PROSTAGLANDIND E_2 AND EXOCYTOSIS OF PROTEINS FROM THE SALIVARY GLANDS OF THE LONE STAR TICK, *Amblyomma americanum* (L.)

By

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I dedicate this thesis to my grandfather, Jiafu Yuan.

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LIST OF ABBREVIATION

A-23187	calcimycin
AA	arachidonic acid
AC	adenylate cyclase
ACU	anticoagulant unit
ADP	adenosine 5'-diphosphate
AH-6809	6-isopropyl-9-xanthone-2-carboxylic acid
APTT	activated partial thromboplastin time
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
cAMP	cyclic adenosine 3', 5'-monophosphate
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
D1	dopamine receptor
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DOC	deoxycholate
EDTA	ethylenediamine tetraacetate
EGTA	ethyleneglycol-bis-(aminoethyl ether) tetraacetate
EP	prostaglandin E_2 receptor
ER	endoplasmic reticulum
FAO	UN food and agricultural organization
G-protein	guanine nucleotide regulatory protein
Gs	stimulatory G protein
GTP-γ-S	guanosine 5'-O-(3-thiotriphosphate)
IC_{50}	50% Inhibition Constant

IgG	immunoglobulin G
IP	inositol phosphate
IP_3	inositol 1,4,5-trisphosphate
MOPS	morpholinopropane sulphonic acid
MT	Malpighian tubules
NBT	nitro blue tetrazolium
ng	nanogram (10^{-9} gram)
nM	nanomolar (10^{-9} molar)
OPC	oleyloxyethylphosphocholine
PGE_2	prostaglandin E_2
PGs	prostaglandins
PI	phosphatidylinositol
PIP_2	phosphatidyl-inositol 4,5-bisphosphate
PI-PLC	Phosphatidylinositide-specific phospholipase C
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
RH	relative humidity
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.M.	standard error of means
TMB-8	8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate
Tris	trishydroxymethylaminomethane
U-46619	9,11-dideoxy-9α, 11α-methanoepoxyprostaglandin $F_{2\alpha}$
U-73122	1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl) amino) hexyl]-1H-pyrrole-2,5-dione
μg	microgram (10^{-6} gram)
μM	micromolar (10^{-6} molar)
V_{max}	maximum velocity of reaction

CHAPTER I

INTRODUCTION

Background and Significance

Ticks are important epidemiologically and economically

Ticks are notorious obligate ectoparasites that infest mammals, birds, reptiles and amphibians. Their infestations cause great economic losses in livestock production globally, especially in South Africa and Australia (Steelman, 1976). The losses were estimated to be $7 billion in 1984 by the FAO (Harrow et al., 1991). Ticks surpass other arthropods in the number and variety of diseases transmitted to domestic animals and rank second only to mosquitoes as vectors of human disease (Sauer et al., 1995). Ticks are major arthropod vectors of arboviruses, rickettsiae, spirochaetes and parasitic protozoa (Kaufman, 1989). The most common tick-borne diseases include Lyme disease, Rocky Mountain spotted fever, ehrlichiosis, encephalitis and babesiosis. Among these, Lyme disease is by far the most important tick-borne disease affecting people globally and the reported incidence of the disease in the USA has increased over recent years (Bowman et al., 1996).

Ticks have a unique and challenging feeding behavior

Adult female ixodid ticks feed on a host for relatively long periods (8-15 days) and imbibe large amounts of blood (1.0 to 1.5 ml) increasing more than 100 times their initial

body weight. During tick feeding, saliva containing excess water and ions, along with many bioactive components and in some cases pathogens are secreted into the host (Sauer et al., 1995). This prolonged and intimate attachment and fluid exchange facilitates transmission of pathogens.

At least three problems could arise from this unique feeding behavior: 1). Local injury caused by penetration of tick mouthpart may cause bleeding, blood clotting, and constriction of local capillaries. 2). Local injury, prolonged feeding and reflux of chemicals from tick saliva into the feeding lesion may cause local inflammation. Inflammation at the feeding site would influence tick feeding efficiency. 3). Prolonged feeding and reflux of foreign proteins into the feeding lesion may provoke the host immune system, which would interrupt the tick feeding process through cellular and humoral immune reactions. However, when feeding on a naive host, ticks do not cause obvious local inflammation and rejection at the feeding site; they can easily feed for two weeks or more. It has been of great interest to find out how ticks overcome hemostasis, inflammation and the immune response of the host.

Tick salivary glands and the importance of salivary secretion

Because salivary secretion is the major route via which pathogenic organisms, toxins, and other regulatory factors access the vertebrate host, the salivary glands are the focus of most medical and veterinary problems associated with ticks (Kaufman, 1989). Tick saliva contains many important bioactive components including cement, various enzymes and inhibitors, histamine agonists and antagonists, prostaglandins, antihemostatic

factors, immuno-modulating factors, procopulatory factors and paralysis toxins (Sauer et al., 1995; Bowman et al., 1997).

All these secretory molecules are very important to the tick's biological success. Cement helps anchor the mouthparts to the host; apyrase in tick saliva inhibits platelet aggregation by breaking down ADP and ATP, which are platelet aggregation stimulators; Esterase and hyaluronidase may increase capillary permeability by breaking down components in mast cell membranes and walls of blood vessels (Geczy, 1971; Neitz et al., 1978); kininase may deactivate bradykinin at the feeding lesion to allow ticks to feed without causing pain to the host (Ribeiro, 1987); anticoagulants and vasodilators assist ticks in maintaining a sufficient blood supply to the feeding site (Ribeiro et al., 1992); the immunosuppressive properties of the saliva include the suppression of the production of interleukin-2 (Ribeiro et al., 1985) and γ-interferon by T-lymphocytes and interleukin-1 and tumor necrosis factor α by macrophages (Ramachandra and Wikel, 1992). Host immuosuppression not only allows ticks to feed successfully, but also benefits establishment of pathogens in the host, development and replication (Wikel et al., 1994; Zeidner et al., 1996).

Prostaglandins play an important role in tick feeding

Prostaglandins have been found in the salivary glands and saliva of at least four species of ticks, including *Amblyomma americanum* (Dickinson et al., 1979; Ribeiro, et al., 1992; Ribeiro, et al., 1985; Shemesh et al., 1979).

Prostaglandins are biologically active and very versatile molecules in both mammals (Smith, 1992) and insects (Stanley-Samuelson, 1994). The exact roles that PGs

play in tick feeding is not known but they exhibit vasodilatory, anti-hemostatic, immunosuppressive and anti-inflammatory effects in mammals (reviewed by Bowman et al., 1996). All these properties could facilitate tick feeding. Besides their effects on the host, PGs appear to have an autocrine or paracrine effect in modulating tick salivary gland function (Qian et al., 1997).

PGE_2 and its biological actions

PGE_2 exhibits a wide spectrum of physiological as well as pharmacological actions in diverse tissues and cells. One important function is regulation of secretion. The roles of PGE_2 in the mammalian kidney is largely confined to water and NaCl reabsorption in the collecting tubule and the adjacent thick ascending limb (Smith, 1989). The role of PGE_2 in regulation of fluid transport is also found in other tissues and systems. In rat gastric mucosa, PGE_2 causes inhibition of gastric acid secretion in the parietal cell but stimulates gastric nonparietal secretion (Soll, 1980; Wollin et al., 1979). In the amphibian cornea, both E and F prostaglandins stimulate Cl^- transport across the epithelium, and thus contribute to the maintenance of corneal transparency (Bentley and McGahen, 1982). Bjerregaard & Nielsen (1987) demonstrated that PGE_2 stimulated an active secretion of Cl^-, Na^+, and K^+ from the skin glands in isolated frog skin. In fish, PGE_1 and PGE_2 reduce ion efflux in seawater-adapted mullet and in the killifish (Brown et al., 1991). Mosquito Malpighian tubules (MTs) are organs of osmoregulation (similar to renal function in mammals). Data indicate that PGE_2 plays a role in the overall regulation of fluid secretion rates of MTs (Petzel et al., 1993).

Tick salivary glands are analogous in some functions to the mammalian kidney and mosquito MTs in osmoregulation. PGE_2 shows powerful actions on regulation of water and ion transport in the latter tissues. However, in the tick salivary gland, PGE_2 does not stimulate fluid secretion itself (Qian et al., 1997) but does stimulate release of anticoagulant molecules from dispersed salivary gland acini (Qian et al., 1998). I hypothesize this stimulation of salivary secretion is triggered by mobilization of intracellular Ca^{2+} via the specific PGE_2 receptor.

PGE_2 receptors

PGE_2, like other prostanoids, exerts its effects by interacting with specific receptors on cell membranes. In contrast to other prostanoids, PGE_2 can induce versatile and opposite cellular responses through its widely distributed receptors coupling multiple signal transduction pathways. Thus PGE_2 receptors are further classified into subtypes in mammalian tissues. At present, four subtypes of PGE_2 receptors, termed EP1, EP2, EP3, and EP4, have been described, based on their responses to some of the natural and synthetic prostanoids as well as various agonists and antagonists (Coleman et al., 1990; Negishi et al., 1993). EP1 receptors are sensitive to the agonist sulprostone but blocked by SC-19220 or AH-6809. Occupation of EP1 receptors has been reported to induce Ca^{2+} mobilization via a phospholipase C signal transduction pathway (Negishi et al., 1993). EP2, EP3, EP4 receptors have been shown to affect intracellular cAMP level via the adenylate cyclase pathway (Coleman et al., 1994). Tick PGE_2 did not affect adenylate cyclase activity in salivary gland membrane (Qian et al., 1997). Exogenous PGE_2 increased the amount of inositol 1,4,5-trisphosphate (IP_3) and the efflux of Ca^{2+} in dispersed salivary

gland dose-dependently (Qian et al., 1998). This evidence suggests that the tick PGE_2 receptor may be an EP1-like receptor. All four PGE_2 receptor subtypes have been isolated and cloned from various mammalian tissues. They possess seven hydrophobic transmembrane domains and belong to the family of G protein-coupled rhodopsin-type receptors (Negishi et al., 1993). The overall homology among these receptors suggests they are derived from different genes though these genes may have evolved from a common ancestor (Negishi et al., 1995).

Qian et al. (1997) identified a specific PGE_2 receptor in the salivary gland membrane of *Amblyomma americanum* and proposed that PGE_2 may implement an autocrine or paracrine function in salivary gland. This is the first PGE_2 receptor identified in invertebrates. It is of great interest to study the PGE_2 receptor further to determine its function and ultimately the mode of action.

Control of salivary secretion and regulated exocytosis

Besides fluid secretion, tick salivary glands also secrete many bioactive components including proteins. Most secretory proteins are believed to be released from the vesicle contents of membrane-bounded granular materials observed in salivary acini types II and III (Fawcett et al., 1986). Little is known about control of the protein secretion (exocytosis) in tick salivary glands.

Exogenous PGE_2 was found to increase the IP_3 level and the efflux of Ca^{2+} dose-dependently in the dispersed salivary gland (Qian et al., 1998), suggesting that the PGE_2 receptor is EP1-like activating a phospholipase C pathway where PIP_2 is hydrolyzed into IP_3 and DAG. IP_3 releases Ca^{2+} from the endoplasmic reticulum, and DAG activates

protein kinase C (PKC) (Berridge, 1987; 1993), which phosphorylates specific proteins and ultimately brings about the functional responses. McSwain et al. (1989) found that phorbol ester, a specific activator of protein kinase C, stimulates the phosphorylation of salivary gland proteins. This suggests that the activation of protein kinase C could be involved in salivary secretion in ticks. PKC is known to be a mediator of PGE_2 EP1 receptor activation and a feed-back negative regulator of EP1 receptors (Negishi, et al., 1995).

Exocytosis, the last stage in the protein secretory pathway, results in the secretion of contents stored in secretory vesicles. It can be divided into two types, regulated and constitutive (Kelly, 1985). Tick salivary secretion is likely regulated because of its coupling with tick feeding (Sauer et al., 1995). Regulated exocytosis in most cells is accompanied by a rise in intracellular Ca^{2+} during cell stimulation (Knight et al., 1989). The mobilization of intracellular Ca^{2+} by PGE_2 (Qian et al., 1998) suggests a possible relationship between PGE_2 and exocytosis of proteins.

The mechanism of exocytosis is complex but highly conserved in vertebrate and invertebrate neural and nonneuronal cells (Augustine et al., 1996; Burgoyne et al., 1996; Edwardson and Marciniak, 1995). Salivary glands are major secretory organs in adult ticks, and very active in exocytosis during tick feeding. They appear to be a good model for studying exocytosis in nonneuronal cells, given the importance of tick salivary secretion in tick feeding and pathogen transmission. Munderloh & Kurtti (1995) suggested that Lyme disease spirochetes and rickettsiae enter and exit tick cells via transcytosis pathways, i.e. they are taken in by endocytosis and released into the host by exocytosis. Understanding the mechanism and regulation of exocytosis in tick salivary glands may

provide background knowledge of how the tick's physiology affects pathogen transmission.

OBJECTIVES AND SPECIFIC AIMS

Tick salivary glands are major secretory organs and very active in exocytosis during tick feeding. Because salivary secretion is the major route for pathogen transmission to the host, the knowledge of how salivary secretion is controlled is of much medical and veterinary importance.

Salivary fluid secretion in ticks is controlled by nerves with dopamine (Sauer et al., 1979). Dopamine stimulates fluid secretion by increasing the intracellular level of cyclic AMP (cAMP) (Schmidt et al., 1982). But little is known of the control of protein secretion in tick salivary gland.

Qian et al. (1997) identified a PGE_2 -specific receptor in the plasma membrane fraction of the salivary glands of *A. americanum* (L.) and found that exogenous PGE_2 increased the amount of inositol 1,4,5-trisphosphate (IP_3) and the efflux of Ca^{2+} in the dispersed salivary gland in a dose-dependent manner (Qian et al., 1998). Because a rise in intracellular Ca^{2+} is associated with regulated exocytosis in most secretory cells (Knight et al., 1989), a relationship between PGE_2 and exocytosis may exist in tick salivary glands. Preliminary studies indicate that PGE_2 dose-dependently stimulates the release of anticoagulant molecules in isolated salivary glands at very low concentration. Anticoagulant molecules, important components of salivary secretion, are used as the marker for exocytosis.

Based on the above, I hypothesize that tick PGE_2 is involved in regulating salivary protein secretion (exocytosis) via a PGE_2 specific, G-protein linked receptor which activates the phosphoinositide signal pathway and increases the intracellular calcium level.

The objective of my research is to investigate the relationship between PGE_2 and exocytosis of proteins in tick salivary glands, and to determine the signal transduction pathway involved. My specific aims are to:

1. Determine the specificity of the effect of PGE_2 on the release of anticoagulant molecules;
2. Characterize the PGE_2 receptor using receptor antagonists and agonists;
3. Study the role of calcium in controlling exocytosis of protein;
4. Further characterize the signal transduction pathway of the PGE_2 receptor.

CHAPTER II

THE SPECIFICITY OF PROSTAGLANDIN E_2 AND ROLE OF CALCIUM IN CONTROLLING EXOCYTOSIS OF SALIVARY GLAND PROTEIN IN IXODID TICKS

ABSTRACT

Previous studies have identified a prostaglandin E_2 (PGE_2) receptor in the salivary glands of partially fed female lone star ticks *Amblyomma americanum* (L.). Subsequent studies suggested that the receptor is coupled to a phosphoinositide signalling pathway and secretion of salivary protein. In the present study, increasing concentrations of the calcium ionophore A-23187 (1 to 100 μM) stimulated release of anticoagulant protein from dispersed salivary glands of partially fed female ticks, while the voltage-gated Ca^{2+} channel blocker verapamil (1 to 1000 μM) did not affect PGE_2 -stimulated secretion of anticoagulant protein. The results support the hypothesis that mobilization of intracellular calcium is the signalling pathway whereby PGE_2 stimulates secretion of salivary gland anticoagulant. Furthermore, the selective PGE_2 EP1 receptor agonist, 17-phenyl trinor PGE_2, was as effective as PGE_2 in stimulating secretion of anticoagulant protein; the selective PGE_2 EP1 receptor antagonist AH-6809 inhibited the secretion at a low concentration, suggesting that the exocytosis mechanism in tick salivary gland is via the EP1-like PGE_2 receptor and the increased Ca^{2+} level. PGE_2 was shown to be much more effective than either $PGF_{2\alpha}$ or the thromboxane A_2 analog U-46619 in stimulating release of

salivary gland anticoagulant protein, in accordance to their respective binding affinities for the PGE_2 receptor. PGE_2 stimulated, and A-23187 caused release of anticoagulant protein from dispersed salivary glands of partially fed male *Dermacentor andersoni* ticks, indicating that prostaglandin receptors linked to mobilization of intracellular calcium and exocytosis occur in other species and in both male and female ixodid ticks.

KEYWORD INDEX: ixodid ticks, salivary glands, exocytosis, prostaglandin, calcium, PGE_2 receptor, EP1 receptor, anticoagulants

INTRODUCTION

The prolonged attachment and extended fluid exchange during tick feeding on host animals allow tick to be an important vector for medical and veterinary pathogens. The tick's salivary glands are crucial to their biological success (Sauer et al., 1995). As feeding progresses, the rate of salivary fluid secretion increases greatly, enabling the tick to concentrate the bloodmeal by returning excess water and ions to the host. At the same time, many bioactive proteins (e.g. anticoagulants, anti-inflammatory and immunosuppressive) and prostaglandins (PGs) are secreted in saliva to help modulate interactions of the tick with its host (Bowman et al., 1997).

Prostaglandins (PGs) are biologically active lipid mediators in both mammals (Smith, 1992) and insects (Stanley-Samuelson, 1994). In mammalian cells, PGs typically serve as local hormones in the same or nearby cells and their effects are elicited by binding to specific transmembrane receptors coupled to signalling pathways which either mobilize

Ca^{2+} or increase or decrease cAMP (Narumiya, 1995). Although most research to date on tick-derived PGs has focused on roles of PGs in facilitating tick feeding through interactions with host cells (Bowman et al., 1996), a G-protein-linked PGE_2 -specific receptor has been identified in the salivary glands of female lone star tick (*A. americanum*) suggesting a functional role for PGE_2 in tick salivary gland physiology (Qian et al., 1997). The PGE_2 receptor's binding specificities for various prostanoids are PGE_2> $PGF_{2\alpha}$> PGD_2 > U-46619 (thromboxane A_2 analog) (Qian et al., 1997). PGE_2 stimulates release of anticoagulant protein from dispersed salivary gland acini suggesting a linkage between PGE_2 stimulation and exocytosis of bioactive proteins during tick feeding (Qian et al., 1998). In this study we sought to compare the effectiveness of various prostanoids on releasing anticoagulants to determine if there is a correlation between prostanoids binding affinities of PGE_2 receptor and the ability of prostanoids to stimulate secretion of anticoagulant protein. Since the selective PGE_2 EP1 receptor antagonist AH-6809 suppresses the stimulatory effect of PGE_2 on anticoagulant release (Qian et al., 1998), we tested the effect of 17-phenyl trinor PGE_2 (a selective EP1 agonist, Johnson et al., 1980) in stimulating secretion of anticoagulant protein to further determine if the exocytosis mechanism is via the EP1-like PGE_2 receptor. PGE_2 does not affect adenylate cyclase activity in membrane preparations of tick salivary glands(Qian et al., 1997), but did increase the amount of IP_3 and the level of intracellular Ca^{2+} in dispersed salivary glands (Qian et al., 1998). Qian et al. (1998) demonstrated that PGE_2 mobilizes intracellular Ca^{2+}. In this study we investigated whether Ca^{2+} from intracellular or extracellular sources is essential for PGE_2-stimulated exocytosis of anticoagulant proteins. We also examined whether PGE_2 is capable of stimulating secretion of anticoagulant protein from dispersed salivary glands

of partially fed male *Dermacentor andersoni* ticks to ascertain the prevalence of the PGE_2 regulatory system for controlling exocytosis in other ixodid tick species and in males.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: Medium-199, lyophilized sheep plasma, activated partial thromboplastin time (APTT) reagent, calcium ionophore A-23187, and verapamil from Sigma Chemical Company (St. Louis, Missouri, USA); PGE_2, and $PGF_{2\alpha}$ were from Biomol (Plymouths Meeting, PA, USA); U-46619 (non-hydrolyzable thromboxane A_2 analog) and 17-phenyl trinor PGE_2 were from Cayman Chemical (Ann Arbor, MI, USA).

Tick rearing

Amblyomma americanum (L.) and *Dermacentor andersoni* ticks were reared at Oklahoma State University's Central Tick Rearing Facility, according to the methods of Patrick & Hair (1975). Immature ticks were fed on rabbits and adult ticks on sheep . All unfed ticks were maintained at 27-28°C and 90% RH under 14:10 (L:D) photoperiod before infestation of the hosts. Partially fed female *A. americanum* and male *D. andersoni* ticks were used within four hours after being removed from the host.

Salivary gland preparation and dispersion

The technique for preparing dispersed salivary gland acini was as previously described (Qian et al., 1998). Briefly, tick salivary glands were dissected out in Medium-199 containing 20 mM MOPS (morpholinopropane sulphonic acid) buffer (pH 7.0), then one of two intact glands from each tick was put in the control group, while the other was put in the experimental group. Four glands per group were used in the study of partially fed female *A. americanum* ticks, and 20 for male *D. andersoni*. Salivary glands were gently teased apart with fine tipped forceps. The dimensions of the dispersed tissue were smaller than 0.5 mm^2. Dispersion was confirmed by viewing under a microscope. The viability of the dispersed gland acini and cells assessed by trypan blue exclusion was estimated to be about 60%. Dispersed tissue was transferred to a microtube, and rinsed 5 times with the same buffer prior to treatment.

Treatment of Salivary glands

Salivary glands were incubated with Medium-199 containing 20 mM MOPS buffer (pH 7.0) containing various drugs of differing concentrations (treatment) or the same medium plus the solvent used to dissolve the drugs (control).

All incubations were performed for 5 min at room temperature except as stated otherwise. Dispersed tissue was gently agitated twice during each incubation. After incubation, the tissue was centrifuged for 1min at 1000g, and two 20 μl aliquots of the supernatant (containing salivary secretion products) were removed and tested for anticoagulant content via the sheep plasma coagulation assay.

Coagulation assay

The coagulation assay was performed as described by Zhu et al. (1997) with some modifications. Activated partial thromboplastin time (APTT, intrinsic pathway assay) was used to examine anticoagulant activities (Evatt et al., 1992). Sheep plasma (50 μl) was incubated with 20 μl samples in each well of a 96-well plate at room temperature for 30 min prior to initiating the coagulation assays. Assay mixture (1:1 APTT reagent: 20 mM $CaCl_2$) (100 µl) was added into each well and then the absorbance was monitored at 405 nm. Time to initiation of coagulation (V_{max}) determined by SOFT Max® PRO software using a Thermo Max plate reader (Molecular Devices, Sunnyvale, CA) was taken as the clotting time. The change in anticoagulant activity was expressed as the percentage of the clotting time difference over the control.

Statistics Analysis

The results are expressed as Mean ± Standard error. The number of repetitions is indicated in the figure legends. The differences of means between the control and experimental treatment were tested for significance by Student's t-test. A P-value (<0.05) was considered significant.

RESULTS

Effect of PGE_2 on secretion (exocytosis) of salivary gland anticoagulant

Tick saliva contains a multitude of bioactive protein molecules including anticoagulants (Zhu, et al., 1997) in addition to prostaglandins (Bowman, et al., 1997).

Since regulated exocytosis in most cells is accompanied by a rise in intracellular Ca^{2+} during cell stimulation (Edwardson *et al.*, 1997), we hypothesized that a major function of Ca^{2+} mobilized by PGE_2 was to stimulate secretion (exocytosis) of salivary protein. Proteins in tick saliva are believed to be released from the vesicle contents of membrane-bounded granular material in acini types II and III in the salivary glands of most ixodid ticks (Fawcett *et al.*, 1986) but little is known about how exocytosis is regulated. PGE_2 concentrations of 1nM to 1μM, with or without indomethacin (inhibitor of prostaglandin biosynthesis), stimulated significant secretion of anticoagulant protein ($p<0.05$) (Fig. 2.1). Lower concentrations of PGE_2 and surprisingly higher concentrations of PGE_2 had no effect on release of anticoagulant protein.

Free prostaglandin precursor arachidonic acid is released during incubation of isolated salivary glands (Bowman *et al.*, 1995a) raising the possibility that some arachidonic acid may be converted to prostaglandins during *in vitro* incubation of salivary gland tissue. It is hypothesized that PGE_2 produced under these conditions could stimulate release of protein in the absence of exogenous PGE_2. Since micromolar amounts of indomethacin have been shown to inhibit prostaglandin biosynthesis in tick salivary gland homogenate preparations (Pedibhotla et al., 1997), dispersed salivary glands were incubated with and without 100μM indomethacin in the absence of exogenous PGE_2. As in Fig. 2.1, there was no effect of indomethacin on the release of anticoagulant but rather indomethacin stimulated a 40% increase in the release of total proteins ($p \leq 0.05$) (data not shown). The significance of the latter finding is unclear.

Effects of different prostanoids on releasing anticoagulant protein

Incubation of dispersed salivary glands with 1 nM to 1 μM PGE_2 significantly stimulated secretion of anticoagulant protein. $PGF_{2\alpha}$ had a stimulatory effect about 40% that noted with PGE_2 and only at a high concentration (1 μM), while U-46619 (Thromboxane A_2 agonist) was ineffective at any concentration tested (Fig. 2.2). Somewhat surprisingly, low concentrations of $PGF_{2\alpha}$ (1 and 10 nM) inhibited anticoagulant release 20% ($P<0.05$) below that observed in the control tissue (Fig. 2.2).

The exocytosis mechanism is via the EP1-like PGE_2 receptor

PGE_2 receptors are classified into four subtypes in mammals (EP1, EP2, EP3 and EP4) on the basis of their responses to natural prostanoids and various inhibitors (Coleman et al., 1990). The effectiveness of 17-phenyl trinor PGE_2 (EP1 agonist) in reversing inhibition of dopamine-induced fluid secretion of OPC (an inhibitor of salivary gland PLA_2, Bowman *et al.*, 1995a; Qian *et al.*, 1997) and evidence that the PGE_2 receptor in tick salivary glands is linked to Ca^{2+} mobilization suggests that the tick salivary gland PGE_2 receptor may be EP1-like. To examine this hypothesis further, 0.1nM to 1μM of the mammalian EP1 receptor antagonist AH-6809 (Coleman et al., 1985) was incubated with dispersed tissue to examine its effect on total protein and anticoagulant release as compared to tissue incubated with the solvent control but without antagonist. The secretion of anticoagulant and total protein were inhibited when tissue was incubated with 0.1 nM AH-6809 ($p<0.05$; Fig. 2.3), suggesting that there may be endogenous PGE_2 in the dispersed tissue. However, a high concentration of AH-6809 (1μM) stimulated

secretion of anticoagulant and total protein ($p<0.05$; Fig. 2.3). AH-6809 inhibited PGE_2 (10nM and 1μM) stimulated secretion of anticoagulant factors at only the 1μM dose and not at higher or lower concentrations (Fig. 2.4). The results indicate that the PGE_2 receptor in tick salivary glands is affected, under some conditions, by a mammalian EP1 receptor antagonist.

While the EP1 antagonist AH-6809 attenuates the stimulatory effect of PGE_2 on stimulating release of anticoagulant protein, selective PGE_2 EP1 receptor agonist 17-phenyl trinor PGE_2 was as potent as PGE_2 at low concentrations (10 to 100 nM) (Fig. 2.5) in stimulating anticoagulant protein release ($P<0.05$), strongly suggesting that the exocytosis mechanism in tick salivary gland is via the EP1-like PGE_2 receptor.

Role of Ca^{2+} in anticoagulant release

The calcium ionophore A-23187 enhanced anticoagulant release from dispersed salivary glands in a dose-dependent manner (Fig. 2.6), implicating increased intracellular Ca^{2+} in the exocytosis of anticoagulant protein. An increase in intracellular Ca^{2+} can come from intracellular stores or from an influx of extracellular Ca^{2+}. The voltage-gated Ca^{2+} channel blocker verapamil had no effect on anticoagulant release in the presence of 10^{-7} M PGE_2 (Fig. 2.7), indicating that PGE_2 probably increases intracellular Ca^{2+} via increasing IP_3 and mobilization of intracellular stores and not an influx of extracellular Ca^{2+}.

Involvement of IP_3 in exocytosis of salivary glands protein

The IP_3 receptor inhibitor TMB-8 at 10^{-6}M abolished the stimulatory effect of PGE_2 on anticoagulant release (Fig. 2.8A), further supporting the hypothesis that IP_3 and its intracellular Ca^{2+} mobilization are crucial for exocytosis of salivary gland proteins. In the presence of exogenous 10^{-8} M PGE_2, TMB-8 treatment (10^{-8}-10^{-4}M) significantly decreased the secretion of anticoagulant (Fig. 2.8B). Without exogenous PGE_2, TMB-8 (10^{-8}-10^{-4}M) also decreased the anticoagulant release (Fig. 2.8C), suggesting the existence of some amount of IP_3 in the tissue possibly caused by endogenous PGE_2.

Prostaglandin receptors in male *Dermacentor andersoni*

10 nM to 1 μM PGE_2 stimulated low amounts of anticoagulant protein release from dispersed salivary glands of male *D. andersoni* (Fig. 2.9A). The calcium ionophore A-23187 also caused release of anticoagulant protein ($P<0.05$) from dispersed salivary glands of male *D. andersoni* (Fig. 2.9B). The results indicate the existence of prostaglandin receptors in male *D. andersoni* salivary glands linked to secretion of salivary gland protein via calcium mobilization, as noted in salivary glands of partially fed female *A. americanum* ticks.

In contrast to the salivary glands in female *A. americanum*, low concentrations of $PGF_{2\alpha}$ (10 nM to 1 μM) were as effective as PGE_2 in stimulating release of anticoagulant protein (Fig. 2.9A), suggesting some differences in receptor types in the two species or differences between male and female ticks.

DISCUSSION

Qian et al. (1997) identified a G-protein-linked, PGE_2 -specific receptor in the plasma membrane fraction of the salivary glands of partially fed female *Amblyomma americanum* ticks, and determined that the affinities of the receptor for prostanoids were in the rank order of $PGE_2 > PGF_{2\alpha} > PGD_2 >$U-46619. Previously, we found that exogenous PGE_2 stimulates secretion of anticoagulant protein from dispersed salivary glands (Qian et al., 1998). In this study we compared the effectiveness of PGE_2 , $PGF_{2\alpha}$, and U-46619 in stimulating release of anticoagulant protein from dispersed salivary glands of *A. americanum*. The rank order of PG effectiveness in stimulating release of anticoagulant protein was similar to that of PG binding affinities to the PGE_2 receptor found in a previous study (Qian et al., 1997). The results suggest that stimulated exocytosis of bioactive proteins is not a general phenomenon of prostanoids but is PGE_2-specific. However, low concentrations of $PGF_{2\alpha}$ (1 and 10 nM) inhibited release of anticoagulant about 20% below that noted in control tissue. It is worth noting that the IC_{50} of $PGF_{2\alpha}$ (316 nM) is 10 times higher than that of PGE_2 (31.6 nM) in competitive displacement ligand binding studies for specific [^{3}H]- PGE_2 binding to the tick salivary gland PGE_2 receptor (Qian, et al. 1997). The possible explanations include a $PGF_{2\alpha}$ specific receptor which inhibits salivary gland protein secretion or, alternatively, that $PGF_{2\alpha}$ competitively antagonizes low levels of endogenous PGE_2. It is interesting to note that a relatively high concentration of $PGF_{2\alpha}$ (1 μM) and low concentrations of $PGF_{2\alpha}$ stimulated release of anticoagulant protein from partially fed female *A. americanum* and partially fed male *D. andersoni* salivary glands respectively.

These results suggest that the low levels of $PGF_{2\alpha}$ may in fact have competed with endogenous PGE_2 in the *A. americanum* experiments. It is also worth noting that low levels of selective mammalian PGE_2 EP1 receptor antagonist AH-6809 inhibits anticoagulant release, but higher concentrations stimulates release (Qian et al., 1998). The inhibitory effect of low AH6809 concentrations suggests contaminating endogenous PGE_2. More experiments are needed to ascertain whether other PG receptors exist in tick salivary glands and the levels of possible endogenous levels of PGs in incubation media.

In mammals, PGE_2 can induce diverse and opposite cellular responses through multiple signal transduction pathways , thus PGE_2 receptors are classified into four subtypes, EP1, EP2, EP3 and EP4 (Coleman, et al., 1990; Negishi, et al., 1993). EP1 receptors are involved in Ca^{2+} mobilization, while EP2, EP3, EP4 receptors have been shown to affect intracellular cAMP levels via stimulation or inhibition of adenylate cyclase (Coleman, et al., 1994). PGE_2 did not affect adenylate cyclase activity in tick salivary gland membrane preparations (Qian et al., 1997) but did stimulate the formation of IP_3 and mobilization of intracellular Ca^{2+} in dispersed tissue (Qian et al., 1998). The selective PGE_2 EP1 receptor agonist, 17-phenyl trinor PGE_2, was as effective as PGE_2 in stimulating secretion of anticoagulant protein; the selective PGE_2 EP1 receptor antagonist AH-6809 inhibited the secretion at the low concentration, strongly suggesting that the exocytosis observed in tick salivary gland is via the PGE_2 receptor EP1-like subtype. This is also consistent with the previous finding of the ability of 17-phenyl trinor PGE_2 to partially reverse the inhibition of salivary fluid secretion by PLA_2 inhibitor oleyloxyethyl-phosphorylcholine (Qian et al., 1997).

Activation of mammalian EP1 receptors leads to an increase in intracellular Ca^{2+} level (Narumiya, 1996). In our results, the calcium ionophore A-23187 enhanced anticoagulant release in a dose-dependent manner and was as potent as PGE_2, suggesting a role for increased intracellular calcium in controlling exocytosis of anticoagulant proteins. The voltage-gated Ca^{2+} channel blocker verapamil had no effect on anticoagulant release in the presence of 10^{-7} M PGE_2, indicating that the increase in intracellular Ca^{2+} needed to stimulate exocytosis is mobilized by PGE_2 from intracellular sources possibly via an increase in IP_3 as previously hypothesized (Qian et al., 1998). That intracellular Ca^{2+} mobilized by PGE_2 is important in regulating exocytosis of salivary gland proteins is consistent with the observation that regulated exocytosis in most secretory cells is caused by a rise in intracellular Ca^{2+} during cell stimulation (Edwardson et al., 1997).

In mammals, the PLC signaling pathway, especially the second messenger IP_3, is thought to be important in secretory cells (Putney, 1988). We previously found that IP_3 and intracellular Ca^{2+} mobilization were stimulated by PGE_2 treatment (Qian, et al. 1998). The IP_3 receptor inhibitor TMB-8 (10^{-6} M) effectively inhibited PGE_2-stimulated anticoagulant release, further supporting this hypothesis. TMB-8 also significantly decreased the secretion of anticoagulant protein in the absence of exogenous PGE_2 providing further evidence for the existence of endogenous PGE_2 in the prepared salivary gland tissue.

Many bioactive proteins such as anticoagulants, anti-inflammatory factors and immuosuppression factors are secreted in tick saliva (Bowman et al., 1997). These proteins may assist pathogen transmission by ticks. Furthermore, membrane-bounded parasites such as spirochetes and rickettsiae may exploit the tick's exocytotic mechanisms

of transport for transmission to the host, since both spirochetes and rickettsiae are believed to enter salivary glands via endocytosis and exit via exocytosis (Munderloh and Kurtti, 1995).

We also examined the possible existence of PG receptors in male *D. andersoni* ticks involved in exocytosis of salivary gland protein. PGE_2 stimulated, and A-23187 caused anticoagulant release in the dispersed salivary glands of male *D. andersoni*. A common mechanism for prostaglandins in controlling regulated secretion may exist in all species of ticks and in both sexes. However, in contrast to female *A. americanum*, low concentrations of $PGF_{2\alpha}$ (10 nM to 1 μM) was as potent as PGE_2 in stimulating secretion of anticoagulant protein in male *D. andersoni*, suggesting possible differences in receptor types in the two species or differences between male and female ticks. More research is needed to clarify possible differences in prostaglandin receptors and their roles in regulated exocytosis in different species of ticks.

Figure 2.1 Effect of PGE_2 on release of anticoagulant from dispersed salivary glands. One anticoagulant unit (ACU) is defined as the activity which delayed the initiation of coagulation of 50μl sheep plasma by 3 seconds at room temperature. Results are expressed as percent change ($\pm$S.E.M.) in ACU release by dispersed salivary glands when incubated with the indicated concentration of PGE_2 as compared to its solvent control. Dispersed salivary glands were incubated with (open circles, n=9) or without (closed circles, n=7)1mM indomethacin. * Significantly different from control (no PGE_2) ($P<0.05$).

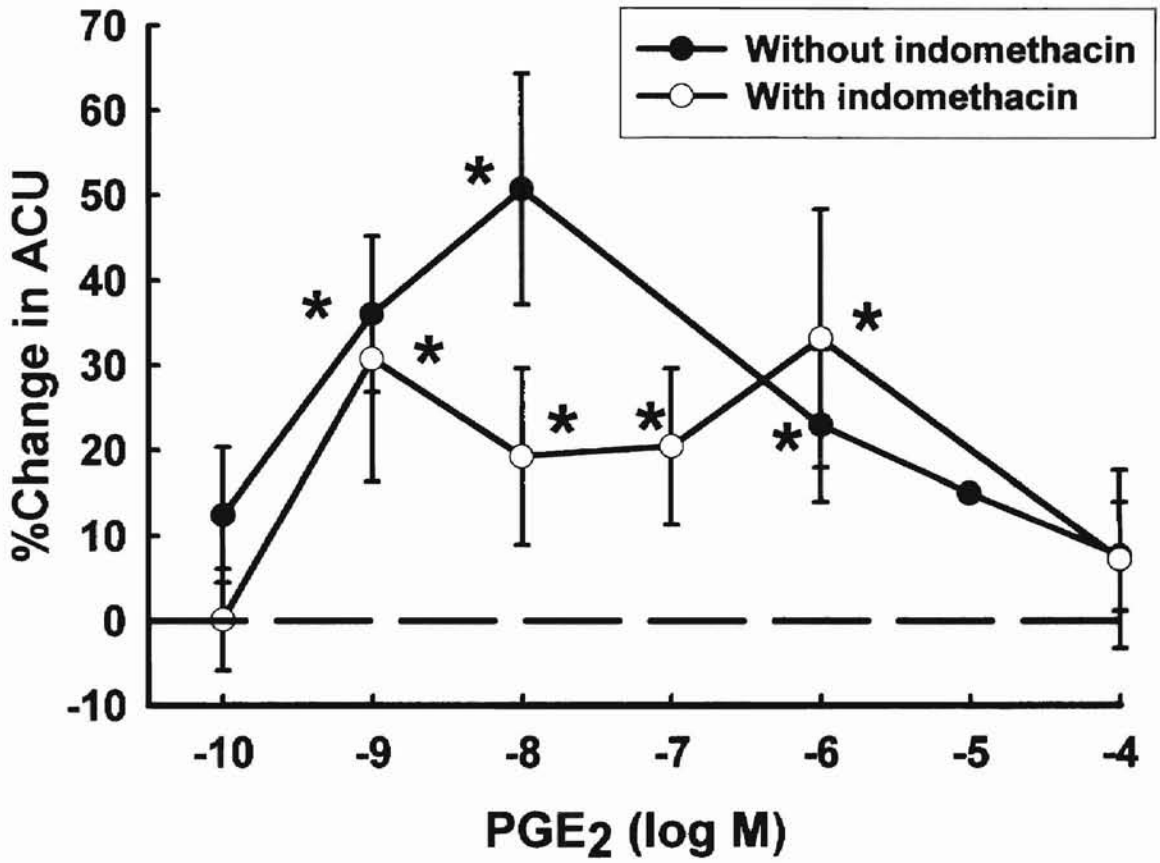

Without indomethacin
With indomethacin
%Change in ACU
70
60
50
40
30
20
10
0
-10
-10
-9
-8
-7
-6
-5
-4
PGE2 (log M)

Figure 2.2 Effects of prostanoids on anticoagulant release from dispersed tick salivary glands. Results are expressed as percent change (±S.E.M.) of anticoagulant activity in secretion of dispersed salivary glands incubated with the indicated concentration of prostanoids over the solvent controls for 5 min at room temperature. For PGE_2, n=7; $PGF_{2\alpha}$, n=3; U-46619, n=4. * indicates a significant difference from the control ($P<0.05$).

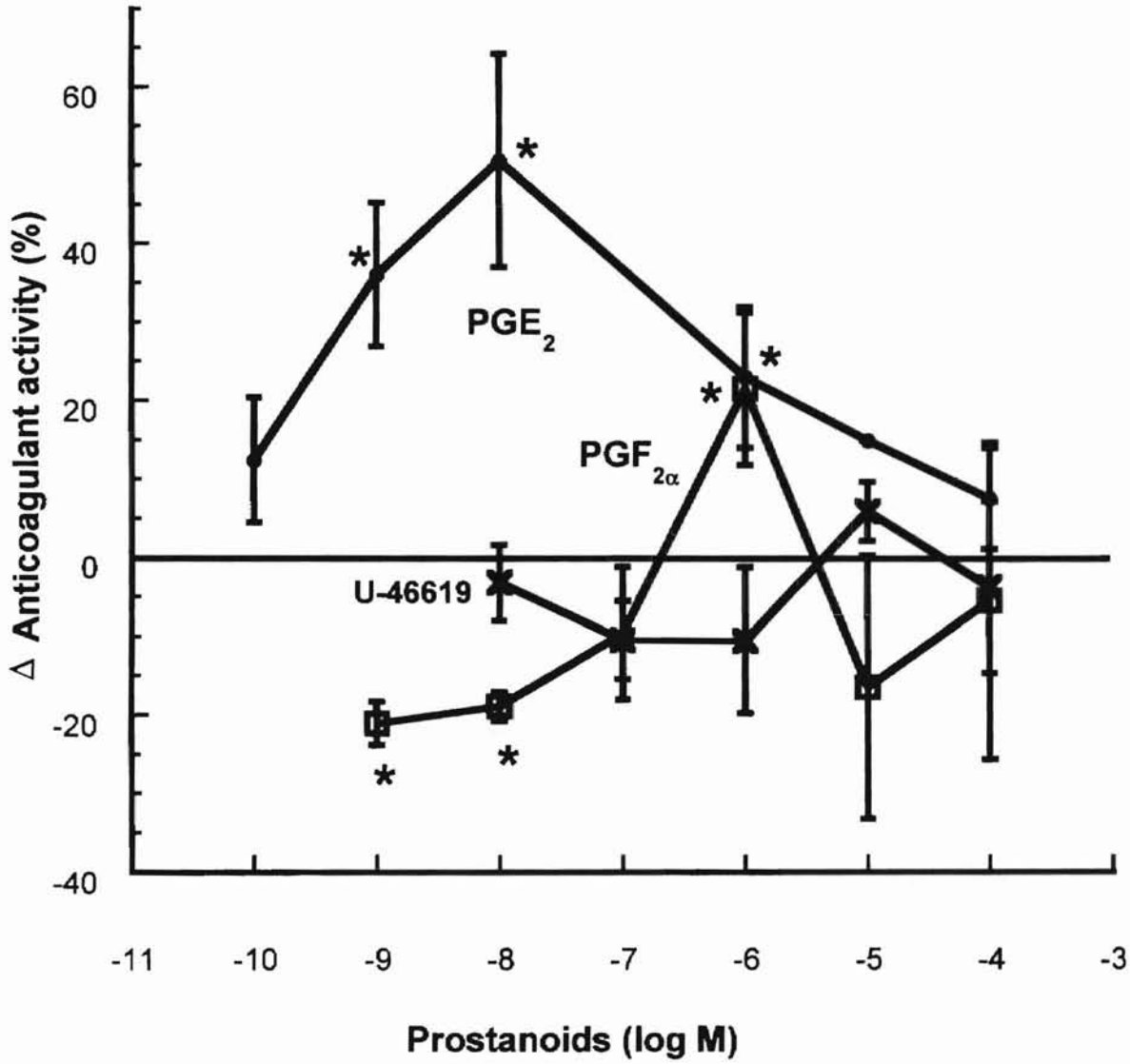

60
40
20
0
-20
-40
Δ Anticoagulant activity (%)
PGE2
PGF2α
U-46619
-11
-10
-9
-8
-7
-6
-5
-4
-3
Prostanoids (log M)

Figure 2.3 Effect of PGE_2, EP1 receptor antagonist AH6809 on percent change (±S.E.M.) in ACU and total protein release by dispersed salivary glands incubated with the indicated concentration of antagonist as compared to its solvent control (n=5).

* Significantly different from control (no AH6809) ($P < 0.05$).

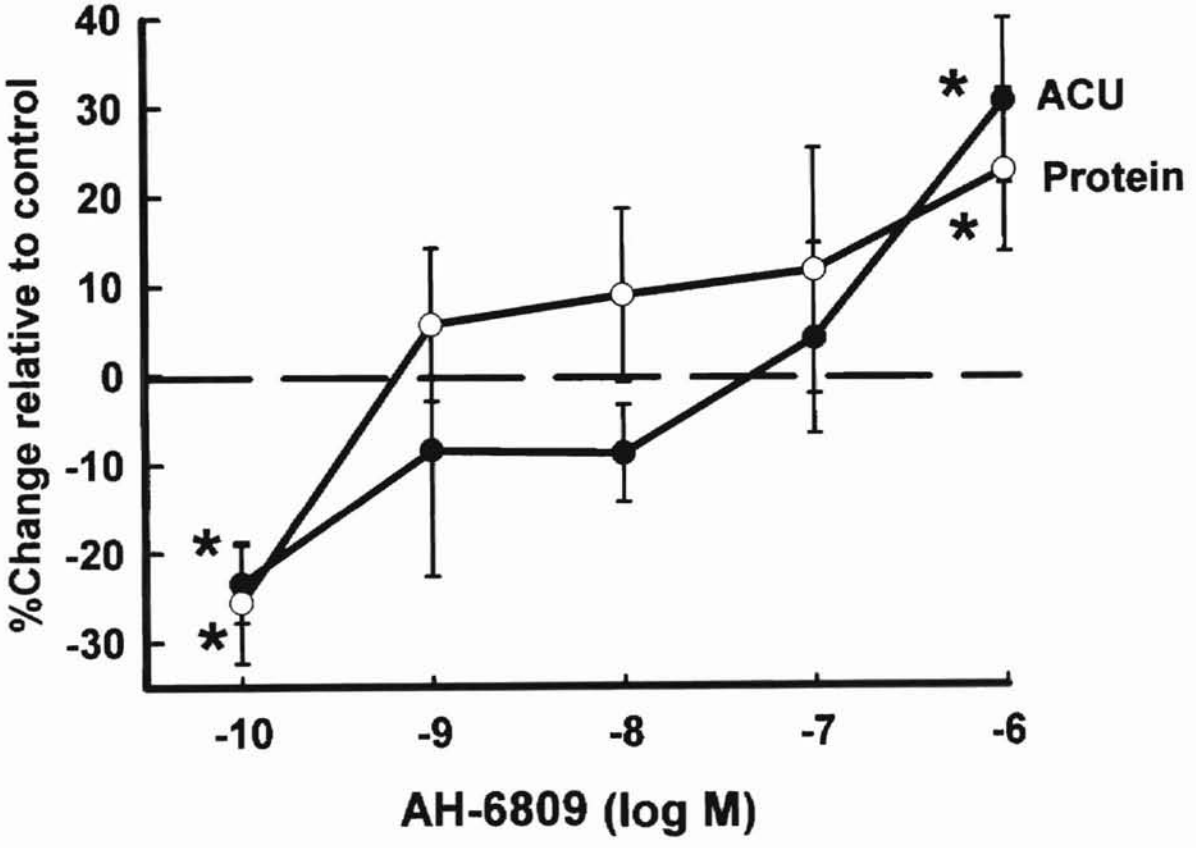

40
30
20
10
0
-10
-20
-30
%Change relative to control
-10
-9
-8
-7
-6
AH-6809 (log M)
ACU
Protein
*
*
*
*

Figure 2.4 Effect of PGE_2, EPI receptor antagonist AH6809 on percent change (± S.E.M.) in ACU release by dispersed salivary glands incubated with the indicated concentration of antagonist and 10^{-8} M PGE_2 (closed circles, n=9) or 10^{-6} M PGE_2 (open circles, n=5) as compared to its solvent control. * Significantly different from control (no PGE_2) ($P<0.05$).

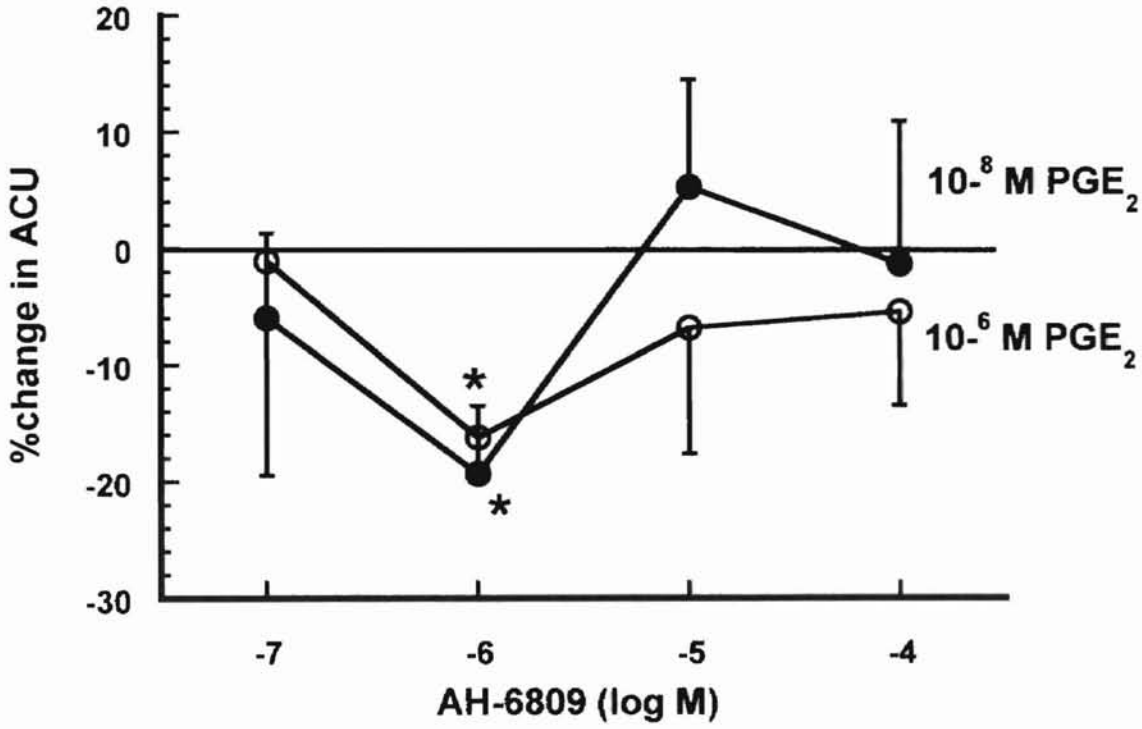

20
10
0
-10
-20
-30
%change in ACU
-7
-6
-5
-4
AH-6809 (log M)
10-8 M PGE2
10-6 M PGE2
*
*

Figure 2.5 Effect of 17-phenyl trinor PGE_2, EP1 receptor agonist, on anticoagulant release from dispersed tick salivary glands. Results are expressed as percent change (±S.E.M.) of anticoagulant activity in secretion of dispersed salivary glands incubated with the indicated concentration of the drug over the solvent control ethanol for 5 min at room temperature (n=4). * Significantly different from the control ($P< 0.05$).

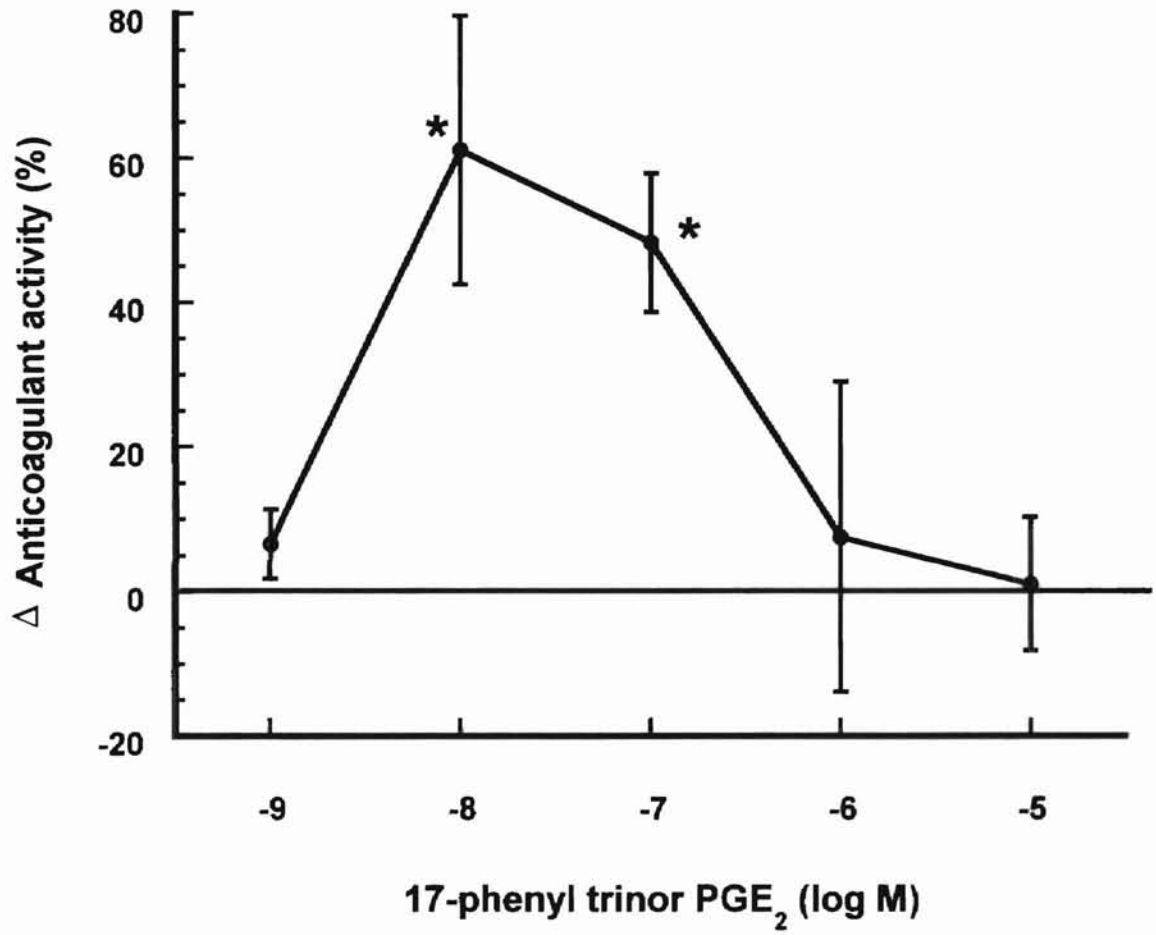

80
60
40
20
0
-20
Δ Anticoagulant activity (%)
*
*
-9
-8
-7
-6
-5
17-phenyl trinor PGE2 (log M)

Figure 2.6 Effect of calcium ionophore A-23187 on anticoagulant release from dispersed tick salivary glands. Results are expressed as percent change (±S.E.M.) of anticoagulant activity in secretion of dispersed salivary glands incubated with the indicated concentration of the drug over the solvent control DMSO for 5 min at room temperature (n=5). * Significantly different from the control ($P< 0.05$).

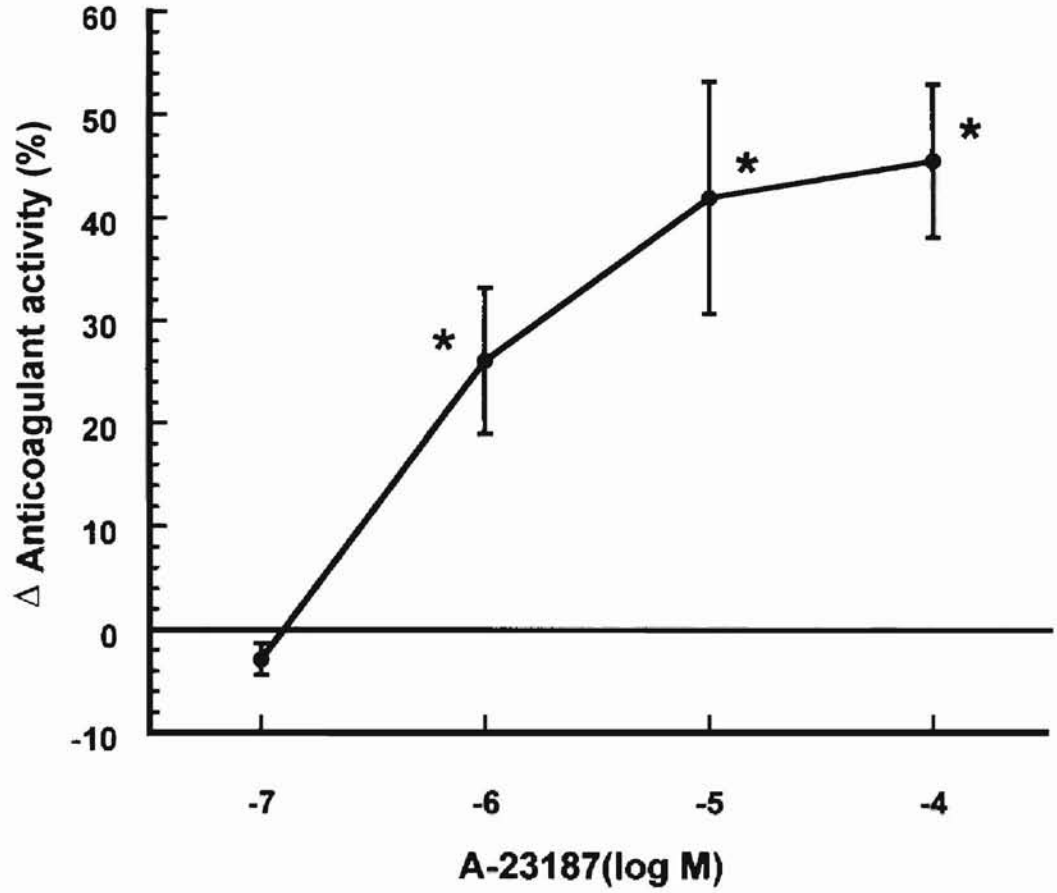

60
50
40
30
20
10
0
-10
Δ Anticoagulant activity (%)
*
*
*
-7
-6
-5
-4
A-23187(log M)

Figure 2.7 Effect of verapamil, voltage-gated Ca^{2+} channel blocker, on anticoagulant release from dispersed tick salivary glands in presence of 10^{-7} M PGE_2. The dispersed salivary glands were preincubated with the indicated concentration of the drug or the buffer control for 30 min, then both were incubated with 10^{-7} M PGE_2 for 5 min at room temperature. Results are expressed as percent change (±S.E.M.) of anticoagulant activity in the secretion (n=8).

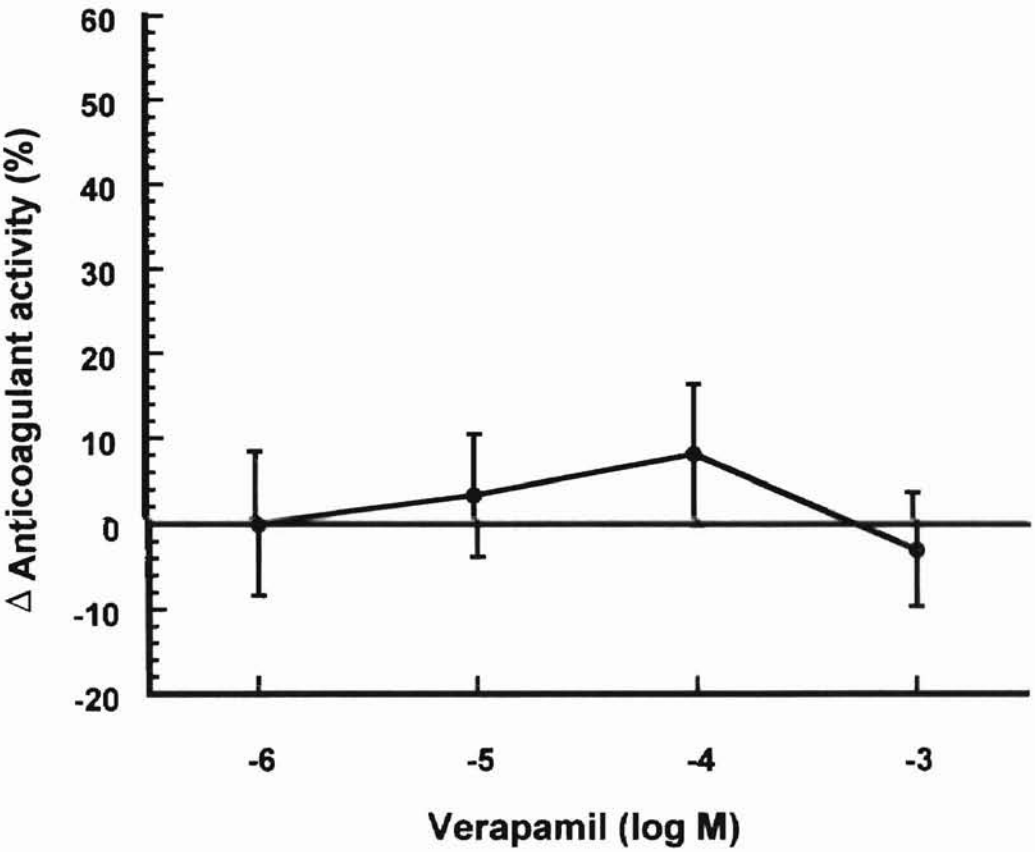
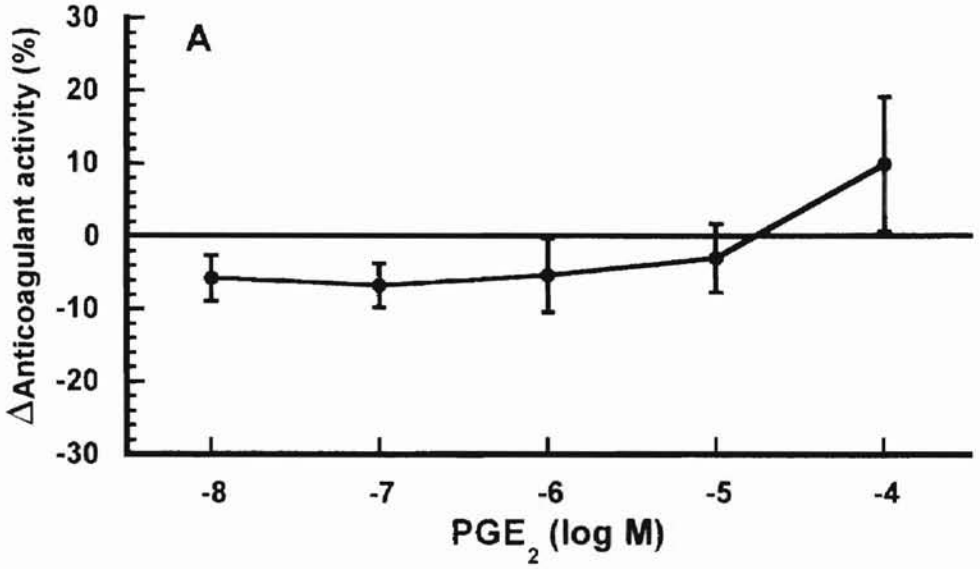
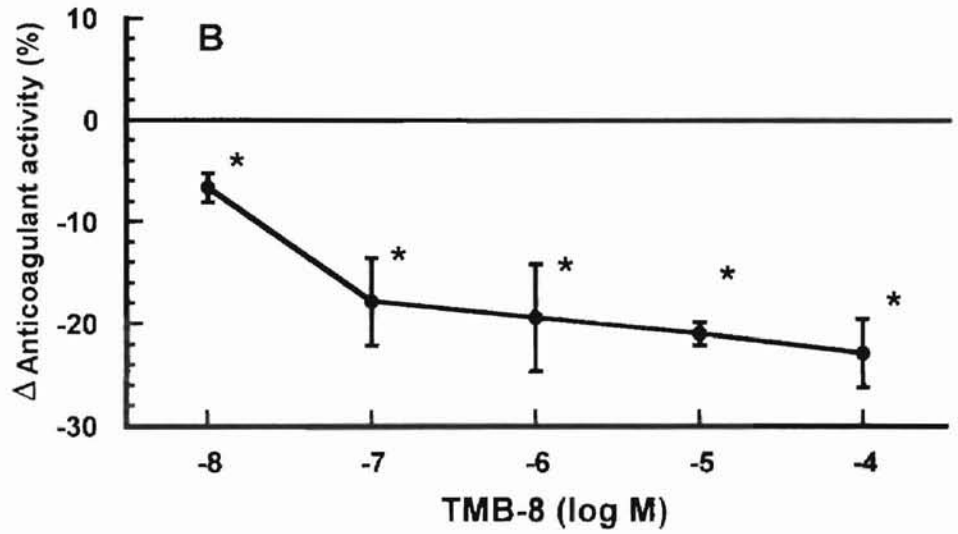

60
50
40
30
20
10
0
-10
-20
Δ Anticoagulant activity (%)
-6
-5
-4
-3
Verapamil (log M)

Figure 2.8 Effects of the selective IP_3 receptor inhibitor TMB-8 on anticoagulant release from dispersed salivary glands. Results are expressed as percent change (±S.E.M.) of anticoagulant activity in secretion of dispersed salivary glands incubated with the indicated concentration of the drug over the solvent control for 5 min at room temperature. (A) different concentrations of PGE_2 in the presence of 10^{-6} M TMB-8, n=7; (B) different concentrations of TMB-8 in the presence of 10^{-8} M PGE_2, n=4; (C) different concentrations of TMB-8 without exogenous PGE_2, n=3. * Significantly different from control ($P< 0.05$).


A
30
20
10
0
-10
-20
-30
ΔAnticoagulant activity (%)
-8
-7
-6
-5
-4
PGE2 (log M)


B
10
0
-10
-20
-30
Δ Anticoagulant activity (%)
*
*
*
*
*
-8
-7
-6
-5
-4
TMB-8 (log M)

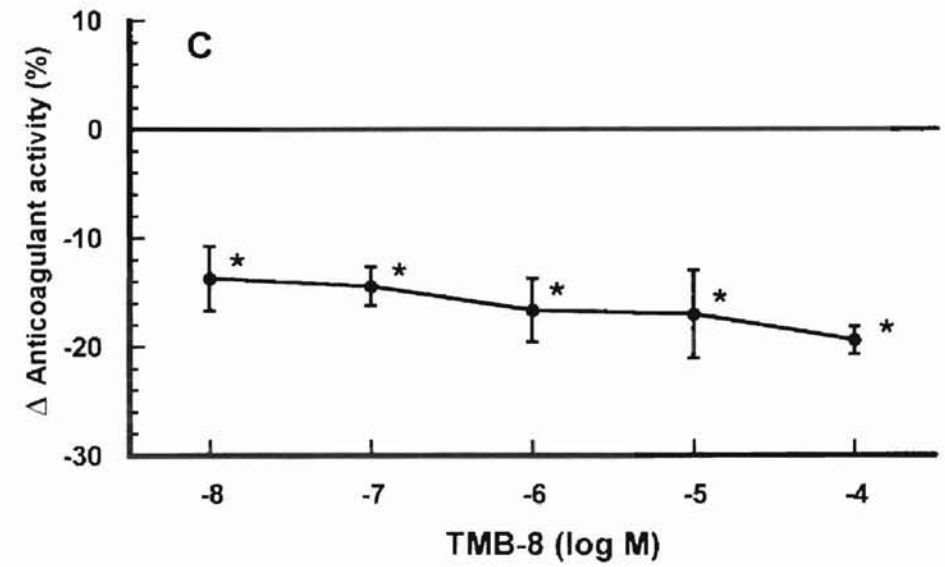
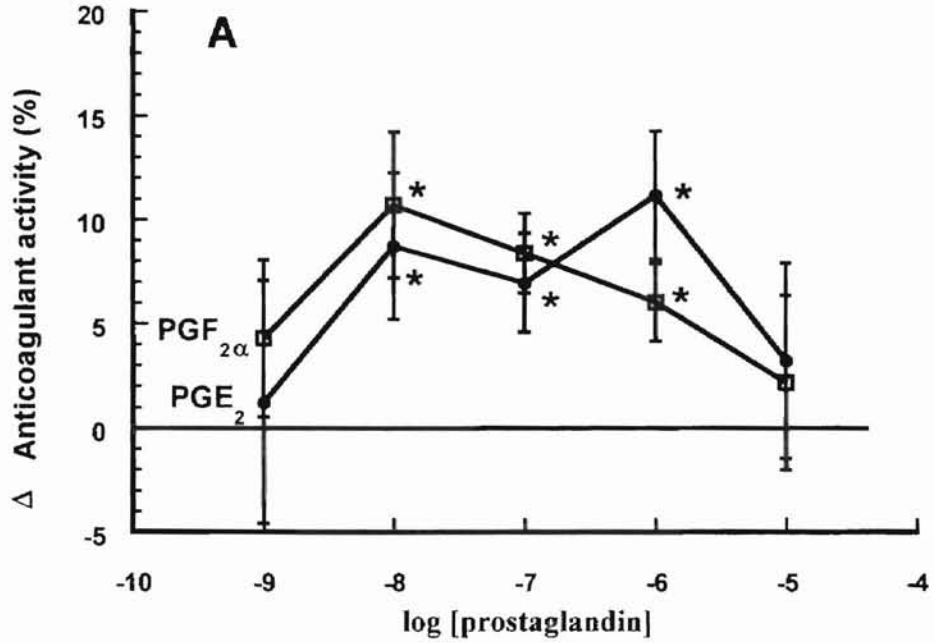
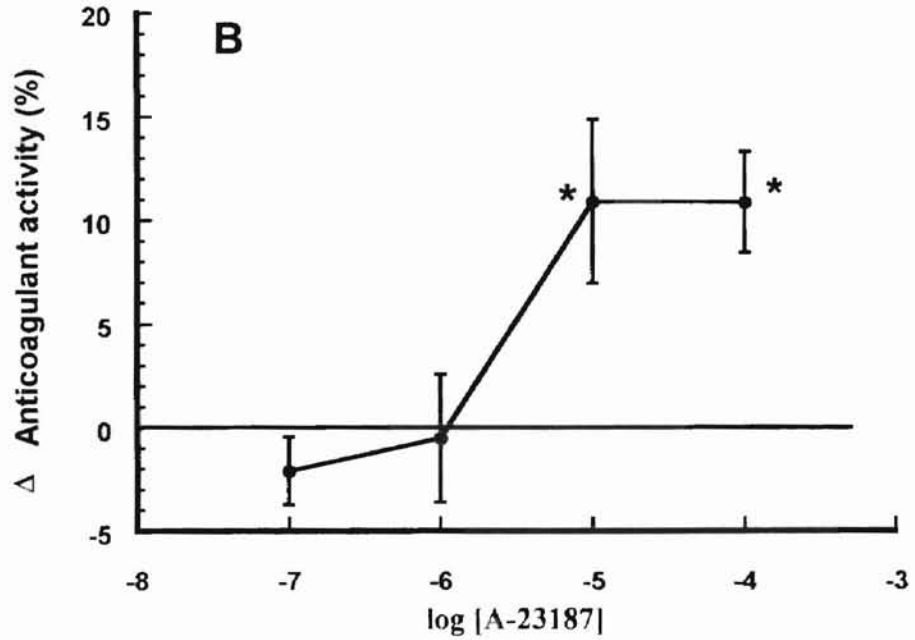

C
10
0
-10
-20
-30
Δ Anticoagulant activity (%)
*
*
*
*
*
-8
-7
-6
-5
-4
TMB-8 (log M)

Figure 2.9 Effects of prostaglandins and calcium ionophore on anticoagulant release from dispersed salivary glands of male *Dermacentor andersoni*. Results are expressed as percent change (±S.E.M.) of anticoagulant activity in secretion of dispersed salivary glands incubated with the indicated concentration of the drug over the solvents control for 5 min at room temperature. (A). PGE_2, n=7; $PGF_{2\alpha}$, n=7; (B). A-23187, n=7.

* Significantly different from the control ($P< 0.05$).


A
20
15
10
5
0
-5
Δ Anticoagulant activity (%)
PGF2α
PGE2
*
-10
-9
-8
-7
-6
-5
-4
log [prostaglandin]


B
20
15
10
5
0
-5
Δ Anticoagulant activity (%)
*
-8
-7
-6
-5
-4
-3
log [A-23187]

CHAPTER III

PHOSPHOLIPASE C IS INVOLVED IN PROSTAGLANDIN E_2-STIMULATED EXOCYTOSIS IN THE SALIVARY GLANDS OF THE LONE STAR TICK, *AMBLYOMMA AMERICANUM* (L.)

ABSTRACT

We previously identified a prostaglandin E_2 (PGE_2) specific, G-protein linked receptor in the plasma membrane fraction of tick salivary glands that increases inositol trisphosphate, mobilizes intracellular calcium and is involved in PGE_2-stimulated secretion (exocytosis) of anticoagulant protein. In this study phospholipase C (PLC) activity (0.3 nmol/min/mg protein) was identified in a crude plasma membrane fraction of salivary glands of the lone star tick (*Amblyomma americanum*). Enzyme activity was linear with time up to 15 min and exhibited a pH optimum of ~7.0. Western blot analysis using an antibody against mammalian PLC γ-1 showed three clear cross-reacting bands at a molecular weight of 220 kD, 70 kD and 55 kD in a plasma membrane-enriched fraction of the salivary glands. PGE_2 did not stimulate an increase in PLC activity in crude membrane fractions. However, activity was inhibited almost 25% by a low concentration (10^{-10} M) of the PGE_2 EP1 receptor antagonist AH6809. The inhibition of PLC activity by AH-6809 was completely reversed by 10^{-7} M PGE_2 and 10^{-5} M GTP-γ-S. Low concentrations (10^{-7}, 10^{-6} M) of the PLC inhibitor neomycin and U-73122 inhibited PGE_2-stimulated secretion of

anticoagulant protein. We conclude that PGE_2 stimulates exocytosis of anticoagulant protein by directly activating a PLC catalyzed phosphoinositide signaling pathway in tick salivary glands.

KEYWORD INDEX: ticks, salivary glands, exocytosis, prostaglandin, phospholipase C, PLC calcium, inositol phosphates, PGE_2 receptor, anticoagulants

INTRODUCTION

Ixodid ticks are obligate ectoparasites and important vectors for many diseases of human and domestic animals (Kaufman, 1989). Prolonged host attachment and anti-hemostatic, vasodilatory, anti-inflammatory and immunosuppressive properties of tick saliva enhance the effectiveness of ticks to transmit pathogens (Bowman et al., 1997; Ramachandra and Wikel, 1992). In addition to many bioactive proteins, tick saliva contains high concentrations of prostaglandins (PGs) especially prostaglandin E_2 (PGE_2) (Ribeiro et al., 1992). To date, most research on tick derived PGs has been focused on their functions in enhancing tick feeding after export to the host (Bowman et al., 1996).

In mammals, PGs typically function as local hormones through interaction with specific receptors on the same or neighboring cells coupled to multiple signalling pathways which either mobilize Ca^{2+} or affect cAMP level (Narumiya, 1995). There are four sub-types of PGE_2 receptors, EP1, EP2, EP3 and EP4 (Coleman et al., 1990). EP1 receptors are involved in Ca^{2-} mobilization, while EP2, EP3, EP4 receptors affect cAMP levels via

stimulation or inhibition of adenylate cyclase (Coleman et al., 1994). Qian et al. (1997) identified a PGE_2 -specific and G-protein-linked receptor from the salivary glands of *Amblyomma americanum*. PGE_2 does not affect adenylate cyclase activity in the plasma membrane fraction of tick salivary gland (Qian et al., 1997), but stimulates an increase in IP_3 and mobilizes intracellular Ca^{2+}. It is thought that the increase in Ca^{2+} by action of PGE_2 stimulates release of proteins from dispersed salivary gland acini (Qian et al., 1998).

Binding of PGE_2 to EP1 receptors may induce Ca^{2+} mobilization via direct activation of a phospholipase C signal transduction pathway (Negishi et al., 1993). Phosphatidylinositide-specific phospholipase C (PI-PLC) is known to be a key enzyme in the signal transduction pathway which regulates many cellular processes in response to extracellular signals such as hormones, neurotransmitters and growth factors (Berridge, 1987). PI-PLC hydrolyzes phosphatidylinositol 4,5- bisphosphate (PIP_2) into two second messengers: diacylglycerol (DAG) and inositol 1,4,5- trisphosphate (IP_3). DAG activates protein kinase C, and IP_3 releases intracellular Ca^{2+} after binding to a specific receptor on the endoplasmic reticulum (ER) (Berridge, 1993). In mammalian cells, PLC has been well studied and classified into four major classes of isozymes (α, β, γ, δ). Five invertebrate PLC-encoding genes (four from *Drosophila melanogaster* and one from brine shrimp, *Artemia*) have been cloned to date (Shortridge and McKay, 1995). A heat- and trypsin-sensitive brain factor increases inositol phosphates in isolated whole tick salivary glands although the function of the factor in salivary gland is unknown (McSwain et al., 1989). IP_3 stimulates a rapid efflux of Ca^{2+} from intracellular stores of the permeablilized whole salivary glands (Roddy et al., 1990). These results, together with the ability of PGE_2 to increase cytosolic IP_3 and mobilize intracellular Ca^{2+} (Qian, et al. 1998), suggest the

presence of a PLC-activated signal transduction pathway in tick salivary glands. However it is unknown if PGE_2 directly activates PLC after binding to its receptor or increases salivary gland IP_3 and Ca^{2+} mobilization indirectly. Results of this study offer evidence that PGE_2-stimulated anticoagulant release follows direct activation of PLC activity after PGE_2 binds to its EP1-like receptor.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: Medium-199, lyophilized sheep plasma, activated partial thromboplastin time (APTT) reagent, phosphatidylinositol (PI), deoxycholate (DOC), Triton X-100, PI-PLC, TMB-8 (IP_3 receptor inhibitor), GTP-γ-S, neomycin, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) / Nitro Blue Tetrazolium (NBT) tablets were from Sigma Chemical Company (St. Louis, Missouri, USA); 6-isopropyl-9-xanthone-2-carboxylic acid (AH-6809, a specific EP1 receptor inhibitor), U-73122 (a specific inhibitor of phospholipase C), and PGE_2 were from Biomol (Plymouths Meeting, PA, USA); L-α-[myo-2- H^3 (N)]- phosphatidylinositol (11Ci/mmol) was from DuPont New England Nuclear (Boston, MA, USA); ScintiSafe™ Econo2 scintillation cocktail was from Fisher Scientific (St. Louis, MO, U.S.A.); antibody to PLC γ–1 (rabbit polyclonal IgG) was from Upstate Biotechnology Inc.(Lake Placid, NY); non-stimulated A-431 cell lysate was a gift from Upstate Biotechnology Inc.; alkaline phosphatase-conjugated donkey anti-rabbit IgG was from Jackson ImmunoResearch

Laboratories, Inc. (West Grove, PA).

Experimental animals

Ticks, *Amblyomma americanum* (L.), were reared at Oklahoma State University's Central Tick Rearing Facility, according to the methods of Patrick & Hair (1975). Immature ticks were fed on rabbits and adult ticks on sheep. All unfed ticks were maintained at 27-28°C and 90% RH under 14:10 (L:D) photoperiod before infestation of the hosts. Partially fed female ticks weighing 80-120 mg were used within four hours after being removed from the host.

Tissue preparation

For the exocytosis assays, dispersed salivary gland acini were prepared as described by Qian et al. (1998). Briefly, salivary glands were dissected in Medium-199 containing 20 mM MOPS buffer (pH 7.0). One gland from each tick was put in the control group and the other in the treatment group. Salivary glands were then gently dispersed by teasing apart with fine tipped forceps. The dimensions of the dispersed tissue were smaller than 0.5 mm^2. Dispersed tissue was transferred to a microtube and rinsed 5 times with fresh buffer prior to treatment.

To prepare tick salivary glands tissue for enzyme assay, 12 glands were homogenized in 200 µl ice-cold buffer (10 mM Tris-EGTA, pH 7.0), then centrifuged at 16,000 g for 20 min at 4°C. The supernatant was removed and considered as the cytosolic fraction. The pellet (membrane-enriched fraction; McSwain et al., 1987) was resuspended with 100 µl of the same buffer. The protein concentration was adjusted to

2.5 μg/μl and used immediately or stored at -80 °C.

Tissue treatment

Salivary glands were incubated with experimental buffer containing the above buffer plus different drugs according to individual experimental designs (see figure captions), or with buffer plus the solvent used to solubilize the respective drugs as a control group.

All incubations were performed for 5 min at room temperature except as stated otherwise. During each incubation, dispersed tissue was gently agitated twice. After incubation, the glands were centrifuged for 1 min at 1000g, and two 20 μl aliquots of supernatant (containing salivary secretion products) were removed and tested for anticoagulant content in the coagulation assay. The centrifuged glands were kept and used to measure phospholipase C activity if necessary.

Coagulation assay

The coagulation assay was performed as described by Qian et al. (1998). The clotting time was determined, and the change in anticoagulant activity was expressed as the percentage of the clotting time difference over the control.

Phospholipase C assay

PLC activity was measured as described by Perrella et al. (1991) and Radallah et al. (1995) with some modifications. Phosphatidylinositol (PI) and [H^3]-PI were used as

substrates and commercially available PI-PLC (Sigma, St. Louis, Mo.) was used as a positive control.

Substrates consisted of a mixture of phosphatidylinositol (PI) (200μM) and [H^3]-PI (1 μCi) in 100 mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid) buffer containing 2 mg/ml deoxycholate (DOC) and sonicated 3 times for 1 min each with 30 sec intervals between each sonication period (Fisher sonic dismembrator, Model 300, frequency 20kHz, power 35 watts). The final concentration in a total 100 μl reaction mixture was: 50 mM HEPES buffer (pH 7.0); 100 mM KCl; 2 mM $CaCl_2$; 1 mg/ml deoxycholate, 100 μM PI and 10,000 cpm of [H^3]-PI. The mixtures were preincubated at 37 °C for 2 min before adding 20 μg enzyme preparation to start the reaction. Typically, the reaction was conducted for 15 min at 37 °C and stopped by adding 0.5 ml chloroform/ methanol/concentrated HCl (100:100:0.6) and vortexed for 40 seconds. An additional 200 μl 1M HCl, 5 mM EDTA was added and vortexed for 40 seconds. After centrifugation at 2,000 g for 5 min at room temperature, a 200 μl aliquot of the upper methanolic phase containing the inositol phosphates (InsPs) was counted for the radioactivity by a Beckman LS6000sc Counter using the scintillation cocktail ScintiSafe™ Econo 2. PLC activity was expressed as nmoles of inositol phosphate released/min/mg protein.

Western immunoblot

The salivary glands from six ticks were dissected, homogenized, and extracted with 200 μl 10mM Tris-HCl Buffer, pH 7.0, 5 mM EGTA, 2 mM PMSF containing 0.2% Triton X-100. After protein determination, the protein concentration was adjusted to 1 μg/μl. Proteins were electrophoresed in a 7.5% SDS-polyacrylamide gel (SDS-PAGE) and

electrotransferred to nitrocellulose. The prestained high molecular weight standard was used. A 20 μg non-stimulated A-431 cell lysate was used as the positive control to identify the position of mammalian PLC. The blotted nitrocellulose was incubated with 10 mM Tris-HCl, 0.9% NaCl, pH 7.4 containing 5% nonfat dry milk at 4 °C overnight. The membrane was then incubated with 1 μg/ml PLC γ–1 antibody (rabbit polyclonal IgG) for 1.5 hour at room temperature, and then 1:10000 alkaline phosphatase-conjugated donkey anti-rabbit IgG as secondary antibody for 1 hour at room temperature. 5-bromo-4-chloro-3-indolyl phosphate (BCIP) / Nitro Blue Tetrazolium (NBT) tablet (substrate of alkaline phosphatase) was used to develop the blot and visualize the protein bands.

Protein assay

The protein concentration was determined by the method of Bradford (1976) with Bio-Rad protein assay dye. Bovine serum albumin was used as the standard protein.

Statistics analysis

Standard errors and the number of repetitions are indicated in the figures. The differences of means between the control and experimental treatment were tested for significance by Student's t-test (Steel et al., 1997). Treatment effect was tested by ANOVA and, if warranted the significance of differences between groups determined by Student-Newman-Keuls' test. A P-value (<0.05) was considered significant.

RESULTS

Phospholipase C activity in tick salivary glands

The rate of phosphatidylinositol hydrolysis in the plasma membrane-enriched fraction was linear with time up to 15 min (Fig. 3.1A) and increased with amount of protein (Fig. 3.1B). The pH optimum was ~7.0 (Fig. 3.1C). The standard assay constituted 15 μg protein, 10 min incubation at 37 °C and pH 7.0. The rate for phosphatidylinositol hydrolysis by membrane fraction from tick salivary glands was 0.3 ± 0.04 nmole/min/mg protein. The activity in the cytosolic fraction of the salivary gland was about 0.25 nmole/min/mg protein.

Western Blotting of PLC

To further verify the existence of PLC in tick salivary glands, the homogenate of tick salivary glands was subjected to immunoblotting with rabbit antibody against the 145 kD bovine PLC γ-1. The negative control which had no first antibody but had secondary antibody showed no band at all. Three distinct bands were detected in tick salivary gland proteins with the molecular weights of about 220 kD, 70 kD, and 55 kD, while the positive control had two faint bands at 145 kD and 55 kD at an equivalent amount of protein (Fig. 3.2). The results suggest that phospholipase C isoforms cross-reacting with the antibody against bovine PLC γ-1 may exist in tick salivary glands.

Activation of PLC by PGE_2

Previous studies demonstrated that PGE_2 stimulates an increase in salivary gland IP_3 (Qian et al., 1998) but it is unknown if PGE_2 activates PLC directly after binding to the G-protein linked receptor. On its own, neither 10^{-7} M PGE_2 nor 10^{-5} M GTP-γ-S (non-hydrolyzable GTP analog) stimulated PLC activity in the membrane fraction. One possible reason why exogenous PGE_2 or GTP-γ-S failed to activate plasma membrane PLC is the presence of endogenous PGE_2 in the fraction. To test this, we treated the salivary gland membranes with a low concentration (10^{-10} M) of the selective PGE_2 EP1 receptor antagonist AH-6809. PLC activity was significantly decreased ($P<0.05$) to ~75% of the basal activity by the receptor antagonist. Furthermore, 10^{-7} M PGE_2 and 10^{-5} M GTP-γ-S completely reversed the inhibition of PLC activity by AH6809 (Fig. 3.3), strongly suggesting that membrane-associated PLC in tick salivary glands is directly activated after PGE_2 binding to its receptor.

Effect of the inhibitors of PLC on exocytosis of salivary glands protein

To further test the hypothesis that phospholipase C is the key enzyme in PGE_2-stimulated exocytosis of salivary gland proteins, we tested the abilities of two PLC inhibitors (U-73122 and neomycin) to block anticoagulant release stimulated by PGE_2. In the presence of 10^{-7} M PGE_2, the specific inhibitor of mammalian PLC, U-73122, slightly inhibited ($P<0.05$) the anticoagulant release from dispersed tick salivary glands at concentrations of 10^{-7} and 10^{-6} M, but concentrations of 10^{-5} and 10^{-4} M greatly enhanced ($P<0.05$) PGE_2 stimulated secretion. Similarly in the presence of 10^{-7} M PGE_2, the non-

specific PLC inhibitor neomycin slightly inhibited ($P<0.05$) the anticoagulant release at the concentration of 10^{-7} and 10^{-6} M, but at 10^{-4} and 10^{-3} M the inhibitor greatly enhanced ($P<0.05$)the PGE_2 stimulated secretion (Fig. 3.4).

DISCUSSION

The present research establishes the existence of a phosphatidylinositide-specific phospholipase C activity in the salivary glands of *Amblyomma americanum* and strongly implicates its involvement in regulated exocytosis. The enzyme activity was detected in both the plasma membrane-enriched and cytosolic fractions of tick salivary gland as has been noted in mammalian tissues (Lee et al., 1987). This is the first direct biochemical evidence of a phospholipase C catalytic activity in the tick although McSwain et al. (1989) demonstrated an increase in salivary gland IP_3 in response to a peptide factor from the ticks synganglion. Western blot analysis with an antibody against bovine PLC γ-1 (the only polyclonal antibody commercially available recommended for Western blotting) showed three cross-reaction bands of tick salivary gland proteins with molecular weight about 220, 70, and 55 kD. This was somewhat surprising, since the molecular masses of PLCs that have been reported in other animals are: 62-68 kD for PLC-α , 150-154 kD for PLC-β , 145-148 kD for PLC-γ and 85-88 kD for PLC-δ (Rhee et al., 1989). Also, it's uncertain if the tick proteins detected in Western blot were PGE_2-activated PLC, since the PLC isoform usually associated with G-protein linked receptors in other animals is PLC-β. However, the combined enzymatic and immunoblot data suggest that phospholipase C

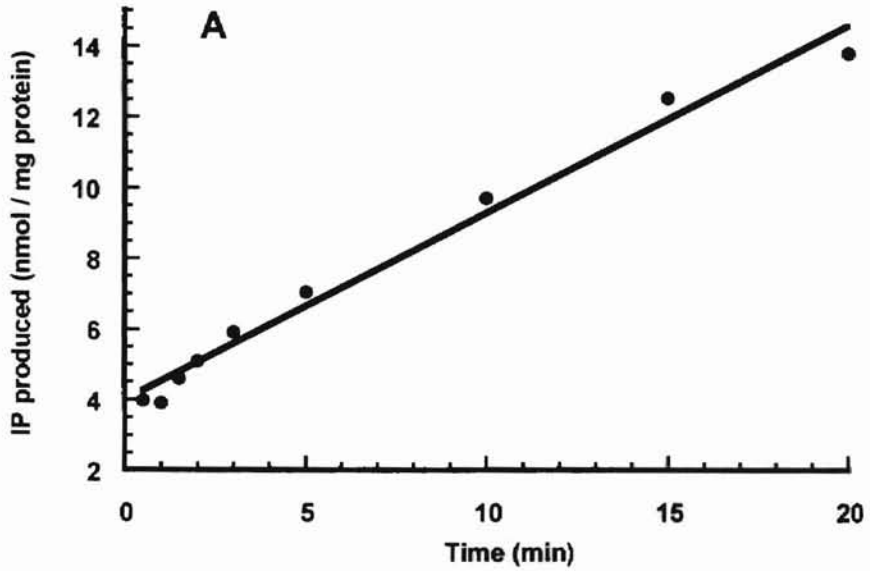
isoforms exist in tick salivary glands. However, the tick forms may differ somewhat from mammalian PLC. To date, only five putative invertebrate PLC-encoding genes have been cloned, four from *Drosophila* (Bloomquist et al., 1988; Emori et al., 1994; McKay et al., 1995; Shortridge et al., 1991) and one from brine shrimp, *Artemia* (Su et al., 1994). These invertebrate PLCs fall into the α, β, γ isoform classification scheme used for mammals, and are structurally and functionally similar to their mammalian counterparts. Moreover, PLC signaling pathways in invertebrates are known to modulate cellular processes such as phototransduction, olfaction, cell growth and differentiation (Shortridge and McKay, 1995).

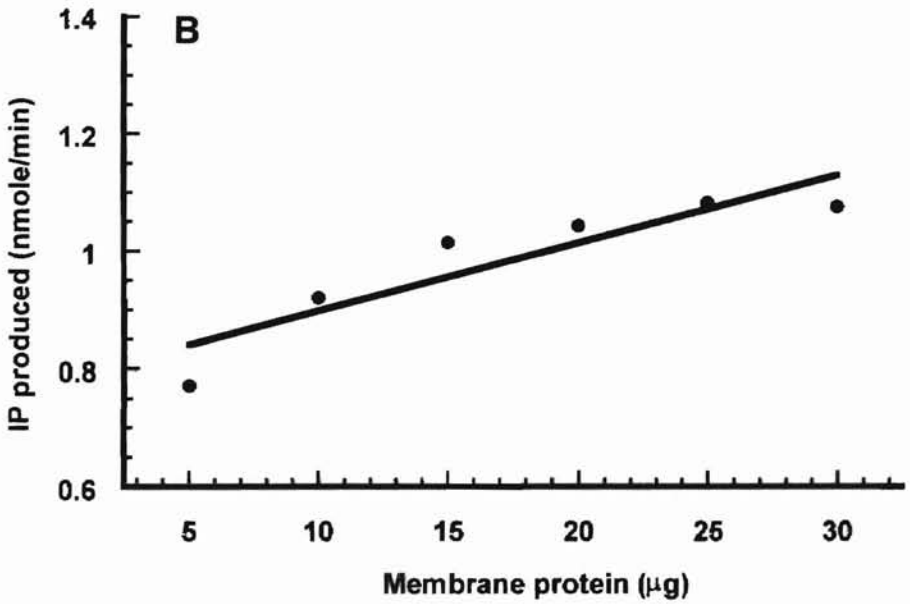
Our previous studies identified a G-protein linked, PGE_2-specific receptor in the plasma membrane fraction of tick salivary glands that increases inositol trisphosphate and mobilizes intracellular calcium (Qian, et al. 1997; 1998). These studies raised the question of whether PGE_2 activates tick salivary gland PLC directly or indirectly. In this study, neither 10^{-7} M PGE_2 nor 10^{-5} M GTP-γ-S affected the PLC activity in tick salivary gland membrane preparation. However, a low concentration (10^{-10} M) of the selective PGE_2 EP1 receptor antagonist AH-6809 suppressed PLC activity in the membrane preparation to ~75% of the control, suggesting the existence of endogenous PGE_2 in the tissue that was activating PLC in the preparation. In support of this, 10^{-7} M PGE_2 and 10^{-5} M GTP-γ-S completely reversed the PLC activity inhibited by AH6809, providing further evidence that PGE_2 directly activates a membrane associated PLC via G-protein in tick salivary glands to increase intracellular IP_3.

The effects of PLC inhibitors on anticoagulant release were also investigated. In the presence of 10^{-7} M PGE_2, both inhibitors (U-73122 and neomycin) inhibited the

anticoagulant release from dispersed tick salivary glands at low concentrations (10^{-7} and 10^{-6} M), but at higher concentrations (10^{-5}-10^{-3} M) the inhibitors potentiated the stimulatory effect of PGE_2. The significance of these results is unclear but the fact that similar results were obtained with two different PLC inhibitors suggests that the results are not artificial. Possibly more than one PLC is involved in regulating exocytosis of salivary gland proteins. In a previous study, low concentrations of $PGF_{2\alpha}$ (1 and 10 nM) inhibited release of anticoagulant protein 20% below that noted in control tissue (Yuan et al., 1999). In mammals, $PGF_{2\alpha}$ receptor typically activates PLC (Narumiya, 1996). Further characterization of PLC isoforms in tick salivary glands and linkage to other receptors should provide greater understanding of their roles in regulating exocytosis of salivary gland proteins.

Figure 3.1 Phospholipase C activity of tick salivary gland membrane proteins determined by its ability to hydrolyze [H^3]-inositol phosphate (IP) from [H^3]-phosphatidylinositol (PI) as a function of (A) incubation time ; (B) amount of enzyme source protein; (C) incubation buffer pH. Each experiment was performed at least three times with similar results.


A
IP produced (nmol / mg protein)
14
12
10
8
6
4
2
0
5
10
15
20
Time (min)


B
IP produced (nmole/min)
1.4
1.2
1
0.8
0.6
5
10
15
20
25
30
Membrane protein (μg)

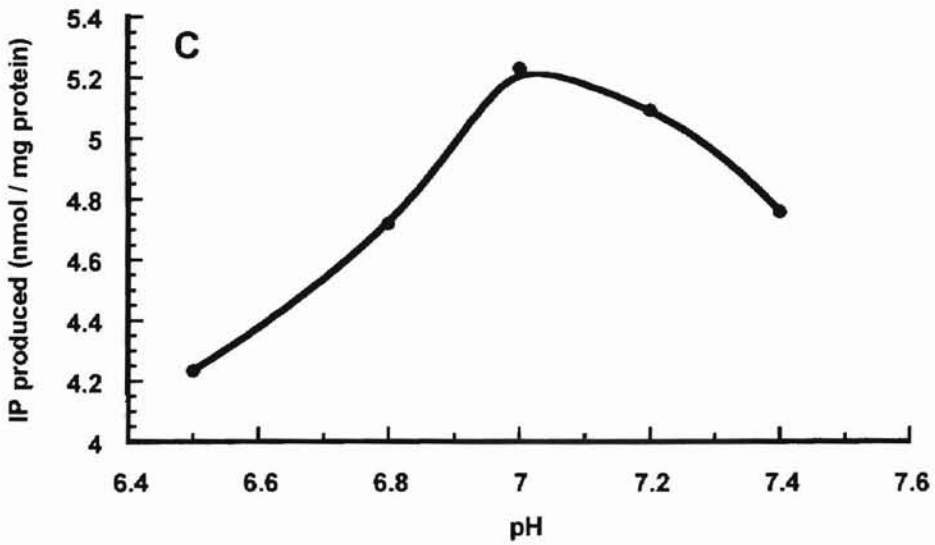

C
IP produced (nmol / mg protein)
5.4
5.2
5
4.8
4.6
4.4
4.2
4
6.4
6.6
6.8
7
7.2
7.4
7.6
pH

Figure 3.2 SDS-PAGE and Western blot of phospholipase C in tick salivary glands. 20 μg tick salivary gland protein (lane 3) and 20 μg non-stimulated A-431 cell lysate (lane 2) were subjected to 7.5% SDS-PAGE gel, stained with Coomassie brilliant blue (A), or transferred to nitrocellulose membrane and probed with an antibody against PLC γ-1 (B). Lane 1 is the prestained high molecular weight standard (from BIO-RAD). The arrow indicates the position of 145 kD bovine PLC γ-1 in the positive control lane (lane 2). The data shown are from one of several representative independent experiments. Standard protein bands in A did not align exactly to those on Western blot (B) due to the shrinkage of gel during the fixation and staining processes.

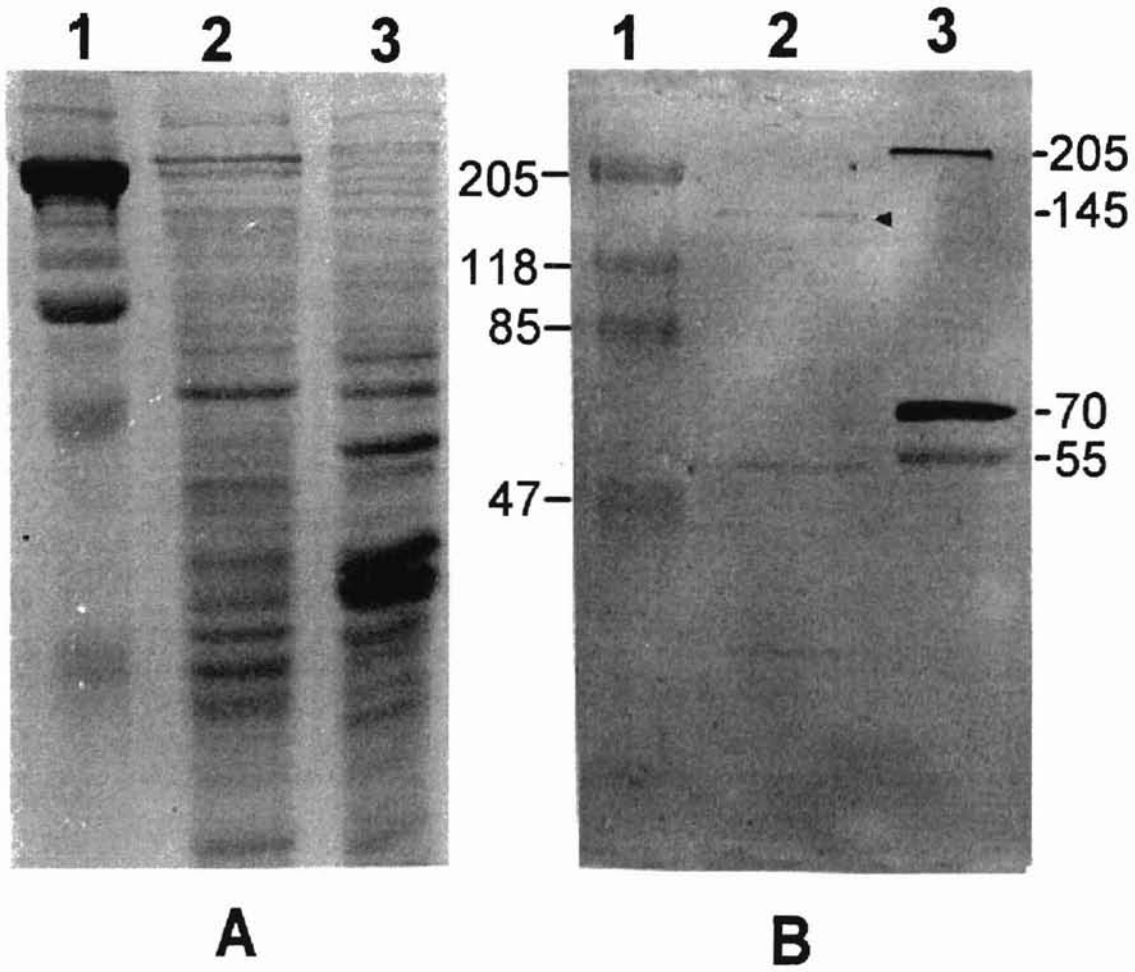

1
2
3
1
2
3
205
118
85
47
-205
-145
-70
-55
A
B

Figure 3.3 Effects of PGE_2 and GTP-γ-S on tick phospholipase C activity. The enzyme activity was expressed as relative percentage over the control (100%). log[AH6809]= - 10; log[PGE2]= - 7; log[GTP-γ-S]= - 5. Mean ±S.E.M. (n=3 except for AH-6809, n=6). * indicates significant difference from the control ($P< 0.05$).

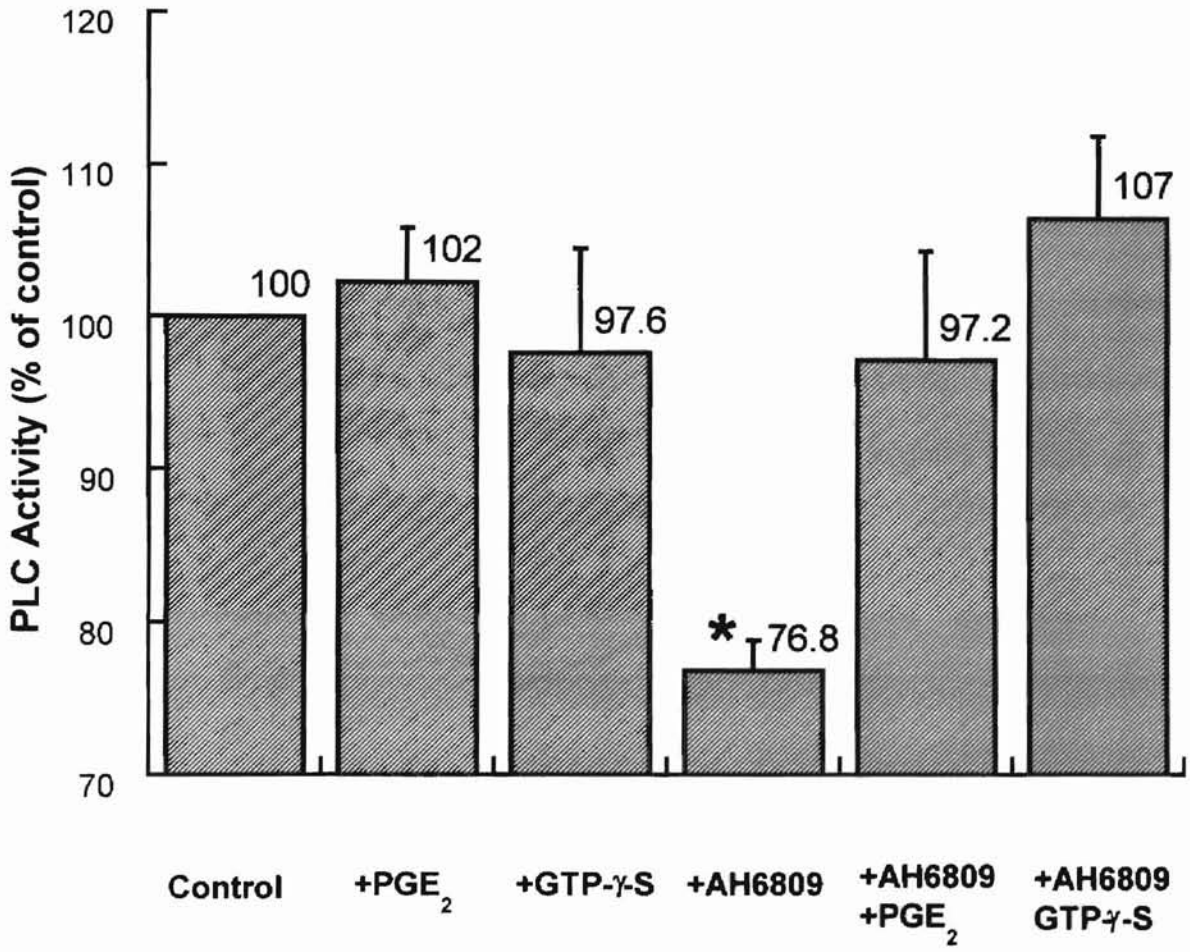
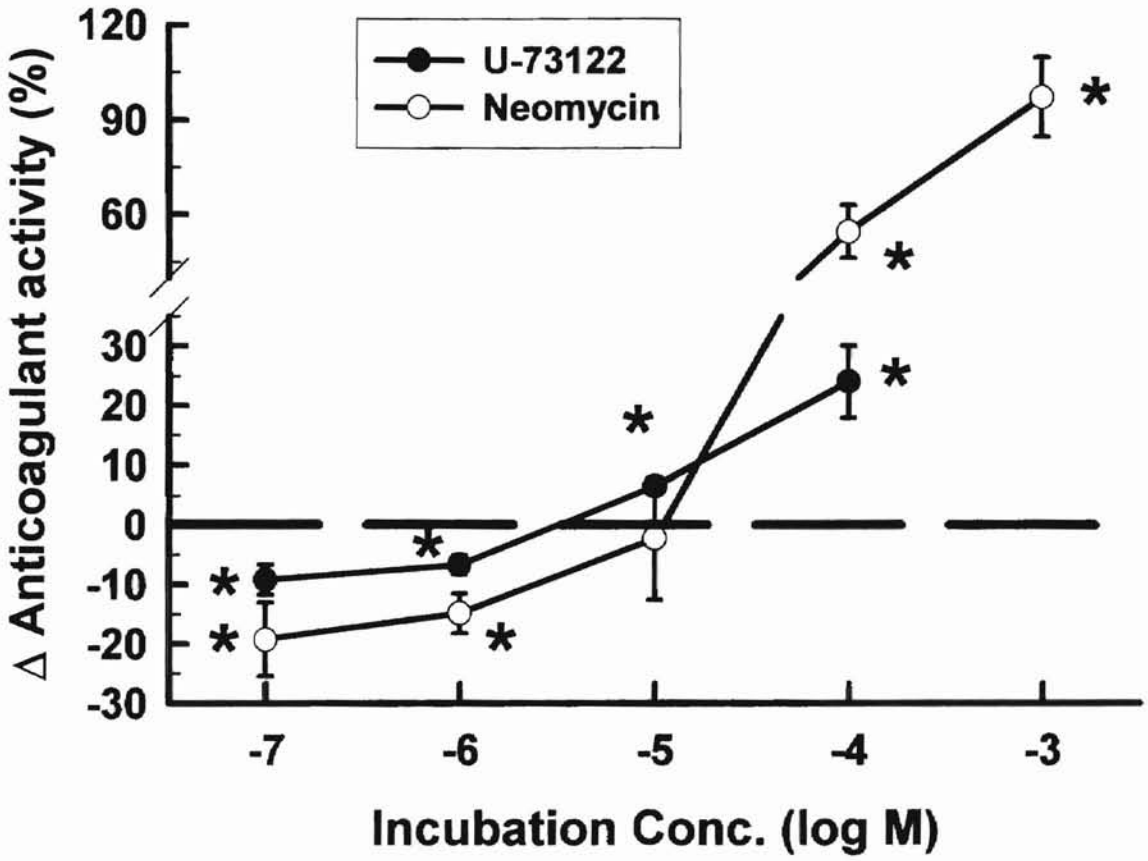

120
110
100
90
80
70
PLC Activity (% of control)
100
102
97.6
*
76.8
97.2
107
Control
+PGE2
+GTP-γ-S
+AH6809
+AH6809
+PGE2
+AH6809
GTP-γ-S

Figure 3.4 Effects of PLC inhibitors on anticoagulant release from dispersed tick salivary glands in the presence of 10^{-7} M PGE_2. Results are expressed as percent change (± S.E.M.) of anticoagulant activity released by dispersed salivary glands incubated with the indicated concentration of the drugs as compared to the solvent control (both plus 10^{-7} M PGE_2) for 5 min at room temperature. (A) U-73122, a specific inhibitor of phospholipase C (n=6) ; (B) neomycin, a non-specific PLC inhibitor (n=3).

* Significantly different from control ($P < 0.05$).


U-73122
Neomycin
Δ Anticoagulant activity (%)
120
90
60
30
20
10
0
-10
-20
-30
-7
-6
-5
-4
-3
Incubation Conc. (log M)

CHAPTER IV

SUMMARY

Tick salivary glands excrete excess water and ions (fluid secretion) back to the host to concentrate the nutrient components of blood meal and to regulate haemolymph volume and ionic balance, they also secrete many bioactive molecules to facilitate feeding (Sauer et al., 1995). Salivary fluid secretion in ticks is controlled by nerves with dopamine being the neurotransmitter leading to an increase in the intracellular level of cyclic AMP (cAMP) (Schmidt, et al., 1982).

Previously our lab identified a PGE_2 specific, G-protein linked receptor in the plasma membrane fraction of tick salivary glands of *A. americanum* (L.) that increases inositol trisphosphate, mobilizes intracellular calcium and is involved in PGE_2-stimulated secretion (exocytosis) of anticoagulant protein (Qian et al., 1997; 1998). Because regulated exocytosis is triggered by a rise in intracellular Ca^{2+} in most secretory cells (Knight et al., 1989), the probable relationship between PGE_2 and exocytosis is implied.

In this study, PGE_2 dose-dependently stimulates the release of anticoagulant molecules in isolated salivary glands at very low concentration. The results also suggest that stimulated exocytosis of bioactive proteins is not a general phenomenon of prostanoids but is PGE_2-specific.

The selective PGE_2 EP1 receptor agonist, 17-phenyl trinor PGE_2, was as effective as PGE_2 in stimulating secretion of anticoagulant protein; the selective PGE_2 EP1 receptor antagonist AH-6809 inhibited the secretion at the low concentration, strongly suggesting that the exocytosis observed in tick salivary gland is via the PGE_2 receptor EP1-like subtype. Activation of mammalian EP1 receptors leads to an increase in intracellular Ca^{2+}

level (Narumiya, 1996). In our results, calcium ionophore A-23187 enhanced anticoagulant release in a dose-dependent manner and was as effective as PGE_2, suggesting a role for increased intracellular calcium in controlling exocytosis of anticoagulant proteins.

In mammals, the PLC signaling pathway, especially the second messenger IP_3 is thought to be important in secretory cells (Putney, 1988). My finding that the IP_3 receptor inhibitor TMB-8 (10^{-6} M) effectively inhibited PGE_2-stimulated anticoagulant release further supports this hypothesis.

In this study phospholipase C (PLC) activity was identified in a crude plasma membrane fraction of salivary glands of the lone star tick, *Amblyomma americanum*. Western blot analysis using an antibody against mammalian PLC γ-1 showed three clear cross-reacting bands at a molecular weight of 220 kD, 70 kD and 55 kD in a plasma membrane-enriched fraction of the salivary glands. 10^{-7} M PGE_2 and 10^{-5} M GTP-γ-S can directly activate the PLC activity. Low concentrations of the PLC inhibitor neomycin and U-73122 inhibited PGE_2-stimulated secretion of anticoagulant protein.

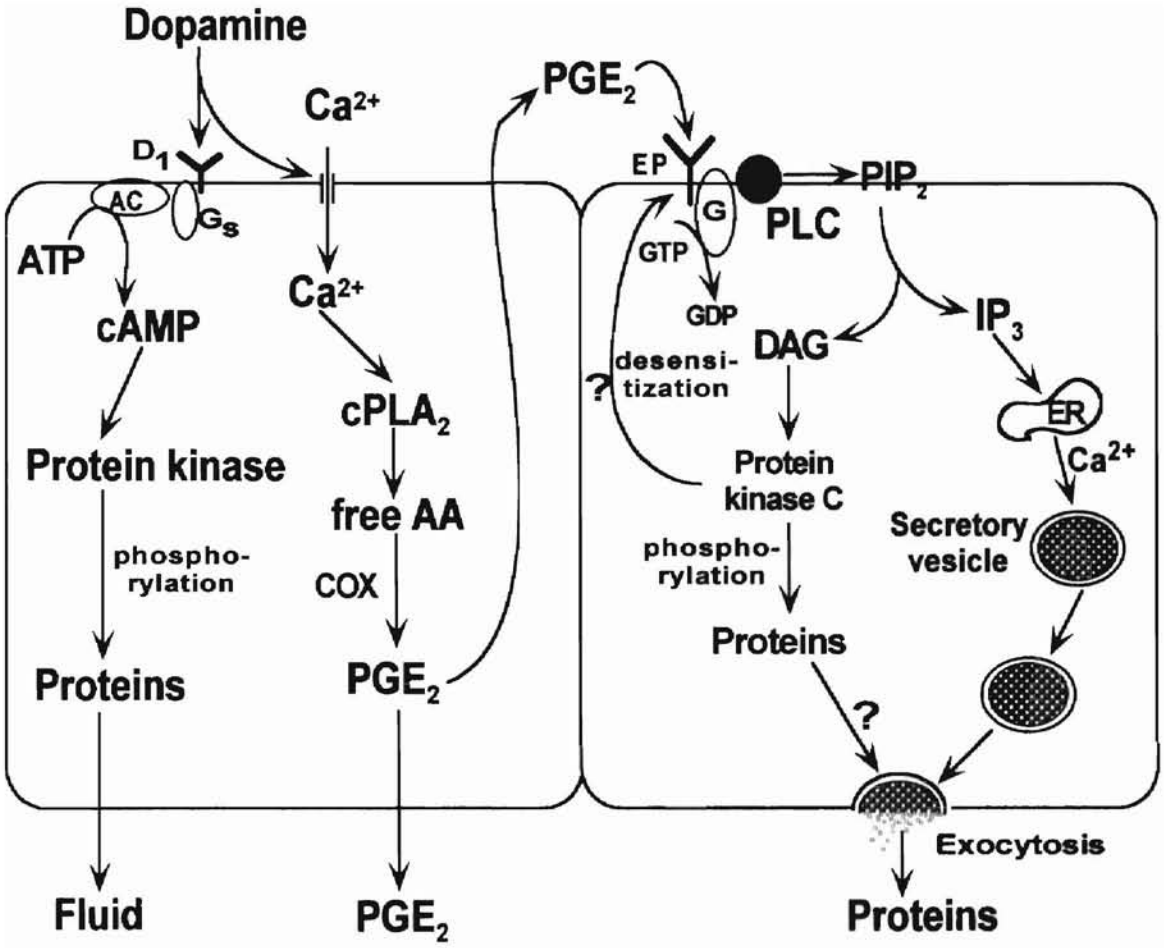
Based on the above data, I propose the following model to explain control of fluid and protein secretion in an ixodid tick salivary gland (Figure 4.1). Dopamine released from a salivary nerve stimulates fluid secretion through an increase in cAMP (Sauer *et al.*, 1995). Dopamine also stimulates an influx of extracellular Ca^{2+}, activating a cytosolic PLA_2 ($cPLA_2$) and the subsequent release of arachidonic acid (Bowman et al., 1995a). Arachidonic acid is converted to prostaglandins (including PGE_2) via a cyclooxygenase pathway (Pedibhota *et al.*, 1997). PGE_2 is both secreted into the saliva and functions as a local hormone by interacting with its PGE_2 receptor (Qian *et al.*, 1997) to increase IP_3. IP_3 mobilizes intracellular Ca^{2+} (Roddy *et al.*, 1990). I hypothesize that the internal Ca^{2+} which is mobilized by PGE_2 is important in regulating exocytosis of salivary gland proteins. In this model, inhibition of prostaglandin synthesis would be inhibitory to secretion of both prostaglandins and proteins and possibly pathogen transmission during

tick feeding. Tick saliva contains many bioactive protein molecules (Bowman et al., 1997) including immunosuppressive protein factors that enhance pathogen transmission (Wikel *et al.*, 1994; Zeidner *et al.*, 1996). Lyme disease spirochetes and some rickettsiae are believed to enter and exit tick salivary gland cells by transcytotic pathways (i.e. they enter by endocytosis, and exit by exocytosis) (Munderloh and Kurtti, 1995). It seems possible that pathogens could exploit exocytotic mechanisms of transport in the tick salivary gland for transmission to the host. Therefore, the mechanism and control of exocytosis in tick salivary glands may be critical both to maintaining a viable host-interface and to pathogen transmission by ticks.

PGE_2, $PGF_{2\alpha}$ and calcium ionophore A-23187 also stimulated anticoagulant release in the dispersed salivary glands of male *D. andersoni*. A common mechanism including a role for prostaglandins in controlling regulated secretion may exist in all species of ixodid ticks and in both sexes with some variations.

Now that the model to explain control of fluid and protein secretion in an ixodid tick salivary gland (Figure 4.1) was proposed, several key molecules in this flow control could be targeted in order to control tick infestations and tick-born diseases. The fact that the PGE_2 receptor in tick salivary gland are EP1-like subtype and tick salivary gland PLC can cross-react with bovine brain PLC γ-1 provides the feasibility to clone these two important components using the probes from mammals. Further comparison of structural and functional difference in PGE_2 receptor or PLC between tick and mammalian host will lead a clue for high efficient drug design.

Figure 4.1 Known and proposed cascade of reactions controlling tick salivary secretion and functions of the PGE_2 receptor. Dopamine released from nerves stimulates fluid secretions through an increase in cAMP (Sauer et al., 1995). Dopamine also stimulates an influx of Ca^{2+} activity on cytosolic PLA_2 (c PLA_2) and the subsequent release of AA (Bowman et al., 1995a). AA is converted via the cyclooxygenase (COX) pathway to prostaglandins including PGE_2 (Bowman et al.,1995b). PGE_2 is either secreted into the saliva or functions as a local hormone by interacting with the PGE_2 receptor (Qian et al., 1997) to increase IP_3 (Fig.1). IP_3 mobilizes intracellular Ca^{2+} (Roddy et al., 1990). Ca^{2+} mobilized by PGE_2 regulates secretions (exocytosis) of saliva anticoagulant (Zhu et al., 1997) and possibly other proteins (Fig. 2.3).


Dopamine
Ca2+
PGE2
D1
EP
PIP2
AC
Gs
G
PLC
ATP
GTP
Ca2+
cAMP
GDP
DAG
IP3
desensi-
tization
?
cPLA2
ER
Protein kinase
Protein
kinase C
Ca2+
free AA
Secretory
vesicle
phospho-
rylation
COX
phospho-
rylation
Proteins
Proteins
?
PGE2
Exocytosis
Fluid
PGE2
Proteins

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