REGULATION OF FREE ARACHIDONIC ACID LEVELS BY SALIVARY GLANDS OF THE LONE STAR TICK, *Amblyomma americanum* (L.)

By

JORGE OMERO LOPEZ

Bachelor of Science

Texas A&M University

College Station, Texas

1997

Submitted to the Faculty of the Graduate College of the
Oklahoma State University in partial fulfillment of the
requirements for the Degree of
MASTER OF SCIENCE
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LIST OF ABBREVIATIONS

A-23187	calcium ionophore
AA	5,8,11,14-Eicosatetraenoic acid (arachidonic acid)
AC	adenylate cyclase
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BHT	butylated hydroxytoluene
BSA	bovine albumin
°C	Celsius
Ca^{2+}	calcium ion
cAMP	cyclic adenosine 3', 5'-monophosphate
CHO	Chinese hamster ovarian cells
cm	centimeter
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
DA	dopamine
D_1	dopamine 1 receptor
D_2	dopamine 2 receptor
G-protein	guanine nucleotide regulatory protein

GTPγ-S	guanosine 5'-O-(3-thiotriphosphate)
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PKC	protein kinase C
dH_20	de-ionized water
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraceticacid
EDTA	ethylenediaminetetraacetic acid
FAA	free arachidonic acid
g	force of gravity
G_α	alpha G-protein subunit
$G_{\beta\gamma}$	beta-gamma G-protein subunit
G_i	inhibitory G protein
G_q	phospholipase C activating G-protein
G_s	stimulatory G protein
$G_{12/13}$	G-protein (function unknown)
GDP	guanosine diphosphate
cGMP	guanosine 3',5'-cyclic monophosphate
GTP	guanosine triphosphate
h	hour

IP_3	inositol triphosphate
K^+	potassium ion
KCl	potassium chloride
kDa	kilodalton
µl	microliter
µm	micrometer
µM	microMolar
mg	milligrams
ml	milliliter
min	minutes
NaCl	sodium chloride
NL	neutral lipid
PI-3 kinase	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PL	phospholipids
PPHT-HCl	(±)-2-(N-Phenethyl-N-propyl)amino-5-hydroxytetralin hydrochloride
PTX	pertussis toxin
R-(+)-SCH23390	mammalian D_1 receptor antagonist

R-(+)-SKF38393	mammalian D_1 receptor agonist
s	second
SE	standard error
v/v	volume per volume
(±)-SKF82958-HBr	mammalian D_1 receptor agonist

CHAPTER I

INTRODUCTION

Background and Significance

Ticks and Mites

Ticks and mites belong to the order Acari and the class Arachnida. The Arachnida constitute the largest class of Chelicerata (approximately 65,000 described species) with about 8,000 in North America (Borror et, al 1992). It has been estimated that over 30,000 species have been described with another half-million still undescribed. The characteristic body shape of the Acari is oval with little or no differentiation of the body regions with 4 pairs of legs, the exception being larvae which have 3 pairs. The Acari have four life stages: egg, larva, nymph and adult.

The suborder Ixodida is one of the suborders of Acari and can be further divided into two main families, the Aragasidae (soft ticks) and the Ixodidea (hard ticks). Ixodid ticks have four stages in their life cycle (embryo, larva, nymph and adult stages) whereas argasid ticks have two or more nymphal stages and feed more rapidly than ixodid ticks.

The general shape of the hard tick is one that is flattened dorsoventrally with an oval shape. Ixodid females are slightly larger than the males and imbibe much larger blood meals. The scutum found in hard ticks (referred to as a shield) is absent in soft ticks. In male hard ticks, the scutum is relatively large and covers most of the dorsal surface. In females, the scutum is smaller and is located posterior to the capitulum. Certain species of ticks may have ornamental or color markings located on the scutum. Each species of hard ticks has variable life cycles and variable seasonal activity. Hard

ticks can be further classified according to the type of host-feeding cycle: one, two or three host ticks.

Ticks play an important role in medical and veterinary medicine throughout the world and vector pathogens that cause diseases such as Rocky Mountain spotted fever and Lyme disease in humans and ehrlichiosis in domestic animals (Service, 1996).

Lone Star Tick; *Amblyomma americanum* (L.)

The lone star tick is a three-host ixodid tick that is an important pest in Oklahoma. This tick can attack man in all three of the feeding/developmental stages. The lone star is commonly found in the Ozarks and southeastern regions of the United States. The lone star tick is considered a three-host tick because it feeds on three different hosts in each of its parasitic life stages. The lone star tick can be found around trails or pathways in natural fields or parks, waiting for a suitable host to pass by and attach to. The adult lone star tick's active period occurs during May and June, continuing on into early July. Larval or "seed ticks" appear in mid- to- late summer parasitizing cattle and potentially man because of their small size and difficulty of detection on clothing. Nymphs are active May through early August, with a peak in activity during May or June. The lone star tick receives its name from the white spot on the scutum of the female. The male has non-connected white markings around its posterior margin. The female ingests an enormous blood meal that may be more than 100 times her own weight.

Tick-host interaction

The lone star tick undergoes a slow or long feeding stage that lasts seven to fourteen days followed by a quick feeding period of 24 to 48 hours. During the slow feeding period, the tick may encounter several defense mechanisms initiated by the host: inflammatory/immune responses, clotting or blood vessel constriction. The salivary glands secrete molecules to counter the host's defense system including a cocktail of proteins and high concentrations of PGE_2 and $PGF_{2\alpha}$ (Bowman et al., 1996). Prostaglandins of the 2-series belong to a group of compounds called eicosanoids. Most eicosanoids are derivatives of the 20-carbon polyunsaturated fatty acid arachidonate and are referred to as "local hormones" functioning primarily on the cells near the point of release or on the same cell. Prostaglandins regulate a variety of physiological processes and can be involved in host parasite interaction (Daugschies and Joachim, 2000). In order for prostaglandins of the 2-series to be synthesized, there has to be free arachidonic acid available for the cyclooxygenase enzyme in the glands. Cyclooxygenase acitivity has been demonstrated in the salivary glands of the lone star tick (Aljamali et al., 2002).

Prostaglandin production and dopamine stimulation

Dopamine belongs to a group of compounds called catecholamines which are derived from tyrosine. Several other members include norepinephrine and epinephrine. These molecules are synthesized in the brain and other neural tissue where they function as neurotransmitters. Dopamine (DA) released from nerves impinging upon the tick salivary glands stimulates salivary secretion. An activated dopamine D_1 receptor stimulates adenylate cyclase, which then converts ATP to cAMP and cAMP is involved in fluid secretion (Schmidt et al., 1981; 1982). Dopamine also stimulates an influx of

Ca^{2+} into the cell, which activates an intracellular phospholipase ($cPLA_2$) (Bowman et al., 1995). Activation of the phospholipase A_2 cleaves arachidonic acid from the sn-2 position on the phospholipid bilayer. Free arachidonic acid is converted to prostaglandins by the cyclooxygenase pathway (Aljamali et al., 2002)

Dopamine Receptors

Dopamine (DA) is the predominant catecholamine neurotransmitter in the mammalian brain (Missale et al, 1998). In mammals, dopamine controls a variety of functions like locomotive activity, food intake and endocrine regulation. Human disorders such as Parkinson's disease, schizophrenia, Tourette's syndrome and hyperprolactinemia have been linked to unnatural control of dopaminergic transmission. Dopamine is known to act through multiple receptors in mammalian cells (Anderson et al, 1990). Work by Spano et al. (1978) demonstrated that DA receptors exist as two discrete populations, (1) positively coupled to adenylate cyclase (AC) and (2) independent of the cAMP system. Kebabian and Calne (1979) found that dopamine interacts with the D_1 receptor and activates AC. From these studies, the activation of AC via a D_1 receptor allowed for the accumulation of cAMP (Zhou et al, 1990). The dopamine, D_1 receptor was first cloned by Zhou et al. (1990). In the early 1980s, the function of the D_2 receptor was shown to inhibit AC (Enjalbert and Bockaert, 1983). From these studies and others, and subsequent cloning of the D_2 receptor its role inhibiting AC was confirmed (Gingrich and Caron, 1993).

Shipley et al. (1996) suggested that both D_1 and D_2 receptors may be present in the salivary glands of *Dermacentor variablis* (Say) based upon dose-dependent dopamine and dopamine receptor agonists and antagonist affects on cAMP accumulation in the

salivary glands of *Dermacentor andersoni*. It was suggested that the D_1 receptor was stimulated by dopamine through activation of AC via a G-protein. This activation then causes the conversion of ATP to cAMP and causes the activation of a protein kinase. The authors presented evidence suggesting that dopamine binding to a D_2 receptor inhibited this activation of AC, thus lowering the amount of cAMP at high concentrations of dopamine.

Arachidonic Acid Release

Piomelli et al., (1991) suggested that arachidonic acid could be released via dopamine interaction with a D_2 receptor in Chinese hamster ovarian (CHO) cells. This observation was further supported when D_2 agonists caused the potentiation of AA release in striated neuron cells in the presence of calcium (Schinelli et al., 1994). Despite work on the mechanism of dopamine-stimulated release, a true understanding is not clear. One suggested mechanism is D_2 receptor linkage to protein kinase C (PKC) activation. It was shown that this activation increases the AA release in the presence of D_2 agonists and a calcium ionophore (DiMarzo et al., 1993). Activation of a D_1 receptor demonstrated that this receptor did not affect the release of AA in CHO cells (Piomelli et al., 1991). This result was confirmed in studies that involved the use of D_1 agonists, which caused an inhibition of calcium-evoked AA release, which was also mimicked by forskolin (Schinelli et al., 1994).

Dopamine D_2 receptors and G-Proteins

In later experiments, D_2 transfected CHO cells activated by DA or a known D_2 agonist R- (+)-3-(3-Hydroxyphenyl)-N-propylpiperidine;(3-PPP) stimulated AA release without requiring the use of a Ca^{2+} mobilizing agent (Nilsson et al., 1998). The same authors were able to show involvement of D_2 receptors in AA release by inhibiting dopamine-stimulated release by a known D_2 antagonist, raclopride. In their findings, AA release was abolished by pertussis toxin (PTX), which suggests that the receptor is linked to a G-protein before releasing AA. Similar results were observed in earlier studies that demonstrated coupling of D_2 receptors to adenylate cyclase inhibition via a G-protein (G_i) in mammalian cells (Cockcroft et al., 1991). With studies involving PTX, D_2 inhibition of AC occurred when the G_i was inhibited. (Cockcroft et al., 1991).

G-proteins

At the cellular level, many hormones, neurotransmitters and other ligands effect cells by binding to G-protein coupled receptors. An important role for membrane-associated trimeric G-proteins is to determine the specificity and temporal characteristics of cellular responses to hormones and neurotransmitters. Trimeric G-proteins serve as a mechanism for information transfer across the lipid membrane (Stryer, 1988). Trimeric G-proteins consist of: α (45 kDa), β (35 kDa) and γ (7 kDa) subunits. There are four main classes of G-Proteins: G_s which activate adenylate cyclase, G_i which inhibit adenylate cyclase, G_q which activate phospholipase C (PLC) and G_{12}/G_{13} whose function is currently unknown (Hamm, 1998).

At rest, trimeric G-proteins are in the GDP-G-protein-inactive state. An exchange of Guanosine triphophate (GTP) for bound GDP causes the G-protein to become active with the heterotrimer dividing into G_{α}-GTP and $G_{\beta\gamma}$ subunits. The disassociated G_{α} subunit is the target of specific bacterial toxins (pertussis and cholera) that have a high affinity for GTP binding sites; and is an effector for intracellular regulatory enzymes like adenylate cyclase (AC) and cGMP phosphodiesterase (Jelsema andAxelrod, 1987). The GTP-bound α subunit's surface changes allowing for a 20-100 fold higher affinity to binding effectors compared to the GDP-bound inactive form(Hamm, 1998).

Effects of $G_{\beta\gamma}$

$G_{\beta\gamma}$ subunits are involved in facilitating the re-association of the complete G protein complex and both are required for specific G-protein interaction with receptor. Currently, not much is known about the exact role of the $\beta\gamma$-dimer upon release from the whole G-protein after ligand-receptor interactions. Originally the main function was thought to be deactivation of the G_{α} subunit (Clapham and Neer, 1997). Later evidence emerged showing that the $G_{\beta\gamma}$ dimer itself could regulate effectors. The first proof came when Logothetis et al. (1987) demonstrated that the $G_{\beta\gamma}$ activated a K^{+}-ion channel in cardiac cells. In the following years, more evidence was presented, supporting the hypothesis that dimeric $G_{\beta\gamma}$ subunits activate numerous effectors. A variety of effectors have been found to be regulated by one or both subunits. Numerically these effectors are regulated by one or two subunits particularly with 20 G_{α}, 6 G_{β} and 12 G_{γ} subunits providing 1440 possible combinations of signal transduction options (Chapman and Neer, 1997).

In recent years, work linking $G_{\beta\gamma}$ to Ca^{2+} mobilization has surfaced. Jelsema and Axelrod (1987) demonstrated that $G_{\beta\gamma}$ stimulated Ca^{2+}-dependent phospholipase A_2 in rod outer segments (ROS) of bovine retina. A direct role for the dimer stimulation of calcium influx was not shown. More recently, Thomason et al., (1994) demonstrated that $G_{\beta\gamma}$ of a G-protein coupled receptor activates PI (phosphoinositide) 3-kinase. PI-3 kinase is a heterodimer of an 85kDa regulatory subunit (p85α,β) and a 160 kDa catalytic subunit (p110α,β) and converts phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) ((Ui et al., 1995; Demirovic et al., 2000). Wortmannin has proven to be an important tool in assessing a role for PI-3 kinase because of its ability to specifically inhibit the enzyme (Ui et at., 1995; Thomason et al., 1994). Significantly, Viard et al., (1999) reported that $G_{\beta\gamma}$ stimulate L-type voltage-dependent Ca^{2+} channels via PI-3 kinase and stimulation is inhibited by wortmannin.

Objectives and Specific Aims

Ticks are important pests of humans and other mammals. When ticks feed, the salivary glands act as the organs of fluid secretion to concentrate the blood meal. Coincidentally the salivary glands serve as a conduit for pathogen transmission to the host. The salivary glands also counter the host's defense system by secreting a cocktail of bioactive molecules, which includes prostaglandins (Sauer et al, 2000). It has been shown that high levels of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ are found in the saliva of the lone star tick (Bowman et al, 1996; Aljamali et al., 2002).

For prostaglandins of the 2-series to be synthesized, the salivary glands must regulate release of their precursor, arachidonic acid. Previous work has shown that dopamine (the primary agonist stimulating secretion) initiates an influx of Ca^{2+} into the glands, activating an intracellular phospholipase (PLA_2) leading to the release of arachidonic acid (Bowman et al., 1995). Arachidonic acid is then converted to prostaglandins via the cyclooxygenase pathway (Aljamali et al., 2002). The goal of my research was to learn more about how AA is released from salivary glands after stimulation by dopamine. As part of this objective, I wanted to see if intracellular "second messenger" molecules are involved in release of arachidonic acid after gland stimulation by dopamine.

My specific aims are:

1. To determine the concentration of dopamine required to release AA.
2. To see if dopamine D_1 and D_2 agonists and antagonists affect the release of AA.
3. To determine the effects of second messenger molecules (Ca^{2+}, cAMP) and signal transduction pathways (protein kinase C) on AA release.
4. To determine if dopamine-stimulated release of AA occurs via a G-protein linked receptor mechanism and whether this G-protein action can be inhibited.

Chapter II

MATERIALS AND METHODS

Tick salivary gland collection

Adult female lone star ticks were reared on sheep according to the methods of Patrick and Hair (1975) at Oklahoma State University's Tick Rearing Facility. The females were removed during the "slow feeding" phase. Salivary glands then were dissected while submerged in ice-cold M199 buffer solution. Upon exposure, the right gland was removed and deemed the experimental gland and the left gland was removed and deemed the control gland. The dissected glands were transferred to microcentrifuge tubes (three pairs per tube) containing 400µl, ice-cold M199 solution. For all the experiments, ticks were used within two hours of their removal from the sheep and the glands were dissected (Bowman et al., 1995). Salivary glands were rinsed twice with 400µl ice-cold M199 with ~20s centrifugation at 5600g in between.

Incorporation of [^{3}H] arachidonic acid and first incubation

Salivary glands were resuspended in 150µl-oxygenated M199 containing 0.15µCi [^{3}H] AA and incubated for 60 min in a shaking water bath at 37°C to label cellular lipids.

Equilibrium Phase

At the end of the first incubation, the salivary glands were removed from the water bath and centrifuged for 20 seconds at 5600g. The labeling solution was discarded and the glands were resuspended in 400µl of M199 medium. Then the glands were

centrifuged for 20 seconds at the same speed as before and wash was repeated four times. At the end of the fourth centrifugation, the supernatant was removed and 150µl of oxygenated, ice-cold M199 added. The glands were then placed in a shaking water bath at 37°C for 60 minutes.

Testing of agonistic or antagonistic compounds

At the end of this "equilibrium period" the supernatant was discarded and the cells rinsed twice with 400µl of M199 medium. The pre-labeled salivary glands were resuspended and incubated, as before, in 150µl oxygenated M199 with and without various agonists and antagonists for 60 minutes. At the end of the incubation period, 325µl ice-cold 120-mM NaCl, 2.7 mM KCl in 10mM phosphate buffer, pH 7.4 containing 5mM EGTA was added to stop the reaction.

Lipid extraction

The cells and supernatant were then transferred to glass/glass homogenizers together with a further 325µl of the phosphate buffer, which was used to rinse the microcentrifuge tube. Lipids were extracted from the combined cells and media according to Bligh and Dyer (1959) and stored in chloroform containing 0.05% butylated hydroxytoluene (BHT) at –20°C.

Lipid analyses

Cellular lipid extracts were separated into neutral lipids, free fatty acids and phospholipids on aminopropyl solid phase extraction columns (500mg) by the method of

Kaluzny et al. (1985) as described by Shipley et al. (1994) and Bowman et al. (1995). Aminopropyl solid phase extraction columns with fitted stainless-steel frits are supplied by Varian, Harbor City, CA. The lipid fractions were dried down under a gentle stream of nitrogen, re-dissolved in 0.5ml methanol: acetic acid (99:1 v/v) and then mixed with 5ml BioCount (Research Products International Corp., Mount Prospect, IL) scintillation cocktail. The radioactivity was determined by liquid scintillation counting (Beckman LS6000SC, Fullerton, CA) employing an automatic quench correction curve.

Lipid extraction confirmation

In some samples, to validate the column solid-phase extraction method for separating and identifying labeled lipid classes, lipid extracts were separated by thin-layer chromatography on 20 x 20 cm silica gel G plates, 250 μm thickness in hexane/diethyl ether/acetic acid (75:25:1, v/v/v). Lipid classes were identified by comparing known lipid standards under iodine vapor. Silica bands corresponding to the lipids were scraped into vials and vortexed with 500μl methanol/acetic acid (99:1) and then 5ml Biocount scintillation fluid. Silica was allowed to settle for at least 5 h before radioactivity was determined by liquid scintillation counting.

Cyclic AMP assay

Lone star tick salivary glands were removed according to procedures previously. Two pairs of left and right glands were placed in separate pools of M199 medium. After removing excess trachea and gut material, the glands were resuspended in 150 μl of M199 medium containing theophylline. The left glands were deemed the control and not

stimulated, while the right glands were deemed the experimental. The experimental samples were stimulated with 10μM PPHT-HCl, a known D_2 receptor agonist. Both sets of glands were placed in a shaking water bath for 60 minutes.

After the 60 minutes incubation, the glands were removed. The control and experimental glands were homogenized in 7ml ground glass homogenizers for 10 minutes. The homogenate of both samples were transferred to two microcentrifuge tubes labeled "C" for control and "E" for experimental and placed in boiling water for 10 minutes. The assay developed by Amersham is based on the competition between unlabelled cyclic AMP versus a fixed quantity of the tritium labeled compound. The two compete for the binding to a protein which has a high affinity and specificity for cAMP. The amount of labeled protein-cAMP complex that is formed is inversely related to the amount of unlabeled cAMP that is created. Separation of bound protein-cAMP from the unbound nucleotide is carried out by charcoal absorption of the free nucleotide.

Reagents were prepared according to protocol outlined by Amersham. A Tris-EDTA buffer, binding protein, charcoal absorbent, labeled tracer and cAMP standard were reconstituted with correct buffers supplied by Amersham. Next, five cyclic AMP standards were prepared in microcentrifuge tubes: 16, 8, 4, 2, and 1 pmol using serial dilution. After incubation the tubes were placed in an ice water bath. 150μl of reagent 1 (Tris-EDTA buffer) was loaded into two microcentrifuge tubes to serve as a blank. 50μl of reagent 1 was loaded into two other tubes to determine the binding of label in the absence of unlabeled cAMP. The next ten tubes were loaded, starting with the lowest level of cAMP standard with 50μl in successive pairs of assay tubes (5-14). Then tubes 15-26 had 50μl of unknown homogenate added to them. Next all assay tubes had 50μl of

reagent 3 (labeled cAMP) added. Lastly tubes 3-26 then had reagent 2 (binding protein) added to them for a final volume of 200µl.

The assay tubes were then vortexed for 5 seconds and placed back into the ice bath. The ice bath was then placed into a 4°C refrigerator for two hours. At the completion of the two hour incubation, 100µl of charcoal suspension was added to all assay tubes. The tubes were then centrifuged for 10 minutes at 15,000g's. Without disturbing the charcoal, 200µl of the supernatant was removed and placed into a scintillation vial with 20ml of scintillation fluid and radioactivity was counted with a Beckman L56000SC liquid scintillation counter.

Protein Assay

To determine the protein concentration in the cAMP assays, a standard protein assay was carried out. Prior to the assay, eight standards were prepared according to the procedure prescribed by BioRad (Hercules, CA). The eight protein standards were composed of BSA/dH_20 and had final concentrations of: 0.025, 0.0375, 0.10, 0.15, 0.20, 0.25 and .20µg/ml. Dye reagent was diluted to a working concentration according to manufacture's instructions.

A Falcon #3915-96 well plate was used to conduct the protein assay. In the first three spaces (moving from left to right) a blank was loaded into the wells. Next 20µl of standard #1 was added into the next three wells. Each standard continuing from 2-8 was added in triplicate with 20µl into each well. After standards were loaded, 180µl of the dye reagent was added. It was previously determined that 5µl of the unknown (control and experimental homogenates) would be loaded in the well with 15µl of dH_2O and

180µl of dye reagent. According to manufacture's instructions, plate must incubate after dye has been added for 5 minutes but no longer than one hour. After incubation, plate was read on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) at 595 nm. Unknown protein concentration was calculated using Softmax® Pro software ver. 1.1 (Molecular Devices, Sunnyvale CA).

Presentation of Data

Data were calculated to show the percent difference in distribution of labeled AA in experimental salivary glands as compared to distribution in control glands. Percent differences were calculated as follows:

1. The total radioactivity found in all three fractions (PLs, NLs, FAA) was determined and the percent radioactive AA of each fraction was calculated.
2. Results in figures are expressed as the differences in the percent of each fraction between the experimental and control glands.

Statistical Analysis

The results are expressed as mean $\pm$ standard error. Percent differences between control and experimental amounts of labeled AA in phospholipids (PL), free fatty acid (FFA) and neutral lipids (NL) were tested for significance by the Student's t-test. A P-value ($p<0.05$) was considered significant.

Materials

Chemicals were obtained from the following sources: Dopamine, Ca^{2+} ionophore A-23187, forskolin, PPHT-HCl, haloperidol, spiperone, R-(+)-SKF-38393, (±)SFK-82958 hydrobromide, R-(+)-SCH-23390, quinelorane, wortmannin, theophylline, M199 medium and phosphate buffered saline solution are purchased from Sigma, St. Louis, MO. Arachidonic acid [5,6,8,9,11,12,14,15-^{3}H(N)]- (100Ci/mmol) was purchased from DuPont®-New England Nuclear, Wilmington, DE. The cyclic AMP (^{3}H) assay system complete with reagents were purchased from Amersham. Aminopropyl solid phase extraction columns with fitted stainless-steel frits were purchased from Varian, Harbor City, CA.

CHAPTER III

RESULTS

Effects of dopamine on the release of labeled arachidonic acid from tick salivary gland lipids.

Previous work demonstrated that stimulation of salivary glands by dopamine evoked release of AA from phospholipids via a verapamil-sensitive mechanism (Bowman et, al 1995). Verapamil is a known inhibitor of voltage-dependent Ca^{2+} channels, suggesting that dopamine stimulates influx of Ca^{2+} and activates a Ca^{2+}-sensitive phospholipase A_2 (PLA_2).

DA stimulated a dose-dependent release of arachidonic acid from salivary glands, that was significant at 1.0 and 10.0 μM, ($p<0.05$) (Figure 1). The amount of AA in the phospholipid fraction decreased with increasing dopamine concentration. There was a corresponding increase in labeled AA in the neutral lipid (NL) fraction. Bowman et al. (1995) have shown a similar shift of labeled AA into the neutral lipid fraction during gland stimulation and that almost all the neutral lipid is in the form of triglyceride (Bowman et al., 1995). As demonstrated before by Bowman et. al. (1995), verapamil blocked the ability of dopamine to release AA but substantially only if glands were pre-incubated with verapamil prior to stimulation with DA (Figure 2). Statistically significant changes were observed in the fatty acid and neutral lipid fractions ($p<0.05$).

The effects of dopamine D_1 and D_2 receptor agonists/antagonists on release of labeled AA.

Stimulation of D_2 receptors in CHO cells elicits release of free AA (Piomelli et al., 1991). This was shown further by Schinelli et al. (1994) who demonstrated AA

release in neurons via D_2-receptor activation. Two mammalian D_1 receptor agonists (SKF-38393 and SKF-82958) stimulated an apparent release of AA but the changes noted were highly variable and not statistically significant (Figure 3).

Previous work demonstrated a dopamine D_1-like receptor in the salivary glands of the lone star tick (Schmidt et al., 1981, 1982). PPHT-HCl, a D_2-receptor agonist in mammals potentiated a significant release of AA from salivary phospholipids ($p<0.05$), with a corresponding increase in AA associated with the neutral lipid fraction ($p<0.05$) (Figure 4). Quinelorane, another mammalian D_2-receptor agonist was ineffective in stimulating re-distribution of AA in lipid fraction (Figure 4). Neither D_2 receptor antagonists (spiperone and haloperidol in combination with dopamine; 10μM) had any affect on release of AA (Figure 5). To investigate further whether a D_2-like receptor is present in the tick salivary gland and involved in release of free AA, a known mammalian D_1 antagonist (SCH-23390; 10μM) was used in combination with dopamine (Figure 6). The D_1 receptor antagonist did not affect significant changes in redistribution of labeled AA as compared to dopamine.

Effects of second messengers on the release of labeled AA from tick salivary gland phospholipids.

Prostaglandin production in the lone star tick relies on the availability of AA. AA is released from phospholipids via the activation of a phospholipase (PLA_2), which cleaves AA from the sn-2 position. Free AA is then converted to prostaglandin via the cyclooxygenase pathway (Bowman et al., 1995). Dillwith et al. demonstrated Ca^{+2} dependent PLA_2 activities in the salivary glands of the lone star tick (unpublished data).

Three different concentrations of the calcium ionophore A-23187 (0.1, 1.0., 10.0μM) displayed an apparent increase in release of free arachidonic acid with release significant at 10.0μM; $p<0.05$ (Figure 7). The next experiment was to see whether verapamil (Ca^{2+} channel blocker) could block AA release stimulated by A-23187, a known calcium ionophore. Surprisingly statistically significant inhibition ($p<0.05$) was recorded in the free fatty acid fraction as compared to that stimulated by 10μM A-23187 (Figure 8).

Verapamil was tested to see if it affected redistribution of AA, as compared to that seen by dopamine D_1 and D_2 agonists alone. Although SKF-82958 on its own did not affect release of AA, verapamil co-incubated with SKF-82958 significantly inhibited release ($p<0.05$) of AA. A similar result was not seen with PPHT-HCl possibly because the salivary glands were not pre-incubated with verapamil prior to adding PPHT-HCl (Figure 9).

As noted a dopamine D_1-like receptor is present in tick salivary glands (Schmidt et al., 1981, 1982). Stimulation of D_1 receptor in tick salivary gland activates adenylate cyclase converting ATP into cAMP. Neither forskolin nor dibutryl cAMP stimulated AA release but instead caused an apparent re-incorporation of labeled AA into the phospholipid fraction. Forskolin was more effective than dibutyrl cAMP in this process (Figure 10).

D_2 receptors have been linked to G_i protein mediated activation of protein kinase C (PKC) in certain cells (Cockcroft et al., 1991). In Chinese hamster ovary (CHO) cells, release of AA occurs via D_2 receptor occupation and coupling to a G_i protein, activation of PKC and inhibition of cAMP levels (Di Marzo et. al., 1993). Experiments involving

the use of phorbol ester, PMA (PKC agonist) and A-23187 together stimulated release of AA from CHO cells (Di Marzo et. al., 1993). In my experiments 0.1μM A-23187 and PMA stimulated a statistically significant release of free fatty acid, $p<0.05$ (Figure 11). The effect of PMA by itself on the release of labeled AA was not investigated. The same concentration of A-23187 on its own had no affect on re-distribution of AA (Figure 7).

Salivary glands were incubated with PPHT-HCl to see whether the known D_2 receptor agonist decreased levels of cAMP production in the tick salivary glands. Surprisingly the D_2 receptor agonist caused a statistically significant increase ($p<0.05$) in cAMP levels (Table 1).

Role of G-proteins on the release of labeled AA from tick salivary glands phospholipids.

To further assess whether dopamine-stimulated release occurs via a G-protein, pre-labeled salivary glands were incubated with varying concentrations of GTPγ-S, a non-hydrolyzable analogue of GTP, pertussis toxin, cholera toxin and wortmannin (fungal metabolite). It is known that wortmannin can inhibit the release of AA by blocking PI-3 kinase (Viard et al., 1999). PI-3 kinase was shown to stimulate L-type calcium channels (Viard et al., 1999).

GTPγ-S potentiated an apparent release of AA but the effect was not statistically significant (Figure 12). However, GTPγ-S together with PPHT-HCl, stimulated a substantial and significant release of free fatty acid from the phospholipid fractions at 10μM; ($p<0.05$) (Figure 12).

Pertussis toxin had no affect on the release of AA; on the other hand, cholera toxin stimulated a significant release of AA from the neutral lipid and phospholipid fractions ($p<0.05$) (Figure 13). Both experiments were compared to a blank control.

To investigate whether G-protein-linked release of AA involves $G_{\beta\gamma}$ salivary glands were pre-incubated with wortmannin for 10 or 60 min prior to stimulation with 10μM of PPHT-HCl and GTPγ-S. Salivary glands pre-incubated with wortmannin for 60 min inhibited release of AA ($p<0.005$) (Figure 14).

In other experiments, salivary glands were pre-incubated with wortmannin for one hour prior to and then stimulated with GTPγ-S in the absence of wortmannin. There was no difference in the amount of AA released in salivary glands pre-incubated with or without wortmannin. On the other hand, salivary glands pre-incubated with wortmannin for one hour, and then stimulated with GTPγ-S and wortmannin together inhibited release of AA as compared to that release stimulated by GTPγ-S ($p<0.05$) (Figure 15).

CHAPTER IV

DISCUSSION

Results indicate that factors controlling release and re-distribution of arachidonic acid in tick salivary glands are linked to a dopamine stimulated, G-protein-linked receptor that evokes an influx of Ca^{2+} and activation of PLA_2. My results confirm that dopamine potentiates release of free arachidonic acid in a dose-dependent manner and that release is inhibited by voltage-dependent Ca^{2+} blocker verapamil. The inhibition by verapamil was most effective if salivary glands were pre-incubated with verapamil before stimulation. Verapamil inhibition of arachidonic acid release stimulated by A-23187 and SKF 82958 is unclear. The inhibition could reflect inhibition of release that occurs as a result of an endogenous activation of Ca^{2+} channels. This hypothesis is supported by substantially more inhibition of Ca^{2+} release than that seen in DA-stimulated glands. A similar result was seen by Bowman et al. (1995). The dopamine results were the basis for further investigation into the type of dopamine receptor in the salivary glands that may be involved in release of free levels of AA. Shipley et al., (1996) suggested that D_1 and D_2 receptors may be present in the salivary glands of *D. variables* ticks based upon changes in levels of cAMP after stimulation of salivary glands with different concentrations of dopamine and use of dopamine receptor antagonists. An interesting observation was made from the D_1-receptor agonist experiment. Both agonists caused an apparent release of labeled AA, but the distribution was not consistent. SKF-82958 had measurable amounts of labeled AA in the NL and FFA fractions while SKF-38393 had measurable amounts of labeled AA in the PL and FFA fractions. The exact reason for this varied

distribution is unclear. However, results suggest that the salivary glands may not have more than one dopamine receptor linked to release of AA. This hypothesis is supported by increased levels of cAMP after stimulation of glands by dopamine, D_2-receptor agonist PPHT-HCl (Table 2). Stimulation of D_2 receptors is expected to inhibit adenylate cyclase and thwart the production of cAMP (Enjalbet and Bockaet, 1983).

An interesting observation from these experiments was the significant findings involving the NL fractions. Whenever DA (or dopamine receptor agonist) was used to stimulate AA release the change in the amount of labeled AA associated with the neutral lipid fraction was significant. I hypothesize that the NLs function as a "sink" or way-station for free AA before transfer to PLs. Evidence in support of this hypothsis comes from the work of Bowman et al. (1995) who demonstrated that labeled AA is first esterfied into PL. Only trace amounts of [^{3}H]-AA become incorporated in the neutral lipid (NL) fraction primarily in the form of triglycerides (TGs). However at higher concentrations of free AA, more labeled AA became esterified in the neutral lipid fraction presumably due to activity of a diacylglycerol acyl transferase (DAT) (Bowman et al., 1995). It was hypothesized that at high concentrations of free AA, the fatty acid was esterfied into TGs due to saturation of lysophosphatide acyl transferase (LAT).

Bowman et al., (1995) also suggested that dopamine stimulates an influx of extracelluar calcium which activates an intracellular phospholipase ($cPLA_2$). My results support the importance of extracellular Ca^{2+} as shown by increasing release of free AA with increasing concentrations of the Ca^{2+} ionophore A-23187. PLA_2 cleaves AA from the sn-2 position of phospholipids freeing the fatty acid which can be converted to prostaglandins via the cyclooxygenase pathway (Aljamali et al., 2002). However, as the

concentration of A-23187 was increased, the amount of free AA increased but little change was seen in labeled AA associated with the NL fraction in contrast to that seen with dopamine. Clearly factors regulating re-distribution of AA between PLs, NLs, and free AA differ depending upon the method of stimulation. A series of experiments were conducted to investigate the role of second messengers (cAMP and PKC) in the release of arachidonic acid. It is known that dopamine stimulates a dopamine D_1-like receptor and adenylate cyclase activity that converts ATP to cAMP (Schmidt et al., 1981, 1982). Two experiments were conducted to investigate whether cAMP potentiates the release of arachidonic acid. Both forskolin (a direct activator of adenylate cyclase, (Schinelli et al., 1994) and dibutryl cAMP were ineffective and instead stimulated an apparent re-esterification of AA into the gland phospholipids. In an experiment to investigate whether PKC activation could potentiate the release of arachidonic acid, phorbol ester was used. I followed the protocol described by Di Marzo et al. (1993), where they used the PKC activator in conjunction with a low concentration of the A-23187 and dopamine. The results were inconclusive. A possible role for PKC in affecting release of AA requires further investigation or possibly testing the effect of phorbol ester on its own or under other conditions.

G- proteins perform an important function in coupling occupied receptors to effector enzymes and cellular signaling. Trimeric G-proteins serve as a mechanism for information transfer across the lipid membrane. Trimeric (heterotrimeric) G-proteins consist of an: α (45 kDa), β (35 kDa) and γ (7 kDa) subunits. Upon stimulation by effector ligands, inactive trimeric G-proteins exchanges GTP for bound GDP, thus activating the G-protein. This activation causes the trimeric G-protein to rapidly separate

into an G_α subunit and a dimeric $G_{\beta\gamma}$ subunit (Hamm, 1998). Axelrod and Jelsema (1987), suggested that G-proteins activated an influx of extracellular Ca^{2+} and cytoplasmic PLA_2 ($cPLA_2$). Viard et al., (1999) reported that dimeric $G_{\beta\gamma}$ stimulates an influx of calcium via activation of phosphoinositide 3-kinase (PI-3 kinase) and downstream activation of voltage-dependent Ca^{2+} channels. PI-3 kinase converts PIP_2 to PIP_3 and is specifically inhibited by the fungal metabolite, wortmannin (Viard et al., 1999). Indirect evidence suggests that PIP_2 is present in the tick salivary glands based upon increased levels of IP_3 after stimulating glands with a protein factor in the tick synaganglion or PGE_2 (McSwain et al., 1989; Qian et al., 1998). In order to test the possibility of G-proteins being involved with AA release, tick salivary glands were incubated with a G-protein activator, GTPγ-S. GTPγ-S is a non-hydrolyzable analogue of GTP which keeps the G-protein in an active state. Incubation with this activator caused an apparent release of labeled AA. In other experiments, salivary glands were co-incubated with GTPγ-S and PPHT-HCl. A statistically significant increase in the FFA fraction was observed in the latter experiment. The cause for this unusually large increase in free AA is unclear. One possibility is that the dopamine receptor agonists stimulate another intracellular component that in conjunction with an activated G-protein, causes the release of labeled AA. Significantly, my results indicate that AA release in response to GTPγ-S and PPHT-HCl is consistently and significantly inhibited by wortmannin when salivary glands were pre-incubated with wortmannin for either 10 or 60 minutes when stimulated with PPHT-HCl and GTPγ-S. Glands pre-incubated with wortmannin, significantly inhibited release of AA stimulated by GTPγ-S alone. The results suggest at least a partial role for dimeric $G_{\beta\gamma}$ in effecting release of AA from

salivary gland PLs hypothesized to be via activation of PI-3 kinase and opening of a Ca^{2+} channel.

To help identify what type of G-protein involved in release of AA in the salivary glands, two toxins known to affect two different kinds of G-proteins were used. An apparent and significant release of labeled arachidonic acid was stimulated by cholera toxin but not petrussis toxin (Figure 12).

Overall, I propose the following tentative model to help explain how free levels of AA are regulated in ixodid tick salivary glands. I suggest that after binding of dopamine to a D_1-like G-protein linked receptors, a $G_{\beta\gamma}$ subunit is produced that causes an influx of calcium via activation of a PI-3 kinase. The influx of Ca^{2+} activates PLA_2, which hydrolyzes arachidonic acid from the sn-2 position of PLs. Free arachidonic acid is then converted to PGE_2 via the COX pathway (Figure 16). Identification of PI-3 kinase and its role in tick salivary glands and possible molecular work on the cholera sensitive G-protein needs further investigation.

CHAPTER V

SUMMARY

Ticks play an important role in medical and veterinary medicine throughout the world. The lone star tick feeds on three different hosts for each of its parasitic life cycle stages. The adult female undergoes slow feeding that lasts seven to fourteen days followed by a more rapid feeding period that lasts 24 to 48 hours. During tick feeding, the salivary glands counter the host's defense system by secreting a cocktail of bioactive molecules. An important component of saliva is prostaglandins (PGs). Our laboratory has identified high concentrations of two PGs: PGE_2 and $PGE_{2\alpha}$ (Aljamali et al., 2002).

For PGs to be synthesized, free arachidonic acid (FAA) must be released and transformed by the cyclooxygenase pathway (COX). Dopamine (DA) released from nerve endings controls tick salivary fluid secretions. DA binds to a D_1-like receptor and stimulates adenylate cyclase (AC) that converts ATP to cAMP. cAMP stimulates fluid secretion (Schmidt et al., 1981; 1982). Related work in the laboratory has shown that DA stimulates an influx of Ca^{2+} into the glands and activates an intracellular phospholipase ($cPLA_2$) that cleaves AA from the sn-2 position of the phospholipids (PLs) (Bowman et al., 1995).

My studies have shown that AA is released in a dose-dependent manner, by dopamine. AA release is inhibited through pre-incubation with verapamil (Ca^{2+} channel blocker) prior to stimulation with DA.

Shipley et al. (1996) suggested that both D_1 and D_2-like receptors might be present in the salivary glands of *Dermacentor variables* (Say). It was believed that the

D_1-receptor (stimulated by DA) linked to a G-protein could activate AC and cause the conversion of ATP to cAMP. Shipley et al. (1996), found that at high concentrations of DA, the amount of cAMP was lowered and suggested that a D_2 receptor was involved in this inhibition. Piomelli et al., (1991) suggested that AA could be released through the activation of D_2 receptors in Chinese hamster ovarian (CHO) cells.

The possibility of two DA receptors envolved in AA release was studied further. A series of experiments was conducted using mammalian D_1 and D_2 receptor agonists. Use of either D_1 receptor agonists invoked release of AA. D_2-receptor antagonists were ineffective in inhibiting the release of AA in the presence of DA. Only the D_1 receptor antagonist SCH-23390 was successful in inhibiting the release of AA stimulated by DA.

The results do not support the hypothesis that an additional dopamine, D_2-like receptor is important in effecting release of AA. Release of free AA via stimulation by either D_1 or D_2 receptors was blocked by pre-incubating salivary glands with verapamil.

Cockcroft et al.(1991) presented evidence that activated D_2 receptors typically inhibit AC and decrease cAMP levels. In my research, salivary glands stimulated with a mammalian D_2-receptor agonist, PPHT-HCl did not cause an increase in cAMP during a radio-immune assay. In fact, the agonist caused an increase in cAMP levels, thus suggesting that PPHT-HCl may work through a D_1-like receptor.

As mentioned before, free AA is converted to PGs via the COX pathway (Bowman et al., 1995) and to release this AA, the presence of Ca^{2+} is needed to activate PLA_2. In unpublished data, Ca^{2+} dependent PLA_2 was seen to have high activity in the salivary glands of the lone star tick. In order to further study this activity of second messengers on the release of AA in the salivary glands, a known calcium ionophore (A-

23187) was used. In these studies, increasing concentrations of A-23187 were able to stimulate release of AA further supporting the theory that release is via activation of a Ca^{2+} dependent PLA_2. Just as in the study with DA-induced release of AA, the calcium channel blocker verapamil was used. In these studies, verapamil was able to block the release of AA in glands that were co-incubated with the ionophore and the calcium channel blocker. An explanation for this blockage could be that verapamil inhibits an endogenous factor that stimulates the release of AA. A similar result was observed by Bowman et al. (1995). Even though these results were comparable to those achieved by the dopamine studies, the involvement of a second messenger on the release of AA is not believed to be direct.

In researching the mechanism of how AA is released in the salivary glands, other cellular membrane pathways were investigated. Axelrod and Jelsema (1987) suggested that activation of a heterotrimeric G-protein produced a dimeric $G_{\beta\gamma}$ that could have a possible role in the release of AA. Viard et al., (1999), found that $G_{\beta\gamma}$ (following ligand binding to a G-protein linked receptor) stimulates voltage-dependent L-type Ca^{2+} channels via activation of phosphotidylinositol-3-kinase (PI-3 kinase). I was able to show release of AA when glands were incubated with a non-hydrolyzable analogue of GTP, GTPγ-S. However, the greatest release achieved occurred when salivary glands were co-incubated with GTPγ-S and PPHT-HCl. I also showed that release of AA was sensitive to cholera-toxin.

In order to see if the $G_{\beta\gamma}$ subunit was linked to the release of AA, wortmannin was used in a series of experiments. $G_{\beta\gamma}$ stimulates PI-3 kinase and wortmannin is a specific inhibitor of PI-3 kinase (Viard et al., 1999). The first series of experiments examined

length of incubation involving wortmannin and the release of AA. Both time periods of 10 minutes and 60 minutes incubations inhibited the release of AA, with significant results in the latter incubation. With release of AA using GTPγ-S, the next series of experiments examined whether or not wortmannin could inhibit the release of AA in the presence of GTPγ-S. It was determined that salivary glands pre-incubated with wortmannin for one hour and then stimulated with GTPγ-S plus wortmannin, significantly inhibited the release of AA as compared to GTPγ-S alone.

Overall, I hypothesize the following: dopamine is released from nerve endings and binds to a G-protein-linked dopamine D_1-like receptor. Upon interation with the ligand occupied receptor, the G-protein separates into G_α and a dimeric $G_{\beta\gamma}$. The $G_{\beta\gamma}$ subunit then activates an influx of Ca^{2+} via a PI-3 kinase stimulation. This influx of Ca^{2+} activates a PLA_2 which cleaves AA from the sn-2 position of the PL. Free AA is then converted to PGE_2 via the COX pathway. My results do not preclude that other intracellular factors may be involved in regulatory re-distribution of AA following salivary gland stimulation by DA. Further work is needed to confirm this hypothesis.

Figure 1. Dose-dependent re-distribution of [^{3}H] arachidonic acid amongst phospholipids (PL), free arachidonic acid (FAA) and neutral lipids (NL) from isolated salivary glands of the lone star tick during 60 minute incubations with dopamine. The results are expressed as changes in the percentage of total incorporated [^{3}H] arachidonic acid as compared to unstimulated control glands. Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

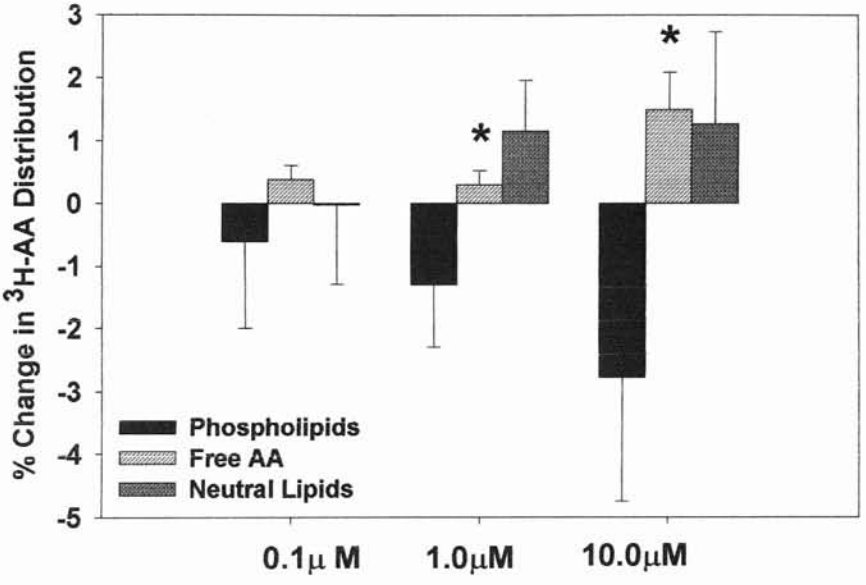
% Change in ^{3}H-AA Distribution
3
2
1
0
-1
-2
-3
-4
-5
*
*
Phospholipids
Free AA
Neutral Lipids
0.1μ M
1.0μM
10.0μM

Figure 2. Effect of 10μM dopamine and 1mM verapamil on the re-distribution of [^{3}H] arachidonic acid in isolated *A. americanum* salivary glands during 60 minute incubations. Salivary glands were either pre-incubated with 1mM verapamil for 7.5 minutes prior to stimulation with dopamine or were not pre-incubated at all. The results are expressed as the percent change of total incorporated [^{3}H] arachidonic acid from glands stimulated with DA and verapamil (experimental) relative to glands only stimulated with DA (control). Values are expressed as the mean $\pm$ SE, n = 4 and statistically significant results are identified with an asterisk.

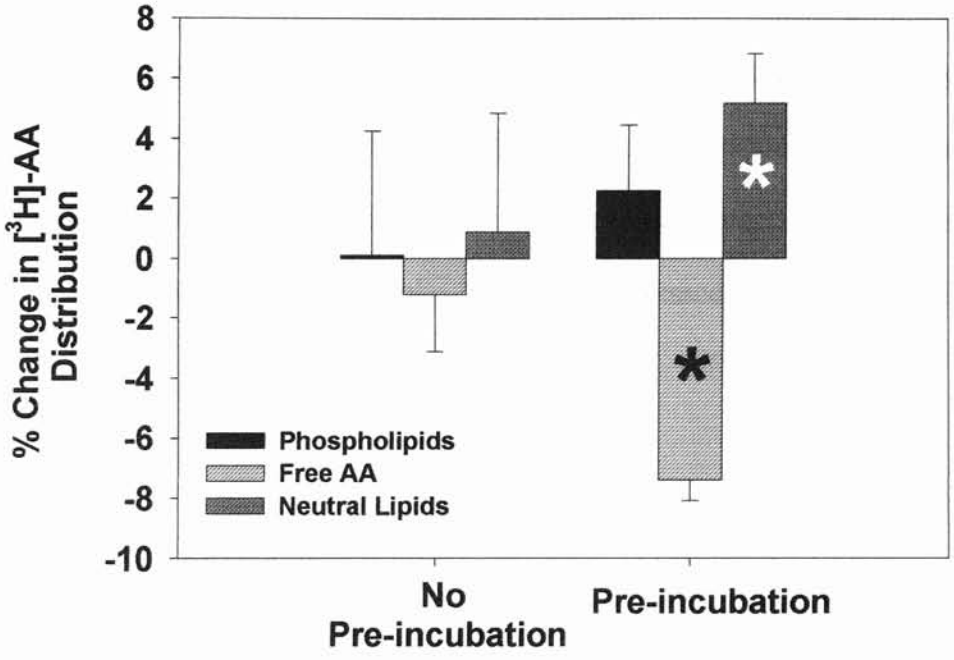
% Change in [3H]-AA Distribution
8
6
4
2
0
-2
-4
-6
-8
-10
Phospholipids
Free AA
Neutral Lipids
*
*
No Pre-incubation
Pre-incubation

Figure 3. The effects of 10μM mammalian dopamine D_1 receptor agonists (SKF-82958 & SKF-38393) on the re-distribution of [^{3}H] arachidonic acid from isolated *A. americanum* salivary glands during 60 min incubations. Results are expressed as percent change of [^{3}H] arachidonic acid incorporated from stimulated glands (experimental) relative to unstimulated glands (control). Values are expressed as the mean $\pm$ SE, n = 8.

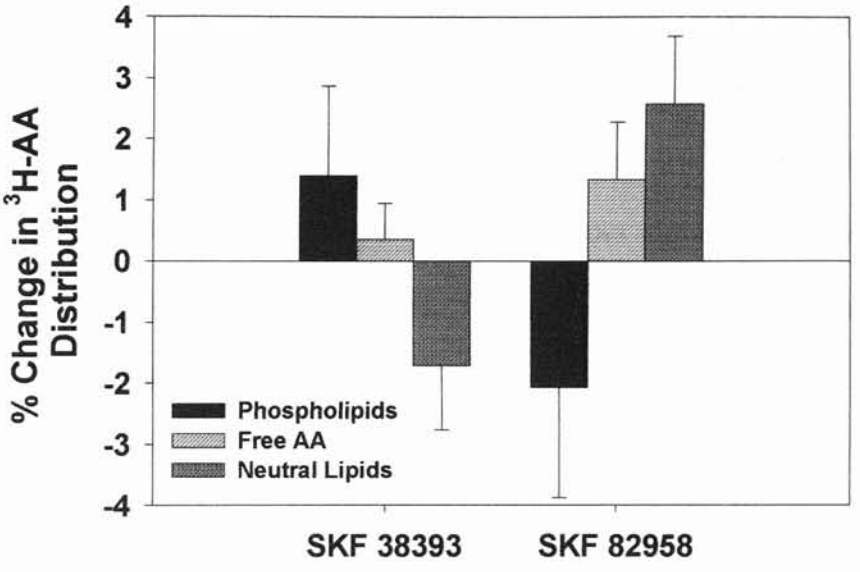
% Change in ^{3}H-AA Distribution
4
3
2
1
0
-1
-2
-3
-4
Phospholipids
Free AA
Neutral Lipids
SKF 38393
SKF 82958

Figure 4. The effects of 10μM mammalian dopamine D_2 receptor agonists (PPHT-HCl & Quinelorane) on the re-distribution of [^{3}H] arachidonic acid from isolated *A. americanum* salivary glands during 60 min incubations. Results are expressed as percent change of total incorporated [^{3}H] arachidonic acid from stimulated glands (experimental) relative to unstimulated glands (control). Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

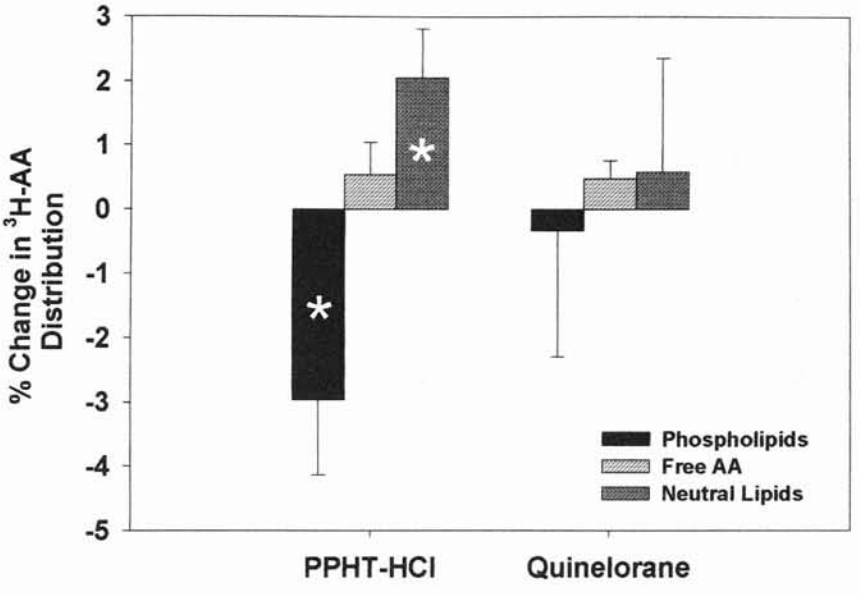
% Change in ^{3}H-AA Distribution
3
2
1
0
-1
-2
-3
-4
-5
*
*
Phospholipids
Free AA
Neutral Lipids
PPHT-HCl
Quinelorane

Figure 5. Effect of 10μM dopamine co-incubated with 10μM of two known mammalian D2 receptor antagonists (spiperone and haloperidol) on the re-distribution of [3H] arachidonic acid in isolated salivary glands during 60 minute incubations. The results are expressed as the percent change of total incorporated [3H] arachidonic acid from co-stimulated glands relative to dopamine stimulated glands (control). Values are expressed as the mean $\pm$ SE, n = 4.

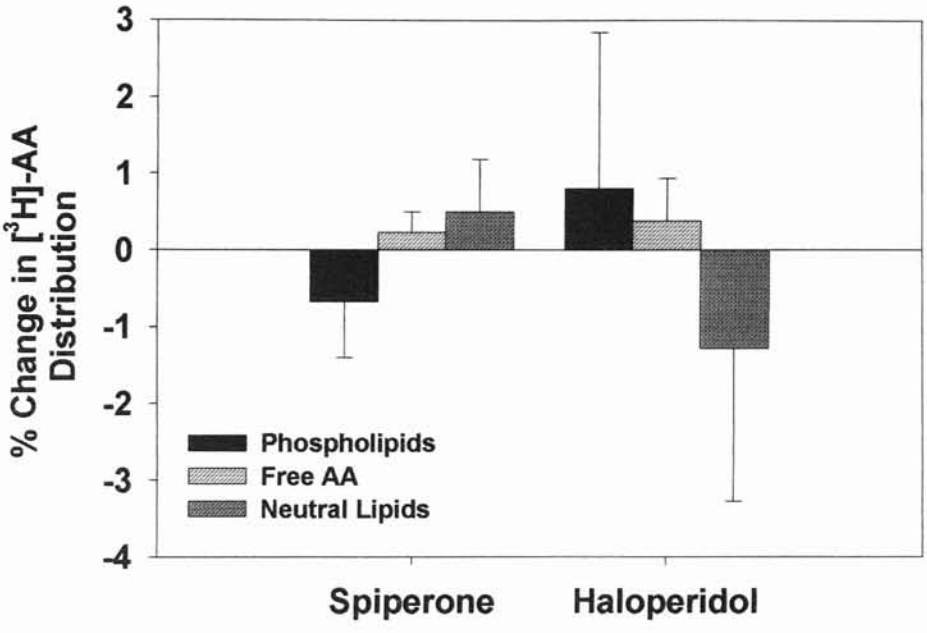
% Change in [3H]-AA Distribution
3
2
1
0
-1
-2
-3
-4
Phospholipids
Free AA
Neutral Lipids
Spiperone
Haloperidol

Figure 6. The apparent re-distribution of [^{3}H] arachidonic acid upon stimulation with 10μM dopamine in the presence of a mammalian D_1 receptor antagonist, 10μM SCH-23390 in isolated A. americanum salivary glands during 60 min incubations. The results are expressed as percent change of the total [^{3}H] arachidonic acid incorporated from glands incubated with dopamine and SCH-23390 (experimental) relative to dopamine stimulated glands (control). Values are expressed as the mean $\pm$ SE, n = 8.

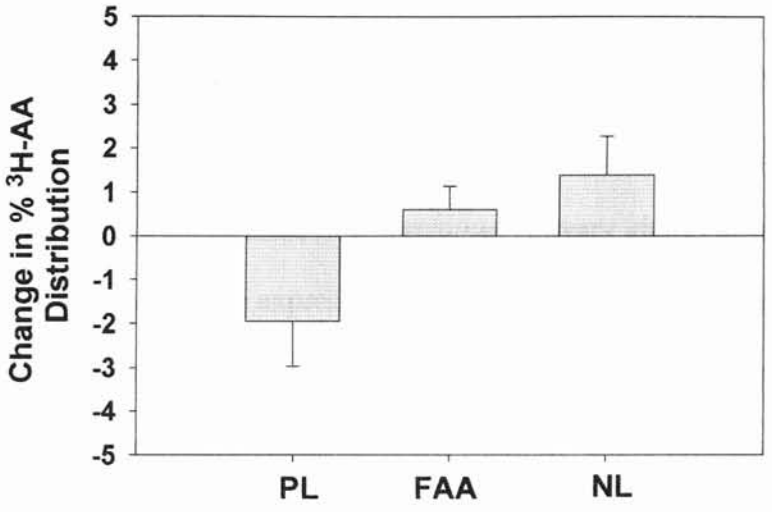
Change in % 3H-AA Distribution
5
4
3
2
1
0
-1
-2
-3
-4
-5
PL
FAA
NL

Figure 7. Dose-dependent effect of calcium ionophore, A-23187 on re-distribution of [^{3}H] arachidonic acid amongst phospholipids (PL), free arachidonic acid (FAA) and neutral lipids (NL) from isolated *A. americanum* salivary glands during 60 min incubations. The results are expressed as percent change of total incorporated [^{3}H] arachidonic acid from stimulated glands (experimental) relative to unstimulated glands (control). Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

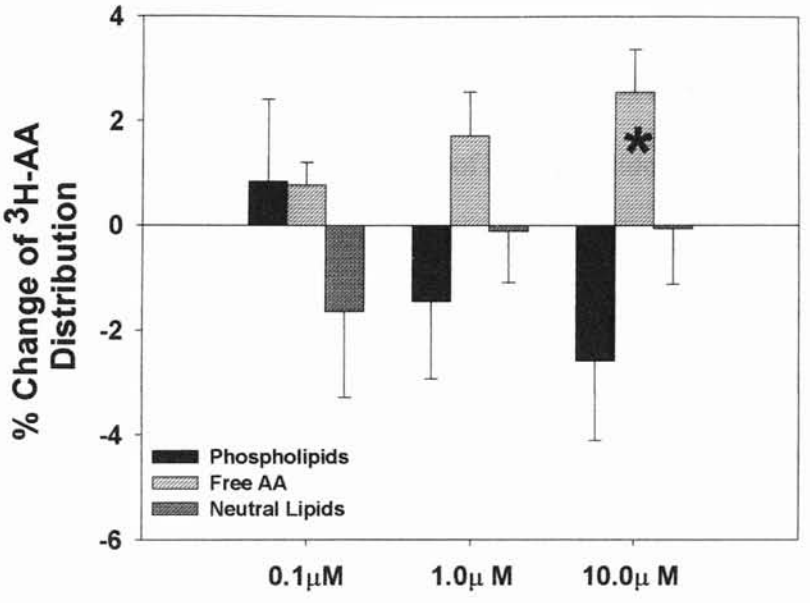
% Change of 3H-AA Distribution
4
2
0
-2
-4
-6
*
Phospholipids
Free AA
Neutral Lipids
0.1μM
1.0μ M
10.0μ M

Figure 8. Effect of 10μM calcium ionophore, A-23187 stimulation in the presence of 10μM verapamil, on the re-distribution of [^{3}H] arachidonic acid in isolated salivary glands of *A. americanum*. Results are expressed as the percent change in total [^{3}H] arachidonic acid incorporated from glands co-incubated with A-23187 and verapamil (experimental) relative to glands incubated only with A-23187 (control). Glands were not pre-incubated with verapamil. Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

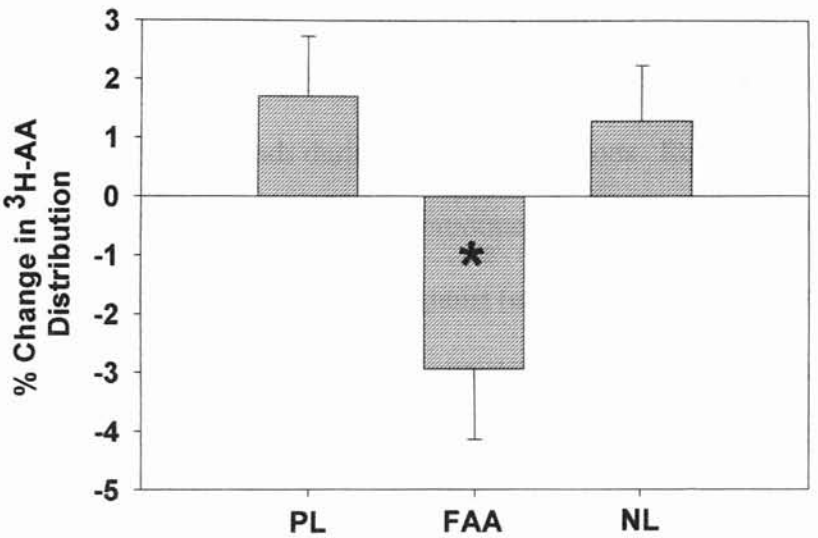
% Change in ^{3}H-AA Distribution
3
2
1
0
-1
-2
-3
-4
-5
*
PL
FAA
NL

Figure 9. Effect of 1mM verapamil and 10μM SKF-82958 and PPHT-HCl **(D_1 and D_2 receptor agonists respectively)** on the re-distribution of [^{3}H] arachidonic acid from isolated *A. americanum* salivary glands during 60 min incubations. Results are expressed as percent total of [^{3}H] arachidonic acid incorporated from glands co-incubated with verapamil and either D_1 or D_2 receptor agonist (experimental) relative to glands stimulated with only dopamine receptor agonists (control). Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

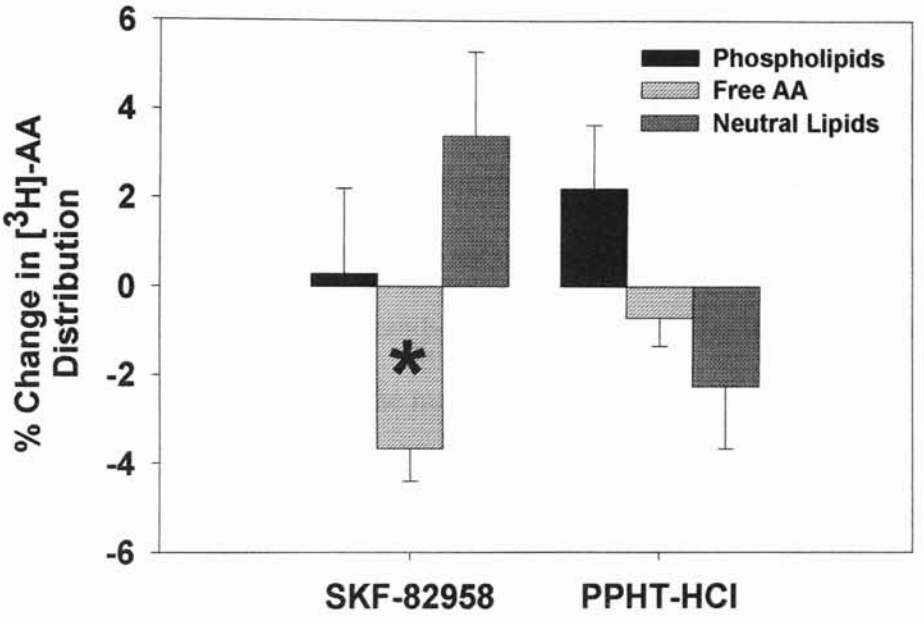
6
4
2
0
-2
-4
-6
% Change in [3H]-AA Distribution
Phospholipids
Free AA
Neutral Lipids
*
SKF-82958
PPHT-HCl

Figure 10. Effect on the re-distribution of [^{3}H] arachidonic acid from isolated salivary glands of *A. americanum* incubated for 60 min with 10μM forskolin and dibutryl cAMP (cAMP analogue). The results are expressed as the percentage of total [^{3}H] arachidonic acid incorporated from stimulated glands (experimental) relative to unstimulated glands (control). Values are expressed as the mean $\pm$ SE, n = 8.

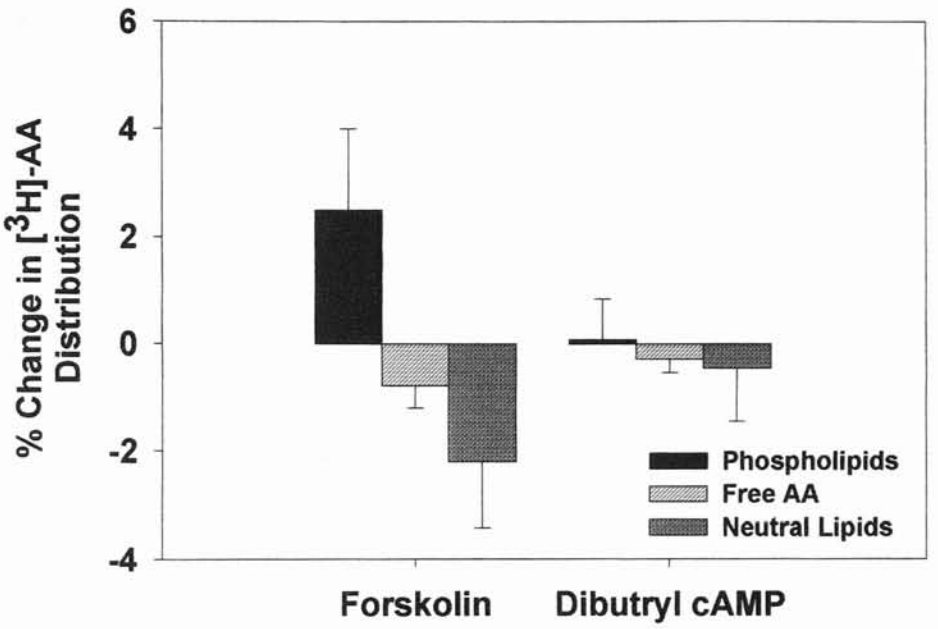
% Change in [3H]-AA Distribution
6
4
2
0
-2
-4
Phospholipids
Free AA
Neutral Lipids
Forskolin
Dibutryl cAMP

Figure 11. Re-distribution of [^{3}H] arachidonic acid from *A. americanum* salivary glands incubated with 0.1μM A-23187, 10μM dopamine and 0.1μM PMA for 60 minutes. Results are expressed as percentage of total incorporated [^{3}H] arachidonic acid from stimulated glands (experimental) relative to unstimulated glands (control). Values are expressed as the mean ± SE, n = 4 and statistically significant results are identified with an asterisk.

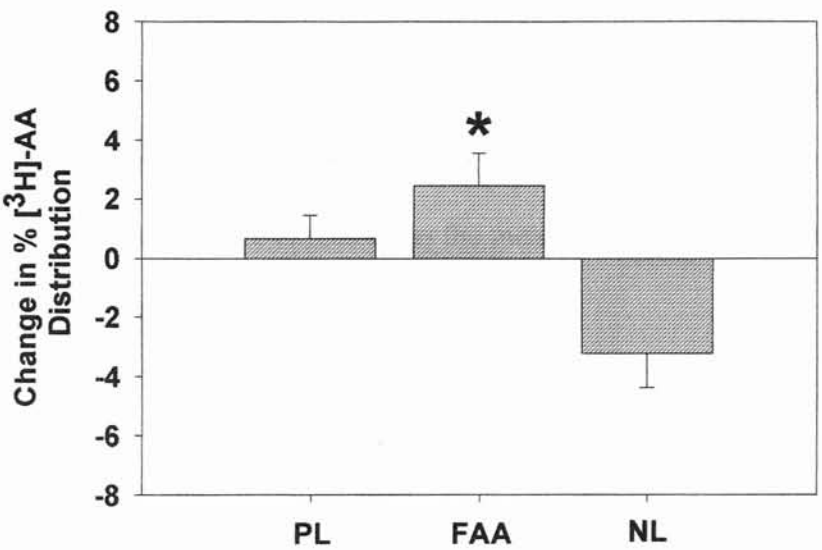
Change in % [3H]-AA Distribution
8
6
4
2
0
-2
-4
-6
-8
*
PL
FAA
NL

Figure 12. Effect of 10μM GTPγ-S (alone) and 10μM PPHT-HCl & GTPγ-S on the redistribution of [^{3}H] arachidonic acid from isolated *A. americanum* salivary glands during 60 minute incubations. Results are expressed as percentage of total incorporated [^{3}H] arachidonic acid from stimulated glands (experimental) relative to unstimulated glands (control). Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

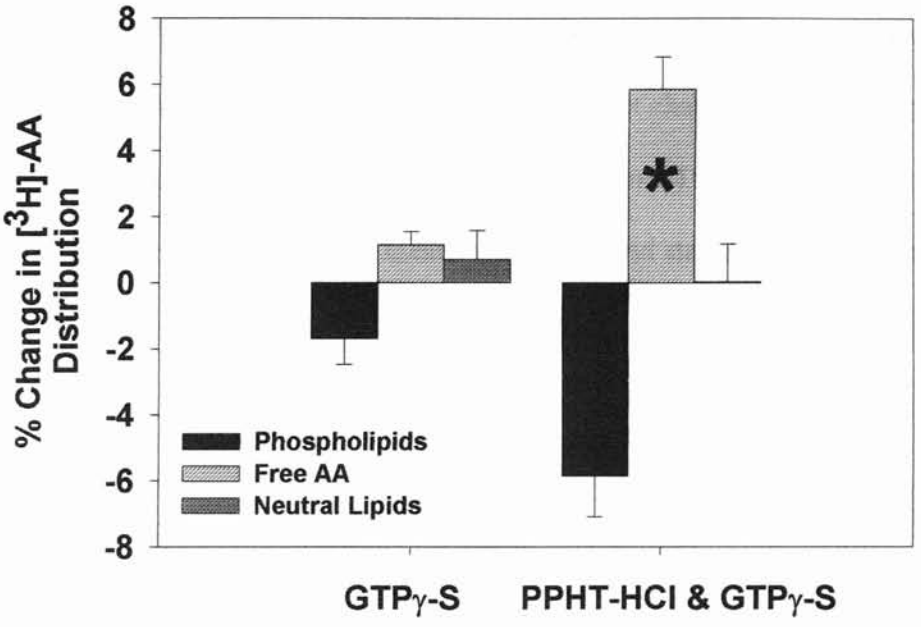
% Change in [3H]-AA Distribution
8
6
4
2
0
-2
-4
-6
-8
Phospholipids
Free AA
Neutral Lipids
*
GTPγ-S
PPHT-HCl & GTPγ-S

Figure 13. Effect of 10μg pertussis toxin or 100μg cholera toxin on the re-distribution of [^{3}H] arachidonic acid in isolated salivary glands of *A. americanum* during 60 min incubations. Results are expressed as the percent change in total incorporated [^{3}H] arachidonic acid from stimulated glands (experimental) relative to unstimulated glands. Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

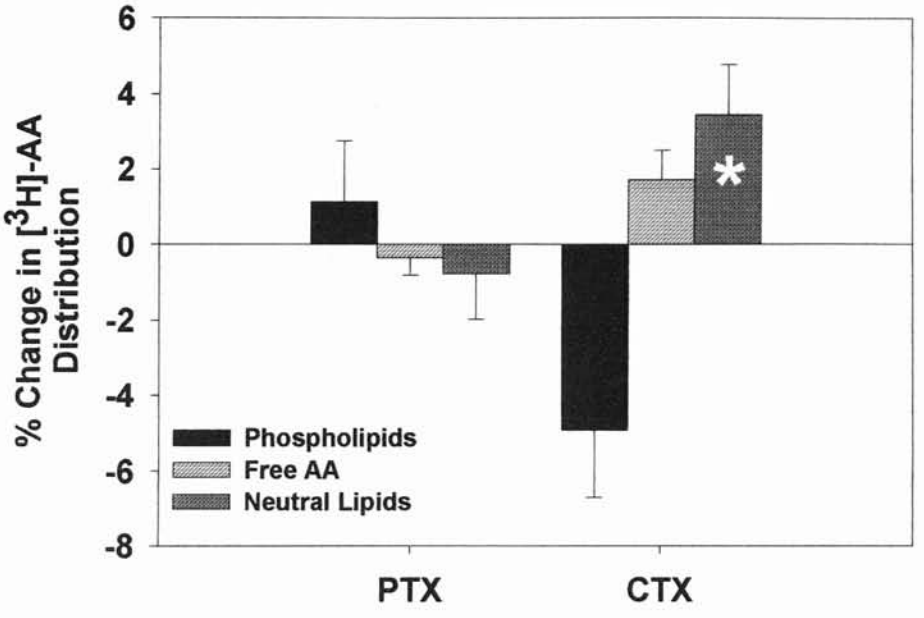
% Change in [3H]-AA Distribution
6
4
2
0
-2
-4
-6
-8
Phospholipids
Free AA
Neutral Lipids
*
PTX
CTX

Figure 14. Effects of 0.1μM wortmannin pre-incubated for 10 min or 60 min on the re-distribution of [^{3}H] arachidonic acid in isolated salivary glands of *A. americanum*. Control and experimental salivary glands were pre-incubated with 10μM wortmannin. Experimental salivary glands were stimulated with 10μM PPHT-HCl & GTPγ-S. Results are expressed as the percent change in total [^{3}H] arachidonic acid incorporated in stimulated glands (experimental) relative to unstimulated glands (control). Values are expressed as the mean ± SE, n = 8 and statistically significant results are identified with an asterisk.

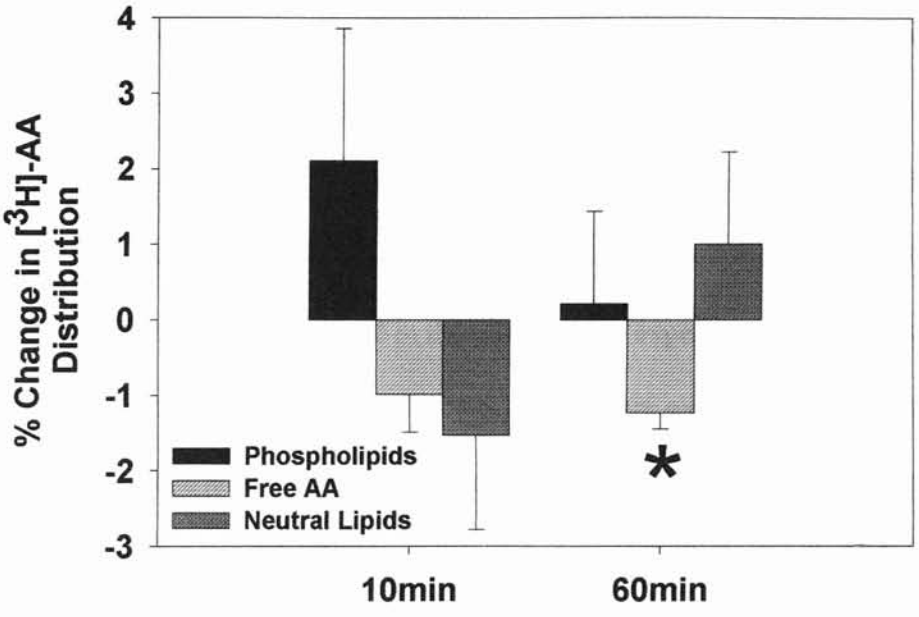
% Change in [3H]-AA Distribution
4
3
2
1
0
-1
-2
-3
Phospholipids
Free AA
Neutral Lipids
*
10min
60min

Figure 15. Effects of 0.1μM wortmannin and 10μM GTPγ-S on the re-distribution of [^{3}H] arachidonic acid in isolated salivary glands of *A. americanum* during 60 min incubations as compared to salivary glands incubated with 10μM GTPγ-S. Experimental salivary glands were pre-incubated for 10 min with wortmannin prior to stimulation by GTPγ-S. In one experiment, experimental glands were incubated with GTPγ-S alone (no co-incubation with wortmannin) and in the other group, experimental glands were incubated with both wortmannin and GTPγ-S. Results are expressed as percentage of total [^{3}H] arachidonic acid incorporated from glands stimulated with wortmannin and GTPγ-S together compared to glands stimulated with GTPγ-S (control). Values are expressed as the mean $\pm$ SE, n = 8 and statistically significant results are identified with an asterisk.

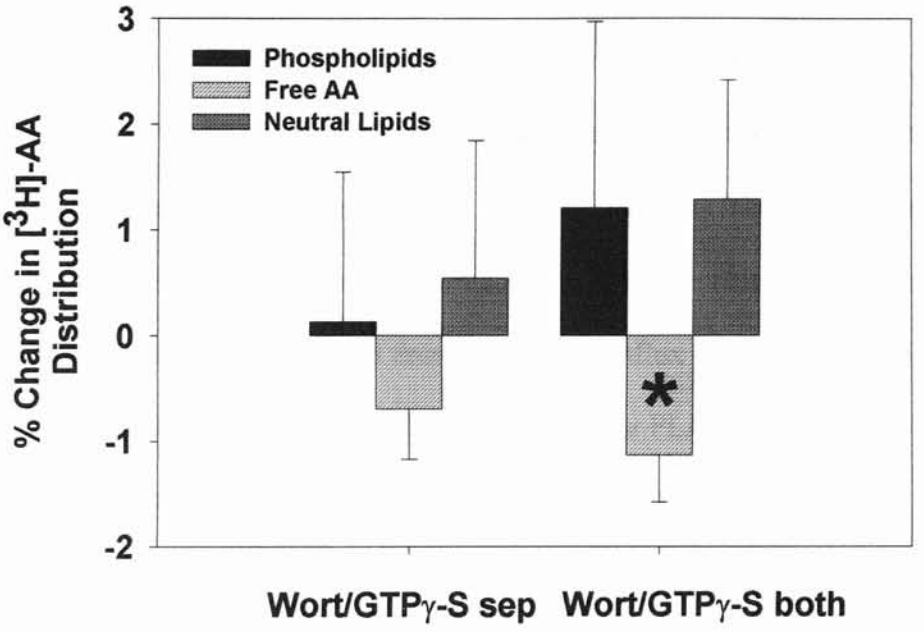
3
2
1
0
-1
-2
Phospholipids
Free AA
Neutral Lipids
% Change in [3H]-AA Distribution
*
Wort/GTPγ-S sep
Wort/GTPγ-S both

Illustration 16. Dopamine released from nerve endings surrounding the salivary glands, binds to a receptor that is bound to heterotrimeric G-protein. Upon binding, the G-protein separates into two subunits: a G_{α} and a $G_{\beta\gamma}$ subunit. The G_{α} subunit could be involved in activation of AC, thus converting ATP to cAMP. This activation needs to be further investigated. The $G_{\beta\gamma}$ subunit then activates an influx of Ca^{2+} via a PI-3 kinase stimulation. This influx of Ca^{2+} activates an intracellular PLA_2 which cleaves AA from the sn-2 position of the salivary gland PL. Free AA is then converted to PGE_2 via the COX pathway.

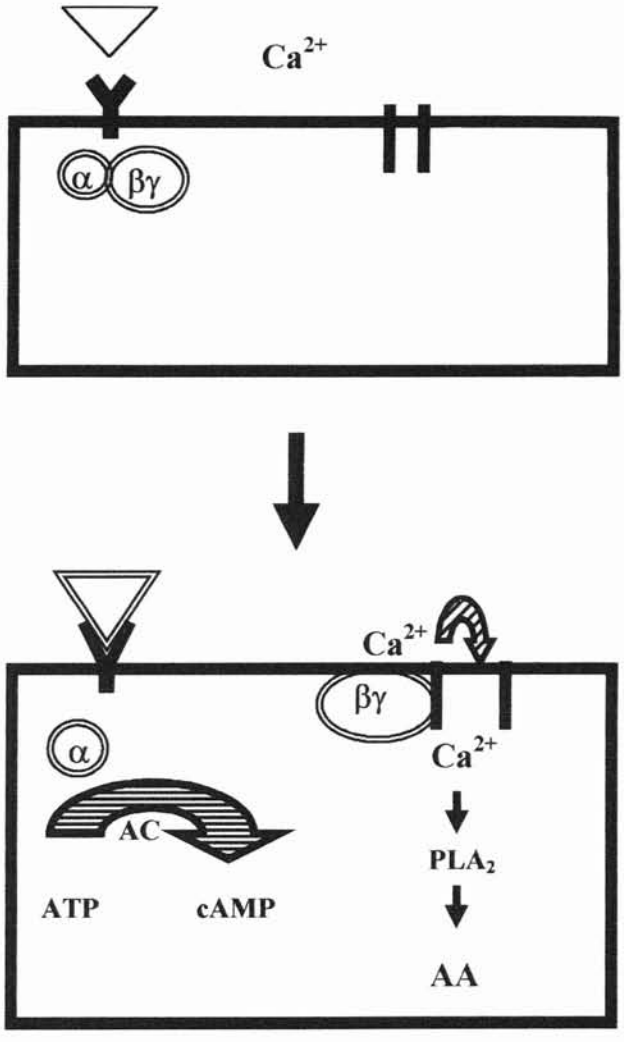
Ca2+
α
βγ
Ca2+
βγ
α
Ca2+
AC
PLA2
ATP
cAMP
AA

Table 1. The amount of cAMP measured through a RIA assay from the salivary glands of the lone star tick that was stimulated with a mammalian D_2 receptor agonist (PPHT-HCl). Salivary glands were incubated in similar conditions as previously reported experiments for 60 min. These results are compared to unstimulated glands (control). Values are expressed as mean $\pm$ SE, n = 18. Findings were statistically significant; $p<0.05$.

Specific activity of cAMP produced in salivary glands of the lone star tick		
(-)	Control	.0743 ± .00585 (pmol cAMP/μg/hr)
(+)	Experimental	.254 ± .1353 (pmol cAMP/μg/hr)

(-) = Unstimulated Glands
(+) = Stimulated Glands with PPHT-HCl (10μM)

CHAPTER VI

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VITA

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