]_i$ ARE DEPENDENT ON EXTRACELLULAR CALCIUM.

B. EFFECTS OF EXOGENOUS ARACHIDONATE ON ELEVATIONS IN $[Ca^{2+}]_i$ ARE NOT AFFECTED BY NDGA.

In Figure 26A, differentiated cells were stimulated with arachidonate in the presence of extracellular calcium, or in the presence of EGTA to chelate extracellular calcium, as described in the text. Measurement of $[Ca^{2+}]_i$ was performed as described in Section 2.9.

In Figure 26B, Fura-2 loaded differentiated cells treated with NDGA (40 μM) as indicated by the arrow. Approximately 2 minutes later, cells were stimuated with 100 μM arachidonate. Results shown are representative of three similar experiments perfomed on different days.

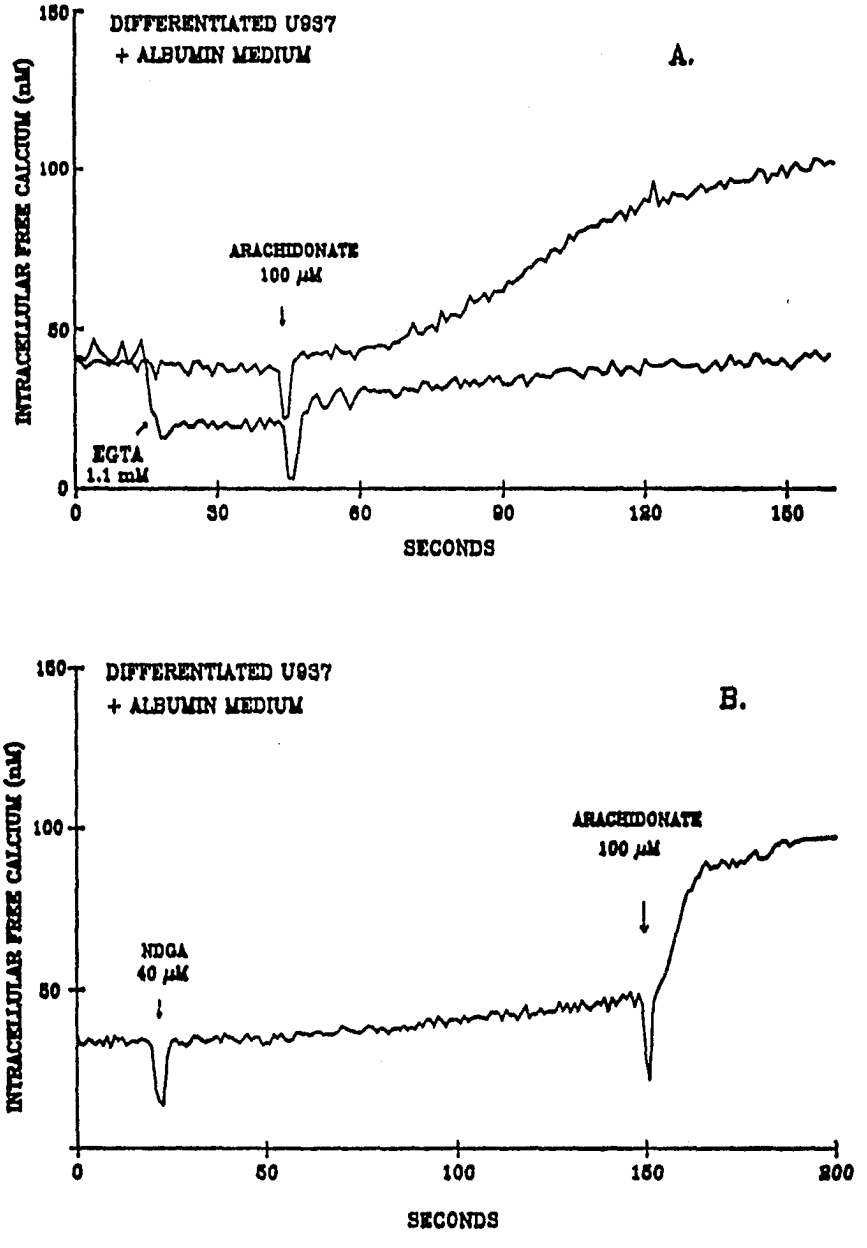


FIGURE 26

FIGURE 27

EFFECTS OF PMA PRE-INCUBATION ON ARACHIDONATE-STIMULATED ELEVATIONS IN $[Ca^{2+}]_i$.

Differentiated cells were loaded with Fura-2, washed, and pre-incubated with or without PMA (10 nM) as described in Methods. Cells were stimulated with arachidonate as described in Figure 25. Results represent the maximum $[Ca^{2+}]_i$ level attained in response to the indicated treatment. Basal levels represent $[Ca^{2+}]_i$ in unstimulated cells. "PMA" indicates basal $[Ca^{2+}]_i$ levels after a ten minute PMA pre-incubation.

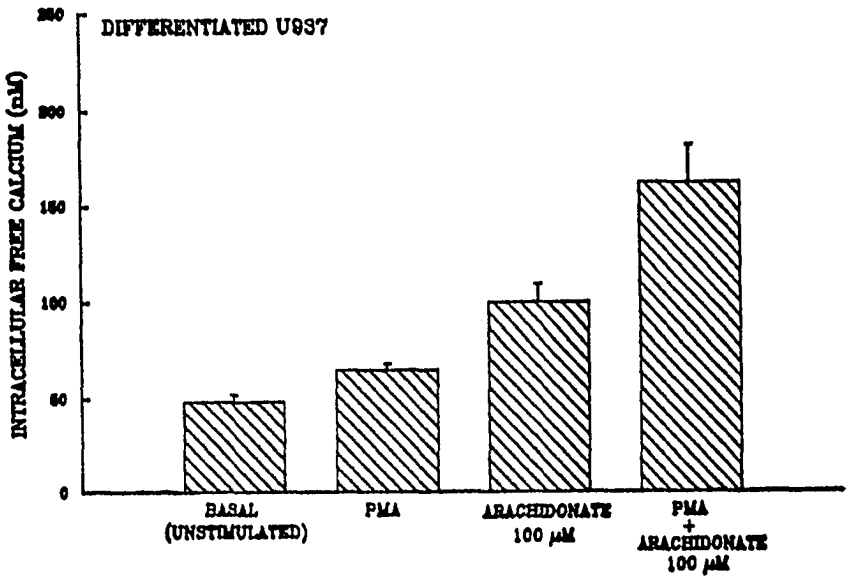


FIGURE 27

5.2.2 Effects of exogenous arachidonate on mobilization of endogenous arachidonate.

The findings obtained thus far suggest that exogenous arachidonate is capable of activating calcium influx in differentiated U937 cells. Results presented in Chapter 4 indicated that $cPLA_2$ activity (and hence release of endogenous arachidonate), occurred in response to elevations in $[Ca^{2+}]_i$ due to both intracellular calcium store depletion, as well as influx of extracellular calcium through SOC. If exogenous arachidonate activates influx of extracellular calcium through SOC, then it should also activate $cPLA_2$ and induce release of endogenous arachidonate.

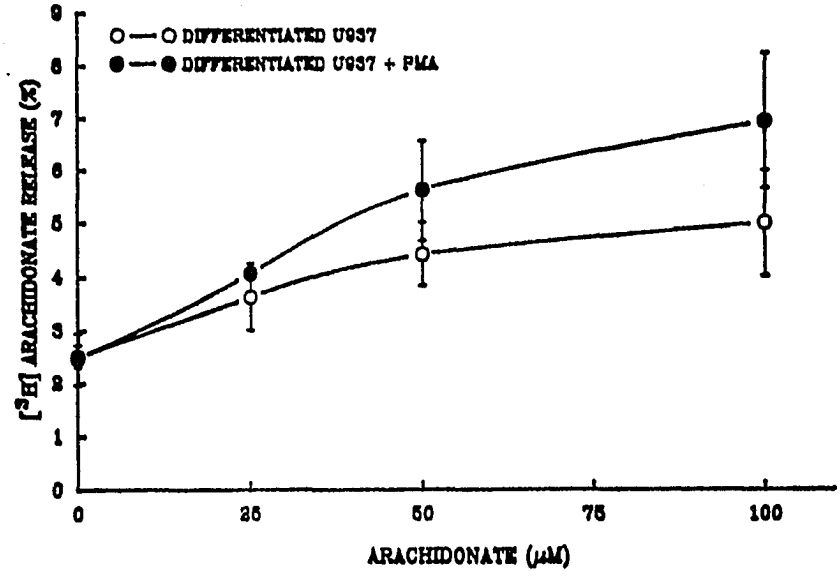
Differentiated and undifferentiated U937 cells were challenged with exogenous arachidonate (25-100 μM) and mobilization of endogenous radiolabeled arachidonate was measured as described in Section 2.4. Undifferentiated cells did not release endogenous arachidonate when stimulated with exogenous arachidonate concentrations of up to 200 μM (data not shown). However, Figure 28 indicates that in differentiated cells, exogenous arachidonate activated mobilization of endogenous arachidonate in a dose dependent manner.

Fatty acid stimulation of endogenous arachidonate release was relatively specific for exogenous arachidonate. As indicated in Figure 29, neither linoleate nor oleate were as effective as exogenous arachidonate. Furthermore, PMA pretreatment potentiated the effects exogenous arachidonate on the release of endogenous arachidonate by 50-60% (Figure 29, filled bars). PMA pre-incubation had no significant effect on linoleate of oleate stimulated release of endogenous arachidonate.

FIGURE 28

EXOGENOUS ARACHIDONATE STIMULATES MOBILIZATION OF ENDOGENOUS ARACHIDONATE.

Differentiated cells were prepared and radiolabeled as described in Methods 2.2-2.3. Arachidonate mobilization in response to stimulation with exogenous arachidonate was as described in Methods 2.4. Results represent the mean ± SE of 3-4 separate experiments performed in duplicate.



FIGUE 28

FIGURE 29

EFFECTS OF EXOGENOUS FATTY ACIDS ON MOBILIZATION OF ENDOGENOUS ARACHIDONATE ARE SPECIFIC FOR ARACHIDONATE.

Radiolabeled differentiated cells were pre-incubated with or without PMA as described in Section 2.4. Cells were stimulated with the indicated concentrations of arachidonate, oleate, or linoleate as described in Figure 28. Results are normalized to percent increase over basal (unstimulated) arachidonate release, and represent the mean $\pm$ SE of 3 separate experiments performed in duplicate.

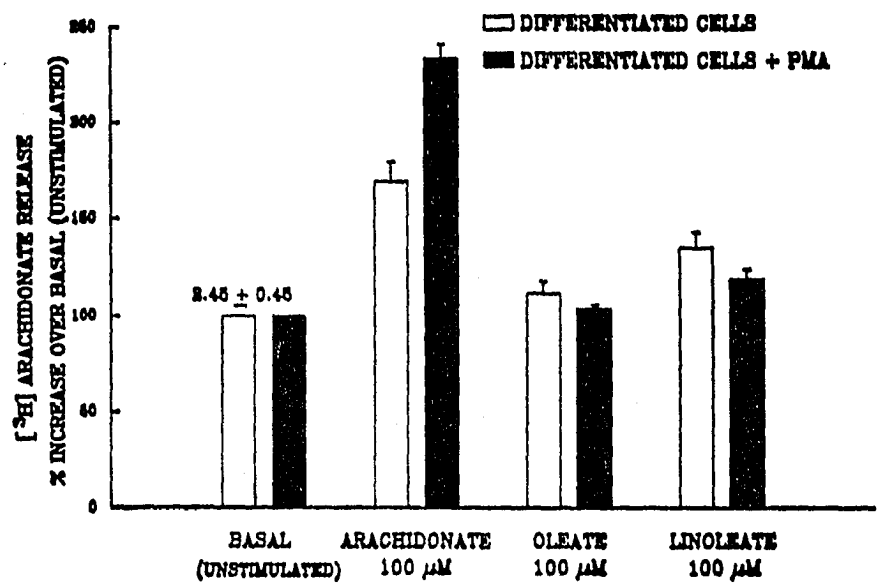


FIGURE 29

5.2.3 Production of arachidonate metabolites by differentiated U937 cells in response to thapsigargin stimulation.

Results thus far suggest that exogenous free arachidonate stimulates both elevation in $[Ca^{2+}]_i$ (*via* influx of extracellular calcium) and release of endogenous arachidonate in differentiated U937 cells. However, it is not clear whether arachidonate itself, or a metabolite of arachidonate, is directly responsible for these observations. Therefore, the products of thapsigargin-stimulated arachidonate release were examined.

For these experiments, differentiated U937 cells were stimulated with 1 μM thapsigargin (± PMA), as described (Section 2.7). The entire incubation volume was acidified and extracted twice with ethyl acetate (Section 2.7). Extracts were chromatographed in ethyl acetate/iso-octane/acetic acid/water (110:50:20:10) (Section 2.7). Thin layer chromatography results indicated that very little arachidonate metabolites were generated during stimulation with thapsigargin or PMA + thapsigargin. Released radiolabel was primarily in the form of free arachidonate (data not shown).

However, the low sensitivity of thin layer chromatography, combined with the extremely labile nature of many arachidonate metabolites, prompted further examination of extracts from thapsigargin-stimulated cells by HPLC. Thus, the same experiments were repeated, and examined via HPLC as described in Section 2.8. As our HPLC system is not presently capable of a positive identification of either prostaglandins or P450 arachidonate metabolites by mass, the column effluent was collected in 1 ml fractions. The presence of [^{3}H]arachidonate metabolites in the fractions was determined by liquid scintillation. Compounds were tentatively identified by their reported retention

times (72). Lipoxygenase products were measured by increases in absorbance at 270 nm (237 nM for 5-HETE) during each HPLC run.

The results shown in Figure 30A indicate that [^{3}H] labeled arachidonate metabolites corresponding to retention times reported for prostaglandins, P450 products, and free arachidonate were produced in response to thapsigargin stimulation. Approximately 61% of the radiolabel was detected as free arachidonate, with 35% and 4% present as prostaglandin and P450 metabolites, respectively. UV absorbance measurements made directly on column effluents confirmed that no lipoxygenase products were produced in differentiated U937 cells (data not shown), consistent with reports that indicate that these cells contain no 5-lipoxygaenase (77). Similar products were obtained in differentiated U937 cells pre-incubated with PMA, however the relative percentage of free arachidonate was increased to 70% (27 % prostaglandins and 3 % P450 products; Figure 30B). Thus, free fatty acid is the primary arachidonate product generated in thapsigargin-stimulated differentiated U937 cells.

To examine the presence of cytochrome P450 arachidonate metabolites, additional incubations were done in the presence of 10 μM indomethacin (with or without thapsigargin ± PMA). Indomethacin inhibits the cyclooxygenase pathway of the arachidonate cascade, thereby inhibiting production of prostaglandin metabolites (11). Thus, arachidonate metabolites formed in response to thapsigargin or PMA + thapsigargin, which were inhibited by incubation with indomethacin, would be cyclooxygenase products. Likewise, any products formed that were not inhibited by indomethacin would be cytochrome P450 metabolites, as lipoxygenase products are not produced by these cells.

FIGURE 30

ARACHIDONATE METABOLITES PRODUCED IN RESPONSE TO THAPSIGARGIN OR PMA + THAPSIGARGIN STIMULATION IN DIFFERENTIATED U937 CELLS.

Differentiated cells were prepared and radiolabeled as described in Sections 2.2-2.3. Cells were pre-incubated with (Figure 30A) or without (Figure 30B) PMA (10 nM), followed by thapsigargin stimulation (Methods 2.8). Combined cells and supernatants from thapsigargin-stimulated incubations were extracted with ethyl acetate and analyzed by reverse phase HPLC in a methanol/water/ phosphoric acid gradient as described in Methods 2.8. Results are representative of 2 separate experiments performed in duplicate.

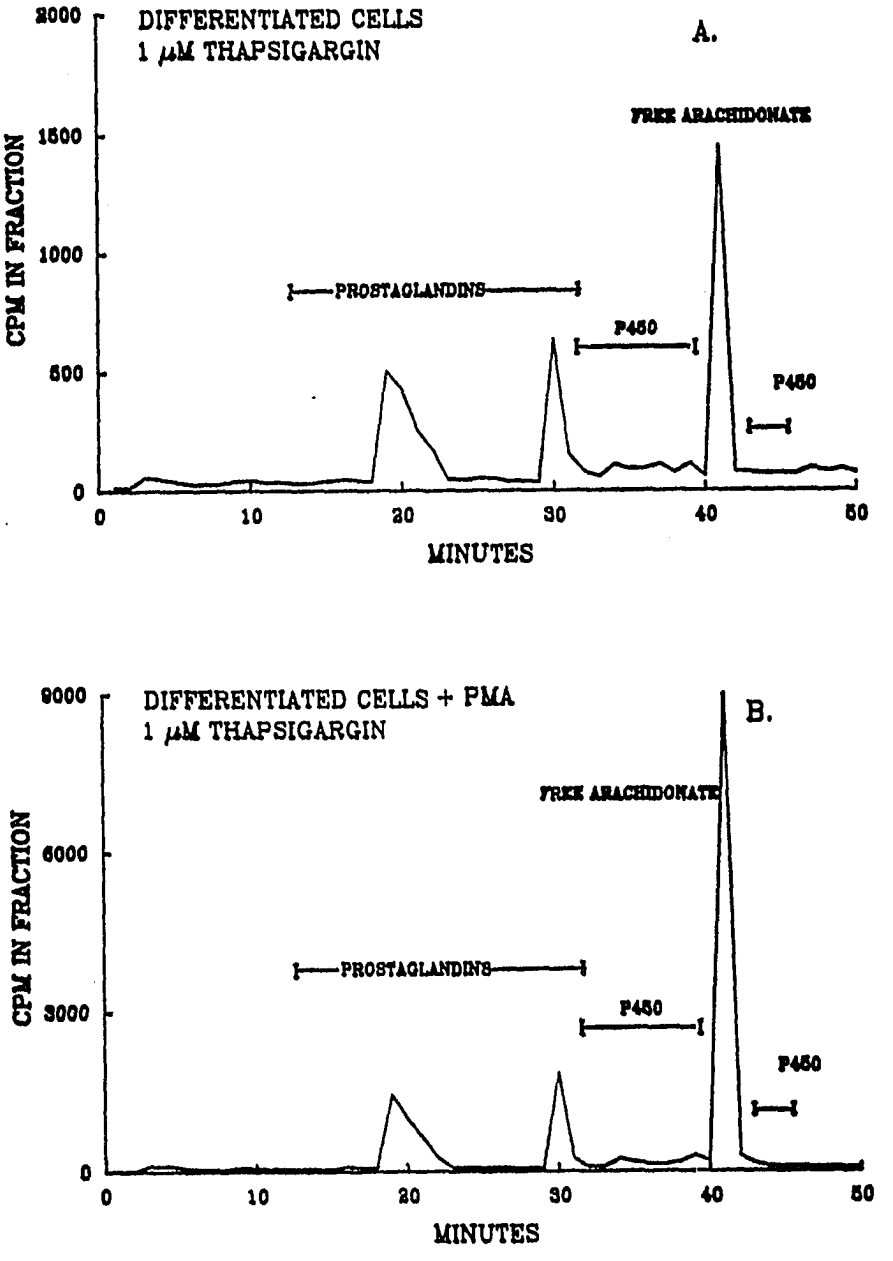


FIGURE 30

Indomethacin inhibited production of [^{3}H] arachidonate metabolites identified as prostaglandins (Fig. 31A). Thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ and arachidonate release were unaffected by indomethacin treatment (data not shown). This precludes a role for prostaglandin metabolites in the capacitative pathway.

In differentiated cells treated with indomethacin, an increase in radiolabel associated with retention times of P450 arachidonate metabolites was detected (Figure 31A). However, if these P450 products were associated with elevation of $[Ca^{2+}]_i$, one would expect that indomethacin would increase thapsigargin-stimulated elevations in $[Ca^{2+}]_i$. Such an increase in $[Ca^{2+}]_i$ was not detected in response to indomethacin.

Furthermore, in indomethacin-treated cells stimulated with PMA + thapsigargin, 97% of the released radiolabel was in the form of free arachidonate. Indomethacin did not potentiate formation of cytochrome P450 metabolites (Figure 31B). Considering that PMA pre-treatment enhances both thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ and arachidonate release, it does not appear likely that a P450 arachidonate metabolite is involved in elevation of $[Ca^{2+}]_i$.

5.3 Discussion

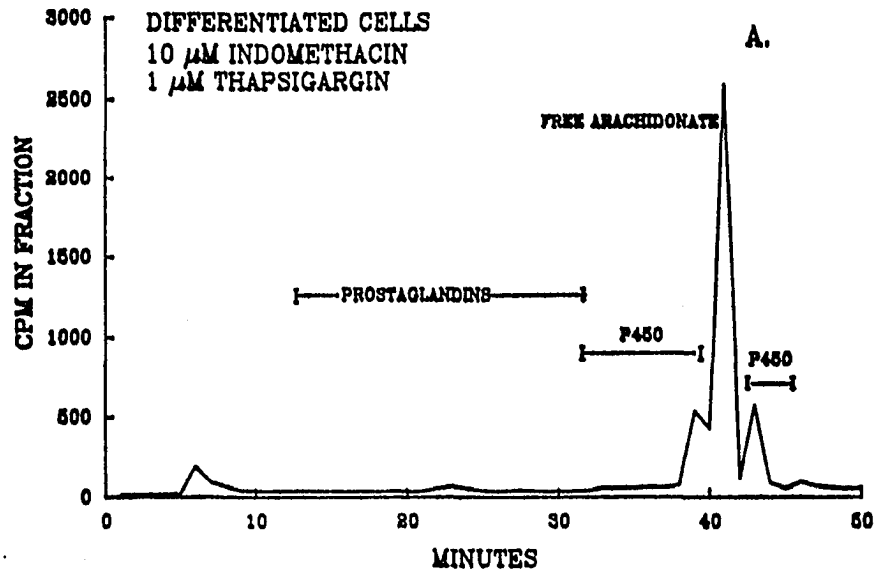
The results presented in Chapter 4 suggested that arachidonate and/or its metabolites may be linked to the generation of a CIF, which functions as a communication link between empty intracellular calcium stores and SOC. The results presented in this Chapter suggest that free arachidonate itself may be a component of the CIF.

Consistent with this hypothesis, exogenous free arachidonate stimulated an elevation in $[Ca^{2+}]_i$ in differentiated U937 cells. In the presence of albumin (2.5 μM)

FIGURE 31

EFFECTS ON INDOMETHACIN ON ARACHIDONATE METABOLITES PRODUCED IN RESPONSE TO THAPSIGARGIN OR PMA + THAPSIGARGIN STIMULATION IN DIFFERENTIATED U937 CELLS.

Differentiated cells were stimulated as described in Figure 30. Indomethacin (10 μM) was added during the pre-incubation period, as described in the text. HPLC was performed as described in Figure 30. Results are representative of 2 separate experiments performed in duplicate. The fractions in which prostaglandins, P450 metabolites, and free arachidonate elute are indicated.



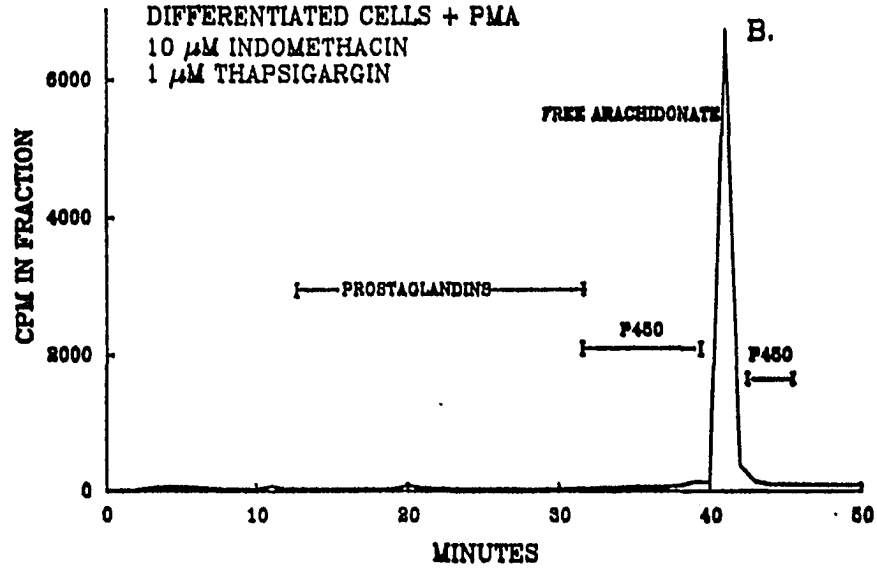


FIGURE 31

arachidonate (100 μM) elevated $[Ca^{2+}]_i$ 2-fold over basal levels. Similar results were observed in thyroid FRTL-5 cells (13). This increase is quite striking, considering that 1.) a substantial portion of the exogenous arachidonate is bound to albumin and unavailable to the cell and 2.) exogenous treatment with arachidonate places a potential intracellular second messenger on the wrong side of the plasma membrane. As it is incorporated intracellularly, it is also subject to metabolic systems such as β oxidation and esterification, which would make the effective concentration of free arachidonate in the cell even lower. Neither linoleate nor oleate were as effective as arachidonate, suggesting that these observations are not due to non-specific effects of fatty acids on membrane permeability.

Undifferentiated cells did not respond to exogenous arachidonate with an elevation in $[Ca^{2+}]_i$. This suggests that free arachidonate is a specific stimulus for elevation of $[Ca^{2+}]_i$ only in those cells with a fully developed capacitative calcium influx pathway. As results in chapter 4 demonstrate that undifferentiated U937 cells do not appear to have such a pathway, it follows that undifferentiated cells do not respond to exogenous arachidonate with an elevation in $[Ca^{2+}]_i$.

Exogenous arachidonate-stimulated elevations in $[Ca^{2+}]_i$ were dependent on extracellular calcium. In the presence of EGTA, exogenous arachidonate had no effect on $[Ca^{2+}]_i$. Thus, exogenous arachidonate appears to elevate $[Ca^{2+}]_i$ downstream of intracellular store depletion, at the level of the calcium influx channel.

Pre-incubation of differentiated U937 cells with PMA potentiated the effects of exogenous arachidonate on elevations in $[Ca^{2+}]_i$. Results presented in Chapter 4 demonstrated that PMA pretreatment potentiated influx of capacitative calcium, but did

not affect intracellular calcium stores. This suggests that PMA enhances capacitative calcium influx downstream of intracellular store depletion and generation of the CIF, at the level of the SOC channel. If arachidonate does, in fact, activate the SOC channel, then its effects should be potentiated by PMA. In support of this hypothesis, PMA pre-incubation potentiated elevations in $[Ca^{2+}]_i$ stimulated by exogenous arachidonate. As PMA activates PKC (50) this could be mediated by effects of PKC on SOC channels. Alternatively, PKC could phosphorylate other components of the CIF, which enhance association with free arachidonate and further activation of influx of extracellular calcium.

Thapsigargin-stimulated $cPLA_2$ activity was initially coupled to depletion intracellular calcium stores. A second phase of arachidonate mobilization was coupled to influx of extracellular calcium (Chapter 4). In these experiments, exogenous arachidonate stimulated influx of extracellular calcium, possibly through SOC. Consistent with the second phase of thapsigargin stimulated $cPLA_2$ activity, exogenous arachidonate also stimulated release of endogenous arachidonate. Thus, consistent with the model for $cPLA_2$ regulation presented in Chapter 4, exogenous arachidonate activated influx of extracellular calcium, which in turn stimulated $cPLA_2$ activity at the level of the calcium influx channel. Furthermore, PMA potentiated exogenous arachidonate-stimulated release of endogenous arachidonate. In Chapter 4, it was demonstrated that PMA potentiation of $cPLA_2$ activity occurred during influx of extracellular calcium, and did not affect that $cPLA_2$ activity coupled to depletion of intracellular calcium stores. Consistent with these observations, PMA potentiation of arachidonate-stimulated release of endogenous arachidonate, also occurs during calcium

influx, downstream of store depletion.

Examination of arachidonate and arachidonate metabolites generated in response to thapsigargin stimulation of differentiated U937 cells indicated that free arachidonic acid itself was the primary product. Although prostaglandin synthesis was evident, inhibition of the cyclooxygenase pathway with indomethacin had no effect on thapsigargin-stimulated elevations in $[Ca^{2+}]_i$ or arachidonate release. Thus, prostaglandin metabolites do not appear to be involved in the capacitative pathway.

Previous experiments with the cytochrome P450 inhibitor, econazole, suggested that a P450 arachidonate metabolite may by involved in generation of the CIF. The amounts of P450 arachidonate metabolites produced during thapsigargin stimulation of differentiated U937 were very low (3-4% of the total radiolabled products) relative to free arachidonate (61-75%). Additionally, pretreatment of differentiated U937 cells with an inhibitor of the P450 system of arachidonate metabolism, NDGA, did not block the effects of exogenous arachidonate. Thus, it does not appear likely that a P450 arachidonate metabolite is involved in the generation of CIF. However, it is possible that some P450 metabolites were not separated from free arachidonate in our HPLC system. Thus, although primary products appear to be free arachidonate, GC/mass spectrometry would be required for positive confirmation.

Taken together, these results strongly suggests that free arachidonate may be a functional component of an intracellular effector system which serves as a link in the communication between intracellular calcium stores and SOC in the plasma membrane. This may occur through a direct action of arachidonate on the membrane environment surrounding calcium channels. Arachidonic acid and its metabolites have been

demonstrated to modify ion channel gating in a variety of cell types (113). Alternatively, arachidonic acid may be a necessary component for the assembly of an effector complex, which in turn regulates the opening of capacitative calcium influx channels. It has been suggested that the signaling link between intracellular calcium stores and plasma membrane calcium channel involves a phosphorylation/dephosphorylation reaction (89, 94, 114); possibly regulated by a low molecular weight GTPase (86, 95). Free arachidonate has been shown to modulate the activity of GAP (115) and the translocation of rac p21s to the plasma membrane (105). Thus, an association between arachidonate and a low molecular weight GTPase or GTPase activating protein, may participate in regulation of capacitative calcium influx. Fasolato (95) suggests that the GTPase sensitive step in capacitative calcium influx may involve transport of vesicles containing calcium channels to the plasma membrane. In a cell free system, membrane fusion was modulated by both GTP and polyunsaturated fatty acids, of which linoleic and arachidonic were most effective (116).

In summary, depletion of intracellular calcium stores and activation of capacitative calcium influx in differentiated U937 cells results in release of significantly higher amounts of free arachidonate than other eicosanoid metabolites. Furthermore, results presented in this chapter suggest that free arachidonate may function as an intracellular second messenger that regulates $[Ca^{2+}]_i$ levels by activation of capacitative calcium influx. This suggests a heretofore unknown role for $cPLA_2$, apart from its well known role in initiation of the arachidonate cascade. In addition to being regulated by low levels of calcium itself, $cPLA_2$ may participate in the regulation $[Ca^{2+}]_i$ of cytoplasmic compartment. Initially, $cPLA_2$ would be activated by a local increase in

$[Ca^{2+}]_i$ at the membrane of intracellular calcium stores in response to agonist-stimulated store depletion. Release of free arachidonate would then activate further increases in $[Ca^{2+}]_i$, through formation of a CIF. Thus, $cPLA_2$ may be have a functional role in regulating intracellular free calcium levels, by mediating release of free arachidonate in response to agonist stimulation.

CHAPTER 6

ARACHIDONATE MOBILIZATION IS COUPLED TO THE CAPACITATIVE CALCIUM INFLUX PATHWAY IN RECEPTOR-MEDIATED EVENTS

6.1 Background

The basic concepts for $cPLA_2$ regulation presented in Figure 22 were derived using thapsigargin as a direct stimulus for intracellular calcium store depletion. In actual receptor mediated events, G-protein or tyrosine kinase linked effector systems activate PLC, followed by generation of the second messenger IP_3 (87, 93). IP_3 binds to a receptor on intracellular calcium stores, stimulating a rapid efflux of calcium (85, 86, 88). In contrast, thapsigargin inhibits the Ca^{2+}-ATPase of intracellular stores (99). Stores are depleted by a slow leakage of calcium, rather than the rapid release of store calcium seen in IP_3 mediated events. Both mechanisms produce similar effects; depletion of store calcium followed by activation of capacitative influx.

If the model presented for regulation of $cPLA_2$ in differentiated U937 is applicable to physiological signal transduction, then the basic components of this

model should occur during actual receptor mediated stimulation. These include the following parameters:

1. Agonists which activate the IP_3 pathway through receptor mediated events should also activate $cPLA_2$.
2. Receptor mediated events that do not elevate $[Ca^{2+}]_i$ via the capacitative pathway should not activate arachidonate release.
3. Mobilization of arachidonate should be initiated in response to depletion of intracellular calcium stores.
4. Mobilization of arachidonate should also accompany capacitative calcium influx.
5. The time course of agonist-stimulated arachidonate release should correlate with the time course of agonist-stimulated elevations in $[Ca^{2+}]_i$.
6. Inhibition of $cPLA_2$ activity should inhibit agonist-stimulated capacitative calcium influx.
7. Arachidonate mobilization observed during receptor mediated events should be consistent with activation of $cPLA_2$.

The experiments presented in this Chapter are designed to address these issues.

Chemoattractants such as fMLP, stimulate both $[Ca^{2+}]_i$ elevation and release of arachidonate in neutrophils (49, 117) and monocytes (118, 119). In both these cell types, fMLP receptors are coupled to an IP_3 dependent signal transduction mechanisms (120, 121). Thus, fMLP results in activation of the capacitative calcium influx pathway. In human neutrophils, calcium influx stimulated by fMLP had identical

properties to that stimulated by thapsigargin (119). Consequently, fMLP was chosen as an agonist for investigation in this study.

The purinergic receptor agonist, ATP, stimulates elevation of $[Ca^{2+}]_i$ in U937 (100, 122), macrophages (96), neutrophils (111,118), and other cells (13, 123). U937 cells have been reported to contain 2 subtypes of purinergic receptors, P_{2U} and P_{2z} (118, 122). The P_{2Z} purinergic receptor activates a ligand gated channel cation channel, permeable to Na^+, K^+, and Ca^{2+} (124). The channel is activated by ATP (but not UTP), and inhibited by Mg^{2+} (124). In the macrophage, activation of P_{2Z} purinergic receptors generates a rapid, almost instantaneous, increase in $[Ca^{2+}]_i$, dependent on extracellular calcium only (96, 124). There is no sustained phase of calcium influx, as is seen in activation of the capacitative pathway. IP_3 is not produced and intracellular stores are unaffected. Rather, receptor-ligand binding results in direct activation of a receptor operated channel (ROC).

In contrast, the P_{2U} purinergic receptor functions via activation of PLC, generation of IP_3, and activation of the capacitative calcium influx pathway. Both ATP and UTP activate this purinergic receptor subtype (123, 124). In the macrophage, P_{2U} receptor activation results in an initial rapid elevation of $[Ca^{2+}]_i$ due to emptying of intracellular stores, followed by a sustained phase of capacitative calcium influx (96). In the case of the P_{2U} receptor, influx of divalent cation is relatively specific for calcium as opposed to sodium and potassium (123-125). As preliminary results indicated that differentiated U937 respond to ATP with an elevation of $[Ca^{2+}]_i$, ATP was also chosen as an agonist for further study in differentiated U937 cells.

6.2 Results

6.2.1 Effects of the chemotactic peptide, fMLP, on elevation of $[Ca^{2+}]_i$ in differentiated and undifferentiated U937 cells.

fMLP (10 μM) had no effect on $[Ca^{2+}]_i$ in undifferentiated cells (results not shown). This is not surprising, as reports indicate that undifferentiated U937 cells do not have receptors for chemotactic peptides (68, 122). fMLP receptors characteristically develop in U937 cells upon monocytic differentiation (68).

In differentiated cells, fMLP produced a dramatic elevation in $[Ca^{2+}]_i$ (Figure 32). fMLP induced an initial rapid increase in $[Ca^{2+}]_i$, followed by a sustained phase during which maximal levels of $[Ca^{2+}]_i$ were attained. Stimulation of differentiated cells in calcium free medium abolished the sustained phase of $[Ca^{2+}]_i$ elevation (Figure. 32, lower trace). Similar results were observed when extracellular calcium was chelated with EGTA prior to fMLP stimulation (Figure 33). However, even in the absence of extracellular calcium, fMLP elevated $[Ca^{2+}]_i$ approximately 3 fold over basal levels. These results are consistent with activation of the capacitative pathway and mobilization of calcium from intracellular stores.

To further dissect fMLP stimulated elevations in $[Ca^{2+}]_i$ with respect to influx of extracellular calcium, differentiated U937 cells were treated with various inhibitors of calcium influx, as described in Chapter 4. Econazole (2 μM), an inhibitor of cytochrome P450, blocked 40-50% of the fMLP-stimulated elevation in $[Ca^{2+}]_i$, (Figure 33) similar to the effects observed in thapsigargin-stimulated cells. Ni^{2+}, which physically blocks calcium channels, also reduced fMLP-stimulated increases in $[Ca^{2+}]_i$

FIGURE 32

EFFECTS OF fMLP ON $[Ca^{2+}]_i$ IN DIFFERENTIATED U937 CELLS.

Differentiated U937 cells were loaded with Fura-2 and stimulated with 10 μM fMLP as described in Methods 2.9. The results shown are representative of 5-6 similar experiments performed on separate days.

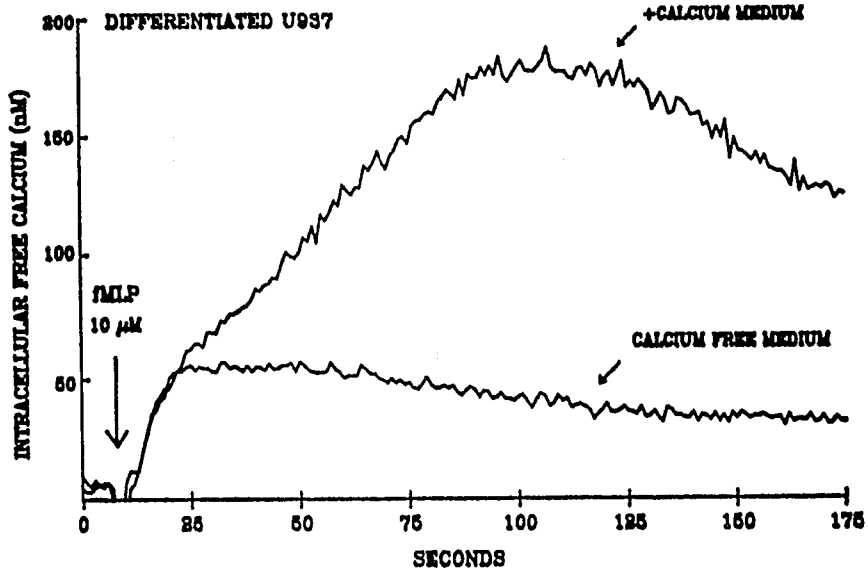


FIGURE 32

FIGURE 33

EFFECTS OF INHIBITORS OF CAPACITATIVE CALCIUM INFLUX ON fMLP-STIMULATED ELEVATIONS IN $[Ca^{2+}]_i$.

Differentiated cells were loaded with Fura-2 as described in Methods 2.9. Inhibitors were added directly to cells in the spectrofluorometer cuvette, at the concentrations indicated in the Figure. fMLP was added after addition of inhibitors, as described in Figure 14. For calcium-free experiments, cells were prepared and stimulated as described in Figure 13. Results were normalized to the maximum $[Ca^{2+}]_i$ level achieved by 10 μM fMLP (279.5 ± 50 nM) and represent the percentage of the 10 μM fMLP response. Data shown represents the mean ± SE of 3-5 separate experiments.

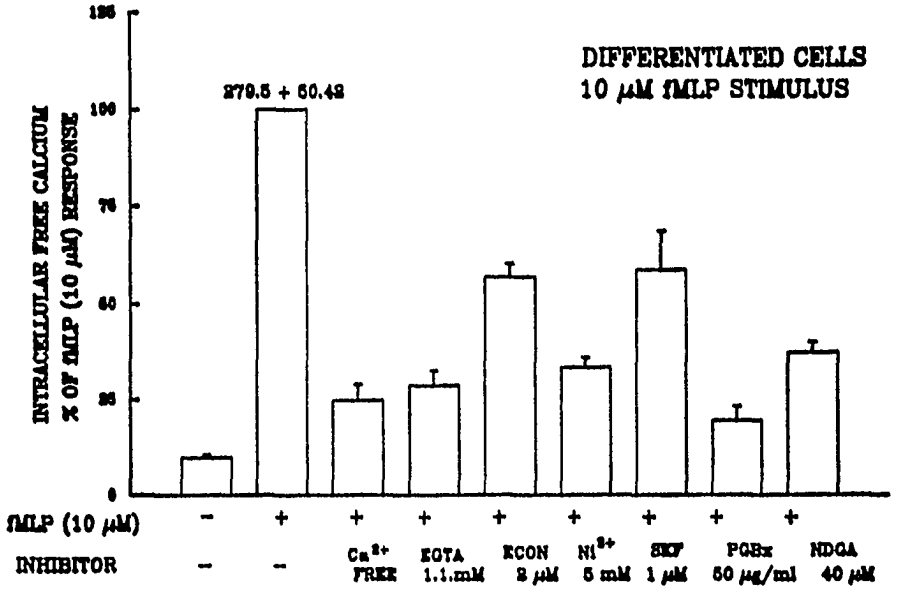


FIGURE 33

by 60% (Figure 33). SKF96365 (1 μM), which specifically blocks capacitative calcium influx channels, inhibited 42% of the fMLP-stimulated increase in $[Ca^{2+}]_i$ response. Thus, consistent with effects observed in thapsigargin-stimulated differentiated cells, inhibitors of capacitative calcium influx inhibited that portion of the fMLP induced $[Ca^{2+}]_i$ elevations associated with influx of extracellular calcium.

Inhibition of $cPLA_2$ with PGBx also inhibited fMLP-stimulated increases in $[Ca^{2+}]_i$. Treatment of differentiated cells with PGBx resulted in an $[Ca^{2+}]_i$ elevation equivalent to that obtained when differentiated cells were stimulated with fMLP in the absence of extracellular calcium (i.e. when $[Ca^{2+}]_i$ elevations were produced solely by store depletion). Thus, inhibition of $cPLA_2$ effectively blocked capacitative calcium influx in fMLP-stimulated cells. This supports the hypothesis that $cPLA_2$ activity may be coupled to generation of a CIF. NDGA also inhibited fMLP-stimulated increases in $[Ca^{2+}]_i$ but only by approximately 40-45%. Thus a portion of capacitative calcium influx was inhibited, but not as effectively as with PGBx.

6.2.2 Effects of fMLP on arachidonate mobilization in differentiated and undifferentiated U937.

For these experiments, arachidonate mobilization was assayed as described in Section 2.4 using 10 μM fMLP as a stimulus. In initial experiments, agonist-stimulated incubations for arachidonate mobilization were conducted for 5-10 minutes. Under these conditions, the observed levels of fMLP-stimulated arachidonate release were weak (only 1-3% above unstimulated levels), and in some cases undetectable. Experiments in Chapter 4 indicate that arachidonate mobilization is maximal at or

slightly before the maximal level of $[Ca^{2+}]_i$ attained in response to stimulation. In the case of fMLP, this was 2 minutes. When incubations were conducted for this shorter time period, significant arachidonate release was observed (8-11% of total incorporated radiolabel). Incubation for longer time periods resulted in re-incorporation of released arachidonate, as evidenced by the appearance of radiolabel in the triglyceride fraction of lipid extractions of stimulated cells. This re-incorporation resulted in underestimation of arachidonate mobilization. Thus, all subsequent agonist stimulations were terminated at the time of maximal $[Ca^{2+}]_i$

As can be seen in Figure 34, fMLP (10 μM) resulted in a 4 fold increase over basal (unstimulated) levels of arachidonate release. Incubation in calcium free medium resulted in inhibition of only 40-45% of fMLP-stimulated arachidonate mobilization. Thus, a significant portion of fMLP-stimulated arachidonate mobilization was coupled to influx of extracellular calcium. However, 55-60% of the fMLP response was sustained in the absence of extracellular calcium, by mobilization of calcium from intracellular stores alone.

To further define the effects of influx of extracellular calcium on fMLP-stimulated arachidonate mobilization, the effects of inhibitors of capacitative calcium influx were examined as in Section 4.2.5. Consistent with effects seen with thapsigargin stimulation, econazole (2 μM) enhanced fMLP-stimulated arachidonate mobilization by approximately 25%. Inhibition of capacitative calcium influx with Ni^{2+} and SKF96365 inhibited 45 and 50 % of the fMLP stimulated response, respectively. Therefore, significant arachidonate release is also coupled to fMLP-stimulated influx of extracellular calcium.

FIGURE 34

EFFECT OF INHIBITION OF CAPACITATIVE CALCIUM INFLUX ON fMLP-STIMULATED ARACHIDONATE MOBILIZATION IN DIFFERENTIATED U937 CELLS.

fMLP-stimulated arachidonate mobilization was determined in the presence of the inhibitors indicated, as described Figure 18. For calcium-free experiments, and experiments involving extracellular strontium, cells were prepared and stimulated as described in Figure 17. The concentrations of inhibitors were as follows : EGTA-1.1 mM; Econazole-2 μM; Ni^{2+}-5 mM; SKF96365-1 μM; PGBx-50 μg/ml; NDGA-40 μM; and Sr^{2+}-1.1 mM. Results were normalized to the release of radiolabel observed in response to 10 μM fMLP (9.09 ± 1.21% of total incorporated cellular radiolabel) and represent the percentage of the 10 μM fMLP response. Data shown represents the mean ± SE of 3 separate experiments.

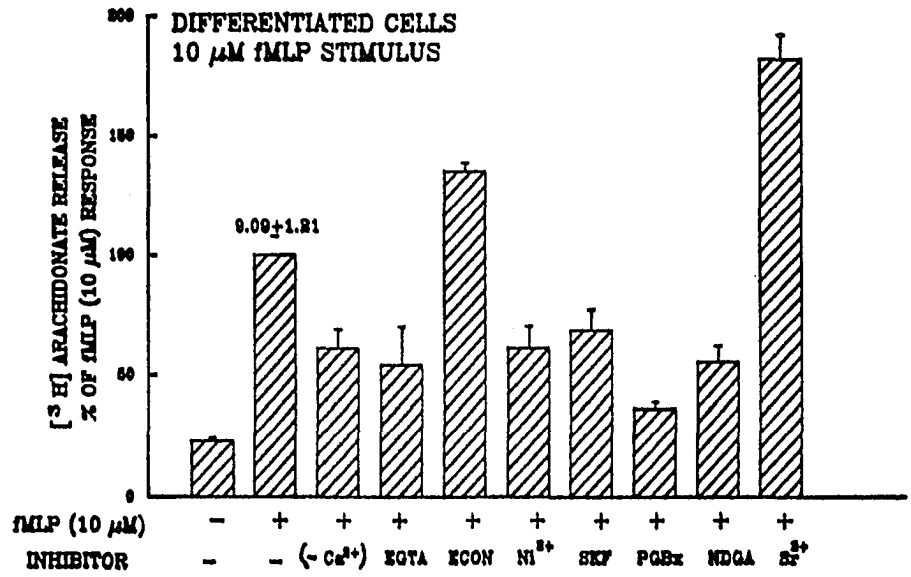


FIGURE 34

fMLP-stimulated arachidonate mobilization was supported in the presence of extracellular strontium, at levels that were approximately 2-fold higher than that observed in the presence of extracellular calcium (Figure 34). $cPLA_2$ can utilize strontium for translocation to membrane substrates, whereas whereas $sPLA_2$ has an absolute requirement for calcium at the catalytic site. Thus, these results suggest that $cPLA_2$ activity is coupled to the intracellular signal transduction mechanism observed in these experiments. Furthermore, PGBx and NDGA both inhibited fMLP stimulated arachidonate release (Figure 34) and elevations in $[Ca^{2+}]_i$ (Figure 33). Therefore, $cPLA_2$ activity appears to be coupled to fMLP-stimulated $[Ca^{2+}]_i$ elevations and arachidonate mobilization.

6.2.3 Time course of fMLP-stimulated $[Ca^{2+}]_i$ elevations and arachidonate release.

If $cPLA_2$ activity is coupled to generation of a CIF, then the temporal release of arachidonate during fMLP stimulation should follow a similar course as the temporal increases in $[Ca^{2+}]_i$. Figure 35 demonstrates that this does occur. Arachidonate release was initiated within the first 10 seconds of fMLP stimulation, corresponding to the time during which calcium is mobilized from intracellular stores. Both $[Ca^{2+}]_i$ and arachidonate release increased until the maximal levels of $[Ca^{2+}]_i$ were attained, at about 90-120 seconds. After this period, calcium levels, as well as free arachidonate levels, decline in parallel (from approximately 3 to 5 minutes post stimulation, results not shown). An initial elevation in radiolabel also appeared in the diglyceride fraction within the first 30 seconds of agonist stimulation ($\leq$ 1% of total

FIGURE 35

TIME COURSE OF fMLP-STIMULATED ARACHIDONATE MOBILIZATION AND ELEVATION IN $[Ca^{2+}]_i$.

The time course of fMLP-stimulated $[Ca^{2+}]_i$ elevation and arachidonate mobilization were measured as described in Figure 19. Results represent mean $\pm$ SE of 3-4 separate experiments performed in duplicate.

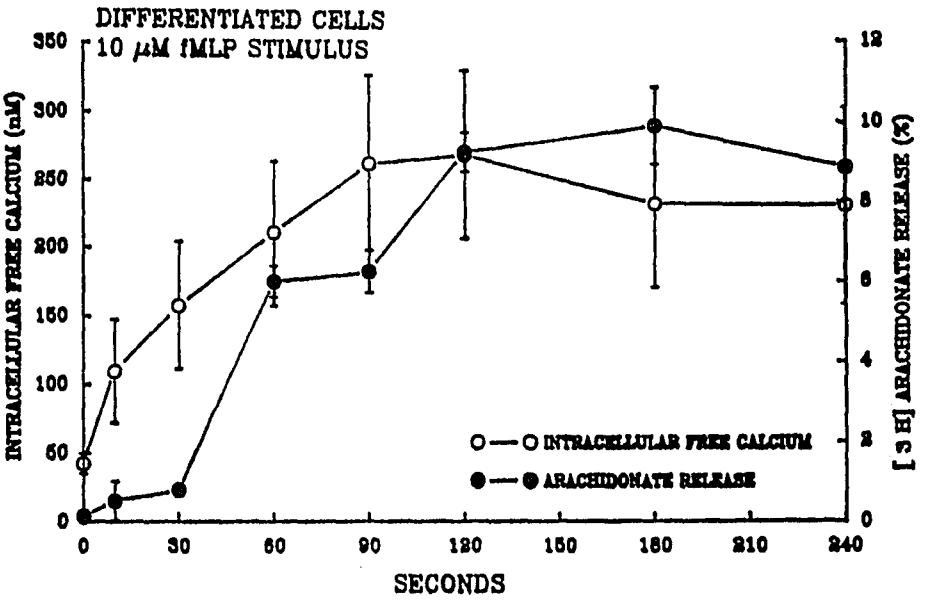


FIGURE 35

incorporated radiolabel), consistent with receptor mediated activation of PLC. However this elevated level of radiolabeled diglyceride remained constant throughout the first two minutes of stimulation.

Further evidence for the coupling of $cPLA_2$ activity to $[Ca^{2+}]_i$ elevation is presented in Figure 36. In this figure, arachidonate release is plotted as a function of $[Ca^{2+}]_i$. Once again, there is a striking correlation between released arachidonate and $[Ca^{2+}]_i$ elevations stimulated by fMLP (correlation coefficient for the regression line in Figure 36 is 0.70).

6.2.4 Effects of ATP on elevation of $[Ca^{2+}]_i$ in both differentiated and undifferentiated U937 cells.

For these experiments, differentiated or undifferentiated cells were loaded with Fura-2 and stimulated with 100 μM ATP. This concentration of ATP has been routinely used in several cell lines to activate calcium influx (13, 96, 122, 126). Figure 37 demonstrates that ATP induced an almost instantaneous elevation in $[Ca^{2+}]_i$ in undifferentiated U937 (first 1-2 seconds), followed by a sustained phase. Upon initial examination, this response appears to be characteristic of capacitative calcium influx (store depletion followed by influx of extracellular calcium). However, stimulation of undifferentiated U937 with 100 μM ATP in calcium free medium abolished these effects (Fig. 37, lower trace). This indicates that in undifferentiated U937, ATP-stimulated elevations in $[Ca^{2+}]_i$ are dependent on influx of extracellular calcium, and are independent of depletion of intracellular calcium stores. These results are characteristic of the P_{2Z} purinergic receptor (124).

FIGURE 36

fMLP-STIMULATED ARACHIDONATE MOBILIZATION AS A FUNCTION OF $[Ca^{2+}]_i$.

Results for arachidonate mobilization obtained in Figure 35 are plotted as a function of $[Ca^{2+}]_i$.

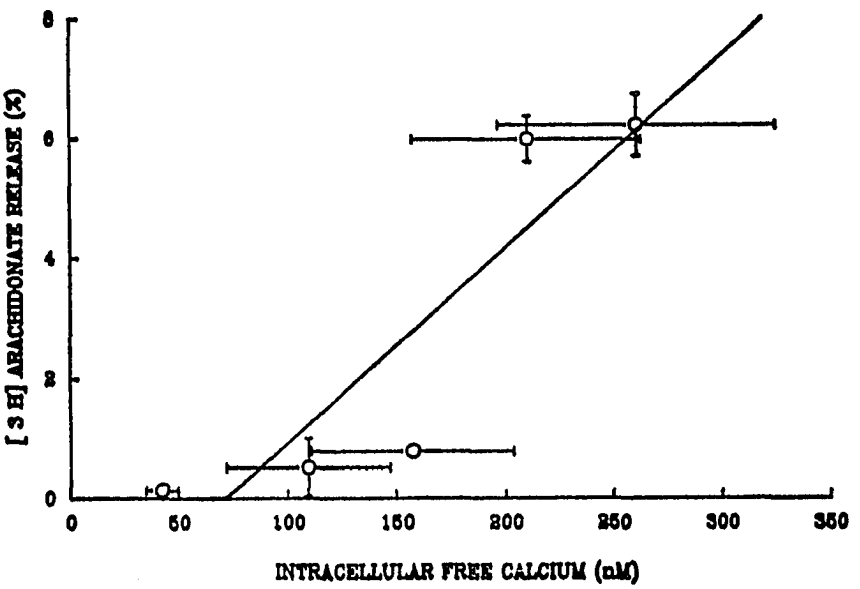


FIGURE 36

FIGURE 37

EFFECTS OF ATP ON $[Ca^{2+}]_i$ IN UNDIFFERENTIATED U937 CELLS.

ATP-stimulated elevations in $[Ca^{2+}]_i$ were measured in Fura-2 loaded undifferentiated U937 cells as described in Methods 2.9. Results shown are representative of 4 similar experiments performed on different days.

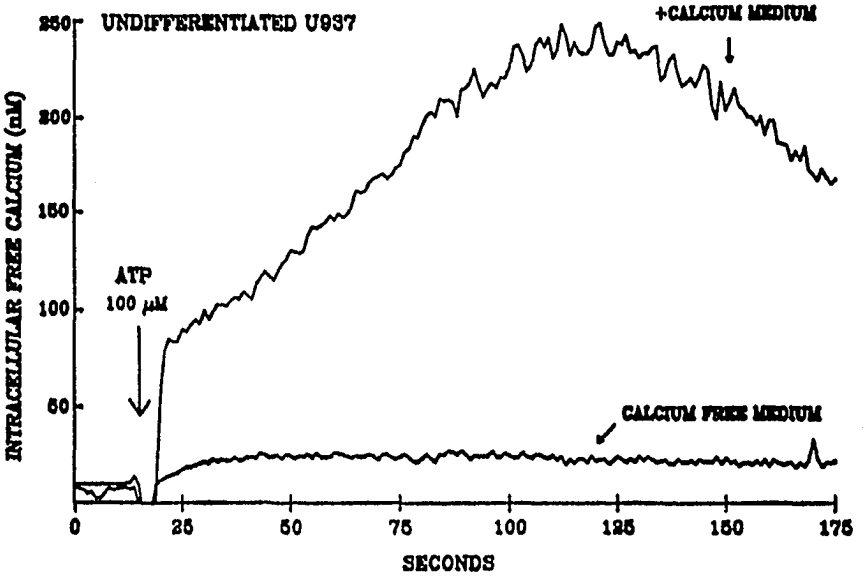


FIGURE 37

ATP (100 μM) also stimulated an elevation in $[Ca^{2+}]_i$ in differentiated U937 cells (Figure 38). However, in the case of differentiated cells, the initial elevation in $[Ca^{2+}]_i$ was not as immediate as that observed in undifferentiated cells (compare $[Ca^{2+}]_i$ elevations during the first 2-5 seconds in Figures 37 and 38). UTP produced similar effects (data not shown). Furthermore, in calcium-free medium, ATP stimulated a 3 fold increase over basal levels of $[Ca^{2+}]_i$ in differentiated cells (Figure 38, lower trace). Similar results were observed when extracellular calcium was chelated in the presence of EGTA (Figure 39B). As extracellular calcium was not present under these conditions, the ATP-stimulated elevations in $[Ca^{2+}]_i$ observed would be due to mobilization of calcium from intracellular stores. Thus, differentiated U937 cells responded to ATP with a depletion of intracellular calcium stores, followed a sustained phase of calcium influx. These results suggest ATP-stimulated increases in $[Ca^{2+}]_i$ in differentiated cells are coupled, at least in part, to the P_{2U} purinergic receptor.

6.2.5 ATP stimulates elevations in $[Ca^{2+}]_i$ through dissimilar pathways in differentiated and undifferentiated U937.

In undifferentiated cells, ATP-stimulated increases in $[Ca^{2+}]_i$ appear to be entirely due to influx of extracellular calcium (characteristic of the P_{2Z} receptor). In contrast, ATP-stimulated increases in $[Ca^{2+}]_i$ in differentiated cells appear to also be coupled to depletion of intracellular calcium stores and activation of capacitative calcium influx (characteristic of the P_{2U} receptor). Further experiments were conducted to dissect the calcium influx component of $[Ca^{2+}]_i$ elevation, as in Section 4.2.3. Figures 39 A and B demonstrate that differentiated U937 attained higher

FIGURE 38

EFFECTS OF ATP ON $[Ca^{2+}]_i$ IN DIFFERENTIATED U937 CELLS.

ATP-stimulated elevations in $[Ca^{2+}]_i$ were measured in Fura-2 loaded differentiated cells as described in Methods 2.9. Results shown are representative of 4 similar experiments performed on different days.

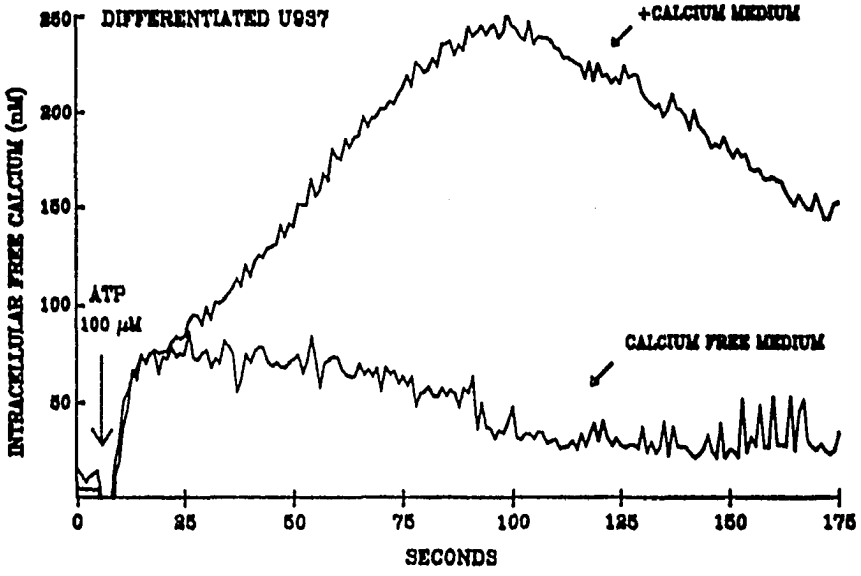
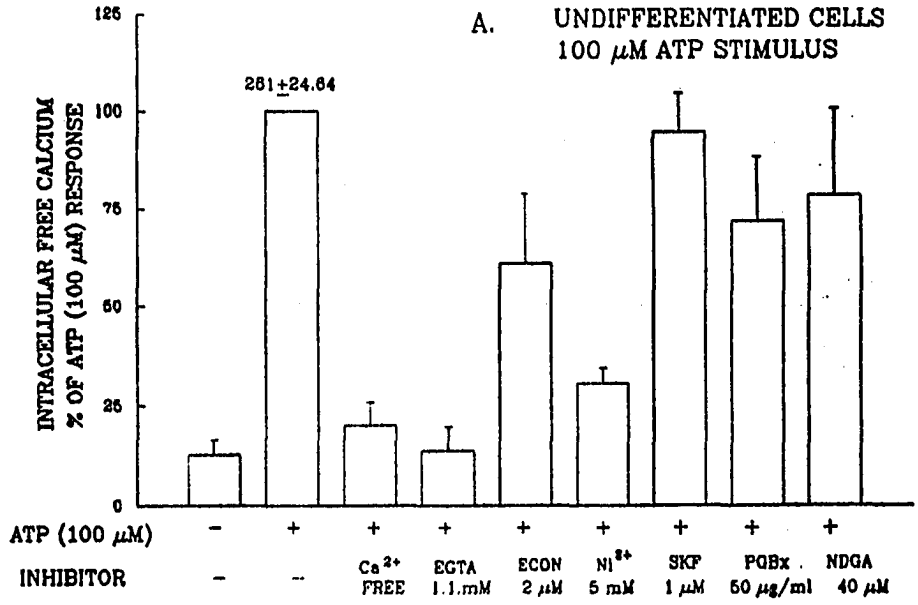


FIGURE 38

FIGURE 39

EFFECTS OF INHIBITORS OF CAPACITATIVE CALCIUM INFLUX ON ATP-STIMULATED ELEVATIONS IN $[Ca^{2+}]_i$ IN DIFFERENTIATED AND UNDIFFERENTIATED U937 CELLS.

Differentiated (Figure A) and undifferentiated (Figure B) cells were loaded with Fura-2 as described in Methods 2.9. ATP-stimulated elevations in $[Ca^{2+}]_i$ in the presence of inhibitors of calcium influx was measured as described for fMLP in Figure 33. Results were normalized to the maximum $[Ca^{2+}]_i$ level achieved by 100 μM ATP (261 $\pm$ 25 nM for undifferentiated cells; 356 $\pm$ 64 for differentiated cells) and represent the percentage of the 100 μM ATP response. Data shown represents the mean $\pm$ SE of 3 separate experiments.



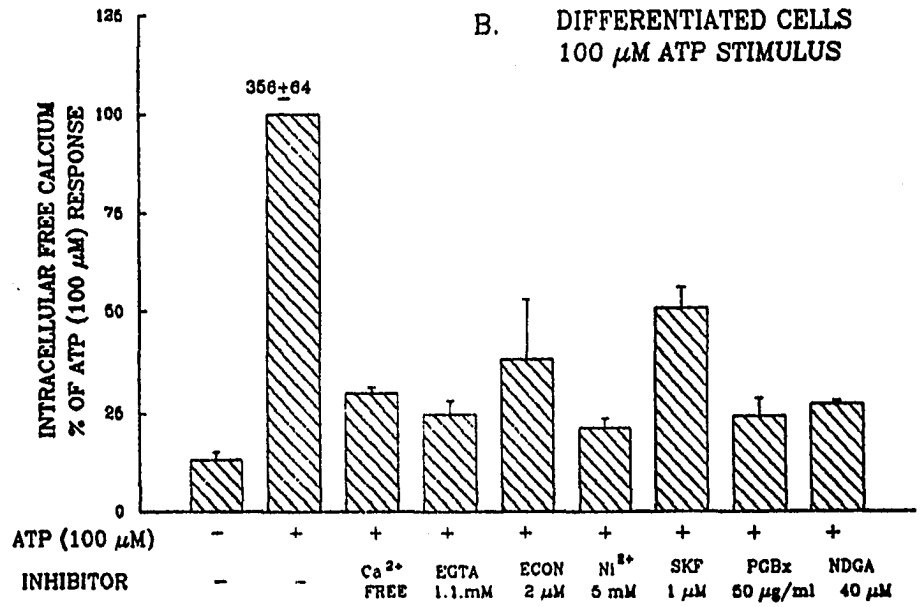


FIGURE 39

elevations in $[Ca^{2+}]_i$ in response to 100 μM ATP than did undifferentiated cells (355 ± 64 vs. 261 ± 25). This suggests that differentiation may induce development of the P_{2U} receptor (and the capacitative pathway), in addition to the P_{2Z}, thereby resulting in enhanced elevations of $[Ca^{2+}]_i$ in differentiated cells.

Econazole (2 μM), the inhibitor of cytochrome P450, was a more effective inhibitor of calcium influx in differentiated as opposed to undifferentiated, U937 cells. As econazole inhibits a cytochrome P450 system believed to be integral to capacitative calcium influx, the weak inhibition observed in undifferentiated cells (only 25-30% as compared to 50-60% inhibition in differentiated cells) may indicate lack of a fully developed capacitative calcium influx pathway in undifferentiated U937 cells. Undifferentiated cells appear to elevate $[Ca^{2+}]_i$ in response to ATP *via* the P_{2Z} purinergic receptor, followed by subsequent activation of ROC for calcium influx. Presumably, this pathway does not involve cytochrome P450, thus econazole does not inhibit calcium influx. Ni^{2+} (5mM) was also a more effective inhibitor of ATP-stimulated $[Ca^{2+}]_i$ elevation in differentiated cells (compare Figs 39 A & B). SKF96365 (1 μM), which blocks capacitative calcium influx channels, had no effect in undifferentiated U937, yet it effectively blocked 50% of the ATP-stimulated $[Ca^{2+}]_i$ elevation in differentiated cells. Taken together, these observations strongly suggest that ATP-stimulated calcium influx occurs by dissimilar pathways in differentiated and undifferentiated U937. Undifferentiated U937 cells appear to respond to ATP by activation of the P_{2Z} receptor and direct influx of extracellular calcium through ROC. Differentiated cells appear to respond to ATP, at least in part, through the P_{2U} purinergic receptor which initiates capacitative pathway for calcium influx.

PGBx (50 μg/ml), which inhibits $cPLA_2$, had no effect on ATP elevations in $[Ca^{2+}]_i$ in undifferentiated cells (Figure 39A). However, in differentiated cells, PGBx inhibited ATP-stimulated elevations in $[Ca^{2+}]_i$ by 75% (Fig. 39B). Similar results were observed with NDGA (Figs 39 A & B). Thus, $cPLA_2$ appears to regulate $[Ca^{2+}]_i$ elevations only in response to agonists which elevate $[Ca^{2+}]_i$ by the capacitative pathway.

Inhibition of $[Ca^{2+}]_i$ elevations by SKF96365, NDGA, and PGBx in differentiated cells stimulated with ATP was not as effective as inhibition observed in differentiated cells stimulated with thapsigargin (Chapter 4). As differentiated U937 cells possess both P_{2U} and P_{2Z} purinerginc receptors (122), it is conceivable that ATP activated both these receptor subtypes in differentiated cells. SKF96365, PGBx, and NDGA would inhibit only that calcium influx associated with SOC channels (i.e. the P_{2U} receptor subtype). Calcium influx through ROC mediated by the P_{2Z} would be unaffected. To identify potential P_{2Z} receptor activity, differentiated cells were challenged with 1 μM thapsigargin for 150 seconds, sufficient time to induce intracellular store depletion. Cells were subsequently stimulated with 100 μM ATP. The results shown in Figure 40 demonstrate that even when intracellular stores were depleted by thapsigargin, ATP induced an immediate rise in $[Ca^{2+}]_i$. These results are consistent with activation of a P_{2Z} receptor, which would produce an additional increase in intracellular calcium by direct influx of calcium through ROC. Thus, differentiated U937 cells respond to ATP by activation of both P_{2U} and P_{2Z} purinergic receptors.

FIGURE 40

DIFFERENTIATED CELLS CONTAIN P_{2Z} RECEPTORS FOR ATP

Differentiated U937 cells were loaded with Fura-2 as described in Methods 2.9. Thapsigargin (1 μM) was added to cells in the spectrofluorometer cuvette as indicated by the arrow. After intracellular stores were depleted, ATP (100 μM) was added, as indicated by the arrow. The tracing shownenter is representative of 3 separate experiments performed on different days.

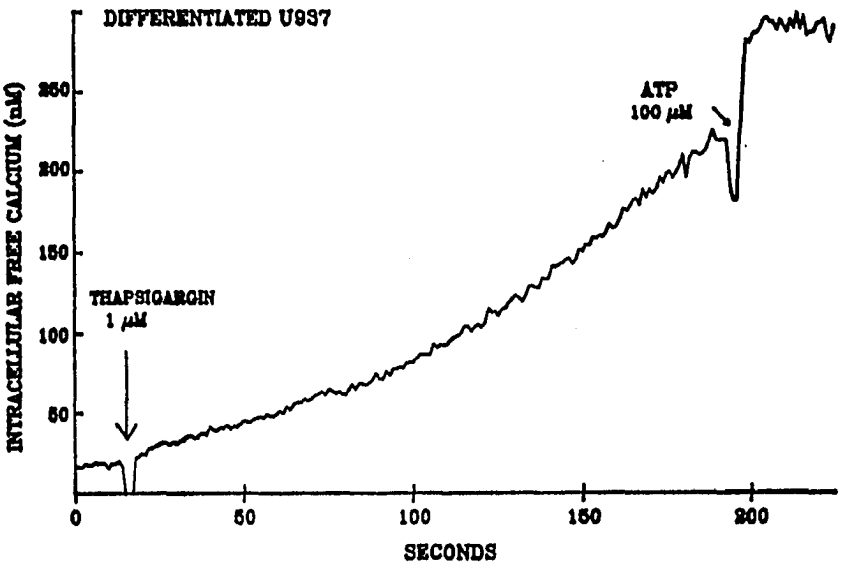


FIGURE 40

6.2.6 Effects of ATP on mobilization of arachidonate in differentiated and undifferentiated U937 cells.

In these experiments, differentiated and undifferentiated cells were challenged with ATP for 2 minutes, as described in Section 2.4. $[Ca^{2+}]_i$ measurements indicated that this was sufficient time for maximal elevations in $[Ca^{2+}]_i$ to be attained.

Undifferentiated cells did not respond to ATP stimulation with increased mobilization of arachidonate (data not shown). This is not surprising, as the ATP-stimulated elevations of $[Ca^{2+}]_i$ were not consistent with activation of the capacitative pathway in undifferentiated U937 cells. In differentiated cells, 100 μM ATP stimulation resulted in low, but significant mobilization arachidonate, approximately 2 fold over basal (unstimulated) levels (Figure 41). In calcium free medium, ATP-stimulated release of arachidonate was inhibited by 30-40%. Figure 38 demonstrated that in the absence of extracellular calcium, ATP stimulated a rise in $[Ca^{2+}]_i$, presumably from emptying of intracellular stores. The results presented in Figure 41 suggest that elevations in $[Ca^{2+}]_i$ stimulated by depletion of intracellular stores are capable of sustaining 60-70% of the ATP-stimulated arachidonate mobilization.

Studies with inhibitors of calcium influx, as described in Section 4.2.5, were conducted to further examine ATP-stimulated arachidonate release as a function of influx of extracellular calcium. Consistent with results observed with thapsigargin and fMLP, inhibition of cytochrome P450 by econazole (2μM) increased ATP-stimulated arachidonate release in differentiated U937 cells. Inhibitors which blocked capacitative calcium influx channels, Ni^{2+} and SKF96365, also blocked 30-40% of ATP-stimulated arachidonate mobilization. Thus, inhibition of capacitative calcium

FIGURE 41

EFFECT OF INHIBITION OF CAPACITATIVE CALCIUM INFLUX ON ATP-STIMULATED ARACHIDONATE MOBILIZATION IN DIFFERENTIATED U937 CELLS.

ATP-stimulated arachidonate mobilization was determined as described for fMLP in Figure 34. The concentrations of inhibitors were as follows : EGTA-1.1 mM; Econazole-2 μM; Ni^{2+}-5 mM; SKF96365-1 μM; PGBx-50 μg/ml; NDGA-40 μM; and Sr^{2+}-1.1 mM. , Results were normalized to the release of radiolabel observed in response to 100 μM ATP (4.02 $\pm$ 0.8 % of total incorporated cellular radiolabel) and represent the percentage of the 100 μM ATP response. Data shown represents the mean $\pm$ SE of 3 separate experiments performed in duplicate.

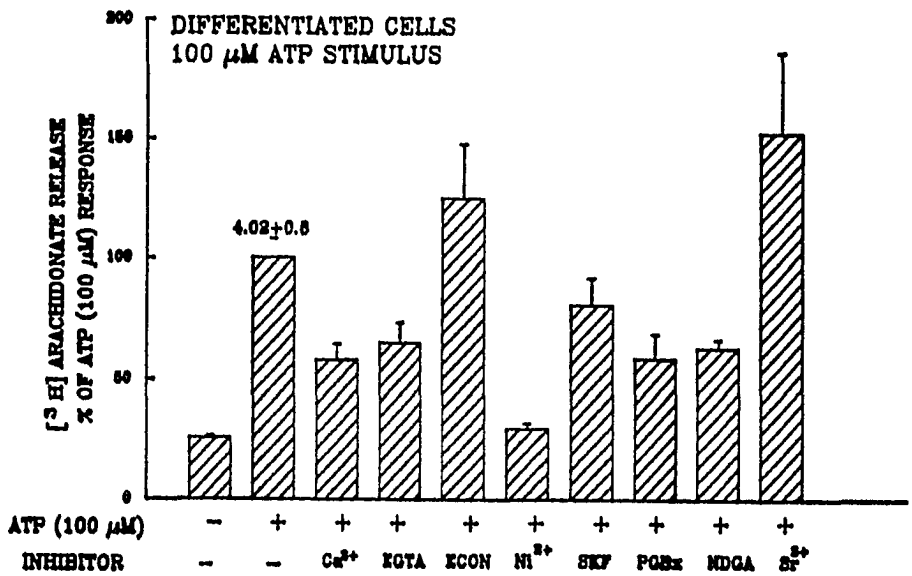


FIGURE 41

influx blocks a portion of ATP-stimulated arachidonate mobilization (i.e., that portion which is coupled to capacitative calcium influx).

In conjunction with their inhibitory effects on increases in $[Ca^{2+}]_i$, both PGBx and NDGA also inhibited 51-55% of ATP-stimulated arachidonate mobilization. PGBx was most effective, as this compound reduced ATP-stimulated arachidonate mobilization to control (unstimulated) levels. These results are consistent with the hypothesis that $cPLA_2$ plays a regulatory role in the capacitative calcium influx pathway.

6.2.7 Time course of ATP stimulated arachidonate release and elevation of $[Ca^{2+}]_i$.

Consistent with both fMLP (6.2.3) and thapsigargin (Chapter 4) Figure 42 demonstrates that ATP-stimulated release of arachidonate parallels ATP-stimulated elevation in $[Ca^{2+}]_i$ for the first two minutes of agonist stimulation. Arachidonate release is increased approximately 2 fold above basal levels within the first 10 seconds of ATP stimulation, and parallels $[Ca^{2+}]_i$ elevations until maximal $[Ca^{2+}]_i$ levels are attained. Thus, arachidonate mobilization is initiated during intracellular store depletion, and continues during the sustained, or calcium influx phase of the ATP response. As $[Ca^{2+}]_i$ decreases, levels of free arachidonate follow. This suggests a correlation between ATP-stimulated mobilization of arachidonate and $[Ca^{2+}]_i$ levels in differentiated U937. Further evidence for a correlation between arachidonate mobilization and $[Ca^{2+}]_i$ is presented in Figure 43. Here, arachidonate mobilization is represented as a function of $[Ca^{2+}]_i$. As can be seen from this graph, release of

FIGURE 42

TIME COURSE OF ATP-STIMULATED ARACHIDONATE MOBILIZATION AND ELEVATION IN $[Ca^{2+}]_i$.

The time course of ATP-stimulated $[Ca^{2+}]_i$ elevation and arachidonate mobilization were measured as described for fMLP in Figure 35. Results represent mean $\pm$ SE of 3-4 separate experiments performed in duplicate.

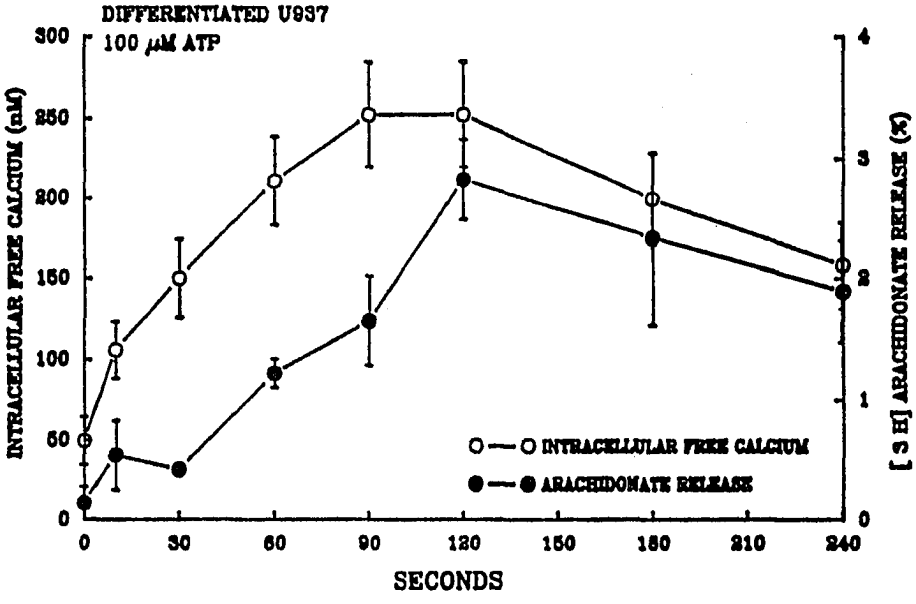


FIGURE 42

FIGURE 43

ATP-STIMULATED ARACHIDONATE MOBILIZATION AS A FUNCTION OF $[Ca^{2+}]_i$.

Results for arachidonate mobilization obtained in Figure 42 are plotted as a function of $[Ca^{2+}]_i$.

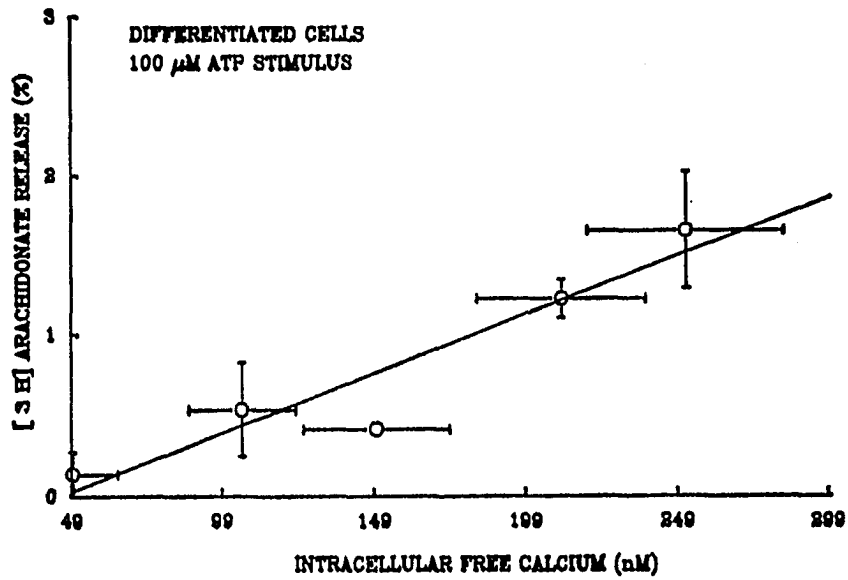


FIGURE 43

arachidonate correlates well with the levels of $[Ca^{2+}]_i$ stimulated during the first 2 minutes of ATP stimulation (correlation coefficient = 0.78). These results strongly suggest that mobilization of arachidonate may regulate capacitative calcium influx.

6.3 DISCUSSION

The results presented in this chapter support the model for $cPLA_2$ activation presented in Chapter 4 (Figure 22) with physiological receptor mediated events. The following criteria have been met:

6.3.1. Agonists which activate the IP_3 pathway through receptor mediated events should also activate PLA_2.

This has been demonstrated in monocytes (97, 127, 128), and many other cell lines (13, 40, 43, 129, 130). Both ATP and fMLP elevate $[Ca^{2+}]_i$ in monocytes and neutrophils via IP_3-mediated activation of capacitative calcium influx (96, 119). Our results confirm that both ATP and fMLP appear to function in this capacity in differentiated U937 cells. Stimulation of differentiated cells with ATP or fMLP resulted in a rapid elevation of $[Ca^{2+}]_i$ that was independent of extracellular calcium, followed by a sustained phase of capacitative calcium influx. This response is characteristic of the capacitative pathway. Furthermore, both ATP and fMLP stimulated influx of extracellular calcium was inhibited by SKF96365, an inhibitor of the SOC of the capacitative pathway. Thus, ATP and fMLP appear to function *via* IP_3 mediated capacitative pathway activation. Consistent with our model, activation of this pathway by ATP and fMLP resulted in arachidonate mobilization in differentiated U937.

2. Receptor mediated events that do not elevate $[Ca^{2+}]_i$ via the capacitative pathway should not activate arachidonate release.

In both differentiated and undifferentiated U937 cells, the purinergic receptor agonist, ATP, induced elevations in $[Ca^{2+}]_i$. However, in undifferentiated cells, $[Ca^{2+}]_i$ elevations appeared to be almost entirely dependent on influx of extracellular calcium. This suggests that in undifferentiated cells, the P_{2Z} purinergic receptor subtype mediates the ATP response. P_{2Z} purinergic receptors exert direct control over a ligand-gated channel (123, 124, 131). Upon activation, influx of cation occurs immediately, without effect on intracellular calcium stores. Further support for the presence of P_{2Z} receptors in undifferentiated U937 cells is demonstrated with the use of SKF96365. This pharmacological agent specifically blocks store-operated calcium influx channels (SOC). However, in undifferentiated U937, SKF96365 had no effect on ATP-stimulated elevations in $[Ca^{2+}]_i$, suggesting that calcium influx is not regulated via the capacitative pathway.

Although undifferentiated cells responded to ATP with increased levels of $[Ca^{2+}]_i$ this increase in $[Ca^{2+}]_i$ was not coupled to arachidonate release, even though these cells have high quantities of functional $cPLA_2$. Thus, receptor-mediated elevation of $[Ca^{2+}]_i$ by mechanisms other than depletion of intracellular calcium stores, does not appear to activate arachidonate release. Similar results were presented in Chapter 4, section 4.2.4. Arachidonate mobilization did not appear to be coupled to direct A23187-mediated calcium transport across the plasma membrane. Rather, it appeared to only be coupled to A23187 mediated depletion of intracellular calcium stores.

In differentiated U937 cells, which possess P_{2U} receptors, the capacitative pathway was activated by ATP. Consistent with our model for $cPLA_2$ regulation, arachidonate mobilization was also activated by ATP in differentiated cells. However, ATP-stimulated arachidonate release was weak, compared to that observed with fMLP. For example, 10 μM fMLP stimulates a maximal $[Ca^{2+}]_i$ level of 280 nM, and release of 9.1% of incorporated radiolabeled arachidonate. ATP (100μM) stimulated $[Ca^{2+}]_i$ elevations of 355 nM, yet maximal arachidonate mobilization was only 4%. Thus, two different agonists generated relatively similar levels of $[Ca^{2+}]_i$ but not of arachidonate mobilization. However, elevation of $[Ca^{2+}]_i$ in differentiated cells stimulated with ATP may occur through more than one pathway. Although results presented here and in Chapter 4 suggest that differentiated cells develop a capacitative calcium entry pathway as a consequence of differentiation, it is also possible that direct receptor operated cation channels such as the P_{2Z} receptor may co-exist. Differentiated U937 cells (100) and macrophages (131) have been reported to contain both P_{2U} and P_{2Z} purinergic receptor subtypes. If $[Ca^{2+}]_i$ elevation occurs by two separate mechanisms, such as P_{2U} and P_{2Z} receptors, only one (P_{2U}) may activate the capacitative pathway. Thus, out of the total increase in $[Ca^{2+}]_i$, only a portion is due to the capacitative pathway. This would account for the decreased sensitivity of arachidonate release to $[Ca^{2+}]_i$ elevations observed in differentiated cells stimulated with ATP, as only calcium originating from activation of the capacitative pathway activated arachidonate mobilization. Thus, the ATP-stimulated calcium response undifferentiated U937 may represent a composite of the activities of these two receptors, only one of which (the P_{2U}) activates $cPLA_2$.

3. Mobilization of arachidonate should be initiated in response to depletion of intracellular calcium stores.

Stimulation of differentiated cells with ATP and fMLP under calcium free conditions produced an elevation in $[Ca^{2+}]_i$ consistent with depletion of intracellular calcium stores. Arachidonate mobilization also occurred under calcium free conditions, indicating that depletion of calcium from stores alone could activate and sustain $cPLA_2$ activity. In fact, 40-50% of the fMLP and ATP-stimulated arachidonate mobilization was maintained, even in the absence of extracellular calcium. Thus, in differentiated U937 cells stimulated with either ATP or fMLP, depletion of intracellular calcium stores was coupled to mobilization of arachidonate. This suggests that a significant portion of $cPLA_2$ activity lies upstream of capacitative calcium influx, even in receptor mediated events.

The amount of arachidonate release coupled to agonist-stimulated capacitative calcium influx appeared to be slightly lower than that observed with thapsigargin (75-80%, Figure 18). However, one must recognize that thapsigargin stimulation of differentiated U937 resulted in $[Ca^{2+}]_i$ elevations of 600-700 nM, most of which was due to influx of extracellular calcium. During agonist-stimulated signal transduction events, IP_3 receptors on intracellular calcium stores are inhibited by $[Ca^{2+}]_i$ elevations of approximately 300 nM (132, 133). Thus, at this level of $[Ca^{2+}]_i$, capacitative calcium influx is essentially "turned off". During thapsigargin stimulation, intracellular calcium stores remain empty, independent of the IP_3 receptor, and there is no feedback mechanism for maintaining $[Ca^{2+}]_i$ below 300 nM. Thus, the amount of $cPLA_2$ activity

stimulated by influx calcium could be much higher during thapsigargin stimulation, as actual calcium influx itself is increased over that normally observed in signal transduction events.

4. Mobilization of arachidonate should also accompany capacitative calcium influx.

In differentiated cells, ATP and fMLP stimulated mobilization of arachidonate were potentiated under conditions which permit capacitative calcium influx (i.e. in the presence of extracellular calcium). Thus, agonist-stimulated influx of capacitance calcium was also coupled to arachidonate release. Arachidonate release coupled to influx of extracellular calcium was inhibited by Ni^{2+} (5mM), which physically blocks a wide range of calcium channels (88), and by SKF96365, which specifically block SOC (101).

In undifferentiated cells, ATP-stimulated influx of extracellular calcium was consistent with activation of the P_{2Z} purinergic receptor subtype. Thus, calcium influx occurred directly through ROC.

5. The time course of agonist-stimulated arachidonate release should correlate with the time course of agonist-stimulated elevation in $[Ca^{2+}]_i$.

The time course for both fMLP and ATP stimulated elevations in $[Ca^{2+}]_i$ correlated with those of arachidonate release. In both cases, arachidonate mobilization was evident (2 fold over basal levels) within ten seconds of agonist stimulation. Thus, arachidonate release occurred as intracellular stores were undergoing depletion.

Release of arachidonate continued until maximal levels of $[Ca^{2+}]_i$ were attained. Taken together with results presented in Chapter 5 regarding the ability of arachidonate to activate influx of extracellular calcium, these results are consistent with a role for $cPLA_2$ in the generation of a CIF. $cPLA_2$ activity, initiated by localized increases in calcium due to depletion of intracellular calcium stores, could generate free arachidonate. Arachidonate in conjunction with a low molecular weight GTPase, may then regulate formation of a CIF which activates SOC calcium influx. Once cells have attained a maximal $[Ca^{2+}]_i$ level, IP_3 receptors on intracellular calcium stores are inhibited. Calcium no longer is available for $cPLA_2$ activity, and the CIF is no longer generated

6. Inhibition of $cPLA_2$ activity should inhibit agonist-stimulated capacitative calcium influx.

In differentiated U937 cells both ATP and fMLP stimulated arachidonate release were inhibited by the PLA_2 inhibitor, PGBx. The ATP and fMLP-stimulated influx of extracellular calcium was also inhibited by PGBx. However, depletion of intracellular store calcium was not affected. These observations support a role for $cPLA_2$ in initiation of capacitative calcium influx in response to intracellular calcium store depletion during receptor mediated events.

7. Arachidonate mobilization observed during receptor mediated events should be consistent with activation of $cPLA_2$.

During both ATP and fMLP-stimulation, intracellular free calcium

concentrations are in the sub-micromolar range. This precludes a role for $sPLA_2$, as this enzyme requires millimolar calcium for catalysis. Furthermore, arachidonate mobilization in response to stimulation by either fMLP or ATP was sustained in the presence of extracellular strontium. As $cPLA_2$ can utilize strontium for translocation and activation, these results further support a role for $cPLA_2$

The results presented in this chapter are consistent with previous reports which demonstrate that $cPLA_2$ is activated by receptor-mediated events that result in activation of PLC and subsequent production of IP_3 (13, 126). However, rather than being a simple consequence of PLC activation, the present results suggest that activation of $cPLA_2$ may be an integral component of an effector system which modulates capacitative calcium influx.

CHAPTER 7

CONCLUSIONS

The conclusions below summarize the results obtained in this study on *in situ* regulation of $cPLA_2$. A cellular system in which $cPLA_2$ can be studied has been identified, and the activity of $cPLA_2$ has been characterized in this system. Furthermore, it was found that the differentiation-induced activity of $cPLA_2$ is coupled to differentiation-induced development of a functional capacitative calcium influx pathway. These experiments have identified the cellular components of calcium dynamics which control $cPLA_2$ translocation and activation by the capacitative pathway. However, in addition to the role of calcium in regulation of $cPLA_2$, $cPLA_2$ itself may be integrally involved in the regulation of agonist-stimulated elevations in $[Ca^{2+}]_i$ levels. Thus, in addition to its role in activation of the arachidonate cascade, this thesis identifies a potential novel role for $cPLA_2$ as an effector system which modulates capacitance calcium influx.

7.1 DMSO-differentiation of U937 cells induces an enhanced capacity to

mobilize arachidonate in response to a calcium signal.

Although undifferentiated U937 cells contain high quantities of $cPLA_2$ which increase only modestly with differentiation, A23187 stimulated arachidonate mobilization *in situ* is low, and relatively non-specific for arachidonate. DMSO-differentiation of U937 cells results in significant enhancement of arachidonate mobilization.

7.2 The arachidonate mobilizing activity observed in differentiated U937 cells is consistent with the *in vitro* characteristics of $cPLA_2$.

a.) $cPLA_2$ can be distinguished by its sensitivity to low calcium and arachidonyl specificity.

$cPLA_2$ has two distinguishing characteristics *in vitro*; sensitivity to sub-micromolar elevations in calcium and a preference for arachidonyl containing phospholipid substrates (22). Consistent with *in vitro* observations, A23187-stimulated arachidonate mobilization in differentiated U937 was activated by nannomolar elevations in $[Ca^{2+}]_i$, and was specific for arachidonate, as compared to oleate. Thus, differentiated U937 appears to be a suitable model system in which to study *in situ* regulation of $cPLA_2$ activity.

b.) Extracellular strontium can be utilized to differentiate $sPLA_2$ activity from $cPLA_2$ activity *in situ*.

In the U937 cellular system, extracellular calcium can be replaced by extracellular strontium without due compromise of biological activity under the incubation conditions used in this study. $sPLA_2$ has an absolute requirement for

calcium, and is inhibited in the presence of strontium (39). In contrast to $sPLA_2$, $cPLA_2$ can utilize strontium as a substitute for calcium. In the absence of extracellular calcium, (and presence of extracellular strontium) thapsigargin stimulation results in influx of extracellular strontium. Strontium also forms a complex with Fura-2, and intracellular concentrations of this divalent cation can be readily measured using the dissociation constant for the Sr^{2+}/Fura-2 complex. Agonist-stimulated arachidonate release observed in the presence of strontium would be due to activation of $cPLA_2$, as $sPLA_2$ is inhibited under these conditions. This represents a novel *in situ* mechanism in which the effects of $cPLA_2$ and $sPLA_2$ can be dissected from each other. In differentiated U937 cells, thapsigargin stimulated both influx of extracellular strontium, and arachidonate release. Thus, thapsigargin stimulation was coupled to $cPLA_2$, rather than $sPLA_2$.

7.3 PMA pre-incubation potentiates A23187-stimulated arachidonate mobilization in differentiated U937 cells.

$cPLA_2$ is phosphorylated *in situ* in response to PMA (33), zymosan (47), bacterial lipopolysaccharides (64), and other agents. Although the unphosphorylated enzyme is active and responds to calcium, evidence suggests that phosphorylation enhances the Vmax for catalytic activity. Pretreatment of differentiated U937 cells with 10 nM PMA for 10 minutes prior to A23187 stimulation dramatically potentiated A23187-stimulated arachidonate mobilization. Thus, our results suggest that phosphorylation events may further potentiate *in situ* arachidonate mobilization in response to a calcium signal.

7.4 Phosphorylation alone is insufficient stimulus for $cPLA_2$ activation in differentiated U937 cells.

In differentiated and undifferentiated U937 cells, PMA alone was insufficient stimulus for arachidonate mobilization, unless combined with a calcium signal. These results suggest that regulation of $cPLA_2$ by phosphorylation is secondary to activation of $cPLA_2$ by an elevation in $[Ca^{2+}]_i$, which permits association of $cPLA_2$ with membrane substrates.

7.5 A23187 and PMA + A23187-stimulated arachidonate mobilization increases during the temporal development of monocytic differentiation in U937 cells.

During DMSO-induced differentiation, A23187 stimulatable arachidonate mobilizing activity developed during two distinct phases, early and late in the differentiation time course. Maximal levels of A23187-stimulated arachidonate mobilization occurred in conjunction with growth cessation and development of the macrophage phenotype. The ability of PMA to potentiate A23187-stimulated arachidonate mobilization occurred early in the differentiation time course (complete by 36 hours) and remained at this level throughout the differentiation process.

As undifferentiated cells contain high levels of functional enzyme, these results suggest that differentiation induces $cPLA_2$ regulatory elements, which are lacking or inactive in undifferentiated cells.

7.6 Differentiation of U937 cells alters intracellular calcium dynamics and

enhances the capacitative calcium influx pathway.

Data presented in Chapters 4 and 6 indicate that U937 cells undergo significant alterations in intracellular calcium dynamics during the differentiation process. Differentiated U937 cells attained significantly higher levels of $[Ca^{2+}]_i$ in response to A23187 and thapsigargin than did undifferentiated cells. This increased calcium responsiveness was due to development of the capacitative calcium influx pathway.

Dissection of the calcium response in differentiated and undifferentiated U937 cells indicates that differentiation induces alterations in both components of capacitative calcium influx, intracellular calcium stores and store operated calcium influx channels (SOC). Differentiated cells had larger thapsigargin-stimulatable intracellular calcium stores than did undifferentiated cells, as evidenced by the higher elevations in $[Ca^{2+}]_i$ attained in response to thapsigargin stimulation in calcium free medium. Additionally, the characteristics of calcium influx channels appeared to be altered with differentiation. Thapsigargin-stimulated calcium influx in undifferentiated cells was not inhibited by econazole or SKF96365, both of which are relatively specific for capacitative calcium influx channels. In contrast, calcium influx in differentiated cells was significantly inhibited by these compounds. This suggests that either a different calcium influx channel is present, or that an existing channel has been structurally modified.

7.7 The enhanced capacity to mobilize arachidonate in response to a calcium signal is coupled to differentiation induced alterations in the capacitative calcium influx pathway.

Undifferentiated U937 cells did not release arachidonate in response to

thapsigargin stimulation. In contrast, differentiated cells responded to thapsigargin with a dose dependent mobilization of arachidonate. Thus, in differentiated cells, activation of the capacitative pathway was coupled to mobilization of arachidonate. In actual receptor mediated events, activation of the capacitative pathway is coupled to receptor-mediated activation of PLC, followed by generation of IP_3. Agonists which function through this signal transduction network, such as ATP and fMLP, also activated arachidonate mobilization in differentiated U937 cells.

7.8 Arachidonate mobilization is coupled to both phases of the capacitative pathway; depletion of intracellular calcium stores, and influx of capacitative calcium.

In differentiated U937 cells, significant thapsigargin-stimulated arachidonate release were observed even in the absence of extracellular calcium, indicating that elevations in $[Ca^{2+}]_i$ resulting from depletion of intracellular calcium stores alone were capable of initiating and sustaining arachidonate mobilization. Arachidonate release coupled to intracellular store depletion was even more evident in receptor mediated events stimulated by fMLP or ATP. Stimulation of differentiated cells with ATP or fMLP in the absence of extracellular calcium resulted in a 2-4 fold increase in arachidonate mobilization over basal (unstimulated) levels. Thus, calcium originating from depletion of intracellular stores alone could both activate and sustain $cPLA_2$ activity.

Additionally, a portion of the total agonist-stimulated arachidonate release was coupled to influx of extracellular calcium. Inhibitors of capacitative calcium influx

(econazole, SKF96365, Ni^{2+}) effectively inhibited arachidonate mobilization coupled to this phase of the capacitative pathway. However, the results presented here suggest that only calcium influx arising from the SOC of the capacitative pathway result in arachidonate mobilization. ATP stimulated calcium influx through P_{2Z} receptor operated channels did not result in mobilization of arachidonate.

This suggests that $cPLA_2$ must somehow be specifically coupled to the capacitative pathway, as it does not respond to intracellular calcium elevations originating from other sources (i.e. receptor operated channels). This may involve potential binding proteins, which physically couple the enzyme to either SOC or intracellular calcium stores, possibly even the CIF itself. Alternatively, depletion of intracellular calcium stores could release some form of inhibitory constraint, thereby coupling $cPLA_2$ activity to the capacitative pathway.

7.9 $cPLA_2$ activity may be coupled to the generation of a calcium influx factor (CIF) which serves as a communication link between empty intracellular calcium stores and store operated calcium influx channels.

The time course of thapsigargin, ATP, and fMLP stimulated elevations in $[Ca^{2+}]_i$ and arachidonate release in differentiated U937 cells indicates that $cPLA_2$ activity is initiated during depletion of intracellular calcium stores, and continues until maximal levels of $[Ca^{2+}]_i$ are attained. Furthermore, inhibition of $cPLA_2$ with the PLA_2 inhibitor, PGBx, inhibited agonist-stimulated elevations in $[Ca^{2+}]_i$, as well as arachidonate release. Results presented in Chapters 4 and 6 suggest that PGBx primarily inhibited capacitative calcium influx, with little effect on calcium store depletion. Thus, inhibition of $cPLA_2$

blocked capacitative influx, supporting a role for $cPLA_2$ in the generation of a CIF. $cPLA_2$ is activated upon intracellular store depletion, and inhibition of $cPLA_2$ blocks capacitative influx.

Further support for this hypothesis comes from evidence presented in Chapter 5. The product of $cPLA_2$ activity, arachidonic acid, was capable of activating calcium influx when exogenously applied to differentiated cells. This effect was relatively specific for arachidonate. Consistent with effects on capacitative calcium influx channels, exogenous arachidonate also activated release of endogenous arachidonate. Thus, arachidonate itself, or in conjunction with other components of a CIF, may regulate store operated calcium influx channels.

7.10 PMA potentiates influx of capacitative calcium, and arachidonate release coupled to influx of capacitative calcium.

PMA potentiated thapsigargin and A23187-stimulated elevation in $[Ca^{2+}]_i$. The effects of PMA were inhibited by staurosporine (1 μM). Results from thapsigargin time course experiments indicate that PMA potentiates $[Ca^{2+}]_i$ *via* increasing capacitative calcium influx. As PMA activates PKC, this suggests that PKC may somehow be involved in regulation of store controlled calcium influx channels. In further support for a role for PKC in regulation of SOC, PMA pretreatment enhanced calcium influx stimulated by exogenous arachidonate.

7.11 PMA elevated basal levels of $[Ca^{2+}]_i$ in both differentiated and undifferentiated U937 cells.

Consistent with a role for PKC in regulation of $[Ca^{2+}]_i$, a 10 minute pre-incubation of differentiated or undifferentiated U937 cells essentially doubled the basal levels of intracellular free calcium. Preliminary experiments in calcium free medium indicated that this effect of PMA required the presence of extracellular calcium. This suggests that PMA may activate isoforms of PKC which regulate calcium channel activity. Alternatively, effects on the plasma membrane Ca^{2+}-ATPase could also be involved. The increase in basal $[Ca^{2+}]_i$ produced by PMA did not occur rapidly, but occurred quite gradually during the ten minute PMA incubation period. Arachidonate release was not activated by this increase in calcium, suggesting that the capacitative pathway may not be involved. This elevation in basal $[Ca^{2+}]_i$ may be one of the mechanisms by which short term exposure to PMA "primes" cells for subsequent agonist stimulated effects.

7.12 PMA may have a dual role in potentiation of arachidonate release

PMA induces phosphorylation of $cPLA_2$ *in situ*, producing increased enzymatic activity of the partially purified enzyme *in vitro*. However our results also indicate that the *in situ* effects of PMA on arachidonate mobilization may also be due to a direct effect of PKC on capacitative calcium influx. As PMA pretreatment enhances influx, more calcium is available for $cPLA_2$ translocation and activation. Thus, PKC may have a dual role in regulation of arachidonate release. It may have effects on calcium influx, as well as on $cPLA_2$ directly. Regardless of the specific mechanism involved, PMA potentiation occurred only during the phase of capacitative calcium influx, suggesting that PKC mediated events lie downstream of calcium store depletion and initial mobilization of arachidonate.

7.13 A cytochrome P450 metabolite of arachidonate does not appear to be involved in generation of a CIF.

Experiments with the imidazole antimycotic, econazole, have suggested that generation of a CIF and influx of capacitative calcium is controlled, in part, by a cytochrome P450 dependent system (98). Furthermore, treatment of differentiated cells with NDGA, an inhibitor of the cytochrome P450 pathway of the arachidonate cascade, blocked both agonist and thapsigargin-stimulated elevations of $[Ca^{2+}]_i$. Thus, we reasoned that a likely candidate for a CIF would be a P450 metabolite of arachidonate. Although NDGA inhibited agonist-stimulated release of arachidonate and elevations in $[Ca^{2+}]_i$, NDGA had no effects on arachidonate release or $[Ca^{2+}]_i$ elevation stimulated by exogenous arachidonate. If a P450 arachidonate product were involved in stimulation of capacitative calcium influx, one would expect that NDGA should block the effects of free arachidonate, as it could not be metabolized to the active P450 compound. This suggest that a P450 arachidonate metabolite is not involved in generation of CIF. HPLC analysis of thapsigargin stimulated arachidonate metabolites further supported this. HPLC revealed that P450 products were not produced in appreciable amounts in response to thapsigargin or PMA + thapsigargin. However, considering the extreme liability of P450 arachidonate epoxide metabolites, one must acknowledge the possibility that some P450 products might have gone undetected in our system. Thus, although results strongly suggest that a P450 arachidonate metabolite is not involved in capacitative calcium influx, we cannot conclusively rule it out.

7.14 The primary product generated in response to thapsigargin stimulation in differentiated U937 cells, was free arachidonate itself.

Thin layer chromatography and HPLC suggested that the principal arachidonate metabolite generated during thapsigargin stimulated mobilization of arachidonate was the free fatty acid itself. Some prostaglandin metabolites were also produced, however these were inhibited by indomethacin without resulting in any inhibitory effects on $[Ca^{2+}]_i$ elevation. Thus, prostaglandin metabolites are not coupled to generation of the CIF.

7.15 Free arachidonic acid may participate in generation of a CIF and activation of capacitative calcium influx.

Exogenous arachidonate stimulated a dose dependent increase in $[Ca^{2+}]_i$ in differentiated U937 cells. The effects of exogenous arachidonate were dependent on extracellular calcium, consistent with a role for arachidonate in activation of capacitative calcium influx. The effects of exogenous arachidonate were not reproduced by either linoleic or oleic acids, indicating that these effects are relatively specific for arachidonate. Furthermore, exogenous arachidonate also stimulated release of endogenous arachidonate, presumably by activation of capacitative calcium entry. Exogenous arachidonate was without effect in undifferentiated cells, suggesting that free arachidonate is only a stimulus in those cells which possess a fully developed capacitative calcium influx pathway. Since $cPLA_2$ activity is coupled to depletion of intracellular calcium stores, and inhibition of $cPLA_2$ activity blocks capacitative calcium influx, it follows that free arachidonate generated by the action of $cPLA_2$ may be a

functional component of an intracellular effector system which serves as a link in the communication between intracellular calcium stores and SOC in the plasma membrane.

7.16 Model for receptor mediated activation of $cPLA_2$.

A model for receptor-mediated activation of $cPLA_2$ is presented in Figure 43. Agonists which stimulate PLC via either G-protein or tyrosine kinase linked receptors result in hydrolysis of PIP_2, yielding diacylglycerol (DAG) and IP_3.

IP_3 binds a receptor on intracellular calcium stores, which results in rapid release of calcium from these stores. $cPLA_2$, present in the cytoplasm (possibly phosphorylated), is translocated to a locally high concentration of calcium surrounding intracellular stores. Calcium dependent association of $cPLA_2$ with membrane substrates then initiates enzymatic activity and release of free arachidonate. This phase of arachidonate mobilization is sustained by $[Ca^{2+}]_i$ elevations due to store depletion alone; influx of extracellular calcium is not required. Free arachidonate liberated in response to depletion of intracellular calcium stores participates in the formation of a calcium influx factor, possibly in conjunction with a low molecular weight GTPase, which directs the opening of SOC in the plasma membrane. Influx of extracellular calcium results in further activation of $cPLA_2$, possibly translocated to the plasma membrane due to a locally high calcium concentration in the vicinity of SOC.

The role of phosphorylation as an *in situ* regulatory mechanism for $cPLA_2$ remains unclear. DAG generated as a consequence of PLC activation, may then activate PKC. PKC may have subsequent effects on either calcium channels or phosphorylation of $cPLA_2$ directly. In the case of tyrosine kinase linked receptors, in

FIGURE 44

REGULATION OF cPLA2 ACTIVITY IN AGONIST-STIMULATED SIGNAL TRANSDUCTION

See text for description.

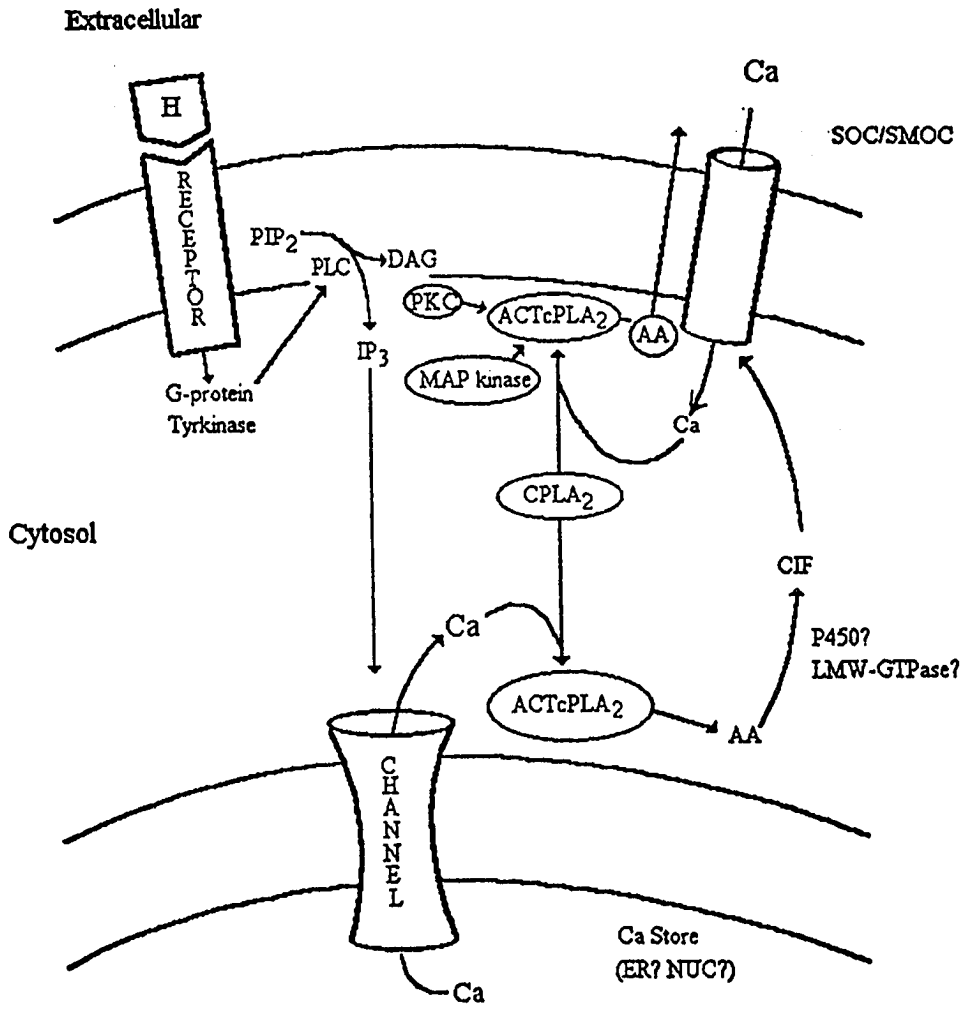


FIGURE 44

addition to activation of PLC γ, activation of MAP kinase may also result in phosphorylation of $cPLA_2$. Evidence suggests that phosphorylation in response to agonists such as PMA, cytokines, and bacterial endotoxin, enhances $cPLA_2$ activity. However, phosphorylation alone does not appear to be sufficient stimulus for arachidonate release, unless coupled to a calcium signal. In differentiated U937 cells, PMA did not appear to affect arachidonate release coupled to intracellular calcium store depletion. PMA potentiation of thapsigargin-stimulated arachidonate release was primarily observed during influx of capacitative calcium. It is possible that phosphorylation stimulates coupling of $cPLA_2$ to plasma membrane calcium influx channels. Alternatively, it is also possible that PMA effects are primarily on calcium influx channels, and arachidonate mobilization is enhanced by increased calcium influx.

In summary, this thesis suggests a novel role for $cPLA_2$ in intracellular signal transduction. Agonists which activate IP_3 dependent pathways are well known activators of $cPLA_2$. However it was assumed that the role of activated $cPLA_2$ was solely to initiate the arachidonate cascade. Thus, $cPLA_2$ is generally considered to be a consequence of IP_3-mediated signal transduction. Results presented in these experiments indicate that $cPLA_2$ activation may be an integral component of IP_3-mediated intracellular signaling. In fact, $cPLA_2$ may be an intracellular effector system for propagation of the signal initiated by IP_3, resulting in capacitative calcium influx.

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EDUCATION

June, 1992 - present:	Eastern Virginia Medical School/Old Dominion University Biochemistry/Biomedical Sciences. Nominated to Phi Kappa Phi. PhD, September 12, 1994.
February, 1978 - September, 1980	New York University Medical Center, Institute of Environmental Medicine, Tuxedo, NY MS in Biochemistry/Toxicology
September, 1973- June, 1977	Rutgers University, New Brunswick, NJ BS in Biology, Cum Laude.

APPOINTMENTS

October 1994	Medical College of Virginia, Richmond, Va Dept. of Pharmacology Research Asst. Prof.
June, 1992 - October, 1994	Ph.D. candidate, Eastern Va. Med. School/Old Dominion University. Recipient of EVMS fellowship award.
April, 1987 - January, 1992.	Environmental Systems Inc., Fair Haven, NJ. CEO/Owner of firm.
September, 1983 - April , 1987	Beth Israel Hospital Medical Center, Dept. of Lipid Research. NY, NY Sr. Research Assistant and Research Assistant.
September, 1980- September, 1983	Brookdale Hospital Medical Center, Lipid Research Lab, Brooklyn, NY Research Scientist.

February, 1978 - September, 1980	New York University Medical Center, Institute of Environmental Medicine, Tuxedo, NY Research Assistant, teaching assistant (Biochemistry), recipient of NIH Fellowship, Teaching Assistant.

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Abstracts (unpublished):

Rzigalinski, B.A., Blackmore, P.F., and Rosenthal, M.D. "Effects of differentiation on agonist stimulated mobilization of intracellular calcium and arachidonate in U937 cells." 28th Annual Southeastern Regional Lipid Conference, 1993. Selected for oral presentation.

Rzigalinski, B.A., Franson, R.C., and Rosenthal, M.D. "Effects of the calcium ionophore A23187 and phorbol myristate acetate on 3H arachidonate mobilization in differentiated and undifferentiated U937 cells." 27th Annual Southeastern Regional Lipid Conference, 1992. Recipient of student research award.