A SPECIFIC PROSTAGLANDIN E_2 RECEPTOR AND ITS ROLE IN MODULATING SALIVARY SECRETION IN THE FEMALE TICK, *AMBLYOMMA AMERICANUM* (L.)

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LIST OF ABBREVIATIONS

ASA	acetylsalicylic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
CTM	clotrimazole
DEX	dexamethasone
DIC	diclofenac
EDTA	ethylenediamine tetraacetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)]tetraacetic acid
ESC	esculetin
ETYA	eicosatetraynoic acid
IBMX	3-isobutyl-1-methyl-xanthine
INDO	indomethacin
IP_3	inositol 1,4,5-trisphosphate
OPC	oleyloxyethyl phosphorylcholine
PGE_2	prostaglandin E_2
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
SDS	sodium dodecyl sulfate

Tris	tris-(hydroxymethyl)aminomethane
VP	verapamil

CHAPTER 1

INTRODUCTION

Ticks belong to the phylum Arthropoda, the class Arachnida, and the subclass Acari that includes mites and ticks (Sonenshine, 1991). There are 2 major families of ticks, i.e., the Ixodidae (hard ticks) and the Argasidae (soft ticks). The lone star tick *Amblyomma americanum* (L.) is a member of Ixodidae. Ticks are considered to be economically important because they are obligate blood sucking arthropods that infest every class of terrestrial vertebrates including mammals and birds, various reptiles and even amphibians (Sonenshine, 1991). They transmit many pathogenic organisms including fungi, viruses, rickettsiae, bacteria, and protozoa and are among the most important vectors of human and animal diseases (Sonenshine, 1991).

The salivary glands are essential to the success of ticks in obtaining a bloodmeal. As feeding progresses, the rate of salivary secretion increases greatly, enabling the ixodid tick to concentrate the bloodmeal by returning excess water and ions to the host. Saliva in feeding ticks is rich in bioactive components that may facilitate prolonged feeding. Bioactive compounds identified in saliva or salivary glands include cement to help anchor the mouthparts to the host, anticoagulants, antihistamine, kininase, apyrase and prostaglandins (Sauer *et al.*, 1995). Prostaglandins, mainly prostaglandin E_2 (PGE_2), have been identified in the saliva of four ixodid species (Sauer *et al.*, 1993).

The prostaglandins are oxygenated derivatives of C_{20} fatty acids, mainly arachidonic acid (Coleman *et al.*, 1990). Arachidonic acid is most often released from esterified stores in the plasma membrane by phospholipase A_2 (PLA_2) and can be converted into a series of compounds called eicosanoids through three major pathways, which include cyclooxygenase, lipoxygenase and cytochrome P-450 epoxygenase. The prostanoids are the products of the first of these to be discovered, the cyclooxygenase pathway, consisting of prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2) and thromboxane A_2 (TxA_2). The lipoxygenase pathway produces the leukotrienes and certain mono-, di- and tri-hydroxy acids, and the cytochrome P-450 'epoxygenase' pathway forms the epoxides (Smith, 1989). The scientific trail leading to the discovery of prostaglandins (PGs) can be traced back over 50 years to the observation by Kurzrok and Lieb (1930) that human semen can cause either uterine contraction or relaxation. The term "prostaglandin" was coined in 1935 by von Euler (1935). After World War II, Bergström demonstrated that semen extracts contained a number of biologically active entities that were unusual in being non-nitrogenous, hydroxylated fatty acids. Application of modern analytical, spectroscopic, and separation techniques in this field led to the elucidation of the prostaglandin structures and the biosynthetic pathways (Andersen *et al.*, 1985).

PROSTANOID BIOSYNTHESIS

Prostanoid biosynthesis has been studied extensively in normal and pathophysiological tissues and is well characterized (Fig. 1.1). It is now clear that prostanoids are not stored, but are synthesized *de novo* and released in response to a stimulus (Coleman *et al.*, 1990).

Arachidonic acid is the most abundant of the prostanoid precursors and its metabolism via the cyclooxygenase pathway leads to formation of the 2-series prostanoids. The principal source of arachidonic acid for prostanoid formation is that esterified at the 2-acyl position in fatty acyl chains of glycerophospholipids. Prostanoid formation requires free, unesterified arachidonic acid. Once liberated, any arachidonic acid not metabolized to prostanoids or other eicosanoids is rapidly reesterified into a lipid by acyltransferase enzymes (Irvine, 1982). Prostaglandin endoperoxide synthase catalyzes the sequential formation of intermediates PGG_2 and PGH_2 (Needleman *et al.*, 1986).The cyclooxygenase activity of this enzyme catalyzes the insertion of two molecules of oxygen into arachidonic acid to give the 15-hydroperoxy compound PGG_2, and the peroxidase activity of the enzyme then reduces the hydroperoxy group to hydroxy, giving PGH_2. The cyclooxygenase and peroxidase activities of endoperoxide synthase reside in a single protein. Formation of the primary prostanoids, PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA_2, is normally catalyzed by specific isomerases or reductases (Smith, 1992). According to Smith (1989), any given prostanoid-forming cell tends to form only one of these compounds as its major product. For example, smooth muscle cells and endothelial cells from large arteries form primarily PGI_2 (DeWitt *et al.*, 1983), platelets form mainly TxA_2 (Hamberg *et al.*, 1975), and PGE_2 is the major product of collecting tubule cells in the kidney (Kirschenbaum *et al.*, 1982). Prostanoids of the 2-series are formed by both vertebrates and invertebrates, but not by plants or bacteria which lack appropriate polyunsaturated C_{20} fatty acid precursors (Smith, 1989).

In ticks, there are considerable data suggesting possible biosynthesis of prostanoids. Prostanoids have been identified in the saliva of at least four ixodid species (Higgs *et al.*, 1976; Dickenson *et al.*, 1976; Shemesh *et al.*, 1979; Ribeiro *et al.*, 1985, 1988, 1992). Higgs *et al.* (1976) confirmed prostaglandin-like activity by bioassay (stimulation of rat and chick smooth muscle preparation) in acid-lipid extracts of saliva from *Boophilus microplus*. Over 80% of the activity in the extracts had the same chromatographic mobility as PGE_2 as assessed by thin layer chromatography. About the same time, researchers in Australia confirmed the existence of prostaglandin activity in the saliva of the same species of ixodid tick (Dickinson *et al.*, 1976). Saliva from partially fed *Ixodes dammini* and *A.americanum* was demonstrated to contain PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (the stable degradation product of prostacyclin) (Ribeiro *et al.*, 1985, 1988; 1992). PGF and PGE_2 were identified by radioimmunoassay (RIA) in the salivary glands as well as in reproductive organs and eggs of *Hyalomma anatolicum excavatum* (Shemesh *et al.*, 1979). Cultured salivary glands accumulated both PGF and PGE_2 with increasing time of incubation suggesting an ability of the salivary glands to synthesize prostaglandins, at least when cultured *in vitro*. Shipley *et al.*(1993) found that salivary glands of *A americanum* female ticks contained relatively high levels of arachidonic acid, the precusor of prostanoids. Bowman *et al.*(1993) also demonstrated that PGE_2, $PGF_{2\alpha}$ and PGD_2 existed with PGE_2 at the highest concentration in secreted saliva of *A. americanum* following labelling of salivary glands *in vivo* with radiolabelled arachidonic acid.

BIOLOGICAL ACTIONS OF PGE_2

PGE_2 is known to have a number of physiological and pathophysiological actions in diverse mammalian tissues. It can mediate contraction and relaxation of smooth muscle from various tissues (Coleman *et al.*, 1990). It also exhibits both inhibition and enhancement of neurotransmitter release. PGE_1 and PGE_2 have been shown to cause inhibition of sympathetic neurotransmitter release in heart, vasculature, spleen, vas deferens and seminal vesicle of various species (Coleman *et al.*, 1990). However, in the guinea pig ileum myenteric plexus preparation, E series prostanoids are found to enhance potently parasympathetic neurotransmission through an increase in the release of acetylcholine (Enrenpreis *et al.*, 1973). In addition, PGE_2 exerts a powerful modulating effect on human platelets, depending on the concentration of PGE_2. At low concentration, PGE_2 antagonizes the antiaggregatory action of PGE_1 and PGI_2 (Bonne *et al.*, 1981). Higher concentration of PGE_2 can inhibit platelet aggregation (Bruno *et al.*, 1974). PGE_2 induces ovulation in rats and rabbits, and ovulation can be prevented by administration of indomethacin, an effect reversed by PGE_2 administration (Lindner *et al.*, 1980). PGE_2 modulates inflammatory (e.g. local vasodilatation, edema and pain) (Milton, 1976), and immune responses (e.g. inhibits both the function and proliferation of T cells) (Lewis, 1983). The role of PGE_2 in regulation of fluid transport is found in a wide range of organisms, including mammals, frogs, fishes, at least two mollusks, and insects (Stanley-Samuelson and Pedibhotla, 1996). In the mammalian kidney, PGE_2 regulates water and NaCl reabsorption in the collecting tubule and the adjacent thick ascending limb (Smith, 1989). PGE_2 has been shown to stimulate the rate of bone resorption induced by the parathyroid hormone (MacDonald, 1986). In rat gastric mucosa, PGE_2 causes inhibition of

gastric acid secretion in the parietal cell but stimulates gastric nonparietal secretion (Reeves and Stables, 1985; Major and Scholes, 1978; 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 (Beitch *et al.*, 1974; Bentley and McGahen, 1982). Bjerregaard and Nielsen (1987) demonstrated that PGE_2 stimulated an active secretion of Cl^-, Na^+, and K^+ from the skin glands in isolated skin of frog . In fish, PGE_1 and E_2 reduce ion efflux in seawater-adapted mullet and in the killfish (Brown *et al.*, 1991). PGE_2 also regulates ion and water transport in invertebrates. Freas and Grollman (1980) reported that PG synthesis increased in gill tissues of the marine bivalve *Modiolus demissus* during hypoosmotic stress. PGs regulate sodium transport in another bivalve, the freshwater mussel *Ligumia subrostrata* (Sanitsing and Dietz, 1983). In insects, PGE_2 has been suggested to influence fluid secretion in Malpighian tubules (MTs) from females of the yellow fever mosquito, *Aedes aegypti* (Petzel and Stanley-Samuelson, 1992) and from workers of the forest ant, *Formica polyctena* (Van Kerkhove *et al.*, 1995). Both of these studies report that eicosanoid biosynthesis inhibitors reduce basal fluid secretion rates in isolated MTs.

PROSTANOID RECEPTORS

The presence of prostanoid receptors is at first inferred from the pharmacological properties of prostanoids. Prostanoids display high potency even at concentrations as low as 10^{-11} M and small chemical modifications can have profound effects on their potency and profile of biological activity, and different prostanoids can have different effects on the

same cell type (Coleman *et al.*, 1990). Distinct receptors have been identified for each of the five naturally occurring prostanoids, PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA_2 by using pharmacological comparisons of agonist and antagonist potency, and biochemical ligand-binding studies, and analyzing PG-induced signal transduction in cells (Coleman *et al.*, 1990). These studies suggest that the prostanoid receptor is a receptor coupling via a G protein to effectors such as adenylate cyclase and phospholipase C. In the case of PGE_2 receptors, four subtypes have currently been identified, namely EP1, EP2, EP3, and EP4 (Coleman *et al.*, 1990, 1994). EP1 subtype is coupled to Ca^{2+} mobilization, EP2 and EP4 are coupled to stimulation of adenylate cyclase and EP3 is coupled to inhibition of adenylate cyclase (Negishi *et al.*, 1995). These four PGE receptor subtypes have been cloned from various tissues (Sugimoto *et al.*, 1992; Honda *et al.*, 1993; Watabe *et al.*, 1993; Adam *et al.*, 1994; Breyer *et al.*, 1994; Namba *et al.*, 1994; Regan *et al.*, 1994; Sando *et al.*, 1994; Yang *et al.*, 1994; Nishigaki *et al.*, 1995). The molecular structures show that they contain seven hydrophobic segments corresponding to the putative transmembrane domains, suggesting that they belong to the family of G protein-coupled rhodopsin-type receptors (Negishi *et al.*, 1995; Narumiya, 1996).

SIGNAL TRANSDUCTION FOR PGE_2 RECEPTORS

Cells respond to external stimuli through transmembrane signaling that stimulates intracellular messenger pathways, such as the inositol 1,4,5-trisphosphate/Ca^{2+} signal, adenylate cyclase/cyclic adenosine 3',5'-monophosphate (cAMP), guanylate cyclase/cyclic guanosine 3',5'-monophosphate (cGMP), diacylglycerol/protein kinase C, voltage-

dependent and -independent Ca^{2+} channels, and growth factors /tyrosine kinase/tyrosine phosphatase (Tsunoda, 1993). These pathways are present in different cell types and impinge on each other for the modulation of the cell function. PGE_2 can cause versatile and opposite action in various tissues and cells due to its receptor subtypes being coupled to a variety of signal transduction pathways (Fig.1.2). PGE_2 is found to stimulate intracellular Ca^{2+} concentration increases in Chinese hamster ovary (CHO) cells expressing the mouse EP1 receptor (Watabe *et al.*, 1993), osteoblast cell line UMR-106 (Yamaguchi *et al.*, 1988), MDCK cells (Aboolian *et al*, 1989) and Swiss 3T3 fibroblast cells (Yamashita and Takai, 1987). The mechanism of the increase in $[Ca^{2+}]_i$ involves a Mn^{2+}-insensitive Ca^{2+} channel, stimulation of phosphoinositide metabolism, and intracellular Ca^{2+} mobilization (Katoh *et al.*, 1995; Negishi *et al.*, 1995). The EP1 receptor is associated with a pertussis toxin-insensitive G protein since guanine nucleotides promote the dissociation of PGE_2 from EP1 receptor (Negishi *et al.*, 1995). It is also found that protein kinase C acts as a negative regulator of EP1 receptors. Protein kinase C induces both short-term and long-term desensitization of EP1 receptors (Negishi *et al.*, 1995). PGE_2 is demonstrated to stimulate adenylate cyclase activity in CHO cells expressing the mouse EP2 receptor (Honda *et al.*, 1993). Guanine nucleotides can reduce the affinity of PGE_2 binding to the EP2 receptor, suggesting that this receptor is coupled to Gs (Negishi *et al.*, 1995). The EP4 receptor, like the EP2 receptor, is positively coupled to adenylate cyclase (Coleman *et al.*, 1994). PGE_2 can strongly inhibit adenylate cyclase in CHO cells expressing the EP3 receptor, and this inhibition can be completely blocked by pertussis toxin (PT) treatment, indicating that EP3 is coupled to a PT-sensitive Gi protein

(Sugimoto *et al.*, 1992). It has also been found that PGE_2 stimulates phosphoinositide metabolism and Ca^{2+} mobilization (Irie *et al.*, 1994), suggesting that EP3 is coupled to multiple signal transduction systems.

OBJECTIVES OF THIS STUDY

Tick salivary glands serve as major osmoregulatory organs and are thus critical to the biological success of ticks during tick feeding. Understanding how salivary secretion is controlled becomes most important in clarifying the mechanism of secretion. Current knowledge about factors controlling salivary secretion is very limited. The best documented agonist of secretion is dopamine, a neurotransmitter released at the neuroeffector junction, to initiate salivary secretion (Sauer *et al.*, 1995). Since dopamine can also stimulate the release of arachidonic acid from salivary gland phospholipids (Bowman *et al.*, 1995a) which may then be metabolized into prostaglandins, can prostaglandins (e.g. PGE_2) function in regulation of tick salivary secretion in the same manner as in other tissues? For example, in the mammalian kidney, arginine vasopressin (AVP) induces the biosynthesis of PGE_2 in the collecting tubule which regulates AVP-induced water and sodium reabsorption in both the collecting tubule and the thick limb (Smith, 1989). The objectives of the present study were:

1. To investigate whether PGE_2 plays a role in regulating tick salivary secretion;
2. To determine whether receptors for PGE_2 exist in tick salivary glands;
3. To study signal transduction pathways following interaction of PGE_2 with its receptor in tick salivary glands.

Figure 1.1 Biosynthetic pathway for prostanoid formation. AA: arachidonic acid. The details are indicated in the text.

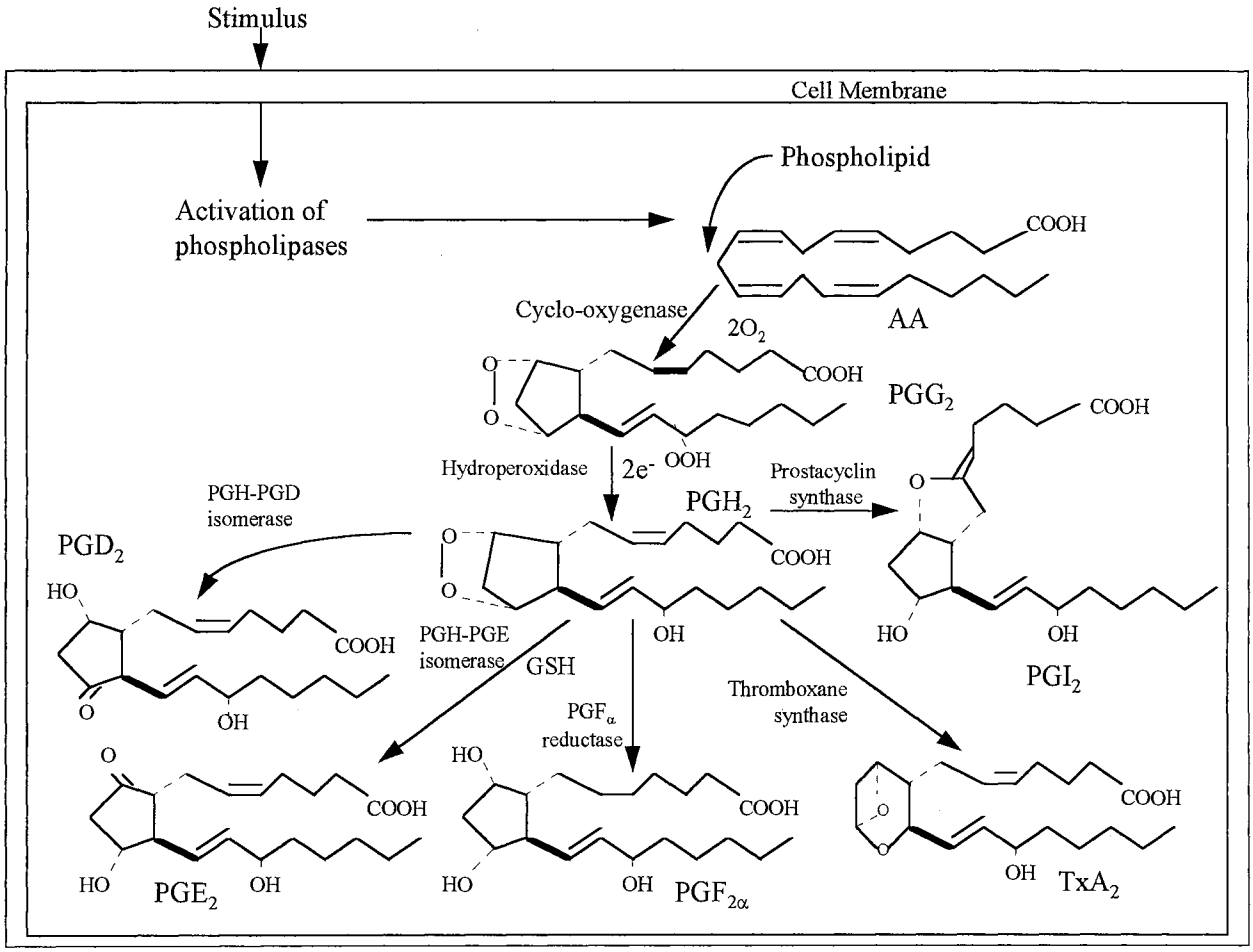
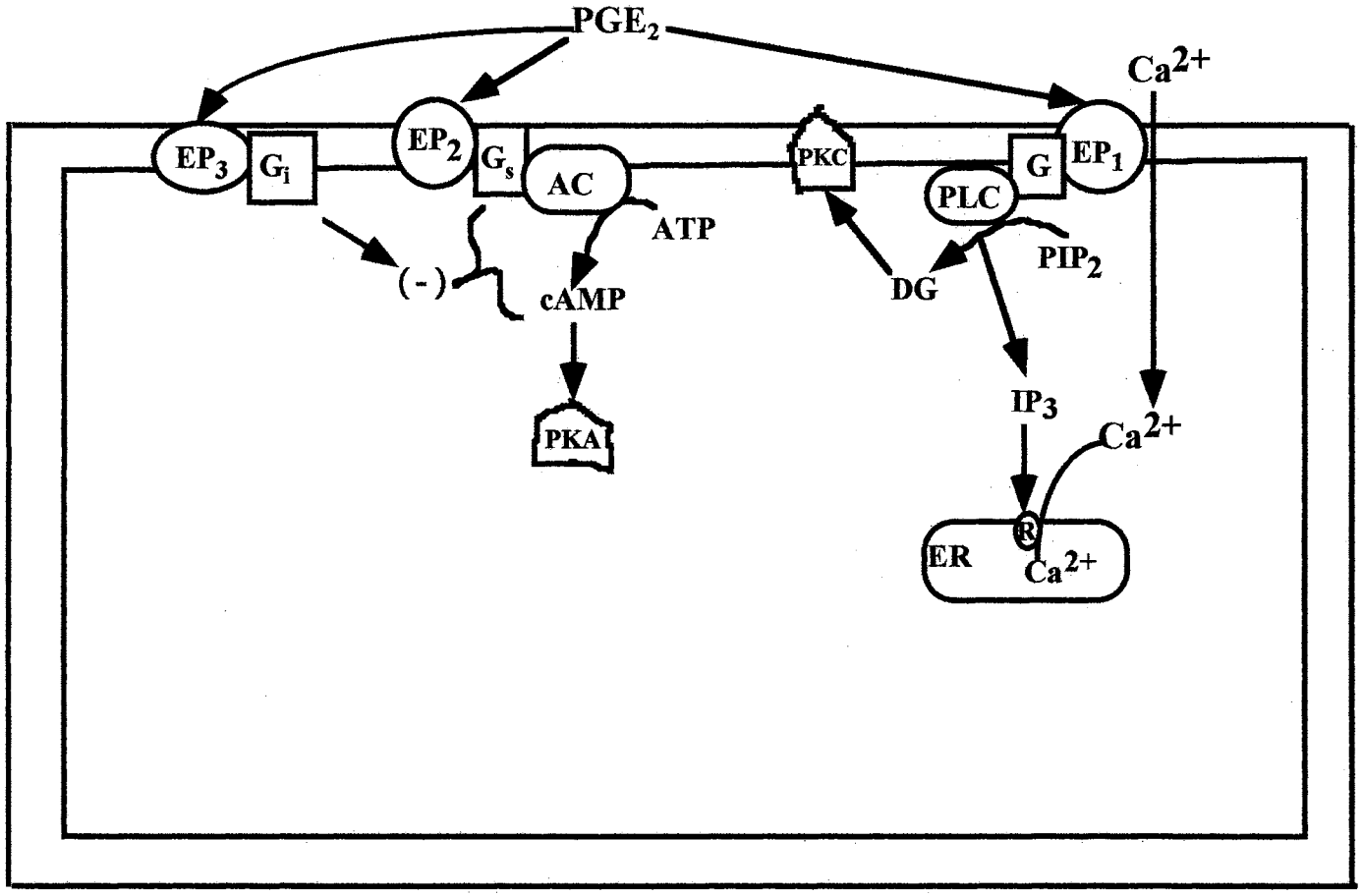

Stimulus
Cell Membrane
Activation of phospholipases
Phospholipid
AA
Cyclo-oxygenase
$2O_2$
PGG_2
Hydroperoxidase
$2e^-$
PGH_2
Prostacyclin synthase
PGI_2
PGH-PGD isomerase
PGD_2
PGH-PGE isomerase
GSH
PGF_α reductase
Thromboxane synthase
PGE_2
$PGF_{2\alpha}$
TxA_2

Fig. 1.2 Signalling transduction utilized by three PGE_2 receptor subtypes (EP1, EP2 and EP3) in mammals. G_S and G_i, stimulatory and inhibitory G proteins, respectively; AC, adenylate cyclase; PLC, phopholipase C; DG, diglyceride; IP_3, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC and PKA, protein kinase C and A, respectively.


PGE2
Ca2+
EP3
Gi
EP2
Gs
AC
ATP
PKC
G
EP1
PLC
PIP2
(-)
cAMP
DG
IP3
Ca2+
PKA
ER
Ca2+

CHAPTER 2

A ROLE OF PROSTAGLANDIN E_2 IN MODULATING THE DOPAMINE-INDUCED SALIVARY SECRETION IN THE FEMALE TICK, *AMBLYOMMA AMERICANUM* (L.)

INTRODUCTION

The salivary glands are the major osmoregulatory organs of ticks during tick feeding. It has been clearly shown that the ixodid tick salivary glands excrete excess fluid back to the host while the tick is attached and feeding (Gregson, 1967; Tatchell, 1967; Kaufman and Phillips, 1973a; Sauer, 1977). Ingested blood is concentrated within the tick gut as excess ions and water from the blood meal are transported across the gut epithelium into the haemolymph and are secreted back into the host via the salivary glands (Tatchell, 1967, 1969; Kirkland, 1971; Kaufman and Phillips, 1973a, b, c; Meredith and Kaufman, 1973; Sauer, 1977). Salivary gland fluid secretion is controlled by nerves (Kaufman, 1979). Salivary glands of several species were observed to be innervated from microscopic studies (Fawcett *et al.*, 1986) and catecholamines were found in nervous tissue and salivary glands using Falck-Hillarp fluorescence (Megaw and Robertson, 1974; Binnington and Stone, 1977). Dopamine is the predominant catecholamine in the salivary glands *of A. hebraeum* (Binnington and Stone, 1977; Kaufman and Harris, 1983). The neurotransmitter dopamine induces fluid secretion when applied to isolated salivary glands (Kaufman 1976; Sauer *et al.*, 1979) and when injected into the tick's haemocoel (Hsu and Sauer, 1975).

The molecular base for the action of dopamine is through an increase in intracellular levels of cAMP (Schmidt *et al.*, 1982).

It is interesting to note that dopamine also stimulates the release of arachidonic acid from phospholipids in the tick salivary glands by presumably activating an intracellular PLA_2 (Bowman *et al.*, 1995a). Free arachidonic acid may be converted into PGs via the cyclooxygenase pathway in tick salivary glands (Higgs *et al.*, 1976; Dickenson *et al.*, 1976; Shemesh *et al.*, 1979; Ribeiro *et al.*, 1985, 1988, 1992; Bowman *et al.*,1993; Shipley *et al.*,1993). The occurrence of PGs in tick salivary glands may indicate an autocrine role for these compounds in ticks. PGE_2 has been shown to modulate fluid transport in a wide range of tissues both in vertebrates and invertebrates. In invertebrates, PGE_2 is believed to regulate overall fluid secretion rates in insect Malpighian tubules (MTs) (Petzel and Stanley-Samuelson, 1992; Van Kerkhove *et al.* 1995). PGE_1 was shown to inhibit 5-hydroxytryptamine stimulated fluid secretion in *Calliphora erythrocephala* salivary glands via an inhibitory effect on adenylate cyclase (Dalton, 1977). It appears that regulation of ion and water transport is a fundamental eicosanoid action in animals (Stanley-Samuelson, 1994). The present work was undertaken to investigate whether PGE_2 participates in regulation of overall fluid secretion in tick salivary glands.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: M-199 medium (Sigma catalogue #, M 0393), oleyloxyethyl phosphorylcholine, esculetin, clotrimazole, indomethacin, acetylsalicylic acid, diclofenac, PGE_2, 17-phenyl trinor PGE_2, and other chemicals were acquired from Sigma (St.Louis, MO). The cyclic AMP assay kit was obtained from Amersham International plc (Amersham, UK). $PGF_{2\alpha}$, PGD_2 and U-46619 were from Cayman Chemical Co.(Ann Arbor, MI).

Ticks

Amblyomma americanum ticks were reared at Oklahoma State University's Central Tick Rearing Facility according to the methods of Patrick and 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 a 14:10 (L:D) photoperiod before infestation of the hosts. Partially fed female ticks weighing from 100 mg to 150 mg were removed by traction and immediately used for all the assays.

Assay of Salivary Secretion

Salivary glands were dissected and secretion was measured as described by Harris *et.al.* (1984) and McSwain *et al.* (1992). Briefly, the dorsal cuticle of a tick was carefully removed by making a circular lateral incision around the tick with a single-edge razor blade. M-199 buffer (pH 7.0) was then added to the tick haemocoel. The gut, reproductive, nervous and tracheal systems, along with any adhering tissues, were carefully removed to fully expose the salivary glands and ducts. The main duct was tied off

with a silk thread (size 4-0) (Ethicon, Inc.) and then carefully cut in front of the knot. For each pair of isolated glands, one was used for the treatment and the other as the control. In assays for examining the effects of various eicosanoid inhibitors on secretion, the isolated salivary glands were weighed and then incubated with the various eicosanoid synthesis inhibitors (treatment gland) or solvent carrier (control gland) for 15 min, followed by incubation for 5 min in 10 μM dopamine dissolved in the previous solutions. After incubation, the glands were rinsed in buffer for 3 min, weighed, and returned to the incubation solutions for another 5 min. The process was repeated. After each designated time interval, the glands were reweighed. The weight changes before and after treatment represent the secretory abilities of the isolated salivary glands. In examining the ability of PGE_2 to reverse inhibition of secretion by inhibitors of PLA_2 or cyclooxgenase, isolated salivary glands were pretreated with the inhibitor for 15 min by the same procedure as in the previous assays, followed by incubation in the same buffer containing 10 μM dopamine, 100 μM prostaglandin, and cAMP phosphodiesterase inhibitor, 10 mM theophylline or 1mM 3-isobutyl-1-methyl-xanthine (IBMX). A control consisted of the same reaction mixture without prostaglandin.

cAMP Assay

Salivary glands were prepared as in the secretion assays. Glands were incubated in M-199 buffer, pH 7.5, containing 1mM IBMX plus the desired effectors (treatment) or solvent carriers (control) for 32 min. The reaction was terminated by placing glands in 100 μl of 50 mM Tris(hydroxymethyl)aminomethane (Tris)/4 mM EDTA buffer, pH 7.5, on ice.

Levels of cAMP were measured with a cAMP kit by Amersham using the protein binding method according to the manufacturer's instructions. This method relies on the competition between [^{3}H]-labelled cAMP and unlabelled cAMP in the sample for a cAMP-binding protein. Free [^{3}H]cAMP was adsorbed by charcoal and removed by centrifugation, and bound [^{3}H]cAMP in the supernatant was determined by liquid scintillation counting. In this assay, the glands were homogenized by sonication for 30 sec, following by heating for 3 min in a boiling water bath to coagulate protein (piercing caps of the microfuge tubes with a syringe needle to prevent the caps from popping off). After centrifugation at 12000*g* for 5 min, the supernatant containing cAMP was used in the cAMP competitive binding assay. 50 μl of the supernatant, 50 μl of [^{3}H]cAMP and 100 μl binding protein solution were mixed together and placed on ice for 2 hour. After incubation, 100 μl charcoal suspension was added to the reaction mixture, followed by centrifugation for 2 min at 12000*g* at 4°C. Free [^{3}H]cAMP was thus removed by charcoal and [^{3}H]cAMP-binding protein complex was in supernatant. The cAMP content of the unknown sample was determined by interpolation from a cAMP standard curve.

Statistical Analysis

The differences between the control and experimental treatment were tested for significance by paired Student's t test. A P value <0.05 was considered significant.

RESULTS

The effects of eicosanoid synthesis inhibitors on dopamine induced secretion by salivary glands *in vitro*.

Various inhibitors of eicosanoid synthesis, including PLA_2 inhibitors oleyloxyethyl phosphorylcholine (OPC) and dexamethasone (DEX), the arachidonic acid analogue eicosatetraynoic acid (ETYA), the inhibitor of PLA_2, cyclo-oxygenase and lipoxygenase, lipoxygenase inhibitor esculetin (ESC) and cytochrome P-450 inhibitor clotrimazole(CTM) as well as cyclo-oxygenase inhibitors indomethacin (INDO), acetylsalicylic acid (ASA), and diclofenac (DIC), were tested and examined for their effects on secretion induced by dopamine in isolated salivary glands. Dopamine is known to stimulate release of arachidonic acid in isolated salivary glands (Bowman *et al.*, 1995) and arachidonic acid may be metabolized under these conditions to prostaglandins. The results showed that treatment of isolated salivary glands with PLA_2 inhibitor OPC or cyclo-oxygenase inhibitor ASA at 10 μM inhibited dopamine-stimulated secretion (Fig. 2.1). An inhibitor of lipoxygenase, ESC, at 100 μM and an inhibitor of cytochrome P-450, CTM, at 10 μM had no significant effect on dopamine stimulated secretion (Fig. 2.1). The inhibition of *in vitro* salivary secretion by OPC, ASA or DIC was dose dependent (Fig. 2.2, 2.3). Other eicosanoid biosynthesis inhibitors ETYA, DEX and INDO also blocked dopamine-stimulated secretion at certain concentrations (Table 2.1). The results suggest that cyclo-oxygenase products may be synthesized and play an important role in enhancing dopamine-stimulated salivary secretion.

Reversal by PGE_2 of the inhibition of dopamine-stimulated salivary secretion by inhibitors of PLA_2 and cyclo-oxygenase

Isolated salivary glands in the presence of 1 mM OPC treated with 100 μM PGE_2 or its analog 17-phenyl trinor PGE_2 increased secretion above the level observed in glands in OPC alone (Fig. 2.4). However, neither 100 μM PGE_2 nor its analog 17-phenyl trinor PGE_2 stimulated secretion above that noted when secretion was inhibited by cyclo-oxygenase inhibitors indomethacin, ASA, and diclofenac. PGE_2 was unable to stimulate secretion at low concentrations. $PGF_{2\alpha}$ (100 μM) was ineffective in stimulating secretion inhibited by OPC (Fig.2.4). Arachidonic acid was unable to recover the secretion inhibited by 1 mM OPC (data not shown). In the absence of dopamine, PGE_2 had no effect on salivary secretion (data not shown).

The effects of inhibitors of PLA_2 and cyclo-oxygenase, and PGE_2 on intracellular cAMP accumulation

Since dopamine stimulates salivary secretion through an increase in cAMP (Schmidt *et al.*, 1982), the effect of PGE_2 on regulation of the fluid secretion stimulated by dopamine was examined by investigating the role of PGE_2 in cAMP metabolism. The PLA_2 inhibitor OPC at 1 mM inhibited dopamine-stimulated cAMP accumulation about 25%. In addition, the cyclo-oxygenase inhibitor indomethacin at 1 mM inhibited cAMP accumulation about 27%. Verapamil, which inhibits dopamine-stimulated secretion by blocking Ca^{2+} influx (Needham *et al.*, 1979) and also blocks arachidonic acid release (Bowman *et al.*, 1995a), also reduced the cAMP accumulation about 65% at 1 mM (Table 2.2). In the presence of

OPC, PGE_2 and its analog 17-phenyl trinor PGE_2 stimulated cAMP accumulation about 20% and 40% above the OPC inhibited control, respectively (Fig. 2.4). However, neither PGE_2 nor its analog 17-phenyl trinor PGE_2 stimulated cAMP accumulation above that inhibited by cyclo-oxygenase inhibitor indomethacin, or verapamil. $PGF_{2\alpha}$ did not stimulate an accumulation of cAMP above that observed in the presence of 1 mM OPC (data not shown). As for secretion, PGE_2 on its own had no effect on cAMP accumulation.

DISCUSSION

Eicosanoids are oxygenated derivatives of C_{20} fatty acids, mainly arachidonic acid. Arachidonic acid is released from phopholipids after stimulation of tick salivary glands by dopamine (Bowman *et al.*, 1995a). Treatment of isolated salivary glands with inhibitors of eicosanoid biosynthesis OPC, ETYA, and DEX, significantly reduced dopamine-induced fluid secretion, suggesting a role for eicosanoids in regulating secretion. In order to probe specific products that participate in regulation of the fluid secretion, pharmaceutical inhibitors of the specific pathways were used in the secretion assays. Inhibition of cyclooxygenase by ASA, INDO, and DIC resulted in reduced fluid secretion. However, an inhibitor of lipoxygenase (ESC) or cytochrome P-450 (CTM) appeared to have no effect on regulation of salivary secretion. Furthermore, PGE_2 or its analog 17-phenyl trinor PGE_2 was able to partially reverse the secretion inhibited by OPC. Our results suggest a role for PGE_2 in regulating tick salivary secretion. Additional evidence in support of this hypothesis comes from the experiments showing the effects of eicosanoid biosynthesis

inhibitors and PGE_2 on cAMP formation in tick salivary glands. Both the PLA_2 inhibitor OPC or verapamil and the cyclooxygenase inhibitor INDO reduced dopamine-stimulated cAMP accumulation. Either PGE_2 or its analog 17-phenyl trinor PGE_2 increased cAMP accumulation above the OPC inhibited control, respectively. In addition, the increase of cAMP accumulation by PGE_2 or 17-phenyl trinor PGE_2 is about the same as the increase of salivary secretion (Fig. 2.4). cAMP is known to be a "second messenger" molecule in the regulation of salivary fluid secretion in ixodid females (Sauer *et al.*, 1976; 1979). Levels of cAMP increase when isolated salivary glands are stimulated by dopamine (Sauer *et al.*, 1979). Exogenous cAMP stimulates glands, in vitro, to secrete fluid (Sauer *et al.*, 1984) and a phophodiesterase inhibitor, theophylline, augments secretion stimulated by low levels of dopamine (Sauer *et al.*, 1979). The stimulatory effect of cAMP is also enhanced by theophylline (Sauer *et al.*, 1979). The specificity of cAMP action resides with various endogenous substrate proteins which are phosphorylated by cAMP-dependent protein kinase which facilitate the fluid secretory process (Greengard, 1978). The present findings suggest that PGE_2 may be involved in regulating salivary secretion by affecting salivary gland levels of cAMP.

However, the results have certain ambiguities. Although a variety of cyclo-oxygenase inhibitors decrease dopamine-stimulated secretion and at least one (indomethacin) inhibits dopamine-stimulated accumulation of cAMP, their effects cannot be reversed by PGE_2. Why this is so for inhibitors of cyclo-oxygenase but not an inhibitor of PLA_2 (OPC) is unclear. In addition, neither PGE_2 nor its analog 17-phenyl trinor PGE_2 stimulates salivary

secretion and intracellular cAMP formation on its own. Overall, the action of PGE_2 in salivary gland physiology seems to be related to events after salivary glands are first stimulated by dopamine and the magnitude of effects is modest.

Table 2.1 Inhibition of salivary secretion by eicosanoid biosynthesis inhibitors. Values represent percentage inhibition of secretion as compared to the control after the isolated salivary glands were incubated in the following pharmaceutical treatments for 16 min. Each value is the mean ± SE. * Significantly different from the control ($P<0.05$). PLA_2: phospholipase A_2; COX: cyclooxygenase; LO: lipoxygenase.

Treatment	Target	Concentration (M)	%Inhibition in secretion	Number
ETYA	PLA_2, COX LO	10^{-4}	39.9 ± 13.9*	4
DEX	PLA_2, COX LO	10^{-5}	17.8 ± 8.2*	16
INDO	COX	10^{-3}	93.2 ± 5.7*	7

Table 2.2 Inhibition of intracellular cAMP formation by inhibitors (1mM) of PLA_2 or cyclooxygenase. Values represent percentage inhibition of cAMP formation as compared to controls after the isolated salivary glands were treated with reagents for 32 min. Each value is the mean ± SE. * Significantly different from the control ($P<0.05$). PLA_2: phospholipase A_2; COX: cyclooxygenase.

Treatment	Target	% Inhibition in cAMP	Number
OPC	PLA_2	25.2 ± 9.5*	12
VP	PLA_2	67.3 ± 5.0*	6
INDO	COX	26.6 ± 6.9*	10

Figure 2.1 Comparison of the effects of various eicosanoid synthesis inhibitors on secretion by isolated salivary glands after 32 min. The control glands were treated with dopamine (10 μM) alone. The experimental glands were treated with dopamine (10 μM) plus the inhibitors as indicated. PLA_2 inhibitor OPC and cyclo-oxygenase inhibitor ASA significantly inhibited secretion about 40% and 33% respectively (*: $P<0.05$). However, no significant difference was observed using cytochrome P-450 inhibitor CTM, or lipoxygenase inhibitor ESC.

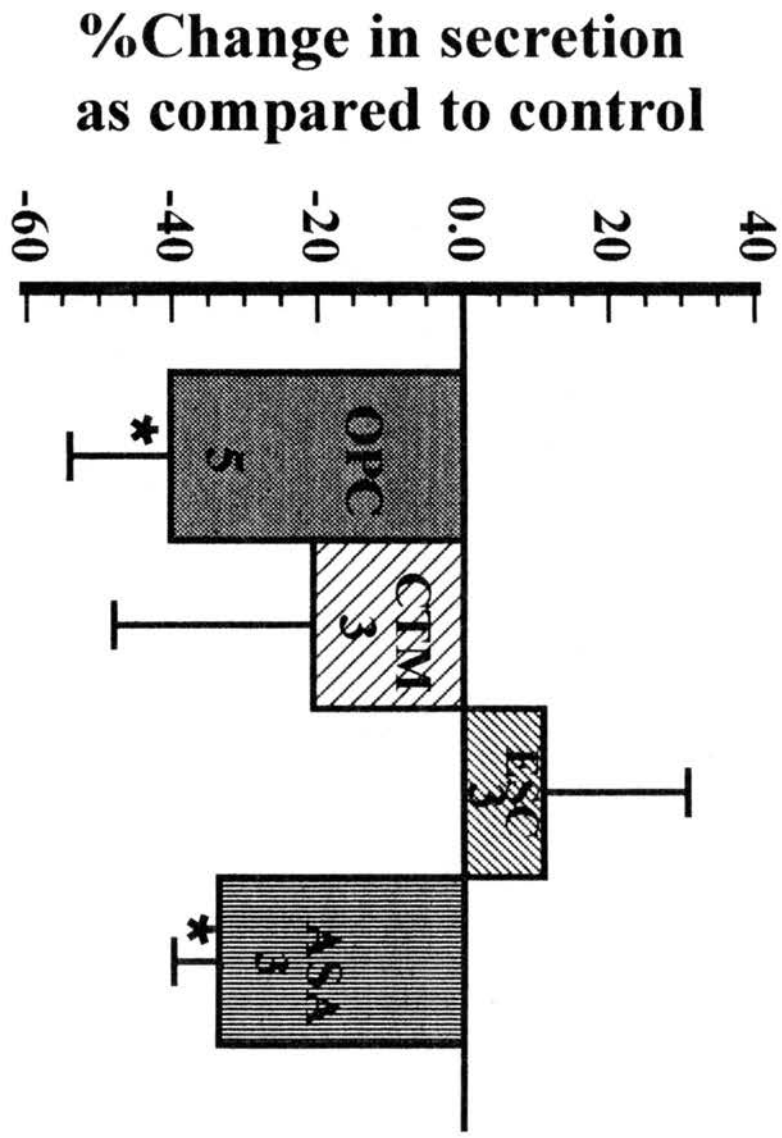
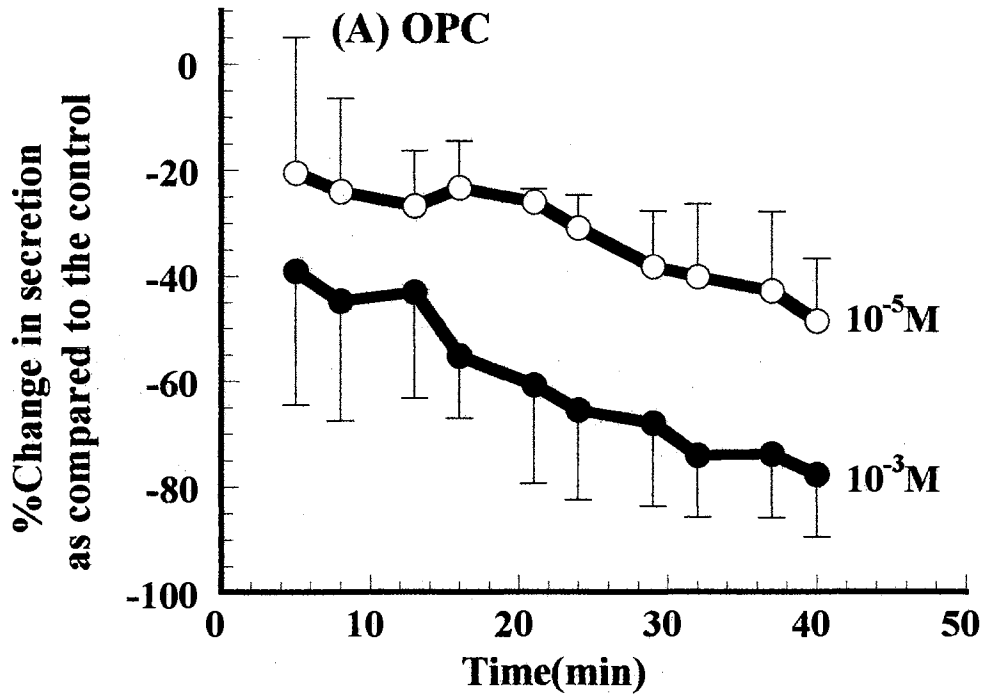

%Change in secretion
as compared to control
-60
-40
-20
0.0
20
40
OPC
5
*
CTM
3
ESC
3
ASA
3
*

Figure 2.2 Inhibition of *in vitro* salivary secretion by different concentrations of (A) PLA_2 inhibitor oleyloxyethyl phosphorylcholine (OPC) and (B) cyclo-oxygenase inhibitor aspirin (ASA) ($n \geq 3$). The experimental glands were treated with dopamine (10 μM) plus the inhibitors as indicated. The control glands were treated with dopamine (10 μM) plus the solvent carriers of the inhibitors.


(A) OPC
%Change in secretion as compared to the control
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
Time(min)
10^{-5}M
10^{-3}M

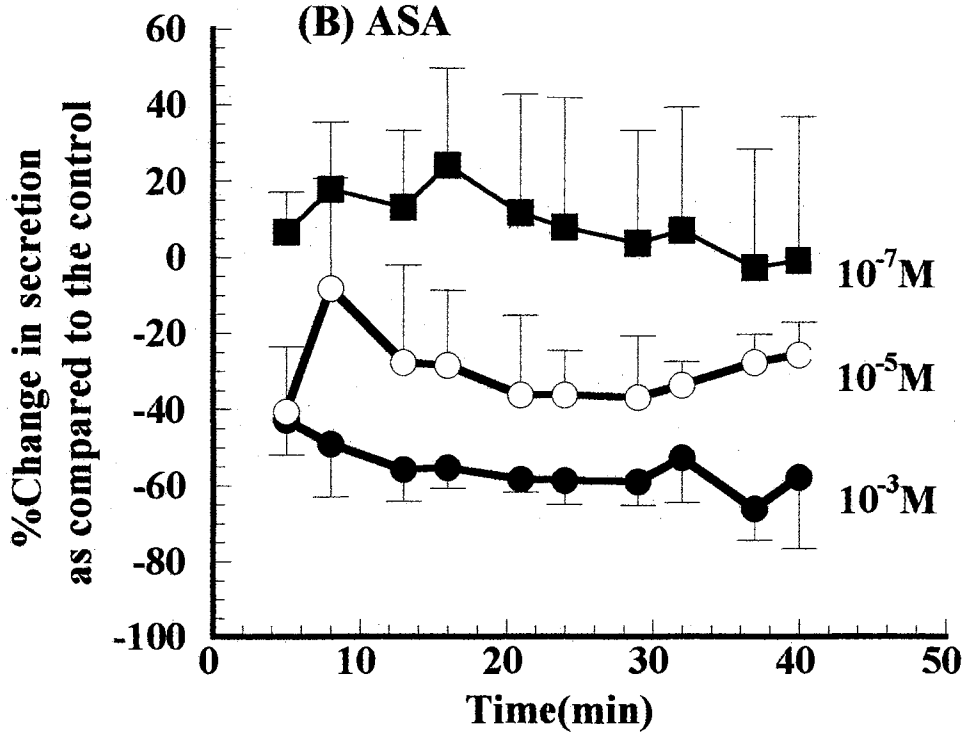

(B) ASA
%Change in secretion as compared to the control
60
40
20
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
Time(min)
10^{-7}M
10^{-5}M
10^{-3}M

Figure 2.3 Inhibition of *in vitro* salivary secretion by different concentrations of cyclo-oxygenase inhibitor diclofenac. The experimental glands were treated with dopamine (10 μM) plus the inhibitor at the indicated concentrations for 15 min. The control glands were treated with dopamine (10 μM) plus the solvent carrier (3% DMSO) of the inhibitor for the same incubation time as the experimental glands. * Significantly different from control ($P<0.05$).

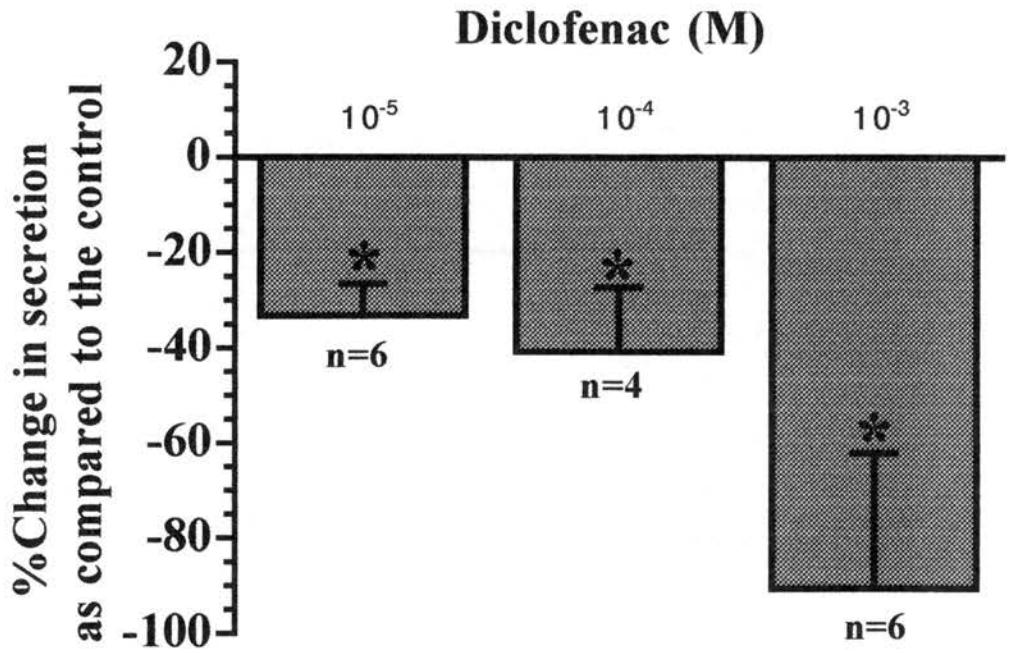
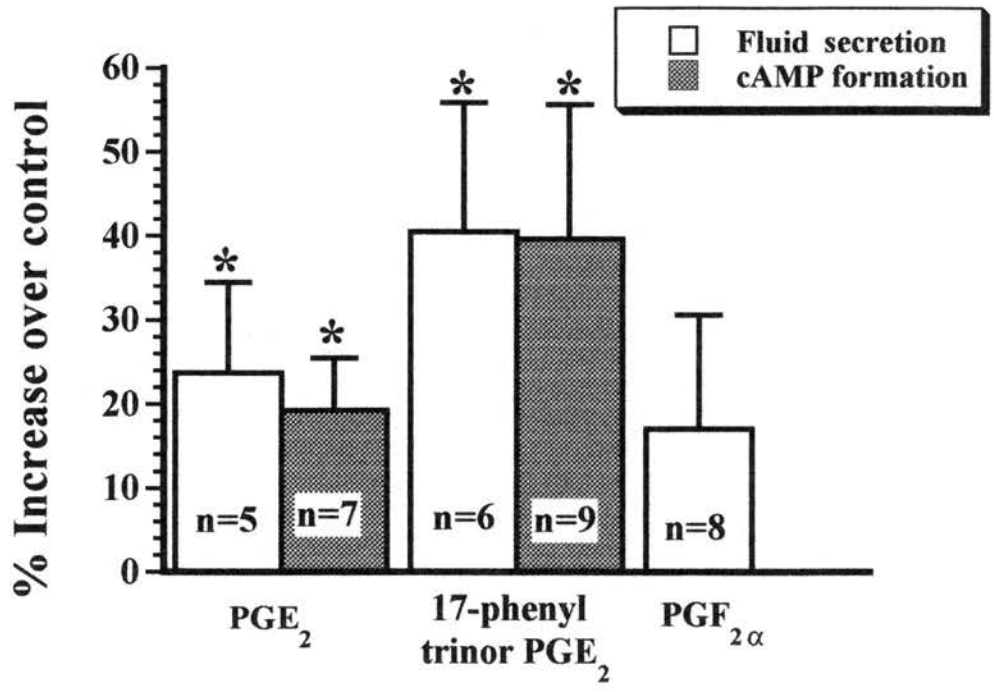

Diclofenac (M)
%Change in secretion as compared to the control
20
0
-20
-40
-60
-80
-100
10-5
10-4
10-3
*
*
*
n=6
n=4
n=6

Figure 2.4 The effects of 100 μM prostaglandins on secretion and intracellular cAMP formation in isolated salivary glands. In secretion assays, the control glands were treated with OPC (1 mM) plus dopamine (10 μM) and the prostaglandin solvent carrier (ETOH) for 16 min. In cAMP assays, the incubation mixtures contained the phosphodiesterase inhibitor theophylline (10 mM) or IBMX (1 mM) in addition to the same components as in the secretion assays (*: $P<0.05$).


Fluid secretion
cAMP formation
% Increase over control
60
50
40
30
20
10
0
*
*
*
*
n=5
n=7
n=6
n=9
n=8
PGE2
17-phenyl trinor PGE2
PGF2α

CHAPTER 3

CHARACTERIZATION OF A SPECIFIC PROSTAGLANDIN E_2 RECEPTOR IN THE SALIVARY GLANDS OF THE FEMALE TICK, *AMBLYOMMA AMERICANUM* (L.)

INTRODUCTION

Prostanoids are thought of as local hormones functioning to co-ordinate effects of those other hormones which induce eicosanoid synthesis in vertebrates (Smith, 1989). Following their intracellular synthesis, prostanoids exit the cell, probably via facilitated diffusion (Smith, 1986; Lam *et al.*, 1990). After exiting the cell, prostanoids act on the parent cell and /or neighboring cells in an autocrine and /or paracrine fashion through specific G protein-linked prostanoid receptors to stimulate or inhibit changes in the levels of second messengers (Smith, 1992). PGE-specific binding sites have been identified in the plasma membranes of various tissues that have a potential receptor function, including adipocytes, corpora lutea, myometrium, kidney, intestinal epithelium, liver, heart, brain, gastric mucosa, erythrocytes, circulating immune cells and platelets (Robertson, 1986; Beinborn *et al.*, 1988; Coleman *et al.*, 1990; Virgolini *et al.*, 1992; Hamon *et al.*, 1993). The function of PGs in regulation of fluid transport has been mostly studied in vertebrates, for example, in the mammalian kidney (Smith 1989; Frazier *et al.* 1992). Low concentrations of PGE_2 ($\leq 10^{-8}$M) are found to inhibit arginine vasopressin (AVP)-induced

water and sodium reabsorption by interfering with AVP-induced cyclic AMP synthesis on the collecting tubule and thick ascending limb. However, higher concentrations of PGE_2 ($\geq 10^{-7}$ M) cause stimulation of water reabsorption by activating adenylate cyclase in both collecting tubule and thick limb cells (Smith, 1989). The opposite actions of PGE_2 are considered to result from the existence of both stimulatory and inhibitory PGE_2 receptor subtypes in the kidney (Watanabe *et al.*, 1986). Recently, Sugimoto *et al* (1994) demonstrated that a high level of EP1 receptor mRNA was expressed in the collecting ducts of the kidney where PGE_2 attenuated the vasopressin-induced osmotic water permeability through Ca^{2+} mobilization. The EP1 receptor is therefore thought to be an important modulator of renal function through opening Ca^{2+} channels in plasma membranes.

Although there are many reports of physiological effects of prostaglandins in invertebrates, there is little evidence of prostaglandin receptors. An exception is a receptor of a prostaglandin A_2 in the gill tissue of a marine bivalve *Modiolus demissus* (Freas and Grollman, 1981).Since PGE_2 has been found to play a role in modulating tick salivary secretion, it is possible that the binding of PGE_2 to a specific receptor may be the initial step leading to its physiological action on the tissue. Therefore, the present work was undertaken to examine if prostaglandin receptors exist in tick salivary gland and characterize the receptors.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: PGE_2, GTP, GDP, ATP, App(NH)p, dithiothreitol, cholera toxin, pertussis toxin, NAD, GTPγS, polyethylenimine and other chemicals were acquired from Sigma (St.Louis, MO). [5,6,8,11,12,14,15-^{3}H(N)]- PGE_2 (171 Ci/mmol) was from Du Pont New England Nuclear (Boston, MA); $PGF_{2\alpha}$, PGD_2 and U-46619 were from Cayman Chemical Co.(Ann Arbor, MI); GF/ F microglass filters(25mm) were from Fisher (St. Louis, MO); Filter-count was from Packard Instrument Company, Inc. (Downers Grove, IL); Thin-layer chromatography plates 20×20 cm, 250 μm thickness channelled silica gel G with a preabsorbent zone were obtained from Analtech (Newark, DE).

Preparation of Membranes

Salivary gland membrane fractions were prepared according to Watanabe *et al.* (1986) and McSwain *et al.* (1987) with modifications. Briefly, salivary glands were dissected at 4°C in a solution of 50 mM Tris-HCl, 100 μM indomethacin, 2 mM EDTA, pH 7.5. The excised glands were homogenized in an all-glass homogenizer. The crude homogenate was centrifuged at 325 *g* for 5 min at 4°C. The supernatant was then centrifuged at 11500 *g* for 10 min at 4°C. The resulting pellet was resuspended in the dissection buffer and membranes collected by centrifugation at 11500 *g* for 10 min twice more. The washed membrane pellet was resuspended in the dissection buffer for use in PGE_2 binding assays. If membrane preparations were not used immediately, they were stored at -70°C. Protein

was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories) using bovine serum albumin as a standard.

Gradient Purification of Plasma Membranes

Gland membrane fractions were further purified by sucrose gradient centrifugation in experiments when effects of guanine nucleotides and exotoxins on the PGE_2 binding activity were investigated. Membranes prepared as above were suspended at a protein concentration of 2-5 mg/ml in 50 mM Tris-HCl, 2 mM EDTA, pH 7.5, containing 1.95 M sucrose. The suspension was overlayed with 0.25 M sucrose and the sample centrifuged at 100,000 *g* for 60 min at 4°C. Material at the interface of 0.25 M and 1.95 M sucrose was withdrawn with a syringe, diluted with about 5 volumes of 50 mM Tris-HCl, 2 mM EDTA, pH 7.5 and centrifuged at 100,000 *g* for 60 min at 4°C. The pellet was washed twice with 50 mM Tris-HCl, 2 mM EDTA, pH 7.5, and suspended in 50 mM Tris-HCl, 2 mM EDTA, pH 7.5. Purified membrane preparations were stored at -70°C until use.

Binding Assays

Direct binding assays of PGE_2 to the plasma membrane fraction were performed with [^{3}H]-lablelled PGE_2 according to Williams and Sills (1990) with some modifications. The principle of this assay is that receptor-ligand (RL) complexes are formed by the incubation of membranes together with radiolabelled PGE_2. Once the RL complexes are formed, the unbound radioactivity can be removed by filtration through microglass filters. After filtration, the radioactivity associated with the microglass filters is quantified by liquid

scintillation counting. Since radioactivity can also be associated with sites that are not related to the receptor, a parallel assay with an excess of unlabeled PGE_2 is included to measure nonspecific binding. Measurement of binding in the absence and presence of unlabeled PGE_2 provides data on total ligand binding and nonspecific binding, respectively. Their difference is the specific binding of PGE_2.

The PGE_2 binding assays used the standard assay mixture containing 50 mM Tris-HCl, 3.84 mM $MgCl_2$, pH 7.5, 3 nM [^{3}H] PGE_2 and 50 µg membrane protein in a final volume of 200 µl. Some variations are indicated in the figure legends. Incubations proceeded for 3 hours at 30°C and were stopped by the addition of 5 ml of ice-cold binding assay buffer and the solution filtered rapidly through a Whatman GF/F microglass filter under high vaccum. 20 glassfilters were soaked in a 2 ml of Tris solution at pH 9.1, containing 0.3% polyethylenimine (PEI) for 1-4 hr before use (Bruns *et al.*, 1983). Pretreatment of glassfilters with PEI was used to reduce non-specific binding of radioligands to the surface of the glassfilters. Each filter was washed three times with 5 ml of ice cold buffer. In all experiments control assays were performed in parallel with 100 µM PGE_2 included in the assay mixture to determine non-specific binding.

PGE_2 Metabolism

The possibility that [^{3}H]PGE_2 might be degraded during the the binding assay was examined according to Bowman *et al.*(1995b). After incubation, the assay suspension (200 µl) was acidified by the addition of 800 µl H_2O that has pH 3-5 adjusted with 3% formic

acid and lipids were extracted into 3 ml ethyl acetate (vigorous vortex for 90 sec). The phases were separated by centrifuging for 5 min at 2500 g. The upper organic layer was collected and the aqueous phase reextracted as before. The pooled lipid extract was dried under nitrogen and mixed with 3.5 µl standard cold PGE_2 and 15 µl ethyl acetate.The control consisted of 0.5 µl [^{3}H]PGE_2, 3.5 µl standard cold PGE_2 and 15 µl ethyl acetate. They were applied to prewashed and activated silica gel G plates with preabsorbent zones. The plates were developed in a solvent system of chloroform-methanol-acetic acid-water (90: 8: 1: 0.8, v/v/v/v) (Salmon and Flower, 1982) in equilibrated developing tanks. The position of PGE_2 was determined following exposure to iodine vapor in a tank. Distribution of the radioactivity was assessed by radioscanning (BioScan 2000, BioScan, Washington, DC).

Treatment of Gland Membranes with Pertussis toxin or Cholera Toxin

Purified membranes were treated with the exotoxins as described by Watanabe *et al.* (1986) and McKenzie (1992) with modifications. The membranes were incubated with 10 µg of activated pertussis toxin at 30°C for 40 min in 0.5 ml of a mixture containing 100 mM potassium phosphate, 5 mM $MgCl_2$, pH 7.5, 10 mM NAD, 1 mM ATP, 1 mM EGTA, 10 mM thymidine, 2 mM dithiothreitol, 100 µM GTP, 3 mM potassium phosphoenolpyruvate and 10 µg/ml of pyruvate kinase. For cholera toxin treatments, membranes were incubated with 100 µg of activated cholera toxin in the same medium as used for the pertussis toxin treatment. Controls were performed without treatment with toxins. Treated membranes were incubated with 10 mM N-ethylmaleimide at 0°C for 30

min. Finally, the treated membranes were washed twice with 50 mM Tris-HCl, pH 7.5, containing 2 mM EDTA and suspended in binding assay buffer. Binding of 3 nM [^{3}H]PGE_2 was then measured in the presence and absence of 1 mM GTPγS.

Statistical Analysis

The differences between the control and experimental treatment were tested for significance by paired Student's t test. A P value <0.05 was considered significant.

RESULTS

Evidence of a Specific PGE_2 Receptor in Tick Salivary Gland Plasma Membranes

To see whether the effects of PGE_2 on secretion and level of cAMP are physiological, I sought evidence of a receptor in the salivary glands through the use of binding assays with [^{3}H]-labelled PGE_2. Thin layer chromatographic analysis indicated no significant metabolism of [^{3}H]PGE_2 during the incubation period for up to 12 hours at 30°C or 6 hours at 37°C (Fig. 3.1). Almost half of PGE_2 was metabolized into PGA_2 during incubation for 10 hours at 37°C.

Comparison of specific binding of PGE_2 to different fractions of the salivary glands, i.e. 11500 *g* pellet, 100,000 *g* supernatant and pellet, found that specific binding of PGE_2 to the 11500 *g* fraction was at least three times that in other tissue fractions (Fig. 3.2). This fraction is known to contain the highest percentage of plasma membranes after tissue fractionation (McSwain *et al.*, 1987). Electron micrographs of this fraction also showed

rich contents of membrane (Fig. 3.3). The 11500 *g* membrane fraction was therefore used in most of the subsequent binding assays.

Specific binding of [^{3}H]PGE_2 to the 11500 *g* membrane fraction was directly related to membrane protein up to 200 µg (Fig. 3.4). The specific binding of [^{3}H]PGE_2 to salivary gland membranes was also related to increasing concentrations of [^{3}H]PGE_2 (Fig. 3.5). The optimum pH for the [^{3}H]PGE_2 binding was 8.5 (Fig. 3.6). In addition, the specific PGE_2 binding activity was enhanced twice by 300 mM $MgCl_2$ as compared to the binding activity in the presence of 3.84 mM $MgCl_2$ in the assay buffer.

The specificity of [^{3}H]PGE_2 binding was investigated by adding increasing concentrations of various unlabelled prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2 and TxA_2) to the assay mixture. [^{3}H]PGE_2 was displaced in the rank order of PGE_2> $PGF_{2\alpha}$> PGD_2 > U-46619 (nonhydrolyzableTxA_2 analog) (Fig. 3.7).The binding characteristic of [^{3}H]PGE_2 to the gland membranes was analyzed by a Scatchard plot of PGE_2 displacement data using LIGAND program (Munson *et al.*, 1980). The best fit of the data indicated a single site model with a $K_D \approx$ 29 nM and $B_{max} \approx$ 1.2 pmoles /mg protein.

The time course of specific binding of [^{3}H]PGE_2 to membranes was studied at various temperatures (Fig. 3.8 A). The rate of specific binding of [^{3}H]PGE_2 to membranes increased with increasing temperatures and reached equilibrium after 4 hr at 37°C and 6 hr at 30°C. Subsequent displacement experiments were carried out at 37°C. 100 µM

unlabelled PGE_2 was added after [^{3}H]PGE_2 had incubated with membranes in assay tubes for 4 hr. Bound [^{3}H]PGE_2 was rapidly dissociated from the membrane fraction during the first 30 min and then the rate of dissociation slowed (Fig. 3.8 B). It is unclear why the bound labelled PGE_2 was only partially displaced from membranes.

Evidence that the PGE_2 Receptor is Associated with a Guanine Nucleotide Regulatory Protein

A series of purine nucleotides was chosen to detect their effects on [^{3}H]PGE_2 binding activity. Data showed that both GTP and its nonhydrolyzable analog GTPγS significantly reduced specific [^{3}H]PGE_2 binding to gland membranes (Fig. 3.9 A). In constrast, ATP and GDP had no significant effects on the binding activity. ATPγS slightly reduced [^{3}H]PGE_2 binding activity. Moreover, 1 mM GTPγS reduced specific [^{3}H]PGE_2 binding to membranes in a PGE_2 dose-dependent manner (Fig. 3.9 B). Results indicate that [^{3}H]PGE_2 binding to membranes is sensitive to GTP. In order to confirm if the PGE_2 binding activity is associated with a guanine nucleotide regulatory protein, I made another observation. Pretreatment of gland membranes with cholera toxin and NAD prevented GTPγS from inhibiting PGE_2 binding activity (Table 3.1). A similar treatment of membranes with pertussis toxin and NAD had no effect on the ability of GTPγS to inhibit PGE_2 binding activity. These results strongly suggest that the PGE_2 receptor in tick salivary glands is linked to a G_S-like rather than a G_i-like GTP-binding protein.

DISCUSSION

[^{3}H]PGE_2 binding assays were performed to determine if a specific PGE_2 receptor exists in tick salivary glands. The Scatchard analysis reveals a single PGE_2 binding site with high a affinity ($K_D \approx$ 29 nM) in tick salivary gland plasma membranes. Time courses of [^{3}H]PGE_2 binding appear to be partially reversible. The incomplete reversibility of binding was also observed by several authors (Schillinger and Prior, 1976; Carsten and Miller, 1981; Sonnenburg *et al.*, 1990; Hamon *et al.*, 1993). Hamon *et al.*(1993) proposed that the incomplete dissociation could have resulted from an alteration of the binding sites or the ligand during the relatively long incubation (covalent binding of the ligand could not be ruled out). Competition studies with different PGs show that PGE_2 is the most effective in inhibiting the binding of [^{3}H]PGE_2 to the gland membranes, while $PGF_{2\alpha}$ and PGD_2 are 10-fold and 90-fold less effective respectively; TxA_2 nonhydrolyzable analog U-46619 is least effective. The result indicates that the receptor site is specific for PGE_2.

The observation that PGE_2 binding to gland membranes is reduced by GTP and its analog (GTPγS) indicates that the PGE_2 receptor is functionally associated with a guanine nucleotide regulatory protein. In general, G protein-coupled receptors exist in two main conformational states, receptor alone having a low affinity for agonists and receptor coupled to the G protein having a high affinity (Graeser and Neubig, 1992). Binding of GTP to a G protein causes dissociation of the G protein from the receptor and thus decreases the affinity of the receptor. GTP-mediated decreases in affinity have been viewed as one of the key processes, along with the covalent receptor modification and G

protein GTPase activity, involved in preventing persistent cellular responses to hormones (Birnbaumer *et al.*, 1985; Sibley and Lefkowitz, 1985).

Further evidence shows that it is a cholera toxin-sensitive G protein because pretreatment of membranes with cholera toxin plus NAD eliminated GTPγS inhibition of PGE_2 binding activity but pertussis toxin did not. Both toxins are bacterial exotoxins which catalyze the ADP-ribosylation of a specific G protein by NAD^+, thereby uncoupling the G-protein from its receptor. To our knowledge, this is the first evidence of a PGE_2 receptor coupled to a cholera toxin-sensitive G protein existing in an invertebrate.

Table 3.1 Effects of cholera toxin and pertussis toxin treatments on the inhibition by GTPγS of PGE_2 binding to tick gland membrane preparations. Salivary gland membranes were prepared and subjected to the indicated treatments and carried out in triplicate for $[^3H]PGE_2$ binding assays in the presence or absence of 1 mM GTPγS. This experiment was performed twice with similar results. *Values were significantly different from values in the absence of GTPγS (P <0.05)

Treatment	Specific PGE_2 Binding (fmoles/mg protein)	
	-GTPγS	+GTPγS
Control	70±15	39±8*
Pertussis toxin	82±14	38±7*
Cholera toxin	51±13	50±20

Figure 3.1 Radio-chromatogram of TLC separated ethyl acetate extracted products. (A) Standard sample (stock [^{3}H]PGE_2), with a stated purity of 97%; (B) Sample from incubation of [^{3}H]PGE_2 with gland membrane fractions for 12 hr at 30°C, about 97% of radioactivity co-chromatographed with authentic PGE_2; (C) Sample from incubation of [^{3}H]PGE_2 with gland membrane fractions for 6 hr at 37°C, about 90% of the radioactivity co-chromatographed with authentic PGE_2.

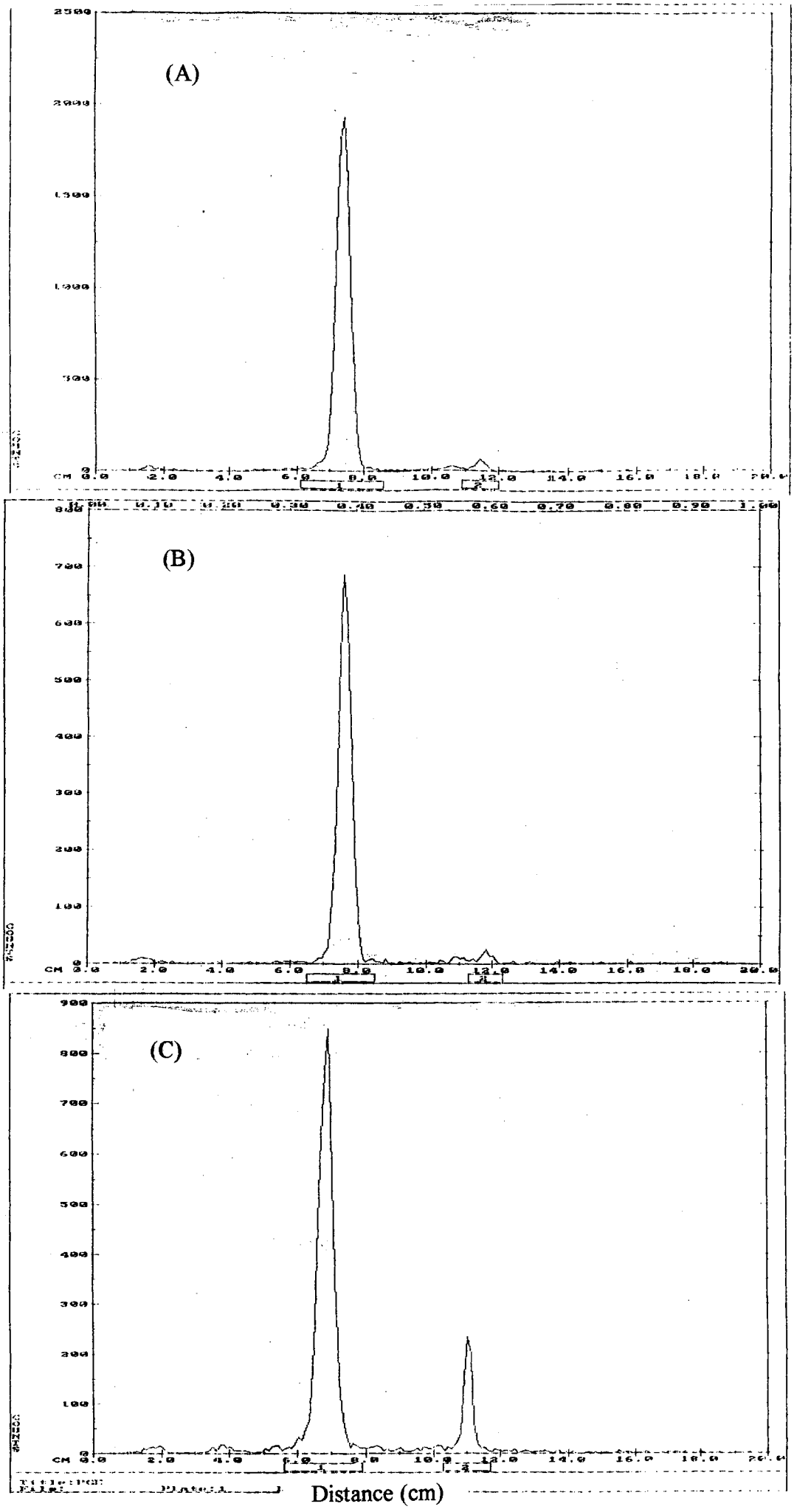

(A)
(B)
(C)
Radioactivity
Distance (cm)

Figure 3.2 Comparison of specific [^{3}H]PGE_2 binding to different fractions of tick salivary gland homogenates. [^{3}H]PGE_2 binding assays were performed at 24°C for 3 hr using 2.75 nM [^{3}H]PGE_2 and 128 μg protein (n>4). * indicates the values significantly different from zero (P <0.05).

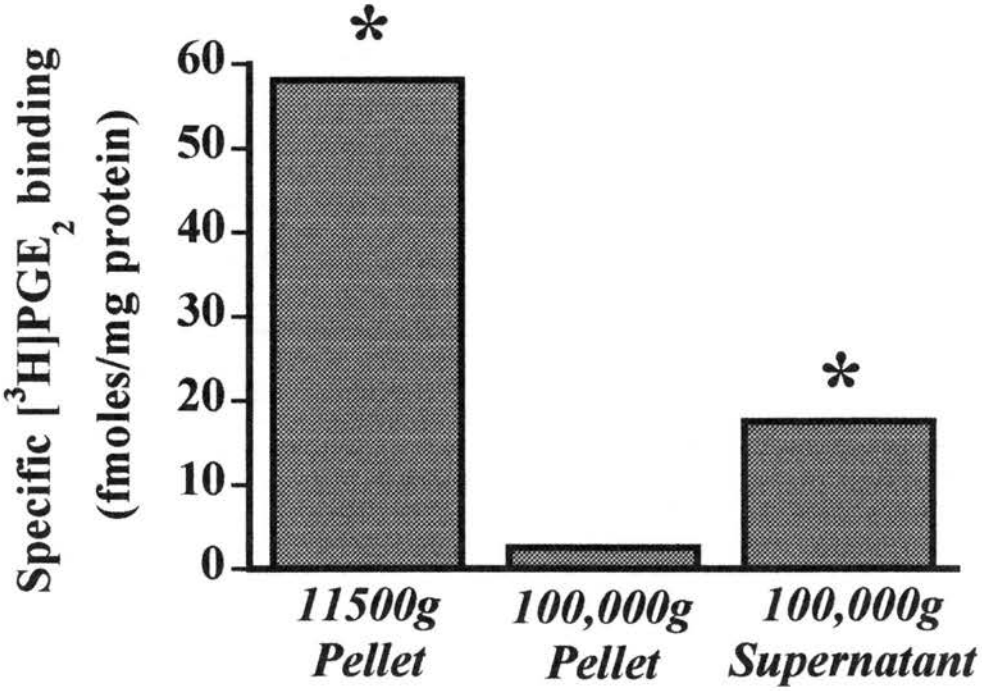

Specific [3H]PGE2 binding
(fmoles/mg protein)
60
50
40
30
20
10
0
*
*
11500g
Pellet
100,000g
Pellet
100,000g
Supernatant

Figure 3.3 Electron micrograph of the 11,500*g* pellet from the salivary gland homogenate of the female tick *A. americanum* taken by Dr. Katherine Kocan. Isolated membranes were pelleted in 1.5 µm microcentrifuge tubes and fixed in 2% glutaraldehyde in a 0.2 M sodium cacodylate buffer overnight and postfixed in 2% osmium tetroxide in 0.2 M sodium cacodylate buffer for 1 hr. The membranes pellet then dehydrated through a graded series of ethanol and infiltrated and embedded in epoxy resin (Dow Epoxy Resin 732), using propylene oxide as the intermediate solvent. Thick sections (1.5 µm) were stained with Mallory's stain (Richardson *et al.*, 1960) for 2 min at 60°C and were examined by light microscopy. Ultrathin (silver-gold reflective) sections were cut with a ultramicrotome (MT 7000, Research and Manufacturing Company, Inc.) and a diamond knife. Sections were collected on 300-mesh copper grids, stained with uranyl acetate and lead citrate (Venable and Coggeshall 1965), and observed and photographed with a transmission electron microscope (JEOL CX 100, JOEL Inc., Boston, MA) operated at 80 kV. An abundance of membranes (Mb) and a few mitochondria (Mt) were seen in the fraction. Magnification ×10,000.

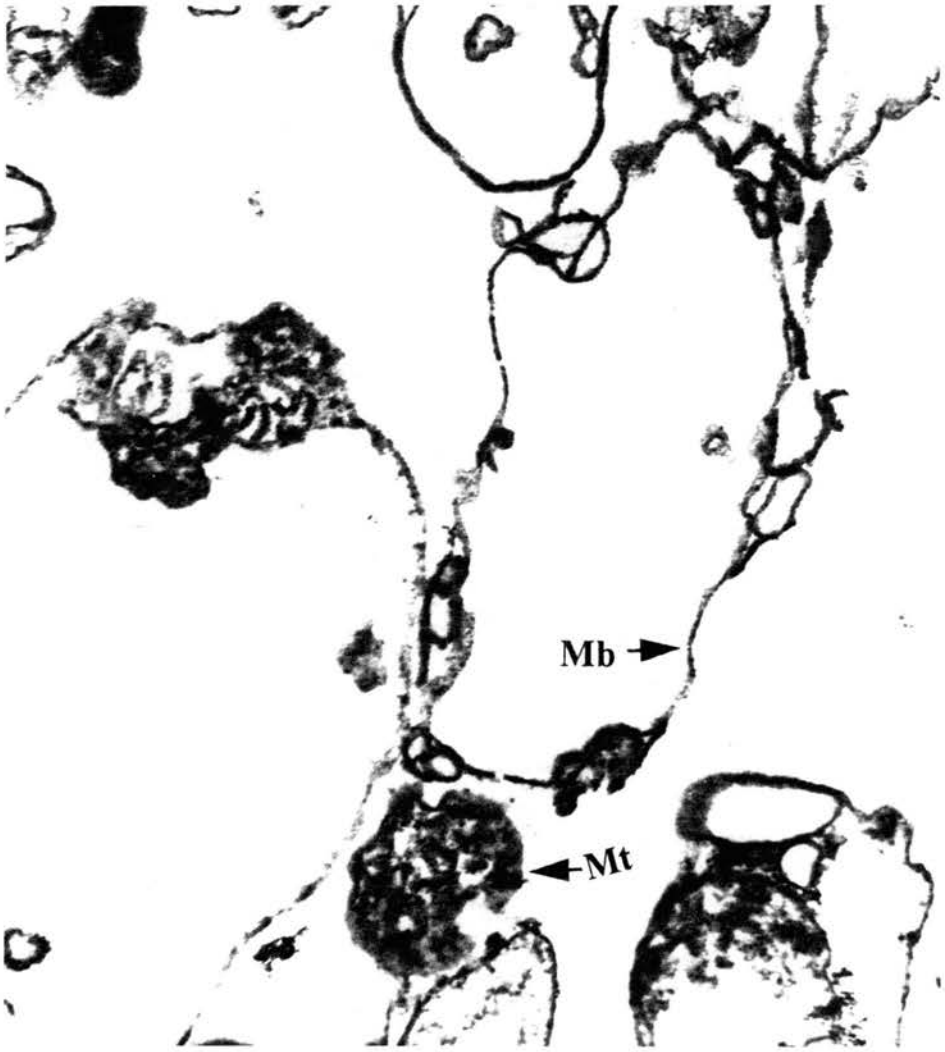

Mb
Mt

Figure 3.4 Specific binding of [^{3}H]PGE_2 to tick salivary gland membranes as a function of membrane protein. The [^{3}H]PGE_2 binding assay was performed in duplicate for 3 hr at 24°C using 3 nM [^{3}H]PGE_2 with the indicated amounts of added membrane protein.

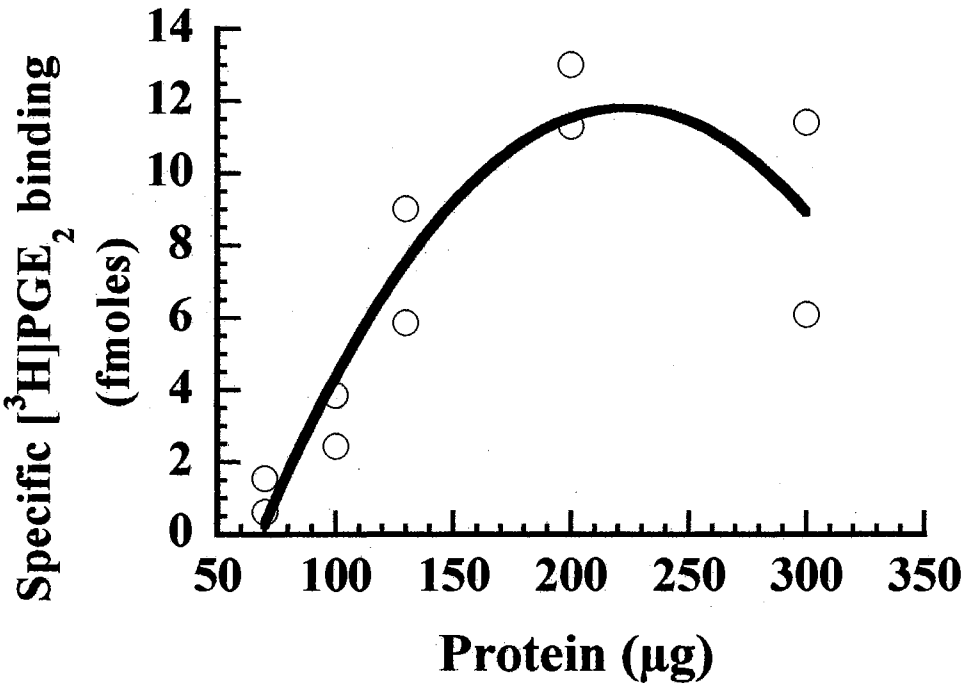

Specific [3H]PGE2 binding
(fmoles)
14
12
10
8
6
4
2
0
50
100
150
200
250
300
350
Protein (µg)

Figure 3.5 Specific binding of [^{3}H]PGE_2 to tick gland membranes as a function of concentrations of [^{3}H]PGE_2. The [^{3}H]PGE_2 binding assay was performed in duplicate for 3 hr at 24°C using 140 μg membrane protein with the indicated concentrations of [^{3}H]PGE_2. The curve was fitted using KaleidaGraph.

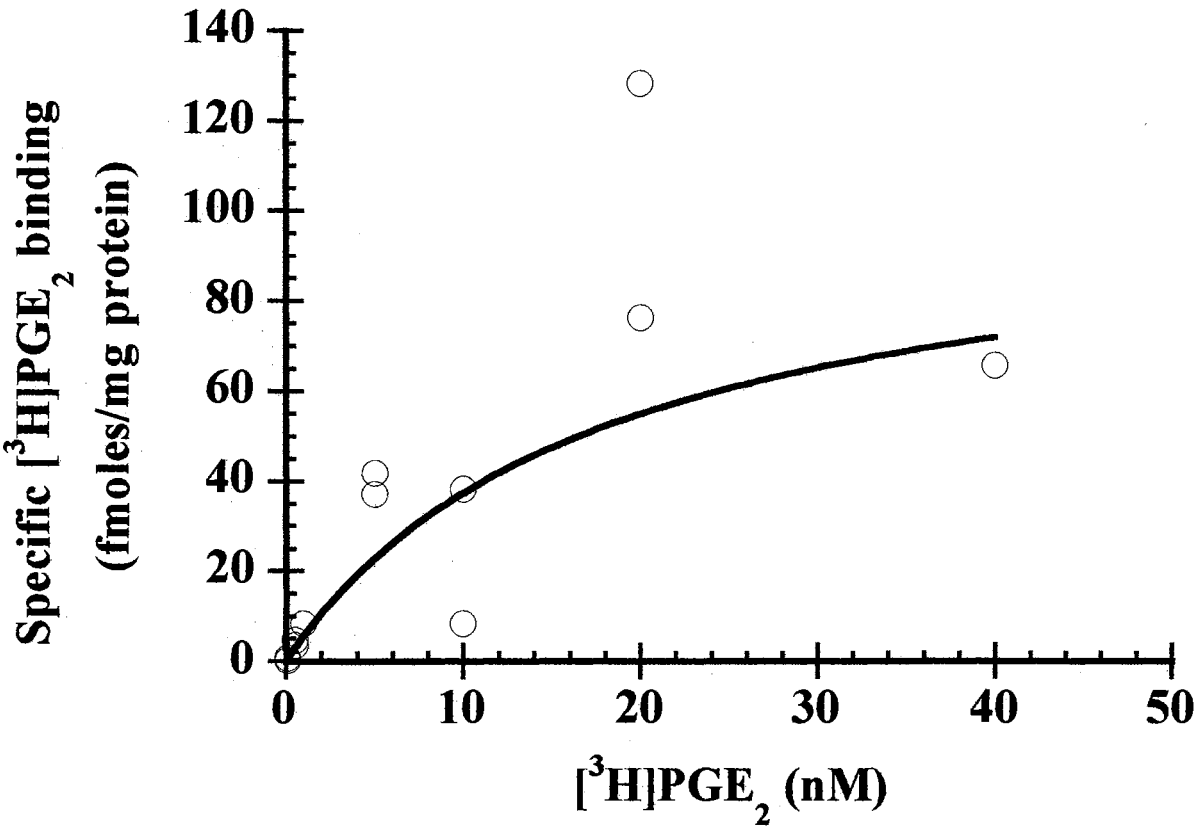

Specific [3H]PGE2 binding
(fmoles/mg protein)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
[3H]PGE2 (nM)

Figure 3.6 Specific binding of [^{3}H]PGE_2 to tick salivary gland membranes at various pH. [^{3}H]PGE_2 binding assays were performed for 3 hr at 24°C using 3 nM [^{3}H]PGE_2 and 150μg membrane protein. The data represent the mean ± SE from three determinations.

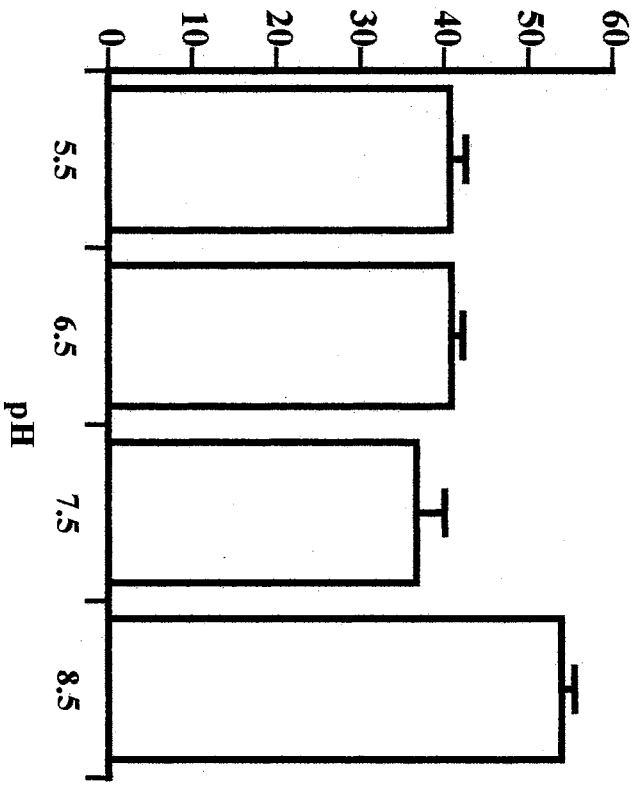

Specific[3H]PGE2 binding
(fmoles/mg protein)
0
10
20
30
40
50
60
pH
5.5
6.5
7.5
8.5

Figure 3.7 Inhibition of specific [^{3}H]PGE_2 binding to gland membranes by various prostaglandins. The binding assays were performed in triplicate using 3 nM [^{3}H]PGE_2 and 50 μg of membrane protein for 3 hr at 24°C (the assays used pH 8.5 and 300 mM $MgCl_2$) in the presence of the indicated concentrations of prostaglandins. Displacement curves were fitted using SigmaPlot (Jandel) and the IC_{50} values were determined as the concentration of prostaglandins necessary to cause 50% inhibition of binding of 3 nM [^{3}H]PGE_2 to gland membranes.

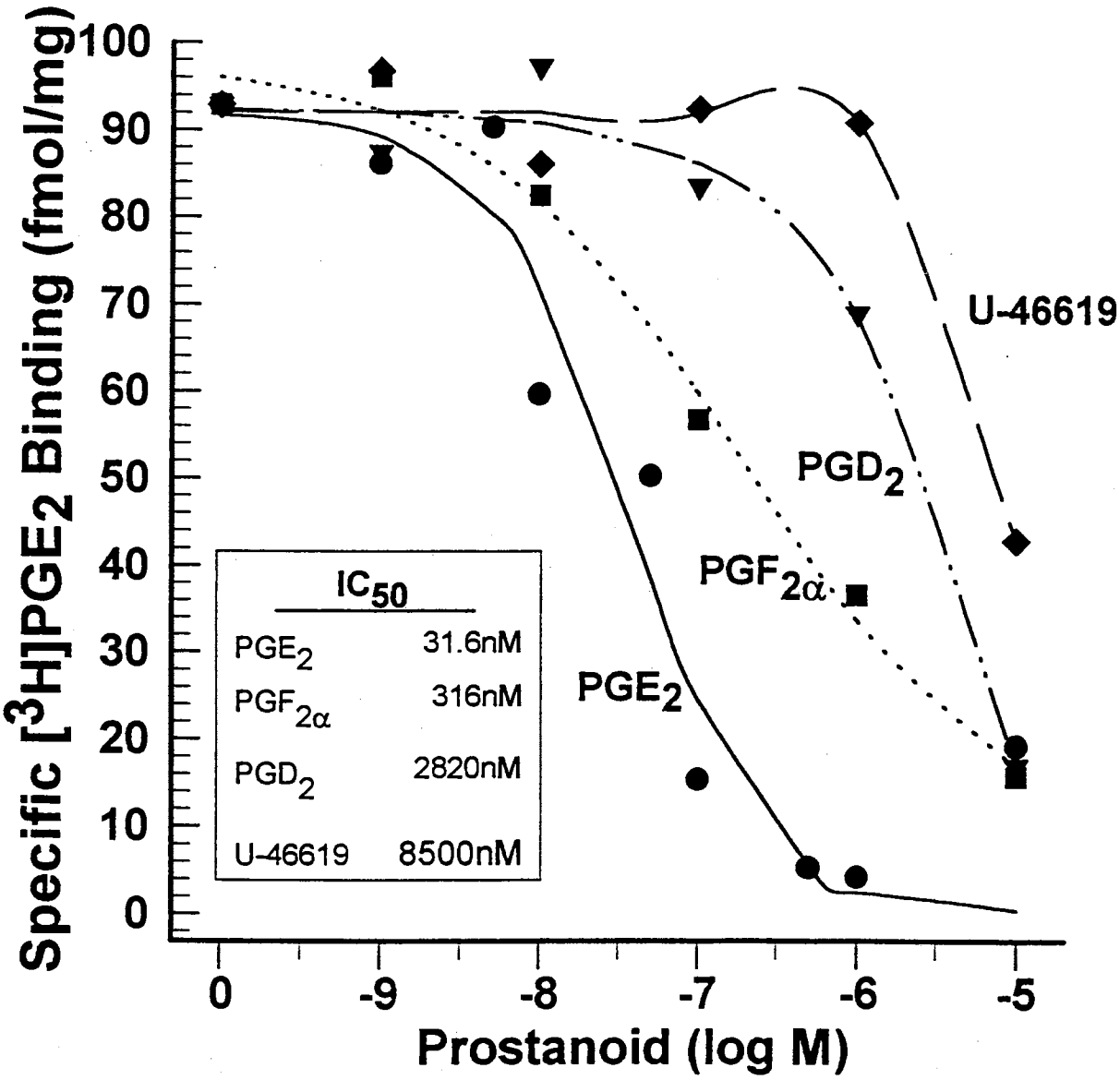
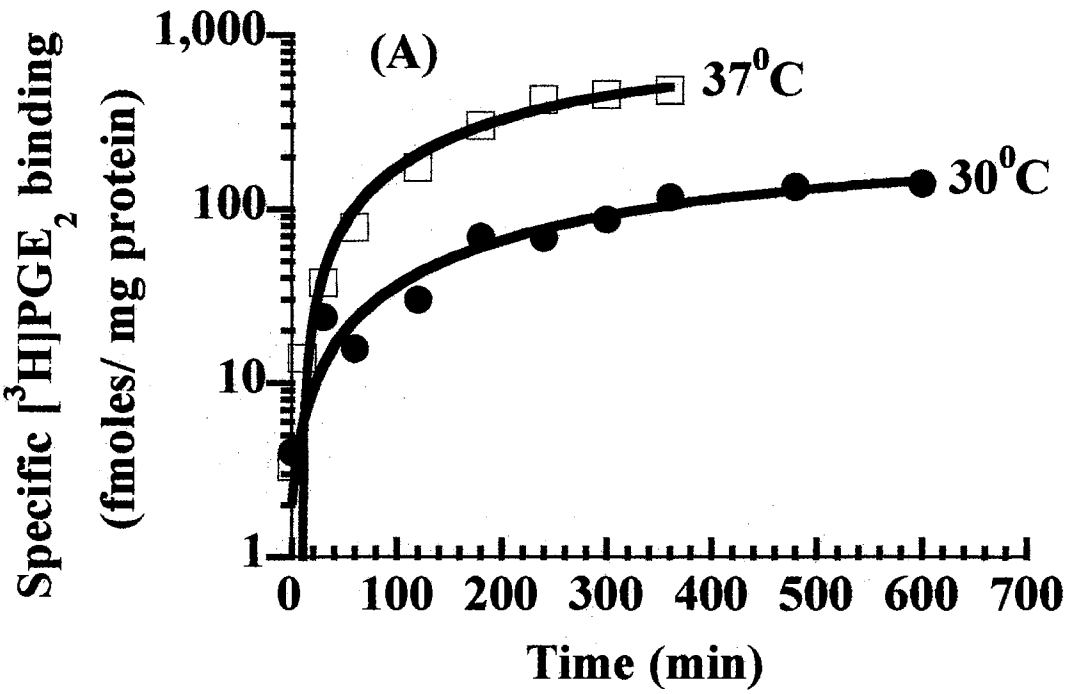

Specific [3H]PGE2 Binding (fmol/mg)
100
90
80
70
60
50
40
30
20
10
0
0
-9
-8
-7
-6
-5
Prostanoid (log M)
U-46619
PGD2
PGF2α
PGE2
IC50
PGE2 31.6nM
PGF2α 316nM
PGD2 2820nM
U-46619 8500nM

Figure 3.8 (A) Time courses of specific binding of [^{3}H]PGE_2 to gland membranes. Binding assays were performed in triplicate at the indicated temperatures for the indicated times using 3 nM [^{3}H]PGE_2 and 50 μg of membrane protein (the assays used pH 8.5 and 300 mM $MgCl_2$). (B) Time courses of displacement of specific [^{3}H]PGE_2 binding to gland membranes. The binding assays were performed in triplicate at 37°C using 3 nM [^{3}H]PGE_2 and 50 μg of membrane protein. Values represent the mean of triplicate determinations ± SE.


(A)
37°C
30°C
Specific [3H]PGE2 binding
(fmoles/ mg protein)
1,000
100
10
1
0 100 200 300 400 500 600 700
Time (min)

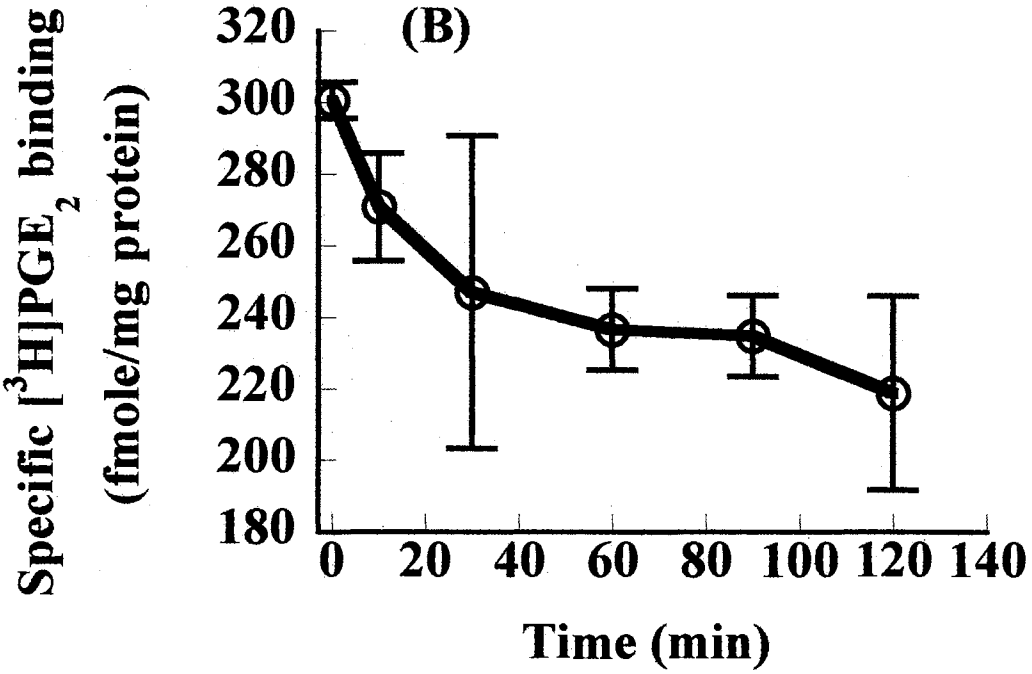
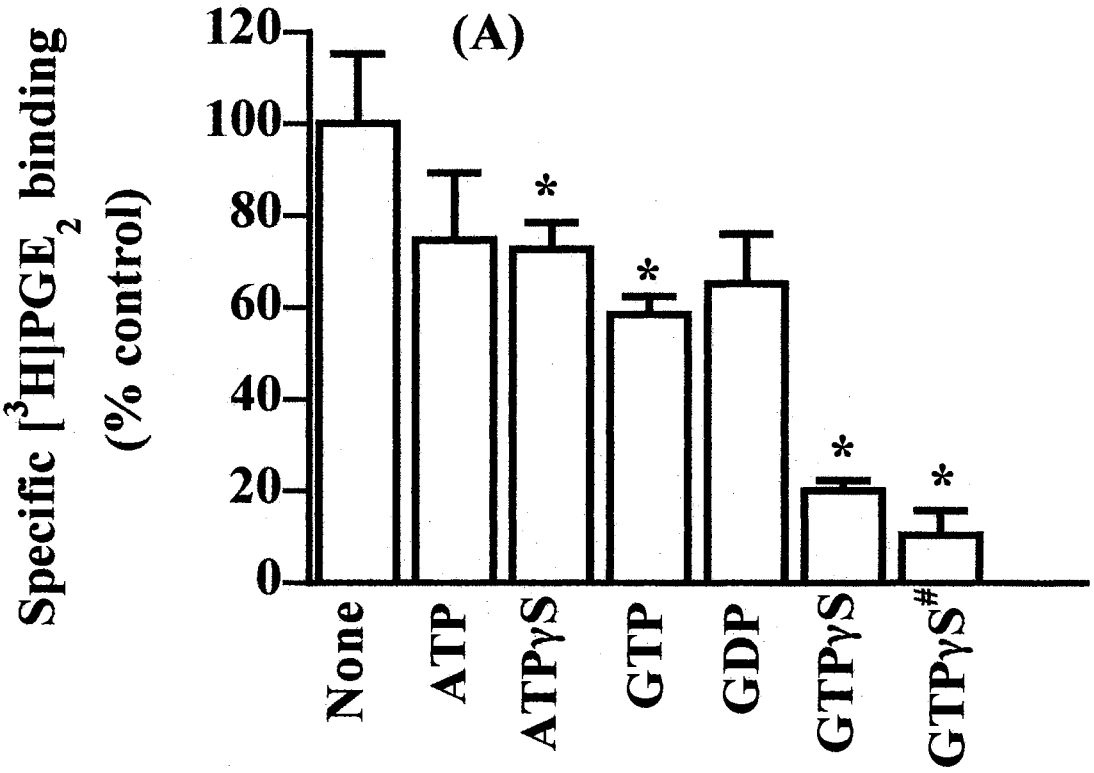
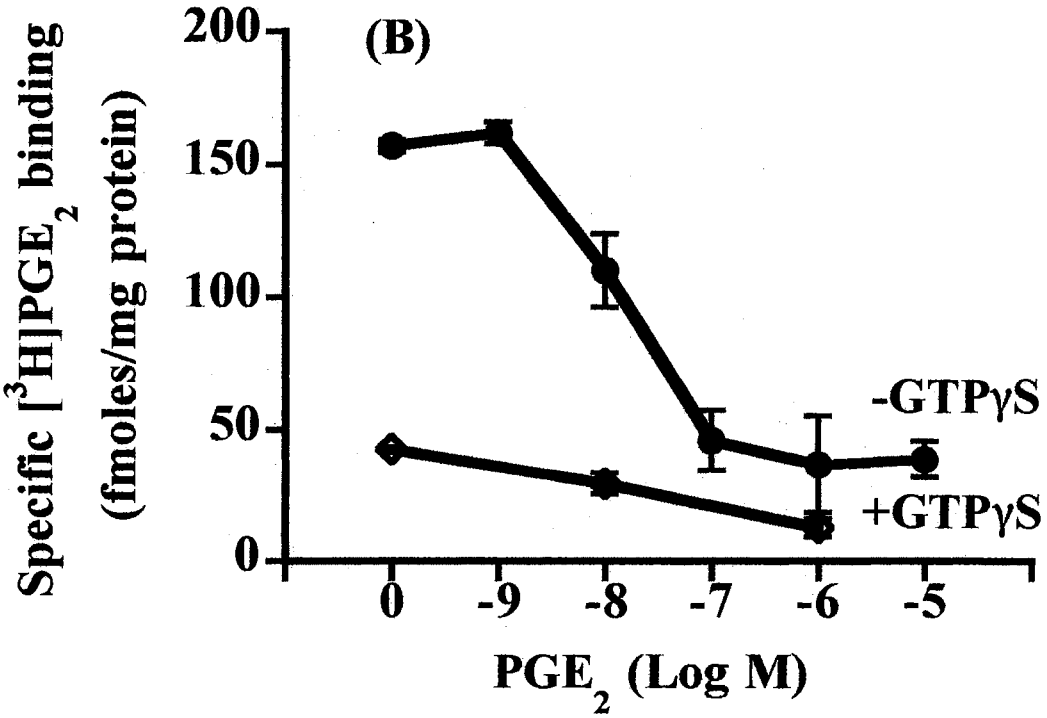

(B)
Specific [3H]PGE2 binding
(fmole/mg protein)
320
300
280
260
240
220
200
180
0 20 40 60 80 100 120 140
Time (min)

Figure 3.9 (A) Effects of purine nucleotides on specific binding of [^{3}H]PGE_2 to gland membranes. Purified membranes were prepared and binding assays were performed in triplicate using 3 nM [^{3}H]PGE_2 and 50 μg of membrane protein in the presence or absence of 1 mM or 5 mM (#) various purine nucleotides at 30°C for 4 hr (the assays used pH 8.5 and 300 mM $MgCl_2$). Values represent the mean of triplicate determinations ± SE. *: Significantly different from control (no purine nucleotide) (P <0.05). (B) Inhibition of specific [^{3}H]PGE_2 binding to gland membranes by 1 mM GTPγS. Membrane preparations and binding assays were performed as in (A). Values represent the mean of triplicate determinations ± SE.


(A)
Specific [3H]PGE2 binding
(% control)
120
100
80
60
40
20
0
*
*
*
*
None
ATP
ATPγS
GTP
GDP
GTPγS
GTPγS#


(B)
Specific [3H]PGE2 binding
(fmoles/mg protein)
200
150
100
50
0
-GTPγS
+GTPγS
0
-9
-8
-7
-6
-5
PGE2 (Log M)

CHAPTER 4

SIGNAL TRANSDUCTION OF THE PROSTAGLANDIN E_2 RECEPTOR IN TICK SALIVARY GLANDS

INTRODUCTION

External signals (e.g. hormones, neurotransmitters) bind to plasma membrane receptors on cell surfaces and are transduced and amplified into "second messenger" molecules and ions within the cell which thus change cellular activities. Various signal transduction pathways have been described for PGE receptors in mammalian tissues, e.g. pathways that cause an increase in intracellular Ca^{2+} and pathways which increase or decrease intracellular cyclic AMP (cAMP) (Narumiya, 1995). An increase in intracellular Ca^{2+} is induced by either or both of two mechanisms, one caused by Ca^{2+} entry from the outside and the other by mobilization of intracellular Ca^{2+}. Calcium and cyclic AMP appear to be important in controlling fluid secretion in tick salivary glands (Sauer and Hair, 1986). Deletion of calcium or the addition of the calcium channel antagonist verapamil to the bathing medium inhibits dopamine-stimulated fluid secretion by isolated salivary glands, implicating an external source of Ca^{2+} in maintaining secretion (Needham and Sauer, 1979). A "second messenger" role for cAMP is indicated in that exogenous cAMP stimulates fluid secretion in isolated salivary gland preparations (Sauer *et al.*, 1979). Furthermore, changing activities of adenylate cyclase and cAMP-dependent phosphodiesterase during tick feeding

are found to correlate with an increase in the rate of salivary fluid secretion as the tick progresses from the slow to rapid stage of feeding (Sauer *et al.*, 1979). Thus, regulation of the enzymes that regulate levels of cAMP is an important mechanism for regulating the magnitude of fluid secretion in salivary glands of feeding ixodid females (Sauer and Essenberg, 1984). The increase in salivary gland cAMP occurs when dopamine released from a salivary nerve interacts with a D_1-like, G protein-coupled receptor, which results in the activation of an adenylate cyclase and stimulates formation of cAMP (Sauer *et al.*, 1995).

Prostaglandins of the 2-series (e.g. PGE_2) are typically synthesized from arachidonic acid (AA) after AA is released from cellular phospholipids following activation of an intracellular phospholipase A_2 (PLA_2). Dopamine stimulates release of AA from salivary gland phospholipids presumably by activating an intracellular PLA_2 (Bowman *et al.*, 1995a). Treatment of isolated salivary glands with PLA_2 inhibitor oleyloxyethyl phosphorylcholine (OPC) or prostaglandin synthetase inhibitors reduces dopamine-induced fluid secretion and cAMP levels in isolated salivary glands. PGE_2 or its analog 17-phenyl trinor PGE_2 partially reverses inhibition of secretion and cAMP level by OPC, suggesting that prostaglandins produced following gland stimulation by dopamine may have an autocrine effect in modulating salivary gland function (Chapter 2). Furthermore, a specific PGE_2 receptor coupled to a cholera toxin-sensitive G protein has been identified in the plasma membrane fraction of the salivary glands (Chapter 3). The following experiments were designed to determine if "second messengers" are mobilized in salivary

gland cells after PGE_2 binds to its specific receptor. The possibility of an effect on adenylate cyclase activity or an effect on an increase in intracellular Ca^{2+} were examined.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: Dowex 50W-H^+ cation exchange resin (200-400 mesh, 8% cross-linkage), neutral alumina (type WN-3), ATP, cyclic AMP, creatine phosphate, creatine phosphokinase, dithiothreitol, bromo-A23187, prostaglandin E_2, indomethacin, trichloroacetic acid (TCA), trioctylamine, 1,1,2-trichloro-1,2,2-trifluoroethane (TCTFE), and other chemicals were acquired from Sigma Chemical Company, St.Louis, MO. [α^{32}P]ATP(3000 Ci/mmol), [2,8-^{3}H]cAMP (30 Ci/mmol), $^{45}CaCl_2$ (40.58 mCi/mg) and the inositol-1,4,5-trisphosphate [^{3}H]radioreceptor assay kit were from Du Pont New England Nuclear (Boston, MA). ScintiSafe™ Econo2 was obtained from Fisher Scientific (St. Louis, MO). Silicone fluids 556 (0.98 g/ml) and 550 (1.07 g/ml) were gifts from Dow Corning Corporation (Midland, MI).

Preparation of Dispersed Salivary Glands

Glands from 20 ticks were dissected and placed in 1 ml of culture medium L-15B (Sigma catalogue #, L4386) at pH 7.8, containing 1 mM indomethacin, 100 units of penicillin and 100 μg streptomycin. The salivary glands were then dispersed mechanically (Ramachandra, personal communication) using two Clay Adams slides (Fisher catalogue#, 3051) which have one end frosted on one side of each slide. The resulting suspension was confirmed for gland dispersion under a 200× microscope (BHS Olympus, Olympus

Optical Co. Ltd., Tokyo, Japan). The viability of the dispersed acini and cells was examined using trypan blue stain and at least 60% of the cells maintained viability after the treatment. The suspension of the dispersed acini and cells was centrifuged at 325g for 4 min. The pellet was washed twice using the medium L-15B and resuspended in the medium L-15B for use in the assays for the formation of IP_3, $^{45}Ca^{2+}$ influx and efflux.

Assay for Adenylate Cyclase Activity

Adenylate cyclase activity was measured as described by Schmidt *et al.* (1982) and Farndale *et al.* (1992) with some modifications. The principle of this method is use of [α^{32}P]ATP to generate [α^{32}P]cAMP followed by a simple two-column chromatography system to separate labeled cAMP from other labeled adenine nucleotides (Farndale *et al.*, 1992). The basic assay mixture contained 25 mM Tris-HCl, pH 7.5, 100 μM[α^{32}P]ATP (10^6 cpm), 5 mM $MgCl_2$, 0.77 mM IBMX, 0.1 mM EDTA, 500 μM cAMP, 20 mM creatine phosphate, 50 units/ml creatine kinase, 1 mM dithiothreitol. Assays were initiated by the additions of PGE_2 and gland membranes (40 μg protein). Reactions were allowed to proceed at 37°C in a water bath for 15min and were stopped by adding a solution containing 2% SDS, 40 mM ATP and 1.4 mM cAMP in deionized water. cAMP was separated from other adenine nucleotides in two steps using Dowex 50 cation exchange and neutral alumina columns. Columns with a total volumn of 10 ml supplied by Bio-Rad (Catalogue #, 731-1150) were used but the plastic sinter supplied was replaced with glass wool to give a satisfactory flow rate. The Dowex column rack was located on top of the alumina column rack so that the Dowex columns drip into the tops of the alumina

columns. The alumina columns in turn drip into scintillation vials. Dowex columns were prepared by pipetting 2 ml of a stirred slurry of 50% Dowex 50×4 200 in 1 M HCl into columns which gave a bed volumn of 1 ml. The Dowex columns were regenerated by washing with 3 ml of 1 M HCl. Alumina columns were prepared by placing 0.8 g of alumina into each column and primed with 10 ml of 0.3M imidazole buffer. Each sample was transferred to Dowex columns primed with 15 ml deionized water and washed with 1 ml of deionized water twice. The Dowex columns were then located on top of the alumina columns and the cAMP was eluted from the Dowex columns onto the alumina columns with 6 ml of deionized water. The alumina columns were washed once with 1 ml of 0.3M imidazole buffer and the cAMP was finally washed from the alumina columns with 1.75 ml of 0.3M imidazole buffer. The eluates were collected in scintillation vials and quantitated by liquid scintillation counting using the scintillant ScintiSafe^{TM}Econo 2. Tracer amounts of [^{3}H]cAMP were added to monitor the recovery of biosynthetic [α^{32}P] cAMP that was isolated by sequential chromatography of the samples on Dowex 50W-X4 and alumina columns (Farndale *et al.*, 1992). cAMP recoveries were 70-80% using this isolation procedure. The [^{32}P]cAMP generated during the assay was calculated as picomoles cAMP per milligram membrane protein by the following formula:

$$\frac{\text{(sample } ^{32}\text{P c.p.m.)} \times (^{3}\text{H cAMP recovery standard c.p.m.)}}{\text{assay constant} \times (\text{sample } ^{3}\text{H c.p.m.-(sample } ^{32}\text{P c.p.m.} \times \text{spillover))}}$$

where the assay constant was defined as the value for multiplying specific activity of [α^{32}P]ATP by the milligrams of membrane protein in the assay. Adenylate cyclase activity was then obtained by dividing this figure by the assay time.

Assay for the Formation of IP_3

Dispersed salivary glands were suspended in 20 mM morpholinopropane sulfonic acid (MOPS) buffer at pH 7.5, containing 177 mM NaCl, 15 mM $KHCO_3$, 3.5 mM $CaCl_2$, 2.8 mM $MgSO_4$, 32 mM glucose, 0.1 g/l bovine serum albumin (BSA), 0.03 g/l penicillin and 0.1 g/l streptomycin sulfate (McSwain *et al.*, 1989). Assays were initiated by the addition of PGE_2 or its solvent carrier, 10 mM LiCl (a myo-inositol 1-phosphatase inhibitor) and 1 mM indomethacin in 20 mM MOPS buffer. Reactions were allowed to proceed at 37°C for 30 seconds, and stopped and IP_3 was extracted with 0.2 volumes of ice-cold 100%TCA solution and vortexed thoroughly. TCA was then removed from extracts with a 3:1 (v/v) solution of TCTFE-trioctylamine. After extraction, the aqueous phase containing IP_3 was removed and stored on ice until assayed for level of IP_3.

The radioreceptor asssay kit consists of membranes derived from calf cerebellum containing the IP_3 receptor. The receptor binds IP_3 and $[^3H]IP_3$ competitively. The amount of $[^3H]IP_3$ bound to the receptor was measured after centrifuging the membranes and counting bound radioactivity in the pellet by liquid scintillation counting using the scintillant ScintiSafe^{TM}Econo 2. Unlabeled IP_3, added to the incubation mixture as either known amounts of standard or the unknown sample, competed with $[^3H]IP_3$ for binding to the receptor. Non-specific binding caused by tracer which was not bound to the receptor but which remained attached to other parts of the membrane or the tube was determined by saturating the receptor with a large amount of cold inositol hexaphosphate (IP_6) (IP_6 produces the same non-specific binding as high concentrations of IP_3). The concentration

of each sample was determined by interpolation from a standard curve derived by using known amounts of IP_3.

The $^{45}Ca^{2+}$-Release Assay

Measurement of $^{45}Ca^{2+}$ efflux was based on the method described by Wilcox *et al.* (1995) with some modifications. The principle involves preloading $^{45}Ca^{2+}$ into intracellular stores and then monitoring release of $^{45}Ca^{2+}$ induced by an agonist. Efflux from preloaded tissue is a function of the free cytosolic ion level available for transport. The dispersed salivary glands were suspended in a buffer whose fivefold stock contained 120 mM KCl, 2 mM KH_2PO_4, 5 mM $(CH_2COONa)_2$, 2 mM $MgCl_2$, and 20 mM HEPES-free acid. Since ATP is relatively unstable in alkaline solutions, it was added to the final buffer just prior to use. The fivefold stock buffer (1 vol) was added to a container with preweighed Na_2ATP (2 mM final), diluted to 5 vol with H_2O, and the pH corrected to 7.2 using 20% (w/v) KOH. 4 μM EGTA was added to this buffer to chelate contaminating Ca^{2+}. The dispersed glands were suspended in the final buffer, and 1 μl of oligomycin (5 mg/ml ethanol) and 10 μCi of $^{45}Ca^{2+}$ in 2 ml of incubation medium. Oligomycin was used to exclude Ca^{2+} uptake into the mitochondrial pool. The mixture was quickly vortexed and placed in a 37°C water bath for 30 min for $^{45}Ca^{2+}$ loading. The mixture was then centrifuged at 325 g for 4 min and the pellet was washed twice to remove unincorporated isotope and resuspended in the final buffer. 50 μl of PGE_2 or its solvent carrier was added to each 50 μl of the suspension, followed by incubation at 37°C for 30 seconds. The reaction was stopped by adding 400 μl of silicon oil mixture (silicone fluids 556 and 555 in 3:2, v/v) to separate the aqueous

phase from cell pellet. The cells were centrifuged through the oil at 12000 g for 5 min. The aqueous phase and most of the silicon oil were carefully removed to avoid disturbing the cell pellet or "smear" on the opposite side of the tube. The tubes were inverted on adsorbent paper underlayered with aluminum foil for at least 1 hour to remove the oil. Finally, the cell pellet was solubilized in 50 µl of 0.15 N NaOH and radioactivity of the retained $^{45}Ca^{2+}$ was measured by liquid scintillation counting using the scintillant ScintiSafe^{TM}Econo 2. The percentage release of $^{45}Ca^{2+}$ was expressed as:

$$\%\ ^{45}Ca^{2+}\text{-release} = (T-Y)\ /T \times 100$$

where T was the total radioactivity in the cells before the addition of agonist, and Y was radioactivity retained in the cells at the end of incubation with agonist. The calcium ionophore bromo-A23187 (20 µM final) was used as an internal control to define the total mobilizable cellular Ca^{2+} pools in the $^{45}Ca^{2+}$-preloaded cells.

Assay of $^{45}Ca^{2+}$ influx

The experiments were performed as described by Tokuda *et al.*(1994) with some modifications. The dispersed salivary glands were suspended in a buffer containing 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 1.5 mM $CaCl_2$, 10 mM HEPES, Tris (pH 7.4), 5 mM glucose, 0.1% bovine serum albumin (BSA) and preincubated at 37°C for 10 min. 200 µl of the cell suspension was then stimulated by 10^{-5}M PGE_2 in the same buffer containing 1 µCi of $^{45}Ca^{2+}$ at various time intervals. After washing three times with 1 ml of the same ice-cold assay buffer without $CaCl_2$ and 0.1% BSA, cell pellets were solubilized

in 50 μl of 0.15 N NaOH, and the cell-associated radioactivity was measured by liquid scintillation counting using the scintillant ScintiSafe™Econo 2.

Statistical Analysis

The differences between the control and experimental treatment were tested for significance by Student's t test. A P value <0.05 was considered significant.

RESULTS

Adenylate Cyclase Activity

Since PGE_2 and its analog 17-phenyl trinor PGE_2 were shown to affect intracellular cAMP formation and the PGE_2 receptor is coupled to a cholera toxin-sensitive G protein which is often linked to activation of adenylate cyclase, I tested whether PGE_2 had a direct effect on adenylate cyclase activity in the tick salivary glands. Although PGE_2 affects cAMP concentrations in whole salivary glands, various concentrations of PGE_2 (10^{-9} M~10^{-5} M) had no effect on plasma membrane adenylate cyclase activity (Table 4.1). High adenylate cyclase activity was observed with dopamine, thus, demonstrating that the assay system was valid.

Effect of PGE_2 on the Formation of inositol 1,4,5-trisphosphate (IP_3)

Thirty seconds of stimulation by PGE_2 increased the amount of IP_3 in dispersed salivary glands in a dose-dependent manner (Fig. 4.1). The maximum effect of PGE_2 occurred at 10 μM. The result suggests that PGE_2 activates a phospholipase C (PLC) and causes the

hydrolysis of phosphatidylinositol 4,5-bisphosphate in tick salivary glands after binding to its receptor.

Effect of PGE_2 on $^{45}Ca^{2+}$-Release

IP_3 is known to mobilize calcium from intracellular stores in a variety of cell types (Berridge, 1993). Since the previous results indicated that PGE_2 stimulated the formation of intracellular IP_3, I examined the ability of PGE_2 to stimulate release of intracellular $^{45}Ca^{2+}$ from pre-labelled salivary glands. PGE_2 increased $^{45}Ca^{2+}$ release from the $^{45}Ca^{2+}$-preloaded cells in comparison to control cells stimulated by the solvent carrier (Fig. 4.2).

Effect of PGE_2 on the $^{45}Ca^{2+}$ Influx

In order to see if PGE_2 increases an influx of calcium, the dispersed salivary gland cells were exposed to PGE_2 (10^{-5} M) for different times at 37°C. The result showed that PGE_2 did not stimulate $^{45}Ca^{2+}$ influx during an extended time period (Fig. 4.3). In contrast, PGE_2 decreased $^{45}Ca^{2+}$ uptake by the salivary glands as compared to the control (solvent carrier of PGE_2 solution).

DISCUSSION

Although PGE_2 has been shown to increase dopamine-stimulated cAMP formation, results indicate that PGE_2 does not directly activate salivary gland adenylate cyclase. The reason for this apparent inconsistency is unclear, but it could be due to an effect of mobilized Ca^{2+}

on adenylate cyclase activity, since Ca^{2+} is known to affect adenylate cyclase in many cells (Tsunoda, 1993).

PGE_2 was shown to stimulate IP_3 formation in tick salivary glands. Cells produce IP_3 after activation of a specific phosphatidylinositol 4,5-bisphosphate phospholipase C (PLC). In some cases, receptors are coupled through G proteins to stimulate phospholipase C-β (PLC-β), whereas in other cells tyrosine kinase-linked receptors are specifically coupled to PLC-γ. These receptors activate PLC to hydrolyse phosphatidylinositol 4, 5-bisphosphate (PIP_2), located in the inner leaflet of the plasma membrane, to yield both IP_3 and diacylglycerol (DAG), which function as second messengers (Berridge, 1993). The pathway of IP_3 formation in tick salivary glands appears to be through PLC-β since the PGE_2 receptor is coupled to a G protein. IP_3 was proposed as a second messenger more than one decade ago (Streb *et al.*, 1983). It is now clear that IP_3 released into the cytosol mobilizes calcium from internal stores, particularly the endoplasmic reticulum, by binding to a specific receptor (Berridge, 1993). IP_3-stimulated release of calcium controls a variety of cellular processes including secretion, metabolism, phototransduction, and cell proliferation (Berridge and Irvine, 1984). A phosphoinositide signaling pathway activated by a peptide from the tick synganglion is known to exist in tick salivary glands (McSwain *et al.*, 1989). The effects of IP_3 on intracellular Ca^{2+} mobilization in permeabilizied tick salivary glands has been observed (Roddy *et al.*, 1990). The effect of PGE_2 on IP_3 formation indicates that IP_3-calcium signalling pathway occurs in response to activation of PGE_2 receptors in tick salivary glands. The result is consistent with the previous findings

of the effectiveness of 17-phenyl trinor PGE_2 on fluid secretion. 17-phenyl trinor PGE_2 is a potent and selective agonist for PGE_2 receptor subtype EP1 in mammals (Johnson *et al.*, 1980). Stimulation of EP1 by PGE_2 induces an increase in intracellular Ca^{2+} concentration in mammalian cells (Narumiya, 1996).

Other experiments were performed to examine if a correlation exists between stimulation of salivary gland cells by PGE_2 and an increase in cytosolic Ca^{2+} concentration. An increase in cytosolic Ca^{2+} concentration could occur via two mechanisms: release from intracellular stores and entry across the plasma membrane through Ca^{2+} channels. I tested the effects of PGE_2 on both of these possibilities. $^{45}Ca^{2+}$ influx assays demonstrate that PGE_2 does not stimulate an influx of external calcium into salivary gland cells. However, PGE_2 does increase $^{45}Ca^{2+}$ release from $^{45}Ca^{2+}$ preloaded salivary gland cells, further demonstrating that the signal transduction for the PGE_2 receptor in tick salivary gland includes activation of a phosphatidylinositol 4,5-bisphosphate PLC, increase in cytosolic IP_3 and mobilization of intracellular Ca^{2+}.

Table 4.1 Comparison of the effects of PGE_2 and dopamine on adenylate cyclase in salivary gland membranes. The data are means determined from three assays. * indicates significantly different from basal level of adenylate cyclase activity (P <0.05). Basal level of adenylate cyclase activity was 197 pmoles/mg/min for measuring the effect of PGE_2 and 112 pmoles/mg/min for measuring the effect of dopamine.

Treatment	Concentration (M)	% of basal activity
PGE_2	10^{-9}	97.6
	10^{-8}	97.6
	10^{-7}	98.6
	10^{-6}	98.3
	10^{-5}	98.6
	5×10^{-5}	94.6
Dopamine	10^{-5}	325*

Figure 4.1 Effect of PGE_2 on IP_3 formation in dispersed salivary glands that were prepared from partially fed *Amblyomma americanum* (L.) females as described in the text. The suspensions were then stimulated for 30 seconds at 37°C with the indicated concentrations of PGE_2, and IP_3 was determined by using an IP_3 [^3H] radioreceptor assay kit. The values represent the mean of four determinations ± SD. *: significantly different from control (no PGE_2) ($P<0.05$).

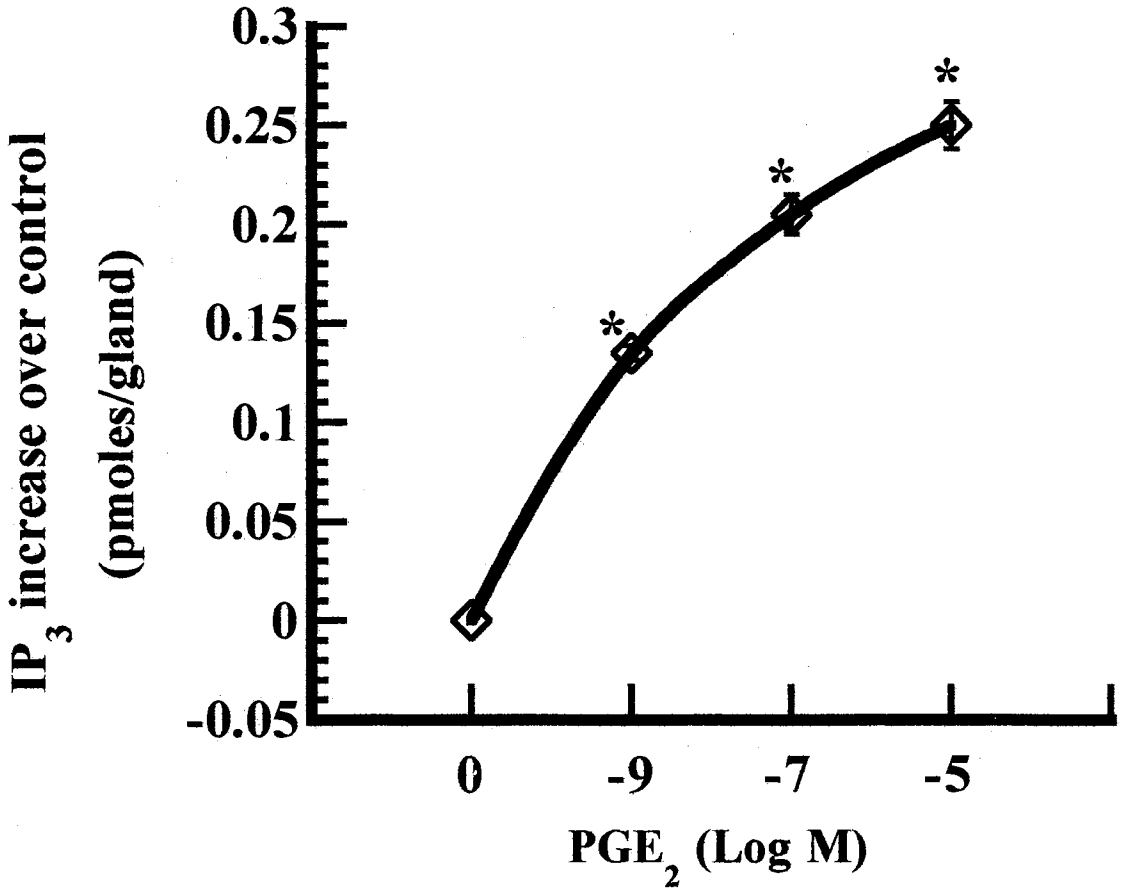

IP3 increase over control
(pmoles/gland)
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
*
*
*
0
-9
-7
-5
PGE2 (Log M)

Figure 4.2 Effect of PGE_2 on $^{45}Ca^{2+}$-release in dispersed salivary glands measured after 30 sec exposure to the indicated agonists. Data are expressed as the mean ± SD of three determinations, each performed in duplicate. *: significantly different from the control (no PGE_2) ($P<0.05$).

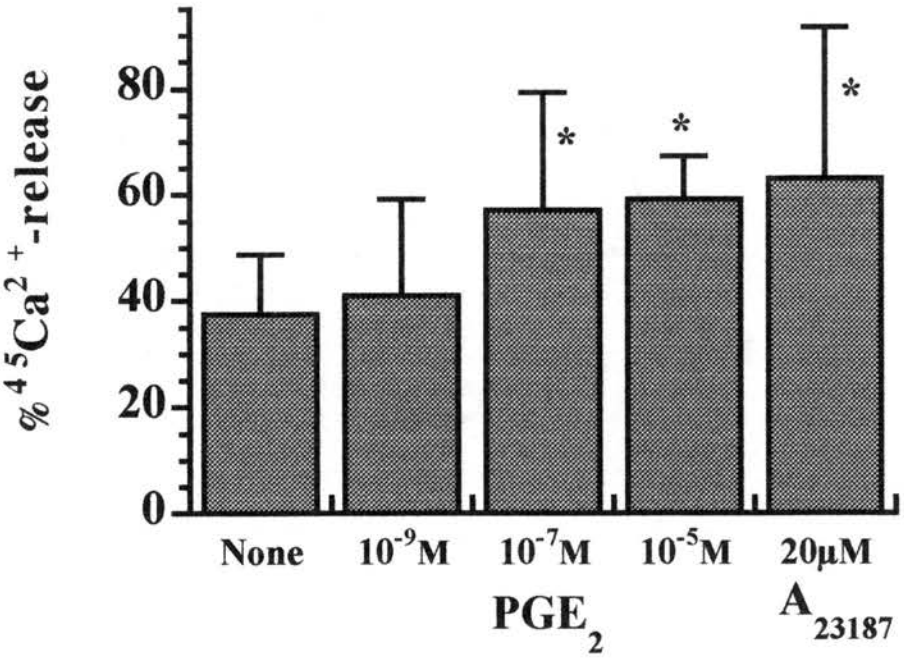
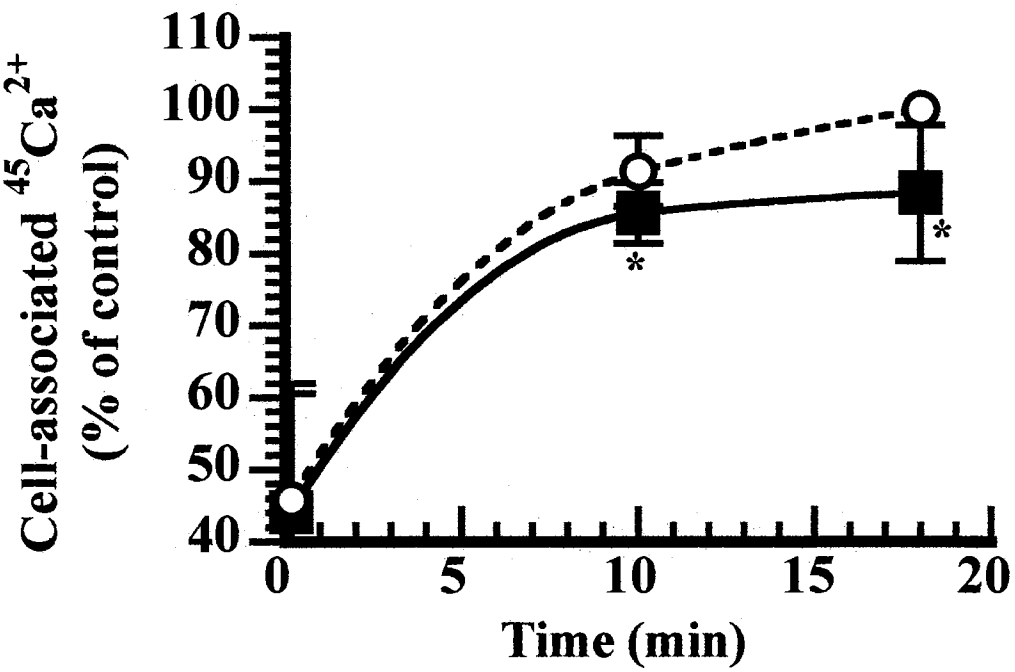

% 45Ca2+-release
80
60
40
20
0
*
*
*
None
10-9M
10-7M
10-5M
20µM
PGE2
A23187

Figure 4.3 Effect of PGE_2 on the uptake of $^{45}Ca^{2+}$ in dispersed salivary glands. Cells were treated with 10^{-5}M PGE_2 (Exp: •) or the solvent carrier (Control: o) for the indicated time periods and the cell-associated $^{45}Ca^{2+}$ was measured. The values are calculated as percent of the uptake of $^{45}Ca^{2+}$ of control cells at 18 min. Each point gives the mean ± SD ($n \geq 4$). *: significantly different from control ($P < 0.05$).


Cell-associated $^{45}Ca^{2+}$ (% of control)
110
100
90
80
70
60
50
40
0
5
10
15
20
Time (min)
*
*

CHAPTER 5

SUMMARY

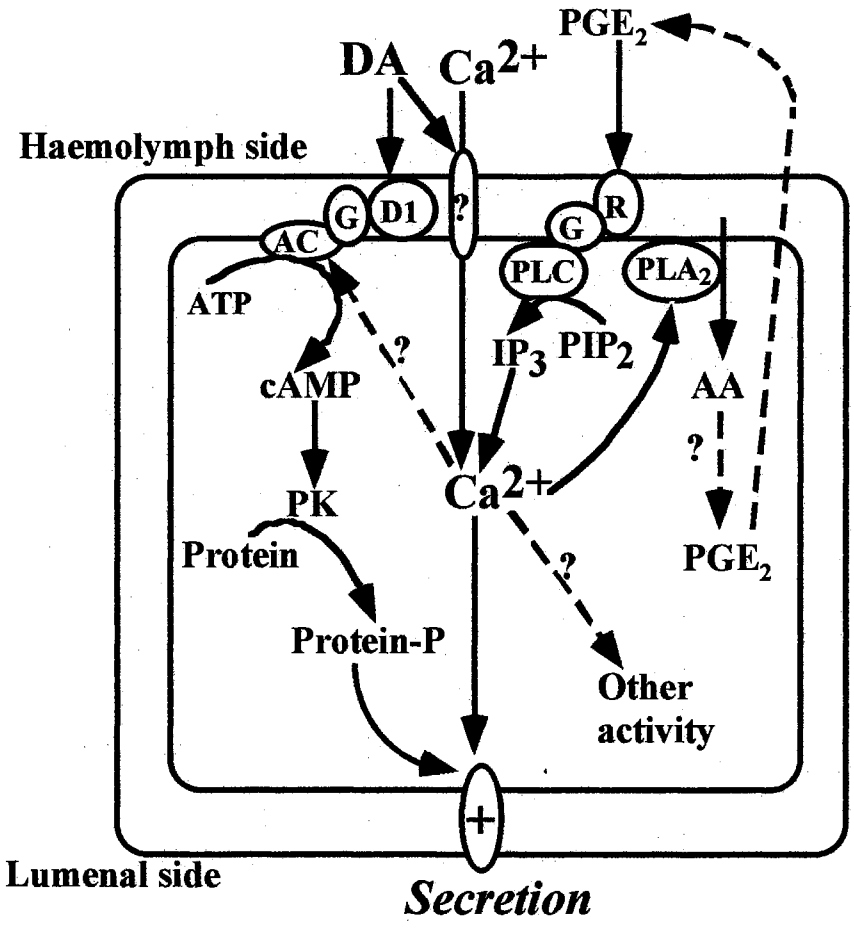
Tick salivary secretion is controlled by the action of the neurotransmitter dopamine through the classic second messenger, cAMP. Dopamine released from a salivary nerve exerts its control via the action of a G protein-coupled receptor (D1 dopamine receptor), which results in the activation of an adenylate cyclase and the formation of cAMP (Sauer *et al.*, 1995). The increased cAMP then leads to augmented fluid secretion of the salivary glands (Needham and Sauer, 1979). Other data indicate that dopamine stimulates release of arachidonic acid presumably by activating an intracellular phospholipase A_2 (PLA_2) (Bowman *et al.*, 1995a). My data provides evidence that the arachidonic acid metabolite PGE_2 plays a role in modulating dopamine-induced fluid secretion in tick salivary glands. Inhibitors of prostaglandin synthesis reduce dopamine-stimulated secretion and exogenous PGE_2 significantly enhances dopamine-induced secretion inhibited by an inhibitor of PLA_2 (OPC). The cellular mechanisms by which PGE_2 enhances dopamine-stimulated fluid secretion have been examined in biochemical studies measuring cAMP generation in isolated salivary glands. PGE_2 increases cAMP accumulation in the isolated salivary glands after dopamine-stimulated accumulation is inhibited by OPC. Furthermore, a high affinity, cholera toxin-sensitive PGE_2 receptor has been identified in tick salivary glands, suggesting that PGE_2 exerts its effects by interacting with its specific G protein-linked receptor on plasma membranes in tick salivary glands. However, PGE_2 does not affect

adenylate cyclase directly but rather increases inositol trisphosphate (IP_3) production, presumably by stimulating phosphatidylinositol hydrolysis. Since diacylglycerol is formed together with inositol phosphates via this signal transduction pathway (Nishizuka, 1984; Berridge, 1987), one would expect that protein kinase-C is also activated when PGE_2 binds to its receptor. Diacylglycerol and Ca^{2+} together are known to activate protein kinase C in cells (Nishizuka, 1988). IP_3 stimulates Ca^{2+} mobilization from the intracellular stores and thus raises the levels of intracellular Ca^{2+}. The increased intracellular Ca^{2+} may contribute to modulating cAMP levels induced by dopamine through its effect on adenylate cyclase (Tsunoda, 1993). Thus, it is conceivable that PGE_2 functions in a positive feedback manner, whereby PGE_2 raises intracellular Ca^{2+} and stimulates dopamine-induced fluid secretion. Other functions for increased levels of Ca^{2+} such as exocytosis seem possible. A model for the action of PGE_2 in modulating tick salivary secretion based on these statements is thus advanced (Fig. 5.1).

Now that the properties of PGE_2 receptor have been mapped out biochemically, future attention should focus on molecular biology of the receptor and other possible functions for increases in cellular Ca^{2+}. Cloning of the PGE_2 receptor and elucidating its molecular structures appear to be feasible given that sequences of prostaglandin receptor genes are known in mammals and the effectiveness of 17-phenyl trinor PGE_2 in mimicking the physiological effects of PGE_2 in tick salivary glands. 17-phenyl trinor PGE_2 is thought to be very specific for the Ca^{2+} mobilizing PGE_2 EP1 receptor in mammals (Negishi *et al.*, 1993), being consistent with the ability of PGE_2 to mobilize Ca^{2+} in tick salivary glands.

Molecular data would enable us to identify the tissue and cell distribution of the receptor's expression. Cellular localization of PGE_2 receptors would help discover new functions for PGE_2 in overall tick salivary gland physiology.

Figure 5.1 Model for the action of PGE_2 in modulating tick salivary secretion. Abbreviations include: DA, dopamine; D1, dopamine receptor subtype; G, G-protein; AC, adenylate cyclase; PK, protein kinase; Protein-P, phosphorylated protein; R, prostaglandin E_2 receptor; PLA_2, phospholipase A_2; AA, arachidonic acid; PGE_2, prostaglandin E_2. PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate. Dash line and "?" indicate uncertainty.


PGE2
DA
Ca2+
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AA
?
Ca2+
PK
Protein
?
PGE2
Protein-P
Other
activity
+
Lumenal side
Secretion

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