CHAPTER 2

SIGNALING PATHWAYS REGULATING PROTEIN SECRETION FROM THE SALIVARY GLANDS OF UNFED FEMALE *Ornithodoros savignyi*

2.1. INTRODUCTION

2.1.1. General anatomy of tick salivary glands

The salivary glands are the largest glands in the tick's body. They are complex, heterogeneous organs. In both argasid and ixodid ticks, the salivary glands consist of a pair of grape-like clusters of acini (alveoli) comprising 2 major types, the agranular and granular acini (Sonenshine 1991). A myo-epithelial sheath surrounds the salivary glands of argasid ticks, while it is absent in ixodid ticks (Figure 2.1). The salivary glands surround the paired salivary ducts, which extend through the basis capituli and open into the salivarium (Sonenshine 1991). It is via these ducts that tick saliva gets secreted into the host's bloodstream and allow for successful feeding.
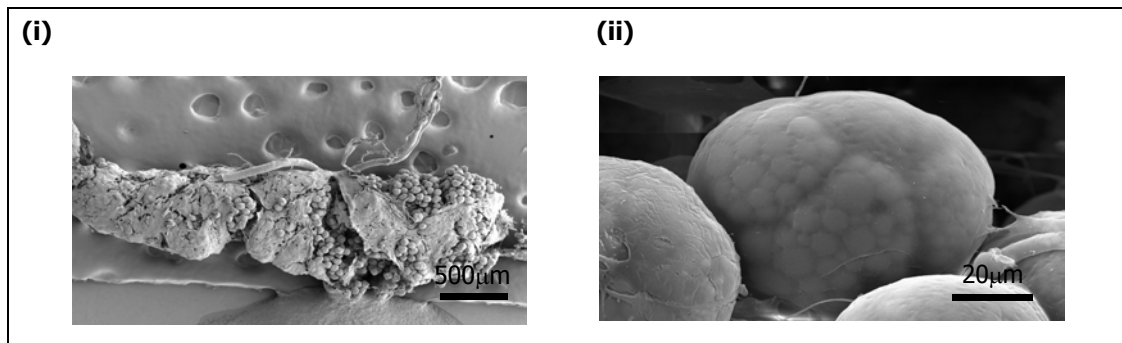

Figure 2.1. SEM analysis of salivary glands from *O. savignyi (Mans 2002a). (i) An intact salivary gland with the myo-epithelial sheath visible, as well as single acini (40-70 μM diameter). Scale bar = 500 μm. (ii) An acini at high electron voltage (30 keV) show the packing of the salivary gland granules through the cell membrane. Scale bar = 20 μm.*

The organization of the salivary glands of ixodid ticks has been best described for the adult female *Rhipicephalus appendiculatus*. In these glands, there are about 1400 acini of three types (I, II, III) in contrast with the 1350 acini of four types (I, II, III, IV) found in males (Sauer and Hair 1986). In both the ixodid and argasid ticks, the agranular acini (type I) occur near the anterior end of the gland and are located adjacent to the main salivary duct. These acini open directly into the main duct. In contrast, the granular types II, III and IV

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acini occur more posteriorly and comprise grape-like clusters surrounding secondary ducts that ramify among lobes. Most of the granular acini open into secondary ducts (Sonenshine 1991). The most important features of these acini are summarized in Table 2.1.

Table 2.1. General features of female ixodid tick salivary gland acini ((Sonenshine 1991).

Acini Type	Function	Location in acinus
I (Agranular)	Osmoregulation	Along the main duct
II (Granular)	Secretion (Granule containing)	Arranged radially around a small lumen
III (Granular)	Secretion (Granule containing)	Terminal branches of the duct at the periphery of the gland

Type I acini consist of a single central cell and a number of peripheral cells, also called the pyramidal cells. Both of these cell types contain an abundance of mitochondria, an inconspicuous Golgi complex, and little or no endoplasmic reticulum. The cytoplasm is poor in ribosomes but contains widely dispersed α and β particles of glycogen. No granules are present but small dense lysosome-like bodies and lipid droplets of various sizes are common. This acinus has been reported to show little change in structure during tick feeding. Support for their function in osmoregulation came from studies by Needham and Coons who indicated significant differences in acini I between dehydrated and rehydrated ticks (Sauer and Hair 1986).

Type II acini contain a bewildering diversity of glandular cell types. Binnington has designated these in 1978 as a, b, c_1, c_2, c_3 and c_4 in the tick *Boophilus microplus* (Sauer and Hair 1986). Their identification at light microscope level was largely based upon size, staining properties and histochemical reactions of their secretory granules. In 1983, Binnington reported that the gland of *R. appendiculatus* was similar to that of *Boophilus* with 6 granular cell types in acinus II (Sauer and Hair 1986). The most important features of these 6 cell types are summarized in Table 2.2.

Type III acini are the best studied, since they are the site of sporogony of *Theileria parva*, the protozoan parasite that causes African East Coast fever. The acinus contains three glandular cell types (d, e and f), and their characteristics are summarized in Table 2.3.

Table 2.2. General features of the cell types found in the type II acinus of the ixodid tick, *R. appendiculatus* (Sauer and Hair 1986).

Cell type	Granule characteristics	Location
a	- Large compound granules visible with light microscope - Large membrane-bound secretory granules (3 μm) with round subunits (0.5 μm) - Subunits are pale and matrix darker - Stain negative with PAS for carbohydrates - Secrete a component of the cement	Hilus of acinus
b	- Stain intensely with PAS - Large secretory granules (up to 2 μm) - Granules exhibit inhomogeneity	Adjacent to a-cell
C_1	- Small, PAS positive granules (up to 0.75 μm) - Affinity for basic dyes - Exhibit most esterase activity in acinus - Lowest density of c-series during electron microscopy - Hypertrophy in course of feeding - May have a very large nucleus with multiple nucleoli	Fundus of acinus
C_2	- Large secretory granules (up to 2 μm) - Pale pink PAS staining reaction - Granules are homogenous and moderately dense	Polar or equatorial region of acinus, adjacent to an a-cell.
C_3	- Granules are smaller than C_2 (up to 1.7 μm) - Stain more intense with PAS - Smooth-contoured and more dense than c2-granules	
C_4	- Small granules (0.75 - 1 μm) - Stain intensely with PAS - Electron dense	Interspersed among C_1-cells at the fundus of the acinus

Table 2.3. General features of the cell types found in the type III acinus of ixodid ticks (Sauer and Hair 1986).

Cell type	Granule characteristics	Location
d	- Secretory granules (up to 3 μm) with large subunits - Density of subunits are inconsistent - Secretory product accumulates during questing. In unfed ticks large numbers of granules are found throughout the cell. - Cytoplasma is rich in mitochondria and narrow cisternal profiles of endoplasmic reticulum. - Golgi is rarely observed - Secrete cement at onset of feeding - Little evidence of continuing synthetic activity during feeding	Around hilus of acinus
e	- Largest granules (4 μm) in the gland - Endoplasmic reticulum cisternae occupy most of cytoplasma - Granules are of low density and composed of closely packed subunits (60 - 100 nm) - Also contain denser granules (100-150 nm diameter) scattered around in cytoplasma - Secrete during feeding and the cells remain active and provide an opportunity to study the role of the organelles in the formation of secretory granules.	Dominant cell type in type III acinus
f	In unfed ticks: - These cells are slender and undifferentiated - Cytoplasm is rich in ribosomes - Few membranous organelles present - No secretory granules are present In feeding ticks: - Cells rapidly enlarge - Differentiate into typical glyco-protein secretory cells - Extensive endoplasmic reticulum becomes visible - Prominent Golgi complex forming dense secretory granules are evident - Only active secretion for 2 days before protein synthesis machinery is dismantled by autophagy. After autophagy: - Body of each f-cell is surrounded by an intricate filigree of slender cell processes forming the ablumenal portion of a very elaborate basal labyrinth. - The ablumenal interstitial cells hypertrophy and undergo proliferation of their mitochondria. They increase in size and elongate, extending towards the periphery of the acinus. - Both the f-cells and the ablumenal interstitial cells terminate in contact with the membrane. - The glandular epithelium of acinus III has thus been converted to an epithelium of unparalleled complexity specialized for fluid transport.	Clustered at the fundus of acinus III

Classical studies performed in 1972 by Roshdy on the glands of *Argas persicus* indicated that at least three granular cell types (a, b and c) are present in the salivary glands of argasid ticks (Roshdy 1972). Roshdy and Coons confirmed this data in 1975 during ultra-structural studies of the salivary glands of *Argas arboreus* (Roshdy and Coons 1975). El Shoura

described similar granular types in 1985 during ultra-structural studies of the salivary glands of *Ornithodoros moubata* (El Shoura 1985).

During recent studies on the argasid tick *Ornithodoros savignyi* four cell types was described (Mans 2002a). Cell type 'a' contains dense core granules (diameter 3 - 5 μm), which stains positive for the proteins apyrase and savignygrin (Figure 2.2.i). The granular content of these cells are released during feeding. Type 'b' cells contain homogenous granules (diameter 4 – 10 μm), which could be immature precursors of the dense core granules. These granules also stained positive for apyrase and savignygrin (Figure 2.2.ii). Type 'c' cells contain smaller granules (diameter 1 – 2 μm) with electron lucent cores. They stained positive for carbohydrates during the Thiery test. Finally, a fourth cell type (d) was described. These cells have a similar morphology and histochemical basis as type b-cells but stained negative for savignygrin.
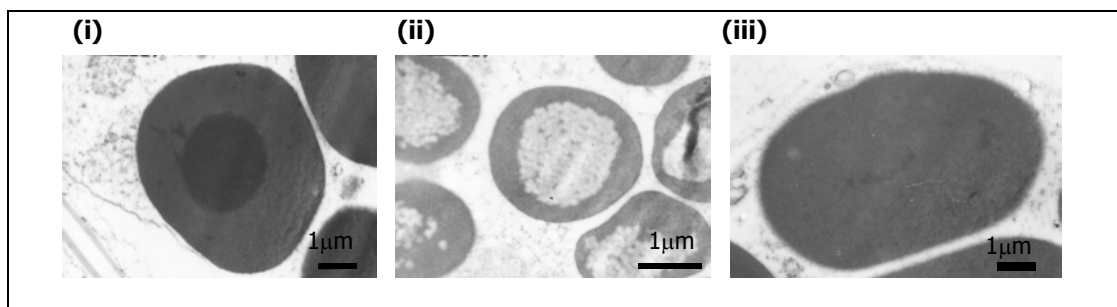

Figure 2.2. TEM micrographs of the granules of type II granular alveoli (Mans 2002a). *(i) Type a cell granules consist of a peripheral carbohydrate region and a dense protein core. (ii) Type b cells are composed of a dense, finely textured material that stains positively for polysaccharides, while the granules show a negative reaction for protein. (iii) Type C cell granules possess electron-dense and electron-lucent zones.*

During feeding of *O. savignyi*, it could be shown that all cells that displayed granule release retained several granules. Also organelles such as mitochondria not previously observed in these cells became prominent. After feeding, the glands resumed the general morphology observed for unfed ticks within one day, except for a dilated lumen that was still visible (Mans 2002a).

2.1.2. Extracellular stimuli

The group of Schmidt, Essenberg and Sauer described the first evidence for neuronal control of fluid secretion from tick salivary glands in 1981, when a D_1-type dopamine receptor was identified in *Amblyomma americanum* (Schmidt *et al.* 1981). To date, 5 types of dopamine

receptors are known (Table 2.4) (Watling 2001). The D_1-dopamine receptor is known as a stimulating G-protein (G_s) that is associated with the activation of adenylyl cyclase (AC) and causes elevated levels of intracellular cAMP upon binding of its ligand, dopamine.

Table 2.4: Structural classification of dopamine receptors (Watling 2001).

Type	D_1	D_2	D_3	D_4	D_5
Structural Information	446 aa (human)	Short: 414 aa (human) Long: 443 aa (human)	400 aa (human)	386 aa (rat)	477 aa (human)
Family	D1	D2	D2-like family	D2-like family	D1-like family
Mechanism	**Gs** Increase cAMP	**Gi** cAMP modulation **Gq/11** Increase IP_3 / DAG	**Gi** cAMP modulation	**Gi** - cAMP modulation - ↑ Arachidonic acid release - Stimulate phospholipid methylation	**Gs** Increase cAMP

Dopamine is a neurotransmitter synthesized from the amino acid tyrosine (Figure 2.3). It is also the substrate for dopamine β-hydroxylase for the synthesis of norepinephrine, which is converted to epinephrine by the enzyme phenylethanolamine N-methyltransferase. AC from the salivary glands of *Amblyomma americanum* is stimulated by several derivatives of phenylethylamine, dopamine, noradrenaline, adrenaline and isoproterenol (a β-adrenergic agonist). Octopamine and L-DOPA have no effect on basal adenylate cyclase activity. Dopamine has the highest potency and the lowest k_a (0.4 μM), followed by adrenaline and noradrenaline (23 μM) and isoproterenol (0.15 mM). The most potent inhibitors of gland AC activity are the dopamine receptor antagonists. The phenothiazine drugs (thioridazine, chlorpromazine and fluphenazine) are more effective than the butyrophenone drug (haloperidol). The k_i for the phenothiazine drugs are 60 nM for thioridazine, 1.9 μM for chlorpromazine and 2.3 μM for fluphenazine. The inhibition of AC activity is specific for the (+) enantiomer of butaclamol (a stereospecific dopamine receptor antagonist), suggesting that the Lone Star tick AC has a D_1 type dopamine receptor (Schmidt *et al.* 1981).
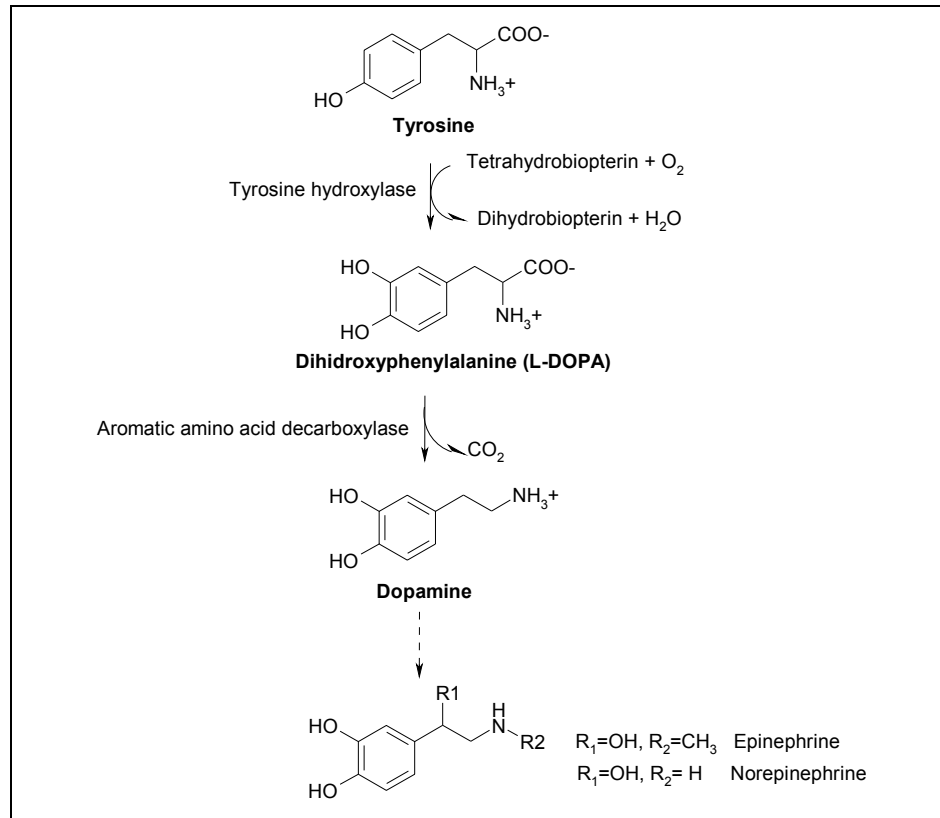
Figure 2.3: Biosynthesis of the physiologically active amines dopamine, epinephrine and norepinephrine (Voet and Voet 1995).

Apart from dopamine, other effectors of signal transduction pathways could also elicit oral secretion from the salivary glands of *A. americanum*. Overall, the volume of oral secretion produced by partially fed ticks in response to the effectors and pharmacological agents varied widely. The volume and rate of oral secretion stimulated by pharmacological agents were: pilocarpine > (dopamine and theophylline) = (dopamine, theophylline and GABA) > (dopamine, theophylline and phorbol 12 myristate 13-acetate; an activator of phospholipase C) (McSwain *et al.* 1992a).

Pilocarpine is often used to induce tick oral secretion, and it is believed to stimulate cholinergic receptors in the tick synganglion, which relay information to nerves innervating the salivary glands. Cholinergic agents fail to stimulate secretion in isolated salivary glands (McSwain *et al.* 1992a). Theophylline, GABA as well as the phorbol esters had no effect when injected individually. Co-injection of the phorbol ester PMA with dopamine and theophylline was inhibitory, indicating an essential role for PKC in eliciting oral secretion.

SDS-PAGE analysis of the secreted proteins showed variable results in response to different agonists. The highest number of proteins was detected from ticks in the earliest stages of feeding. In some ticks, proteins were identified at one collection time but not at another time in secretions from the same tick stimulated by the same agent. One such an example is pilocarpine, which stimulated secretion of different proteins in the same tick after multiple injections. In the case of dopamine, theophylline and GABA, protein secretion was consistent (McSwain *et al.* 1992a).

Other evidence indicates that Ca^{2+} is essential for secretion. Removal of Ca^{2+} from the support medium greatly inhibits dopamine and cAMP-stimulated secretion from isolated salivary glands (McSwain *et al.* 1992a). This is similar to results obtained in salivary duct cells of the cockroach, *Periplaneta americana*. In this study, dopamine evoked a slow and reversible dose-dependent elevation in $[Ca^{2+}]_i$ in salivary duct cells. The dopamine-induced elevation in $[Ca^{2+}]_i$ is absent in Ca^{2+}-free saline and is blocked by La^{3+}, indicating that dopamine induces an influx of Ca^{2+} across the basolateral membrane of the duct cells. Stimulation with dopamine depolarizes the basolateral membrane and is also blocked by 100 μM La^{3+} and abolished when the Na^+ concentration in the solution is reduced from physiological concentrations to 10 mM (Lang and Walz 1999).

GABA, as mentioned before, does not stimulate secretion by itself (McSwain *et al.* 1992a). The enhancing effect of GABA on dopamine-stimulated fluid secretion of isolated salivary glands occurred only at high concentrations (Lindsay and Kaufman 1986). Activation of another receptor by GABA may potentiate secretion by another, but poorly understood, mechanism. A model was proposed and experimentally investigated by Linday and Kaufman in 1996 (Figure 2.4). Their data indicated that GABA and spiperone binds to the same receptor, and that this reaction can be inhibited by sulpiride without diminishing the effect of dopamine. Another receptor, the so-called ergot alkaloid sensitive receptor, which can also be blocked by sulpiride without altering the response to dopamine, was also detected but its function remains unknown. In conclusion, the authors suggested that GABA might play an important neuromodulatory role in salivary fluid secretion (Lindsay and Kaufman 1986). Interestingly, spiperone is also a subtype selective antagonist for the 5-HT$_{1A}$ serotonin receptor, and it has been reported that serotonin, a known agonist of salivary secretion in the insect, *Calliphora erythrocephala*, inhibits basal cyclase activity (Schmidt *et al.* 1981;

Watling 2001). Therefore, the possible inhibitory effect of serotonin on the GABA activated signaling pathway should be investigated.
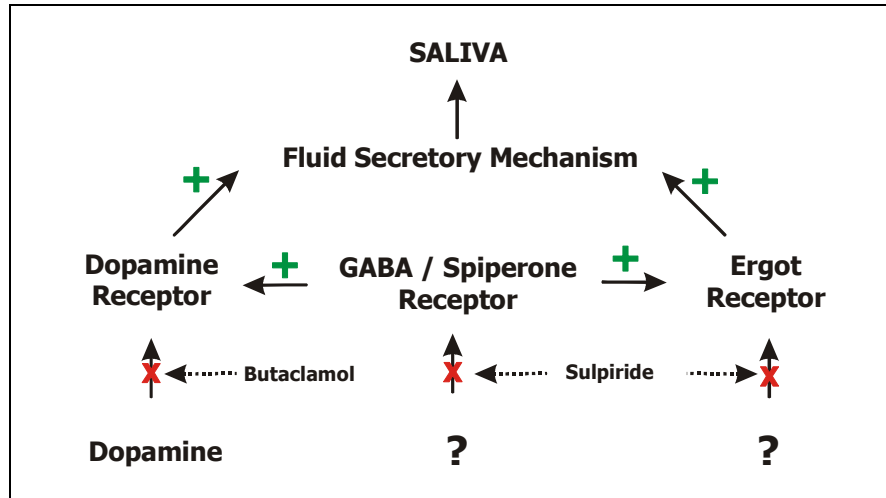

Figure 2.4. A model to demonstrate the receptors involved in salivary fluid secretion in ixodid ticks (Lindsay and Kaufman 1986). *A spiperone receptor in some way modulates the dopamine and ergot alkaloid receptors, resulting in an increased maximum response. Because spiperone has no intrinsic activity, its receptor has no direct link to the fluid secretory mechanism.*

2.1.3. Adenylyl cyclase and cAMP

As mentioned before, it has been demonstrated in ixodid ticks that the dopamine D_1-receptor activates adenylyl cyclase (AC) activity (Schmidt *et al.* 1981). This activation is most likely through the dopamine surface receptor linked via heterotrimeric ($\alpha\beta\gamma$) stimulatory (G_s) guanine nucleotide-dependent regulatory proteins (G proteins). To date, ten distinct adenylyl cyclase isozymes have been identified, and all but one isozyme is activated by $G\alpha_s$. Activation by $G\alpha_s$ occurs through its interaction with the C_2 domain of AC, yielding the active enzyme GTP•α_s•C. Inhibition by G proteins may occur by a direct effect of $G\alpha_I$ with the C_1 domain of AC or by the recombination of $\beta\gamma$ with $G\alpha_s$ (Watling 2001).

A substrate binding cleft is formed between the C_1•C_2 domains upon activation of AC (Watling 2001). The active site catalyzes a cation-dependent attack of the 3'-OH on the α-phosphate of a nucleoside triphosphate (ATP), with pyrophosphate as the leaving group. cAMP subsequently binds to a cAMP-dependent protein kinase (R_2C_2) whose catalytic subunit (C), when activated by the dissociation of the regulatory dimer as R_2•cAMP$_4$, activates various cellular proteins by catalyzing their phosphorylation (Figure 2.5).
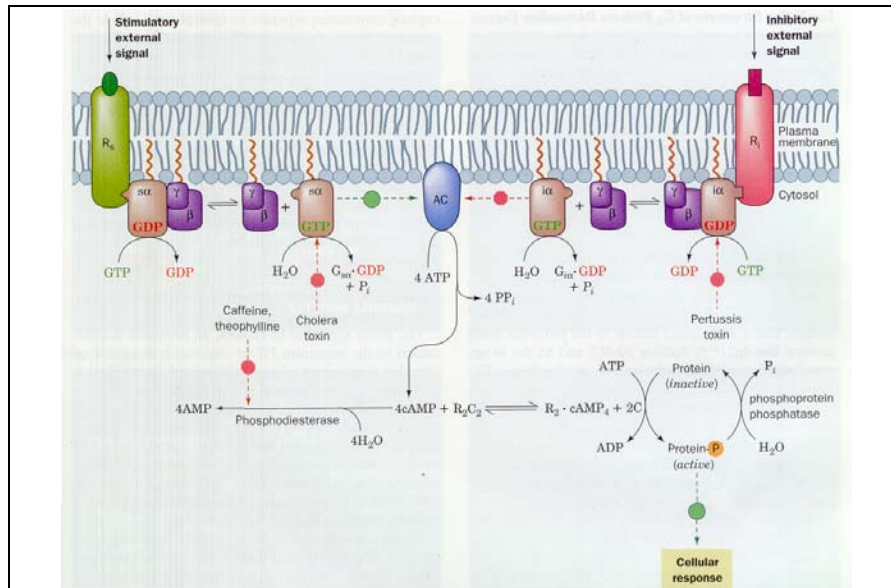
Figure 2.5. The mechanism of receptor-mediated activation / inhibition of adenylyl cyclase (Voet and Voet 1995). *The binding of hormone to a stimulatory receptor (Rs) induces it to bind Gs protein, which in turn stimulates the Gsα subunit of this hetero-trimer to exchange its bound GDP for GTP. The Gsα•GTP complex then dissociates and stimulates adenylyl cyclase to convert ATP to cAMP. Binding of hormone to an inhibitory receptor (Ri) triggers an almost identical chain of events except that the Giα.GTP complex inhibits AC from producing cAMP. R_2C_2 represents a cAMP-dependent protein kinase whose catalytic subunit (C), when activated by dissociation of the R_2•cAMP complex, activates various cellular proteins by means of phosphorylation.*

Studies regarding the cAMP mediated phosphorylation of endogenous proteins in the ixodid tick *A. americanum* indicated 12 proteins. Ten of these were dephosphorylated after dopamine stimulation was attenuated with the dopaminergic antagonists thioridazine and d-butaclamol. The most prominent proteins were those with molecular weights of 62, 47 and 45 kDa. By comparing the proteins phosphorylated upon stimulation with respectively, dopamine and cAMP, 7 proteins (148, 102, 62, 55, 47, 45 and 37 kDa) were identical. Therefore, it could be concluded that a dopamine-sensitive AC is present (McSwain *et al.* 1985). Later studies by Mane *et al* in 1985 and 1987 resulted in the identification of the cAMP-dependent protein kinase, as well as the kinetics of the phosphotransferase reaction of the catalytic subunit of the kinase from the salivary glands of *A. americanum* (Mane *et al.* 1985; Mane 1987).

In most eukaryotes, cAMP-dependent kinases (PKA or cAPK) occur as two enzymes (type I and II). These exist as tetramers (R_2C_2), consisting of two regulatory- and two catalytic subunits (Table 2.5). In the absence of it's activating ligand cAMP, PKA exists as an inactive holoenzyme, which can be anchored to specific compartments via interaction of their

regulatory subunits with specific PKA anchoring proteins. Upon binding of four cAMP molecules to the regulatory subunits, the catalytic subunits are released. These then catalyze the transfer of the γ-phosphate of ATP to serine and threonine residues of many cellular proteins containing the –R-R/K-X-S/T consensus sequence. It must be noted that exceptions to this consensus sequence have been observed (Watling 2001).

Table 2.5: Structural classification of Protein kinases A / cAMP-dependent kinases (Watling 2001).

Name	PKA I (RI$_2$C$_2$) and PKA II (RII$_2$C$_2$)		
Structural Information	Tetramer of 2 regulatory (R) and two catalytic (C) subunits		
	RI RIα: 381 aa RIβ: 381 aa	**RII** RIIα: 404 aa RIIβ: 418 aa	**C** Cα: 351 aa Cβ: 351 aa Cγ: 351 aa
Subcellular localization	Cytoplasm	Cytoskeletal structures, Organelles, membranes	_
Activators	cAMP	cAMP	_

Three isoforms of cAMP-dependent kinase catalytic subunits have been identified in the tick, *A. americanum,* to date (Palmer *et al.* 1999). These are believed to be the product of alternative RNA processing of a single cAPK-C gene. The cDNAs contain unique N-termini of variable lengths, which are linked to a common region containing the αA helix, catalytic core, and a C-terminal tail (Figure 2.6). The common region is highly similar to both insects and vertebrate cAPK-Cs. Although three isoforms have been identified, only a single cAPK isoform (i.e. cAPK-C) is expressed in the salivary glands of both unfed and feeding female ticks (Palmer *et al.* 1999).
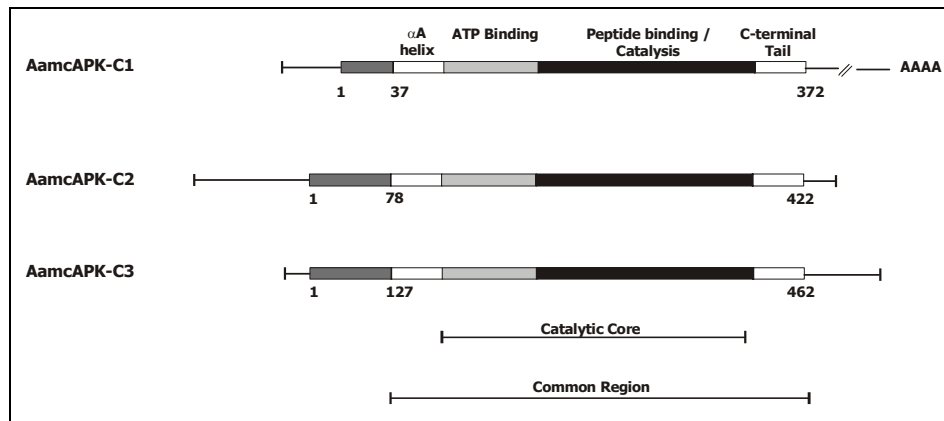
Figure 2.6. Schematic representation of *A. americanum* cAPK-cDNAs and proteins (Palmer *et al.* 1999). *Structure of the three AamcAPK-C cDNAs containing unique 5' termini linked to a common region that includes 1108 bp of open reading frame and the 3' UT sequences. The translated sequences contain unique amino termini of 37, 78 and 127 amino acids that precede a common region containing the catalytic core. Shown above are the αA helix that precedes the catalytic core, the regions involved in ATP binding and peptide binding and catalysis, and the C-terminal tail.*

2.1.4. Prostanoids

Prostanoids were first discovered in the 1930s, when it was shown by Von Euler that semen compounds are able to lower blood pressure in animals (Versteeg *et al.* 1999). Today it is known that prostanoids comprise prostaglandins (PGs), thromboxanes (Txs) and the most recent addition, isoprostanes. The synthesis of prostanoids is shown in Figure 2.7, from which it is evident that synthesis is regulated by the release of the precursor lipid arachidonic acid (AA) from plasma membrane phospholipids. Either cytosolic phospholipase A_2 ($cPLA_2$) or the combined action of phospholipase C, a diglyceride- and a monoglyceride lipase, produces AA (Versteeg *et al.* 1999). Since a cytosolic phospholipase A_2 ($cPLA_2$) is essential for exocytosis from the ixodid tick *A. americanum*, some characteristics of the class is given in Table 2.6.

Upon its release, AA is converted by cyclooxygenase (COX) to PGG_2, and subsequently to PGH_2, which is converted to other prostanoids via a specific prostaglandin synthetase. The isoprostanes are synthesized through non enzymatic conversions of arachidonic acid. Five primary prostanoids are distinguished: PGD_2, PGE_2, PGI_2, $PGF_{2\alpha}$ and TXA_2 with the exact metabolites synthesized dependent on the tissue type and stimulus. For instance, TXs, PGD_2, PGI_2, $PGF_{2\alpha}$ are major metabolites in platelets, mast cells, endothelial cells, and kidney glomerulus cells, respectively. In contrast, a broad range of cell types synthesize PGE_2 (Versteeg *et al.* 1999).

Table 2.6: Structural classification of the phospholipases A$_2$ (Watling 2001).

Group	Secreted / Cytosolic	Molecular Weight (kDa)	Cofactor
IA	Secreted	13-15	mM Ca^{2+}
IB	Secreted	13-15	mM Ca^{2+}
IIA	Secreted	13-15	mM Ca^{2+}
IIB	Secreted	13-15	mM Ca^{2+}
IIC	Secreted	15	mM Ca^{2+}
III	Secreted	16-18	mM Ca^{2+}
IV	Cytosolic	85	μM Ca^{2+}
V	Secreted	14	mM Ca^{2+}
VI	Cytosolic	80-85	None
VII	Secreted	45	None
VIII	Cytosolic	29	None
IX	Secreted	14	< mM Ca^{2+}
X	Secreted	14	mM Ca^{2+}

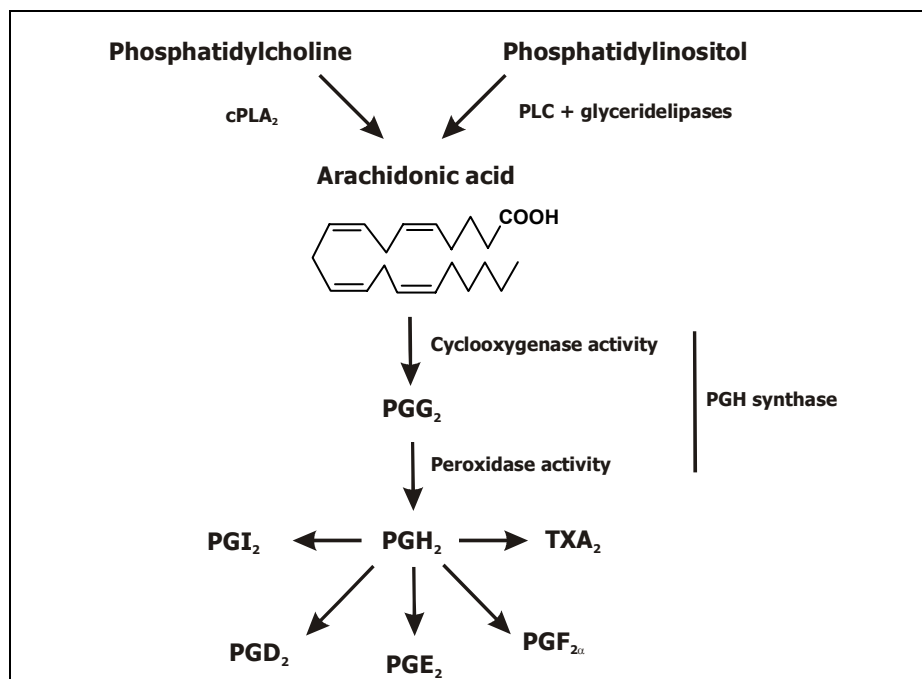**Figure 2.7. Schematic representation of the prostanoid synthesis pathway (Narumiya *et al.* 1999; Versteeg *et al.* 1999).** *Either a cytosolic PLA$_2$ (cPLA$_2$) or the combined effect of phospholipase C (PLC) and glyceridelipases releases arachidonic acid from the plasma membrane. The conversion of arachidonic acid to PGG$_2$ and then to PGH$_2$ are catalyzed by PGH synthase and subsequent conversion of PGH$_2$ to each prostaglandin or thromboxane is catalyzed by the specific prostanoid / thromboxane synthase.*

Prostanoids mediate their effect on cells via prostanoid receptors (see Table 2.7). In general these receptors belong to either the superfamily of the G-protein coupled rhodopsin type receptors, or to the superfamily of nuclear steroid/thyroid hormone receptors (Versteeg *et al.* 1999). All prostanoid-induced effects are transduced either through modulation of the activity of adenylyl cyclase or inositol phospholipid hydrolysis and calcium mobilization (Watling 2001).

Table 2.7: Structural classification of prostanoid receptors (Watling 2001).

	EP1	EP2	EP3	EP4
Structural Information	402 aa (human)	358 aa (human)	4 splice variants 390, 388, 365, 374aa (human)	488 aa (human)
Receptor selective prostanoid	PGE_2	PGE_2	PGE_2	PGE_2
Receptor selective antagonists	None	None	None	None
Signal Transduction Mechanisms	$G_{q/11}$ Increase IP_3/DAG	G_s Increase cAMP	$G_{q/11}$ Increase IP_3/DAG G_i cAMP modulation	G_s Increase cAMP
	DP	**FP**	**IP**	**TP**
Structural Information	359 aa (human)	358 aa (human)	386 aa (human)	343 aa (human)
Receptor selective prostanoid	PGD_2	PGF_{2a}	PGI_2	TxA_2
Receptor selective antagonists	Various	None	None	Various
Signal Transduction Mechanisms	G_s Increase cAMP	$G_{q/11}$ Increase IP_3/DAG	G_s Increase cAMP $G_{q/11}$ Increase IP_3/DAG	$G_{q/11}$ Increase IP_3/DAG

Studies regarding changes in the lipid content of the salivary gland of *A. americanum* during feeding indicated that most of the fatty acids were associated with the phospholipids, with the most abundant phospholipids molecules at all feeding stages being phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

During feeding it was found that arachidonic acid (AA, 20:4) levels increased dramatically, up to 40 times. Interestingly, virgin female ticks (which do not increase to more than 40 mg during feeding) had a percentage AA content similar to fed/mated females (250 mg) despite the fact that mating is required for females to acquire a large bloodmeal (Shipley *et al.* 1993). Similar to other organisms, the majority of 20:4 fatty acids (>83%) were found to be stored in cell membranes esterified at glycerol C2 of phospholipids (the diacyl phospholipids subclass). Shipley et al showed in 1994 that [^{3}H]-20:4 were primarily incorporated into the *sn-2* position of diacyl PC > PE, with some incorporation into triglycerides (Shipley 1994). Later, Bowman *et al.* investigated the origin of AA since most arthropods studied to date are able to synthesize AA from dietary linoleic acid (18:2). It was found that ticks have the ability to synthesize mono-unsaturated fatty acids, but not poly-unsaturated fatty acids such as AA, which are sequestered from the host bloodmeal (Bowman 1995a; Sauer *et al.* 2000). This inability to convert linoleate to arachidonate is highly unusual, and has only been described for the domestic cat, rainbow trout and mosquito, *Culex pipiens* (Bowman *et al.* 1995b). Furthermore, when ticks were fed [^{3}H] arachidonic acid, the radioactivity was incorporated solely into the PC and PE phospholipids fraction of the salivary glands (Bowman 1995a).

The next question involved the biosynthesis of salivary prostaglandins from the obtained bloodmeal arachidonic acid. This is important, since prostaglandins are postulated to aid the tick in overcoming hemostasis, inflammatory responses and host immunity. For example, PGE_2 and prostacyclin (PGI_2) can prevent platelet aggregation and cause vasodilation, which maintains a generous supply of blood flowing to the tick during feeding. Furthermore, PGE_2 and PGI_2 also inhibit mast cell degranulation (which minimizes release of inflammatory mediators) and suppress secretion of interleukin-1 and –2, causing inhibition of T-cell clonal expansion (Shipley *et al.* 1993). In 1993, it was described that *A. americanum* possesses a calcium-sensitive PLA_2 which is capable of increasing free AA levels in the SG following calcium ionophore stimulation (Bowman *et al.* 1993). Prostaglandin synthetase (PGS) activity was also detected in dopamine-stimulated glands. PGE_2 was always the major product together with appreciable quantities of $PGF_{2\alpha}$ and PGD_2 (Bowman 1995d). A later study by Pedibhotla *et al.* describes the subcellular localization of prostaglandin H synthase and prostaglandin synthesis in *A. americanum* (Pedibhotla *et al.* 1995).

Since PGS activity could only be detected after dopamine stimulation, the mechanism for regulating the concentration of free AA by dopamine was investigated by Bowman *et al.* It

was found that dopamine was as effective as the calcium ionophore A23187 in stimulating PLA_2 activity. This effect was abolished in the presence of the calcium channel blocking agent, verapamil, and the PLA_2 substrate analogue, oleyloxyethyl phosphorylcholine, in a dose dependent manner. It was concluded that free AA levels are increased through activation of a type IV-like PLA_2 (due to its insensitivity to merthiolate), following an increase of intracellular calcium caused by the opening of a voltage-gated calcium channel upon dopamine stimulation (Bowman 1995c).

In 1997 a specific PGE_2 receptor was identified in the plasma membrane fraction of the salivary glands of *A. americanum*. The receptor exhibited a single, high affinity PGE_2 binding site with a $K_d \sim 29$ nM, which is saturable, reversible, PGE_2 specific and coupled to a cholera toxin sensitive guanine nucleotide regulatory protein (Qian *et al.* 1997). Since PGE_2 was found not to affect adenylyl cyclase activity similar to EP1 and EP3 (see Table 2.7), it was suggested that the PGE_2 receptor stimulates an alternate pathway. Data obtained from using GTPγS strongly suggested that the tick PGE_2 receptor is linked to a G_s-like, rather than a G_i-protein, but later studies indicated that PGE_2 have no effect on plasma membrane adenylyl cyclase activity (Qian *et al.* 1997).

Since PGE_2 receptors in mammals are known to affect either adenylyl cyclase or the mobilization of calcium, the effects of PGE_2 and inositol 1,4,5-triphosphate (IP_3) were investigated. The results of Qian *et al.* indicated that exogenous PGE_2 does not stimulate an influx of calcium, but resulted in significant secretion of anticoagulant protein at concentrations ranging from 1 nM to 1 μM PGE_2. Lower or higher concentrations of PGE_2 had no effect on protein secretion (Qian *et al.* 1998). Protein secretion was also possible using the PGE_2 EP1 receptor antagonist, AH-6809, indicating an EP1-like salivary gland PGE_2 receptor. Upon PGE_2 stimulation, a rise in IP_3 and intracellular calcium (released from microsomes) were observed. Therefore, the authors hypothesized that PGE_2 is both secreted into the saliva and functions as a local hormone by interacting with a PGE_2 receptor to increase IP_3. IP_3 mobilizes intracellular calcium, which is important for regulating exocytosis of salivary gland proteins (McSwain 1992b; Qian *et al.* 1998). This hypothesis was supported by data obtained in 2000 by Yuan *et al*. They were able to show that the effect of PGE_2 can be inhibited by TMB-8, an antagonist of IP_3 receptors (Yuan *et al.* 2000). Also, it was shown that the PGE_2 receptor increases IP_3 levels via activation of a cholera-sensitive G-protein coupled phospholipase C (PLC).

2.1.5. Phospholipase C and intracellular calcium

The hydrolysis of a minor membrane phospholipid, phosphatidyl inositol 4,5-bisphosphate (PIP_2), by a specific PLC is one of the earliest key events in the regulation of various cell functions by more than 100 extracellular signaling molecules. This reaction produces two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which mediate the activation of protein kinase C (PKC) and intracellular calcium release, respectively (Figure 2.8).
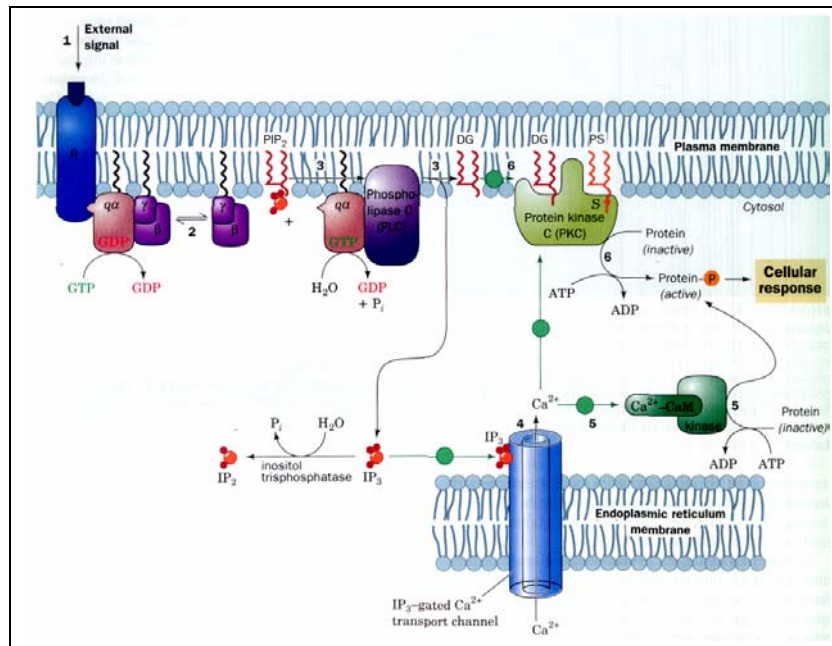
Figure 2.8. A schematic representation of the activation of PLC and the role of PIP_2 in intracellular signaling (Voet and Voet 1995). *Binding of an angonist to a surface receptor activates PLC through a G-protein. In some cases it is via a receptor tyrosine kinase or a non-receptor tyrosine kinase. PLC catalyzes the hydrolysis of PIP_2 to diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3). IP_3 stimulates the release of calcium from the endoplasmic reticulum, which in turn activates numerous cellular processes through CaM and its homologues. DG remains membrane associated where it activates PKC (only in the presence of phosphatidylserine and calcium), which in turn phosphorylates numerous cellular proteins.*

A decrease in PIP_2 itself is an important signal because PIP_2 is an activator of phospholipase D and phospholipase A_2, modulates actin polymerization by interacting with various actin-binding proteins, and serves as a membrane attachment site for many signaling proteins that contain pleckstrin (PH) domains. Consequently, the activity of PLC is strictly regulated through several distinct mechanisms that link PLC isoforms to various receptors (Watling 2001).

In *A. americanum*, a trypsin-sensitive factor from tick synganglion was found to stimulate phosphoinositide hydrolysis in tick salivary glands (McSwain *et al.* 1989). Diacylglycerol, the other product of PLC, activates salivary gland protein kinase C (PKC) in the presence of calcium and phosphatidylserine. Activators of PKC, phorbol 12-myristate 13-acetate (PMA) and 1-oleoyl-2-acetyl-sn-glycerol (OAG) did not stimulate fluid secretion and scarcely affected the ability of dopamine to stimulate fluid secretion and intracellular levels of cAMP (McSwain 1992b; Sauer *et al.* 2000).

2.1.6. Current model for the control and mechanism of secretion in ixodid ticks

A current model for the control and mechanism of secretion from the salivary glands of ixodid ticks is depicted in Figure 2.9. Dopamine is released at the neuroeffector junction and interacts with D1-subtype receptor, which is coupled to a stimulatory guanine nucleotide protein (Gs) that activates adenylyl cyclase to synthesize cAMP. In response to cAMP, a cAMP-dependent protein kinase is activated and numerous gland proteins are phosphorylated, e.g. Aquaporin (AQP-5). The latter then translocates to the plasma membrane and is responsible for fluid transport. Dopamine also opens a voltage-gated calcium channel, allowing the influx of extracellular calcium. This influx of calcium stimulates a cytosolic phospholipase A_2 (cPLA$_2$) to liberate free arachidonic acid (AA). AA is then converted into a variety of prostaglandins, including PGE$_2$, via the cyclooxygenase pathway (COX). High levels of PGE$_2$ are secreted into the host via saliva. Additionally, PGE$_2$ also interacts with a EP1-subtype receptor on the same or neighboring cells. Upon activation of the EP1 receptor, PLC is activated by a G-protein and generates diacylglycerol (DAG) and IP$_3$. The IP$_3$ interacts with and opens the IP$_3$-receptor channel in the endoplasmic reticulum releasing calcium into the cytosol. The increased calcium appears to regulate the exocytosis of anticoagulant protein from secretory vesicles into the saliva.
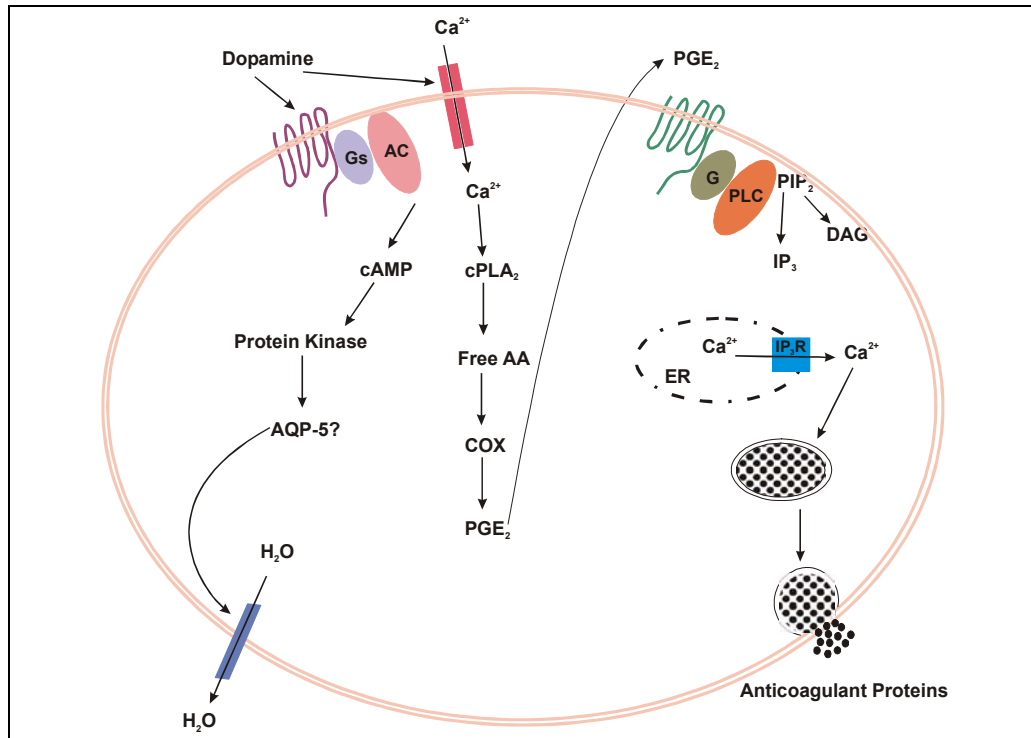
Figure 2.9. Known and hypothesised factors and events controlling secretion in ixodid female salivary glands (Sauer *et al.* 2000). Dopamine released at the neuro-effector junction interacts with a dopamine (D1-subtype) receptor coupled to a stimulatory guanine nucleotide protein (Gs) that activates adenylate cyclase leading to increased levels of cAMP. Numerous gland proteins are phosphorylated by a cAMP-dependent protein kinase. It is speculated that a water channel protein (AQP-5) is phosphorylated, moves to the cell membrane and is responsible for fluid transport observed in dopamine- and cAMP-stimulated glands. Dopamine also opens a voltage-gated Ca^{2+} channel allowing an influx of extracellular Ca^{2+}, thus, increasing intracellular Ca^{2+} levels and stimulating a cytosolic phospholipase A_2 (cPLA$_2$). The free arachidonic acid (AA) liberated by the cPLA$_2$ is converted by the cyclooxygenase pathway (COX) into a variety of prostaglandins, including PGE$_2$. Additionally, on the same or neighbouring cells, PGE$_2$ interacts with an EP1-subtype receptor coupled to a guanine nucleotide protein (G) that activates PLC and generates diacylglycerol (DAG) and IP$_3$. The IP$_3$ interacts with and opens the IP$_3$-receptor channel in the endoplasmic reticulum releasing Ca^{2+} into the cytosol. The increased Ca^{2+} appears to regulate the exocytosis of anticoagulant protein from secretory vesicles into the saliva.

2.2. HYPOTHESIS

- The signaling mechanisms involved in controlling protein secretion from ixodid ticks are similar to those involved in the argasid tick, *Ornithodoros savignyi*.

2.3. AIMS

- To compare key molecules involved in the regulated exocytosis of proteins from large dense core granules between ixodid and argasid ticks using various agonists and/or antagonists.

2.4. MATERIALS

Ticks were collected from the North West Province of South Africa by sifting of sand and were kept in sand at room temperature. Dopamine, isoproterenol, carbachol, oleyloxyethyl phosphorylcholine (OPC), digitonin, verapamil, Ouabain, N-ethylmaleimide, anti-phosphoserine, anti-phosphothreonine, phosphatase inhibitor cocktail, Wortmannin, U73122, cytochalasin D, colchicine and HEPES were purchased from Sigma-Aldrich Co. Theophylline was a kind gift from Prof. J. R. Sauer, Oklahoma State University, USA. PGE_2, cAMP, $GTP\gamma S$, ATP, IP_3 and glucose were from ICN (Separations). NaCl, ethylene diamine tetra-acetic acid (EDTA), glycine, ammonium persulphate Tris(hydroxymethyl)aminomethane, N,N,N′,N′-tetramethyl-ethylenediamine (TEMED) and methanol were obtained from Merck, Germany. Acrylamide, bisacrylamide, sodium dodecyl sulphate (SDS), $CaCl_2$, MgCl were from BDH Laboratory Supplies LTD., England. Hybond-P membranes were from Amersham Pharmacia Biotech. Super Signal® chemiluminescent substrate and the Protein Assay kit were from Pierce, USA (Separations). X-ray film, photographic developer and fixer were from Konica. Low molecular weight marker proteins were purchased from Pharmacia, USA. All materials were of analytical grade and double distilled, deionized water was used in all experiments.

2.5. METHODS

2.5.1. TICK SALIVARY GLAND DISSECTION

Female ticks were embedded in molten wax with their dorsal sides visible. The integument was removed by lateral dissection of the cuticle with a scalpel under a 0.9% NaCl solution using a binocular stereomicroscope (10 x magnification). Salivary glands were removed with fine forceps and placed on ice in calcium free Hanks balanced salt solution (HBSS, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM Glucose, 10 mM HEPES, pH 7.2).

2.5.2. APYRASE ACTIVITY ASSAY

In female *O. savignyi* ticks the enzyme apyrase has been localized to the 'a' cell types where it is stored in large dense core vesicles / granules (LDCV, 3-5 μm) and released during feeding (Mans 2002a). Therefore, by investigating the release of apyrase, one can gain insight into the mechanisms involved in regulated exocytosis of LDCVs. In order to create a higher throughput system and also compensate for the numerous variables between individual salivary glands (such as size, age, amount of active apyrase etc.), we created a 96-well assay that is shown and described below (Table 2.8).

Table 2.8. Schematic presentation of the micro-titer plate setup in the secretion assay.

		Assay #1			Assay #2			Assay #3			Assay #4		
		1	2	3	4	5	6	7	8	9	10	11	12
A	Glands	SG	SG	SG	SG	SG	SG	SG	SG	SG	SG	SG	SG
B	Secreted	$10\mu l$											
C		$10\mu l$											
D		$10\mu l$											
E		$10\mu l$											
F	Dilutions of crude	Crude											
G		1/10											
H		1/10											

In the 96-well test, we were able to perform 4 individual assays, each in 12-fold (i.e. n=12), on a single plate. Briefly, glands were dissected and cut in half. Four randomly selected halves were placed in the wells of row A in 100 μl of HBSS containing the appropriate compound of interest (see 2.5.3). After stimulation, 10 μl of the medium was removed and placed in rows B-E containing 90 μl of apyrase reaction medium (20 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, pH 7.6) per well. The remaining salivary glands were sonified with a Branson Model B-30 sonifier (Branson Sonic Power Co.) at 10 pulses at 10% duty cycles at an output control of 2. Fractions (10 μl) were taken and diluted into 90 μl apyrase reaction medium in row F, and subsequently diluted twice in rows G and H. The plate was covered and incubated at 37°C for 30 min. The reactions were terminated by the addition of 33 μl molybdic acid mixture to each sample or standard orthophosphate sample (100 μl) in a microtiter well (25 ml of 2.5% molybdate and 13.3% concentrated sulphuric acid were added to 8 ml of a 1% ascorbic acid solution and mixed thoroughly). The reactions were shaken for 10 minutes and the absorbance determined at 620 nm using a SLT 340 ATC scanner (SLT Labinstruments). The amount of phosphate released was calculated from an orthophophate standard curve and one enzyme unit (U) was defined as 1 μmole of inorganic phosphate released per minute. The apyrase activity secreted, as well as the apyrase activity remaining in the salivary glands (crude) after stimulation were determined. Data is expressed as the % apyrase secreted (i.e. apyrase secreted / apyrase remaining in the glands x 100) in order to normalize data obtained from various sizes and metabolic states of the salivary glands. Data was statistically evaluated by means of the student's t-Test.

2.5.3. AGONIST AND ANTAGONIST TREATMENT

Salivary glands were made accessible for treatment with various compounds by dividing the glands into half using a sterile blade, hence rupturing the surrounding myo-epithelial sheath. Two halves of non-paired glands were placed in a microtiter plate well containing 100 μl calcium-free HBSS. By using a calcium specific electrode (Model 93-20, Orion Research, USA), the free calcium concentration in HBSS was determined to be less than 0.1 μM (Micro-processor ionalyzer Model 910, Orion Research, USA). The latter concentration is the lower detection limit of the electrode. Glands were washed three times with 100 μl HBSS and excess fluid was removed using a sterile 25G x 1" needle linked to a vacuum pump. Permeabilized cells were prepared by incubating the salivary gland sections in HBSS containing digitonin (40 μg / ml HBSS) at 4°C for 5 minutes. Digitonin-containing HBSS was removed by washing the cells three times with HBSS prior to incubation with the molecule of interest. Various conditions were investigated and these are described in the result section.

2.5.4. PHOSPHORYLATION ASSAY

Ten salivary glands were dissected, cut in half and placed on ice in 100 μl HBSS containing 0.1 mg/ml penicillin, 0.25 mg/ml fungizone, 0.1 mg/ml streptomycin sulphate, 1 μg/ml leupeptin, 20 μg/ml aprotonin, 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and 5 μl phosphatase inhibitor cocktail (Sigma). Intact salivary glands were stimulated for 10 minutes at 37ºC with 10 μM dopamine, while broken cells (hand homogenated using a teflon pestle, on ice for 2 minutes) were stimulated with 10 μM cAMP and 10 mM theophylline. A 5 μl aliquot of sample was removed and used to determine the protein concentration of the various fractions. The phosphorylation reaction was terminated by adding 30μl stop buffer (6 μl of 1M Tris, pH 6.8, 10 μl of 20% SDS, 8 μl of 80% glycerol, 5 μl mercapto-ethanol, 1 μl of 1% bromophenol blue) and boiling the samples for 10 minutes. Equal amounts of protein from each sample was subjected to SDS-PAGE and Western Blotting (as described in chapter 3) using monoclonal mouse-anti-phosphothreonine IgG$_{2b}$ or mouse-anti-phosphoserine IgG$_1$ as the primary antibody and a goat-anti-mouse IgG (heavy and light chain) coupled to horseradish peroxidase as secondary antibody.

2.6. RESULTS AND DISCUSSION

2.6.1. DOPAMINE / ISOPROTERENOL / CARBACHOL

It is well established that tick salivary glands (ixodid and argasid) are stimulated by dopamine, a neurotransmitter secreted at the neuroeffector junction (Sauer *et al.* 2000). In order to determine the optimal concentration of dopamine required for stimulation of salivary glands of *O. savignyi*, intact glands were stimulated in the presence and absence of added extracellular calcium in HBSS (see sections 2.5.2 and 2.5.3). In the presence of an excess added calcium (1.3 mM), maximal stimulation was obtained with 0.5 μM dopamine, in contrast to the 10 μM dopamine required in the absence of extracellular calcium (Figure 2.10). This 20-fold difference clearly indicates that the intracellular effect of dopamine is tightly regulated by extracellular calcium, which has been indicated for ixodid ticks as the opening of a voltage-gated calcium channel by dopamine. The k_a for dopamine in ixodid ticks has been established as 0.4 μM, which is in a range similar to that observed in this study, i.e. 0.5 μM. Free calcium levels in the HBSS buffer were not affected by the addition of salivary glands (see section 2.5.3).
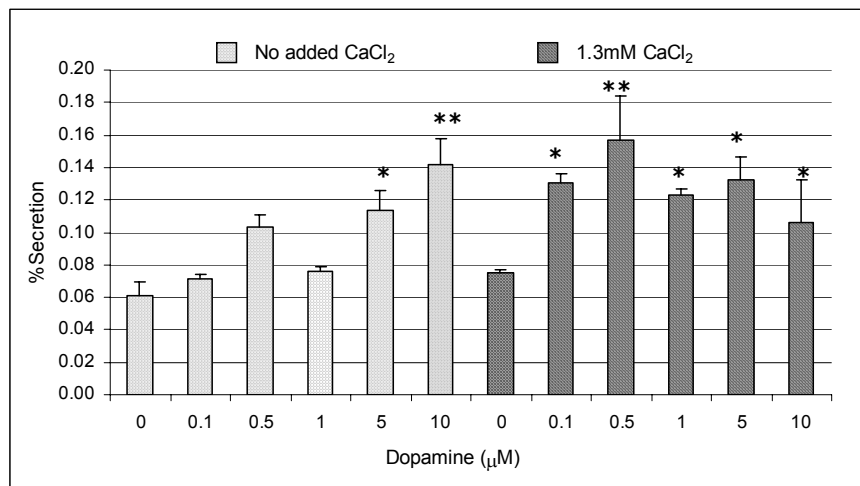
Figure 2.10. The effect of dopamine and extracellular calcium on apyrase secretion from the salivary glands of *O. savignyi*. *Salivary glands were incubated in HBSS without added calcium (dotted bars) or HBSS containing 1.3 mM CaCl$_2$ (striped bars) and stimulated with various concentrations of dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.005, (**) p<0.001.*

Upon testing the effect of isoproterenol (a β-adrenoceptor agonist) in concentrations similar to that of dopamine (in the presence of extracellular calcium), no significant stimulation above baseline was detected (p>0.05 of a t-test assuming unequal variance; Figure 2.11).

The absence of apyrase secretion in the presence of isoproterenol can be explained by the high k_a described for isoproterenol in ixodid ticks, i.e. 0.15 mM, which greatly exceed the concentrations tested in this study.
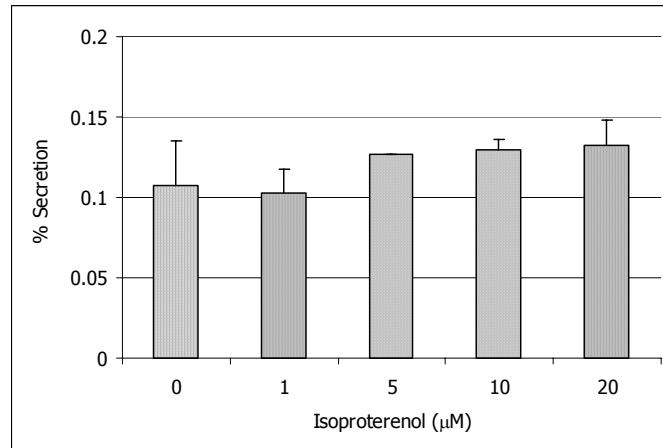

Figure 2.11. The effect of isoproterenol on apyrase secretion from the salivary glands of *O. savignyi*. *Salivary glands were incubated in HBSS containing 1.3 mM $CaCl_2$ and stimulated with various concentrations of isoproterenol. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. In all cases p>0.005.*

Injecting ticks with pilocarpine (a nonselective muscarinic acetylcholine receptor agonist) has been described for inducing salivation. In ixodid ticks it is believed that pilocarpine does not stimulate the salivary glands directly, but rather acts upon the synganglion, which then in response stimulates secretion from the salivary glands (personal communication with Prof. J.R. Sauer). By using the non-selective cholinergic agonist, carbachol (carbamyl chloride), the activation of cholinergic receptors on the salivary glands were investigated. The results indicated no stimulation, but rather inhibition of secretion (Figure 2.12). One explanation could be the observation that carbachol causes apoptosis in some cells (Sigma-Aldrich 2002). Upon apoptosis, cell caspases are processed into proteolytically active forms that initiate the 'caspase' cascade of cell destruction, including the collapse of the cytoskeleton and degradation of proteins involved in DNA replication and repair (Watling 2001).
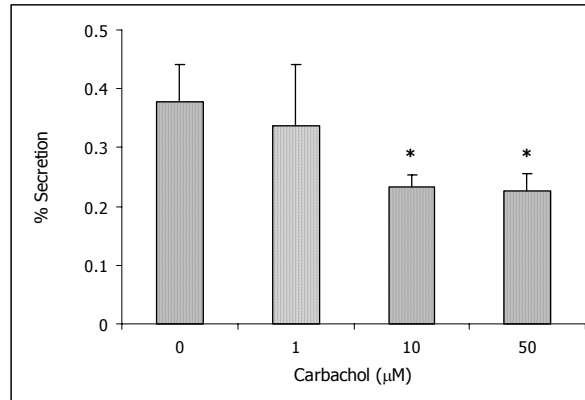
Figure 2.12. The effect of carbachol on apyrase secretion from the salivary glands of *O. savignyi*. *Salivary glands were incubated in HBSS containing 1.3 mM CaCl$_2$ and stimulated with various concentrations of carbachol. Error bars represent SD with n=12. (*) p<0.005.*

2.6.2.   INTRACELLULAR CALCIUM

In most, if not all secretory cells, the final step of regulated exocytosis is triggered by a burst of intracellular calcium. The effect of intracellular calcium in salivary glands was investigated by permeabilizing glands and placing them in a calcium-free buffer containing an excess of EGTA (5 mM), which will sequester any free calcium released intracellularly upon dopamine stimulation. In the presence of EGTA, a 1.5 fold increase in signal was observed when compared with the baseline (i.e. unstimulated permeabilized cells). In contrast, a 2.6 fold increase was observed in the absence of EGTA (Figure 2.13). Firstly, this indicates that an increase in intracellular calcium is essential for secretion from argasid salivary glands. Secondly, it also indicates that intracellular calcium is released in response to dopamine.
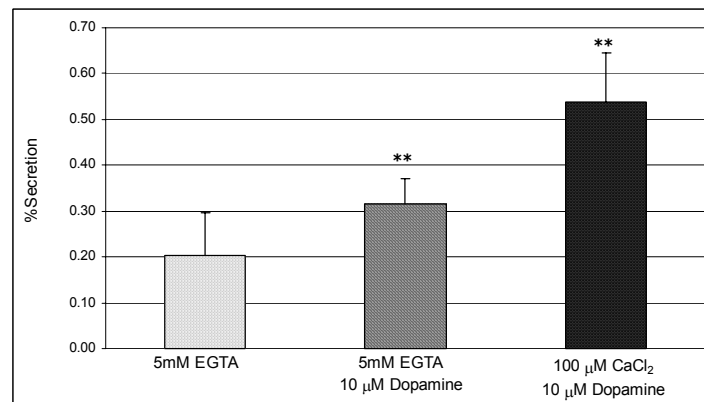
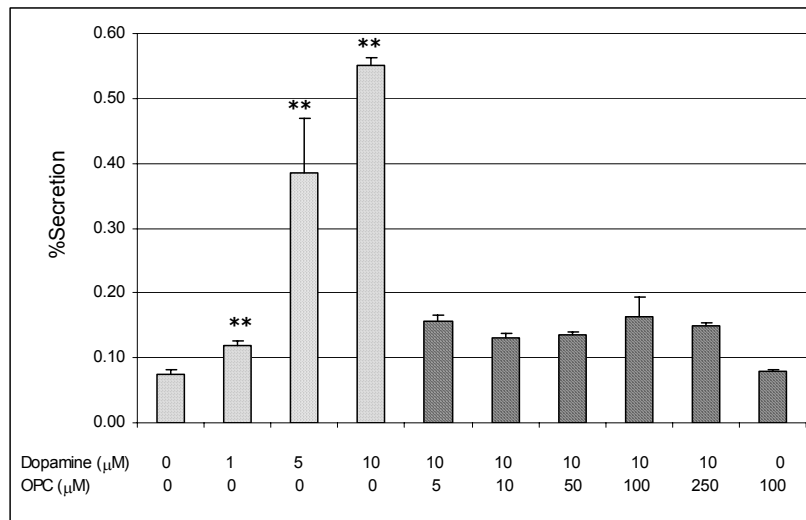**Figure 2.13. The effect of intracellular calcium on dopamine-stimulated apyrase secretion from permeabilized salivary glands of *O. savignyi*.** *Salivary glands were permeabilized with digitonin and incubated in HBSS with 5 mM EGTA (bars 1 and 2) or HBSS with 100 μM CaCl$_2$ (bar 3). Cells were stimulated with 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (**) p<0.001.*

2.6.3.   PROSTAGLANDINS

In the case of *A. americanum* it has been proven that PGE_2 plays an important role in stimulating protein secretion from the salivary glands (Sauer *et al.* 2000). It has been shown that activation of a PGE_2 receptor on the plasma membrane results in activation of PLC, production of IP_3 and a rise in intracellular calcium (Qian *et al.* 1998). PGE_2 in the ixodid salivary glands are synthesized from arachidonic acid via two key enzymes, a cytosolic phospholipase A2 (cPLA2) and PGH synthase that is essential to the COX-pathway. In order to investigate whether the synthesis of prostaglandins are essential for exocytosis from *O. savignyi* salivary glands, glands were permeabilized and treated with the PLA_2 inhibitory substrate analogue oleyloxyethyl phosphorylcholine (OPC), similar to the studies performed by Qian *et al.* (Qian *et al.* 1997). In the case of cells treated with OPC, apyrase release was greatly abolished (3,5 fold) in the presence of as little as 5 μM OPC (Figure 2.14, bars 4 and 5). This indicates that dopamine cannot bring about exocytosis without an active PLA_2, and hence it seems that liberation of free arachidonic acid from the plasma membrane and possibly prostaglandin synthesis is necessary for exocytosis from argasid salivary glands.

Figure 2.14. The effect of OPC on dopamine-stimulated apyrase secretion from permeabilized salivary glands of *O. savignyi*. *Salivary glands were permeabilized with digitonin and incubated in HBSS containing 1.3 mM $CaCl_2$ and various concentrations of OPC. After washing the cells, they were stimulated with 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (**) p<0.001.*

The following question is whether PGE_2 by itself can bring about apyrase secretion in argasid salivary glands. Since the PGE_2-receptor of *A. americanum* is localized on the plasma

membrane and directly accessible to PGE_2 in intact glands, intact *O. savignyi* salivary glands were incubated with HBSS containing calcium and stimulated with various concentrations of PGE_2. The results obtained (Figure 2.15) indicate that exogenous PGE_2 is unable to induce apyrase secretion.
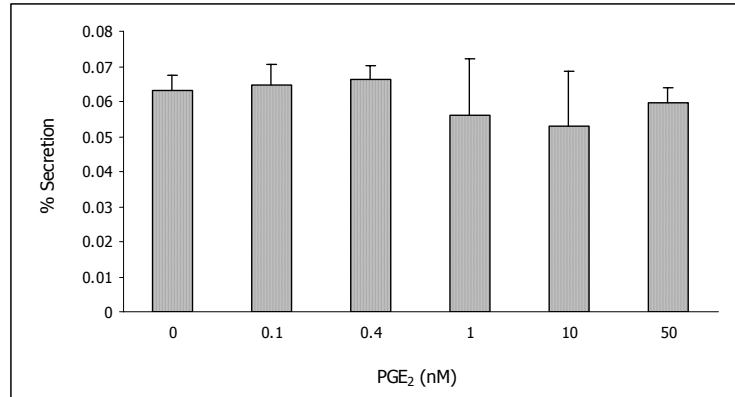

Figure 2.15. PGE_2 stimulated apyrase secretion from intact salivary glands in the presence of HBSS with calcium. *Salivary glands were incubated in HBSS containing 1.3 mM $CaCl_2$ and stimulated with various concentrations of PGE_2. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12.*

In order to remove excess endogenous PGE_2 (as suggested by Prof. J.R. Sauer) and to also investigate the possibility of an intracellular PGE_2 receptor, glands were permeabilized with digitonin for 15 minutes and washed extensively. Glands were subsequently incubated with various concentrations of PGE_2. The results indicated that PGE_2 was unable to stimulate apyrase secretion (Figure 2.16). In contrast, PGE_2 had an inhibitory effect on secretion at concentrations as low as 10nM. We were also unable to rescue cells treated with OPC by incubating cells with PGE_2 (Figure 2.17). Therefore the possibility of side effects of OPC must be further investigated as well as rescue studies using arachidonic acid. If an active PLA_2 is however required, one can deduce that generating free AA (arachidonic acid) and its metabolites are essential for secretion. A suitable positive control (such as *A. americanum* salivary glands) should also be investigated to confirm that PGE_2 is bioactive.
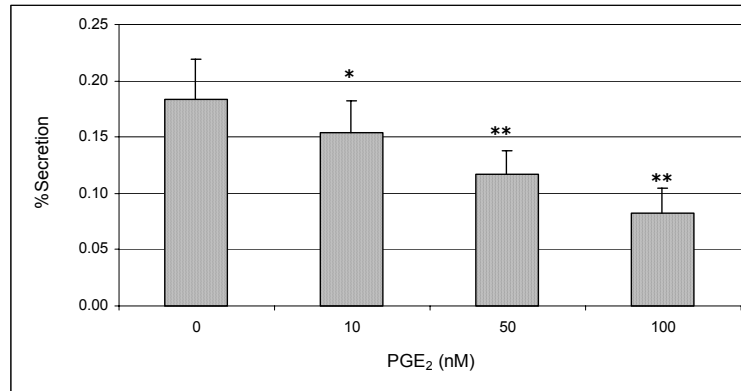
Figure 2.16. PGE$_2$ stimulated apyrase secretion from permeabilized salivary glands in the presence of HBSS with calcium. *Salivary glands were incubated in HBSS containing 1.3 mM CaCl$_2$ and digitonin. After washing the cells, they were stimulated with various concentrations of PGE$_2$. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.05, (**) p<0.001.*
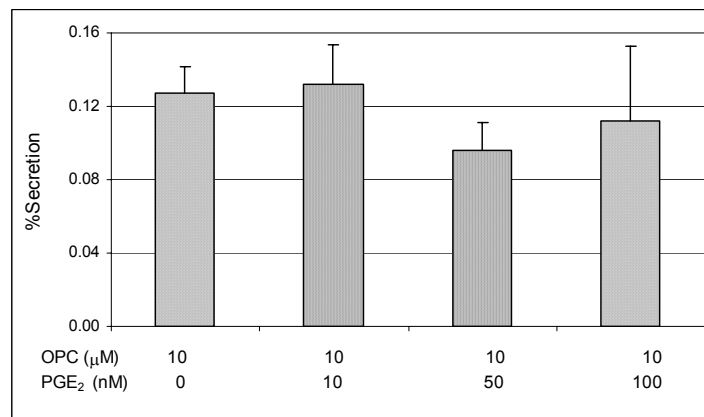

Figure 2.17. Rescue of OPC treated cells with PGE$_2$. *Salivary glands were permeabilized with digitonin and incubated in HBSS containing 1.3 mM CaCl$_2$ and 5 μM OPC. After washing the cells, they were stimulated with various concentrations of PGE$_2$. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12.*

2.6.4.   cAMP

cAMP is a widely used secondary messenger synthesized by adenylyl cyclase (AC). In ixodid tick salivary glands it has been shown that although cAMP levels are elevated during dopamine stimulation (via activation of AC), it is important for fluid secretion and not for protein secretion (Schmidt *et al.* 1981). Similar to the studies performed by McSwain *et al.*, intact argasid salivary glands were stimulated with milli-molar concentrations of exogenous cAMP (in HBSS with calcium) (McSwain *et al.* 1985). Limited stimulation of the glands was detected, i.e. apyrase secretion, was observed at 1 and 5 mM (Figure 2.18).
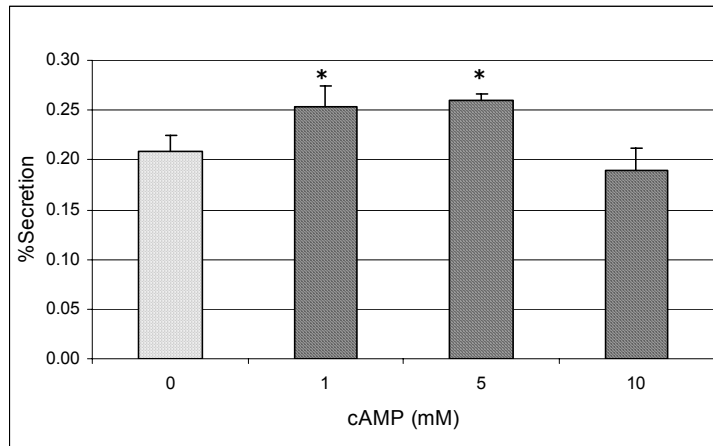
Figure 2.18. The effect of elevated extracellular cAMP levels on apyrase secretion from intact salivary glands of *O. savignyi*. *Salivary glands were incubated in HBSS containing 1.3 mM CaCl$_2$ and stimulated with various concentrations of exogenous cAMP. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.002.*

In the case of stimulating digitonin-permeabilized salivary glands with lower (micro-molar) concentrations of cAMP (in HBSS containing calcium), an inhibition of apyrase secretion was detected (Figure 2.19). The effect of intracellular cAMP on dopamine-stimulation of permeabilized cells was also investigated. In the presence of Ca^{2+}, cAMP inhibited dopamine-stimulated secretion of apyrase (Figure 2.20).
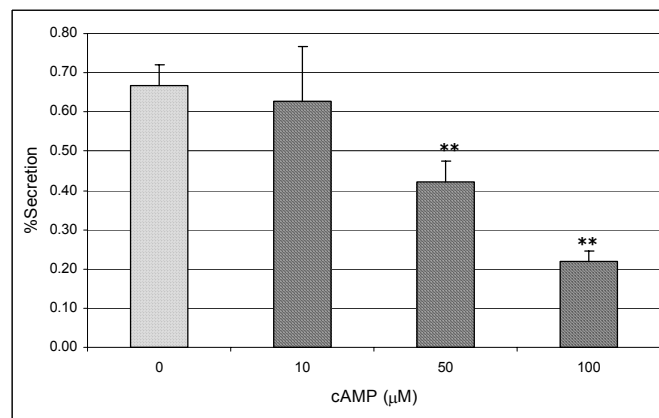

Figure 2.19. The effect of elevated intracellular cAMP levels on apyrase secretion from permeabilized salivary glands of *O. savignyi*. *Salivary glands were permeabilized with digitonin and incubated with HBSS containing various concentrations of cAMP. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (**) p<0.001.*
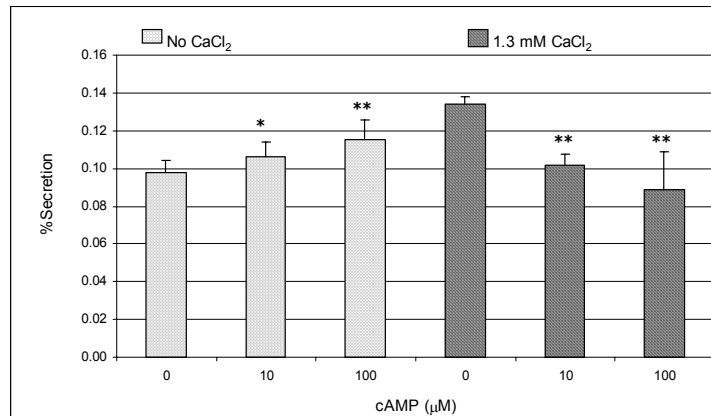
56

Figure 2.20. The effect of elevated intracellular cAMP levels on dopamine-stimulated apyrase secretion from permeabilized salivary glands of *O. savignyi*. *Salivary glands were permeabilized with digitonin, washed, incubated with HBSS containing various concentrations of cAMP and stimulated with 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.05, (**) p<0.001.*

This inhibition of secretion by intracellular cAMP can be explained firstly by the change in amplitude and duration of the signal, i.e. change in normal physiological signal. Secondly, the increase in cAMP will increase PKA activity resulting in the phosphorylation and change in activity of numerous proteins. Thirdly, cAMP-regulated guanine (GTP/GDP) exchange factors of small G-proteins, as well as cAMP-regulated ion channels, will be activated. All of the above could contribute to the inhibition of apyrase secretion.

These results do, however, indicate that elevated levels of cAMP are not sufficient to bring about apyrase secretion. Whether adenylyl cyclase and the production of cAMP are essential for exocytosis should be addressed by inactivating adenylyl cyclase. It has, however, been shown that cAMP-dependent kinase phosphorylation of proteins are essential for protein secretion, since they regulate phosphorylation of secretory proteins such as SNAP25 (Risinger and Bennett 1999), α-SNAP (Hirling and Scheller 1996) and rabphillin (Lonart and Sudhof 2001) in neurons, syntaxin 4 in non-neuronal cells (Foster *et al.* 1998), secretory granule membrane proteins of rat parotid glands (Marino *et al.* 1990) and t-SNAREs in yeast (Marash and Gerst 2001).

2.6.5. VERAPAMIL

The phenylalkylamines (e.g. verapamil) have long been used to distinguish between the various calcium channels (Jeziorski *et al.* 2000; Watling 2001). Only the L-type is verapamil

sensitive, while the T, N, P, Q and R-types are all insensitive (Watling 2001). Both the L and T type of calcium channels are found on endocrine cells. Upon stimulating intact salivary glands with dopamine in the presence of verapamil (in HBSS, with calcium), apyrase secretion was inhibited. At a concentration of 1, 50 and 100 μM verapamil, secretion was inhibited by 14%, 22% and 53%, respectively (Figure 2.21). This indicates an important role for a L-type calcium channels in argasid tick secretion, and also explains the need for extracellular calcium for optimal dopamine-stimulated secretion. Since these studies were performed on intact salivary glands, the L-type channels is deduced to be localized on the plasma membrane.
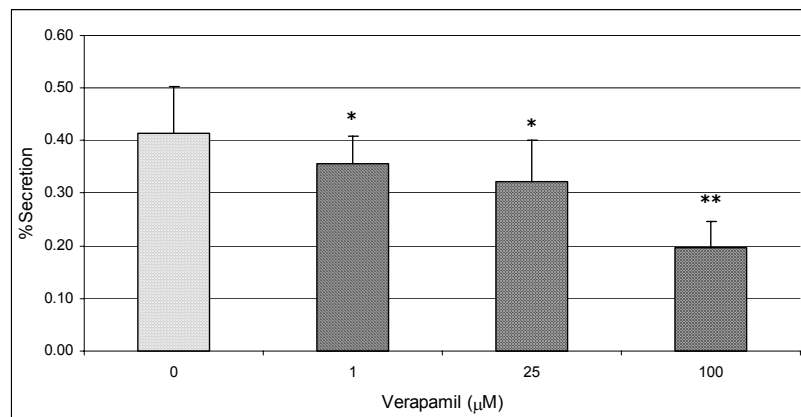

Figure 2.21. The effect of verapamil on dopamine-stimulated apyrase secretion from intact salivary glands. *Salivary glands were incubated in HBSS containing 1.3 mM CaCl$_2$ and various concentrations of verapamil. After washing the cells, they were stimulated with 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12*. *(*) p<0.05, (**) p<0.001.*

The possible activation of CaM via this influx of extracellular calcium should also be investigated, since this is involved in the phosphorylation (and thus the metabolic control) of numerous proteins, the regulation of adenylyl cyclase, inactivation of voltage-gated calcium channels and cyclic nucleotide phosphodiesterases (Watling 2001).

2.6.6.   OUABAIN

Na$^+$-K$^+$-ATPases of plasma membranes were first isolated in 1957 by Jens Skou (Voet and Voet 1995). The enzyme is often referred to as the Na$^+$-K$^+$-Pump because it pumps Na$^+$ out of and K$^+$ into the cell with the simultaneous hydrolysis of intracellular ATP. This extrusion of Na$^+$ enables animal cells to control their water content osmotically. Without functioning Na$^+$-K$^+$-ATPases, cells lacking cell walls (like animal cells) would swell and burst. In neuronal

cells, the electrochemical potential gradient generated by Na^+-K^+-ATPases is responsible for the electrical excitability of nerve cells, while in other cells (such as intestinal epithelium and erythrocytes) it provides the free energy for active transport of glucose and amino acids.

Ouabain is a glycoside that blocks the effective efflux of Na^+ and reuptake of K^+ by blocking the movement of the H5 and H6 transmembrane domains of Na^+-K^+-ATPases (Sigma-Aldrich 2002). In ixodid ticks it has been described that the correlation to fluid secretion and activities is highest in the salivary glands of mated, rapidly feeding females (Sauer *et al.* 2000). Furthermore, it has been shown that the Na^+-K^+-ATPases are sensitive to Ouabain and that the volume of saliva secreted is dependent on an active Na^+-K^+-ATPase. This indicates that Na^+-K^+-ATPases in ixodid ticks are involved in regulating cell volume. In argasid ticks we were able to show that Ouabain also inhibits apyrase secretion. In the presence of 10, 20 and 50 μM Ouabain secretion was inhibited by 12%, 41% and 47%, respectively (Figure 2.22).
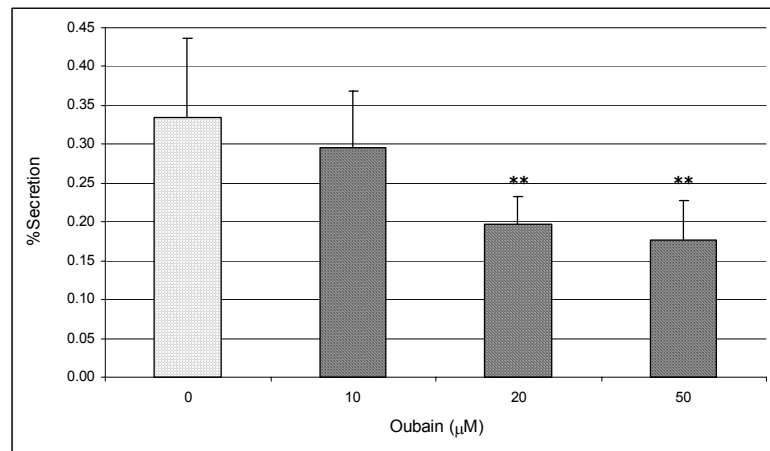

Figure 2.22. The effect of Ouabain on dopamine-stimulated apyrase secretion in HBSS with calcium. *Salivary glands were incubated in HBSS containing 1.3 mM CaCl$_2$ and various concentrations of Ouabain. After washing the cells, they were stimulated with 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (**) p<0.001.*

In various cells it has been found that by inactivating Na^+-K^+-ATPases, exocytosis is also affected (Finkelstein *et al.* 1986; Troyer and Wightman 2002). In ixodid ticks, the inactivation of the Na^+-K^+-ATPase did not affect protein secretion, but the volume of saliva secreted was affected. Therefore it was hypothesized that Na^+-K^+-ATPases are involved in maintaining cellular osmolarity. By inhibiting Na^+-K^+-ATPases with Ouabain in the salivary

glands of *O. savignyi* we observed 41-47% inhibition of apyrase secretion when using concentrations between 20-50 μM. This indicates that an active Na^+-K^+-ATPase is required for protein secretion from argasid salivary glands. The current hypothesis for the role of Na^+-K^+-ATPases in exocytosis are based on vesicle / granule swelling which occurs due to the displacement of associated cations in the granule with hydrated cations such as Na^+ from the external solution (Troyer and Wightman 2002). Whether osmotic swelling is a driving force in argasid tick salivary gland exocytosis must be further examined by determining the effect of high and low osmolarity solutions on apyrase secretion, similar to the studies done on mast- and chromaffin cells (Troyer and Wightman 2002).

2.6.7. EXTRACELLULAR AND INTRACELLULAR CONDITIONS (Membrane potential)

It has long been known that elevating the extracellular K^+ concentration (thereby depolarizing the membrane) can bring about neurotransmitter release from neuronal cells (Adam-Vizi 1992). In order to investigate the effect of depolarizing the salivary gland plasma membrane potential, the effect of dopamine-stimulated secretion was investigated under conditions reflecting an extracellular environment (high Na^+, low K^+, high $Ca2^+$, low ATP; HBSS) and conditions reflecting an intracellular environment (low Na^+, high K^+, low $Ca2^+$, high ATP; AISS) similar to the studies performed by Chaturvedi *et al*. (Chaturvedi *et al.* 1999). From the results it is evident that membrane depolarization of salivary glands is not sufficient to bring about optimal apyrase secretion (Figures 2.23 and 2.24). Therefore, the mechanism underlying apyrase secretion is not identical to that of neurotransmitter release. Also, in the case of HBSS with calcium, again it could be seen that 0.5 μM of dopamine stimulated optimal secretion of apyrase (Figure 2.23). In the case of AISS, five times less (0.1 μM) dopamine was sufficient to stimulate optimal apyrase secretion (Figure 2.24).
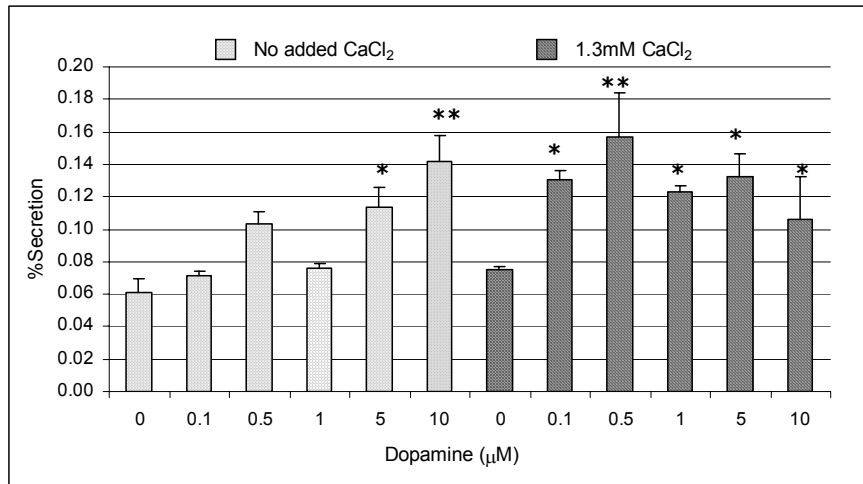
Figure 2.23. The effect of dopamine and extracellular calcium on apyrase secretion from the salivary glands of *O. savignyi* in HBSS. *Salivary glands were incubated in HBSS without calcium (dotted bars) or HBSS containing 1.3 mM CaCl$_2$ (striped bars) and stimulated with various concentrations of dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.005, (**) p<0.001.*
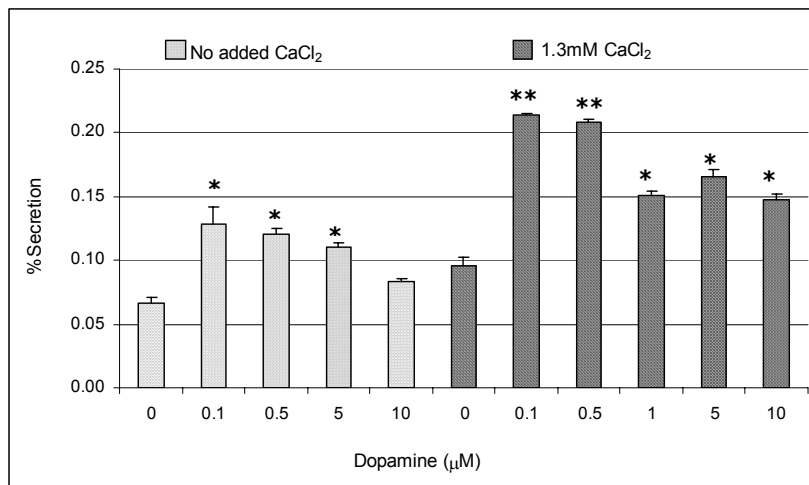

Figure 2.24. The effect of dopamine and extracellular calcium on apyrase secretion from the salivary glands of *O. savignyi* in AISS. *Salivary glands were incubated in AISS without calcium (dotted bars) or HBSS containing 1.3 mM CaCl$_2$ (striped bars) and stimulated with various concentrations of dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.005, (**) p<0.001.*

2.6.8.   N-ethylmaleimide (NEM)

N-ethylmaleimide is a well-known alkylating agent. During studies on the exocytotic machinery of various cells, a NEM-sensitive fusion protein called NSF (N-ethylmaleimide sensitive factor) was identified and isolated. NSF was shown to be a homo-oligomeric

ATPase. It's ATP-dependent activity is involved in rearranging soluble NSF attachment protein (SNAP) receptor (SNARE) protein complexes during ATP-dependent priming (Banerjee 1996a).

By incubating permeabilized salivary glands with NEM, we were able to observe a 41% decrease in apyrase secretion at 100 μM NEM (Figure 2.25). At concentrations of 500 μM and 1000 μM NEM, apyrase secretion was inhibited by 53% and 74%, respectively. Only concentrations lower than 1 mM was tested since higher concentrations have been described to have cellular side effects (Chaturvedi *et al.* 1999).
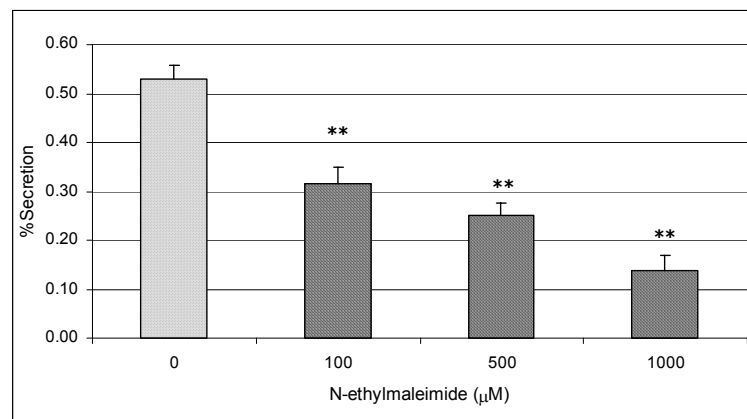

Figure 2.25. Effect of N-ethylmaleimide on dopamine-stimulated apyrase secretion. *Salivary glands were permeabilized with digitonin, washed, incubated with HBSS containing various concentrations of NEM and stimulated with 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (**) p<0.001.*

2.6.9.   GTPγS

GTPγS is a non-hydrolyzable GTP analog, known for its role as a G-protein and GTPase activator. In order to test the effect of GTP-binding proteins in apyrase secretion, cells were incubated and stimulated with HBSS containing GTPγS. The results obtained (Figure 2.26) indicate that activation of GTP-binding proteins is sufficient for inducing apyrase secretion without further extracellular stimulation. At concentrations of 50, 100 and 250 μM GTPγS, we observed a 19%, 61%, 91% increase in secretion from the baseline values.
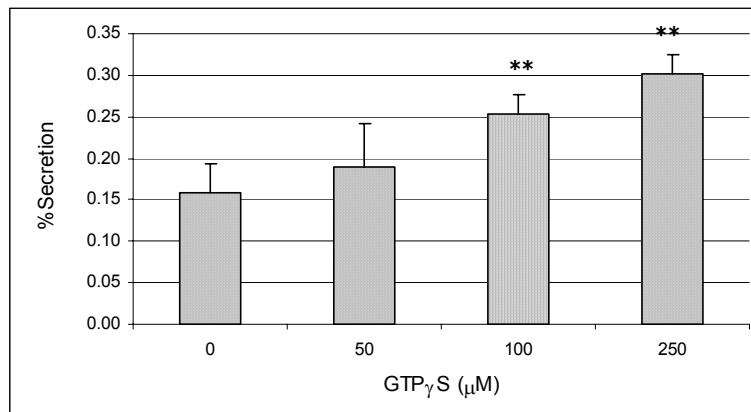
Figure 2.26. Effect of GTPγS on apyrase secretion. *Salivary glands were permeabilized with digitonin, washed, incubated with HBSS (with calcium) containing various concentrations of GTPγS. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (**) p<0.001.*

2.6.10. cAMP-DEPENDENT PHOSPHORYLATION

In ixodid ticks dopamine activates a D1-type receptor linked to a Gs-protein that activates adenylyl cyclase to produce cAMP (Schmidt *et al.* 1981). This increase in cAMP activates a cAMP-dependent kinase that mediates the phosphorylation of various proteins. In studies performed by McSwain *et al.*, it was shown that the increase in cAMP activates a cAMP-dependent kinase that mediates the phosphorylation of 12 endogenous proteins in *A. americanum*. Upon attenuating dopamine stimulation with dopaminergic antagonists, ten of these proteins were dephosphorylated, indicating the presence of a dopamine-sensitive AC (McSwain *et al.* 1985).

We investigated the effect of dopamine and cAMP (in the presence of the phosphodiesterase inhibitor theophylline) stimulation on the phosphorylation of endogenous proteins in the salivary glands of *O. savignyi* by means of Western blotting with both anti-phosphothreonine and anti-phosphoserine antibodies, similar to the studies of McSwain *et al.* (McSwain *et al.* 1985). In all cases, equal amounts of protein were subjected to SDS-PAGE in order to allow for comparison. No phospho-proteins could be detected using the anti-phosphoserine antibodies. We were able to identify 6 proteins that are phosphorylated via threonine residues in response to both dopamine and cAMP (Figure 2.27 lane 2 and 3). The molecular masses of these are 76, 68, 52, 37, 27 and 22 kDa, respectively. By comparing the molecular masses of the proteins phosphorylated in *A. americanum* (masses indicated in brackets) and *O. savignyi*, the masses are similar for the 76 (74), 68 (62), 52 (55), 37 (37) and 27 (29) kDa proteins, respectively (Table 2.9). These results indicate that a dopamine activated

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cAMP-dependent kinase is present, and that dopamine therefore activates adenylyl cyclase similar to the model proposed for ixodid ticks. The possibility of phosphorylated tyrosine residues as well as dephosphorylation studies on the identified phoshothreonine proteins must still be investigated.
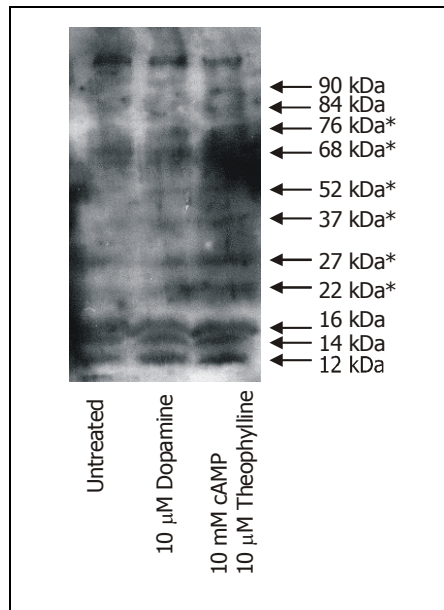

Figure 2.27. Western blotting of dopamine and cAMP treated salivary glands using a monoclonal anti-phosphothreonine IgG. *Lane 1 indicates intact untreated salivary glands, lane 2 indicate intact salivary glands treated with 10 μM dopamine and lane 3 homogenated salivary glands treated with 10 μM cAMP, 10 μM theophylline. Bands that increased in density in response to stimulation is indicated with *.*

Table 2.9. Molecular masses of proteins phosphorylated by a dopamine-sensitive cAMP-kinase in the salivary glands of the ixodid tick *A. americanum* (McSwain *et al.* 1985) and the argasid tick *O. savignyi*.

A. americanum	*O. savignyi*
93.5	
74	76
62	68
55	52
49	
47	
45	
37	37
34	
29	27
	22

2.6.11.   PI-3-KINASE INHIBITOR (WORTMANNIN)

In the tick *A. americanum*, a trypsin-sensitive brain factor was identified that induced the formation of inositol phosphates in tick salivary glands (McSwain *et al.* 1989). In secretory cells an increased turnover of inositol phospholipids correlate with exocytosis (Huijbregts *et al.* 2000). To date, 3- and 4-phosphorylated inositol lipids have been indicated in membrane trafficking. 4-Phosphorylated inositol lipids are believed to function in membrane trafficking e.g. priming of dense core granules in neuroendocrine cells by activation of a calcium-dependent activator protein for secretion (CAPS). 3-Phosphorylated inositol lipids are not only involved in both regulated exocytosis and protein sorting, but by interacting with FYVE-domain containing proteins (such as EEA1, early endosome associated antigen), the PtdIns-3-P signals are integrated with the activation of Rab GTPases (Huijbregts *et al.* 2000). The fact that phosphoinositides regulate membrane trafficking suggests that enzymes dedicated to phosphoinositide turnover will also exert modulatory effects. One such an enzyme is synaptojanin, which contains a catalytic domain with both inositol 3- and 4-phosphatase activity, and is required for endocytosis (Huijbregts *et al.* 2000).

During this study we investigated the possible role of PI-3-kinases type I, which is involved primarily in the synthesis of PtdIns(3,4,5)P$_3$. Our interest in PI-3-kinases type I came from the recent studies indicating a role for this enzyme in exocytosis and also the fact that PI-3-kinases are activated by the βγ-subunits of G-proteins. The reason for the latter is the observation that PLCβ (1,2, and 3 subfamilies) could also be regulated by βγ dimers (Bonacci *et al.* 2005). If one can indicate the presence of active PI-3-kinases, it can be hypothesized that βγ dimers are activators in argasid ticks and thus the possibility of PLC activation by βγ dimers must be investigated.

Wortmannin (from *Penicillium funiculosum*) is a potent and specific phophatidylinositol 3-kinase (PI3-K) inhibitor (Sigma-Aldrich 2002). In cells there are two types of PI3-kinases (type 1A and 1B) that are affected by Wortmannin (see Table 2.10). Since the type 1B is only localized in hemopoietic cells, one can assume that by investigating the effect of Wortmannin on salivary glands, one would determine the effects of a Type 1A phophatidylinositol 3-kinases (Watling 2001; Sigma-Aldrich 2002). A summary of the functions of the various kinases as well as the reaction inhibited by Wortmannin is shown in Figure 2.28.

Table 2.10. Characteristics of Type 1A and 1B phophatidylinositol 3- kinases sensitive to Wortmannin (Watling 2001).

| Kinase	Type 1A PI3K	Type 1B PI3K
Reaction *in vivo*	PtdIns(4,5)P$_2$ > PtdIns(3,4,5)P$_3$	PtdIns(4,5)P$_2$ > PtdIns(3,4,5)P$_3$
Structure	p85 (α,β) or p55γ regulatory subunit; p110 (α,β or γ) catalytic subunit	p101 regulatory subunit p101γ catalytic subunit
Control/Comments	- P85 SH2 domain interacts with P-Tyr residues - P110 activated by p85 and by activated Ras -Activated by insulin receptor/ growth factor receptors and $\beta\gamma$-complexes liberated from activated Gs proteins	- P101 complexes activated by $\beta\gamma$-complexes liberated from activated Gi and/or Go proteins. - Receptors that influence motility and the bacterial oxidative burst of neutrophils
Localization	Various cells	Hemopoietic cells
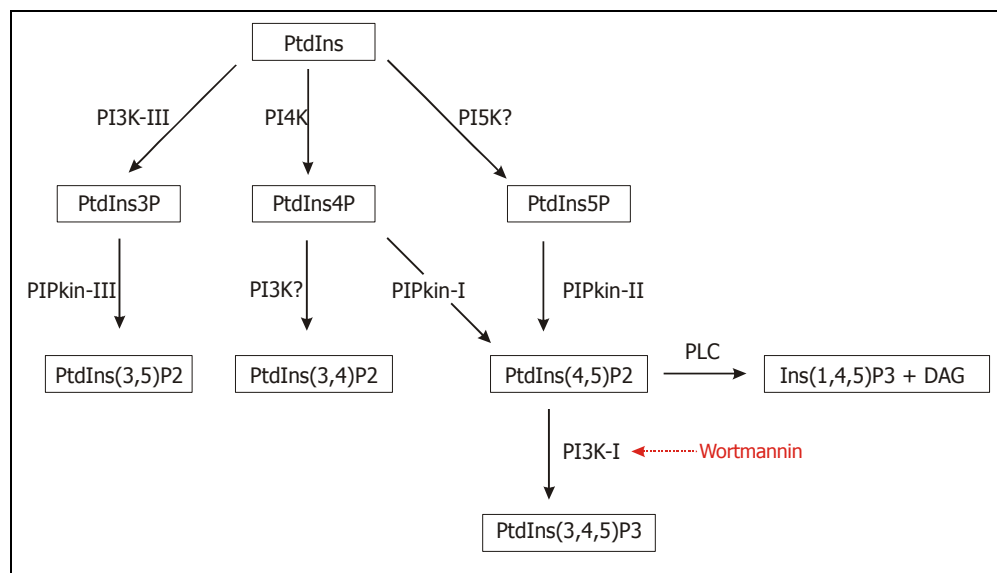
Figure 2.28. A schematic presentation of the functions of the various reactions catalyzed by cellular phosphoinositide kinase isozymes (Watling 2001). *The reaction affected by Wortmannin is indicated in red.*

PtdIns(3,4,5)P$_3$, which is the product formed by PI3K-I, is a remarkable membrane-associated second messenger molecule. It appears to have many direct target proteins, each interacting with highly PtdIns(3,4,5)P$_3$-selective pleckstrin (PH) domains. Some of these are protein kinases, while others include regulators of small GTPases, e.g. GTP/GDP exchange factors and GTPase-activated factors (Watling 2001). Recent work indicated that PI3-kinases may be one of the important regulatory exocytotic components involved in the signaling cascade controlling actin rearrangements (blocking of actin disassembly) required for

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catecholamine secretion from chromaffin cells by affecting myosin-actin ATPase activity (Chasserrot-Golaz *et al.* 1998; Neco *et al.* 2003). Inhibitors of PI3-kinase also significantly decrease heterophil degranulation (Kogut *et al.* 2002). PI3-kinases are also required for the efficient routing of proteins through FYVE-domain proteins such as EEA1. Inhibition of PI3-kinase activity by agents such as Wortmannin effects release of EEA1 from endosomal membranes, and correspondingly inhibits endosome fusion (Huijbregts *et al.* 2000).

Upon stimulating permeabilized salivary glands in the presence of 1, 10 and 100 nM of Wortmannin we observed a 15, 29 and 36% inhibition of apyrase secretion, respectively (Figure 2.29). Since inhibitor concentrations used were in the nano-molar range we can conclude that other enzymes such as myosin light-chain kinase, PLA_2 and some PI4-kinases were not affected. The latter have been reported in literature to be affected by Wortmannin when used in the micro molar range (Chasserrot-Golaz *et al.* 1998). This indicates that a dopamine activated (direct / indirect) G_s-$\beta\gamma$ subunit is most likely involved in activating PI3-kinases and that $PtdIns(3,4,5)P_3$ is an important signaling lipid during regulated exocytosis of apyrase. Furthermore, we hypothesize that these PI3-kinases are regulated by $\beta\gamma$-dimers, similar to all other secretory cells. The role of these $\beta\gamma$-dimers must be investigated by future studies to determine their role in activation of PLC.
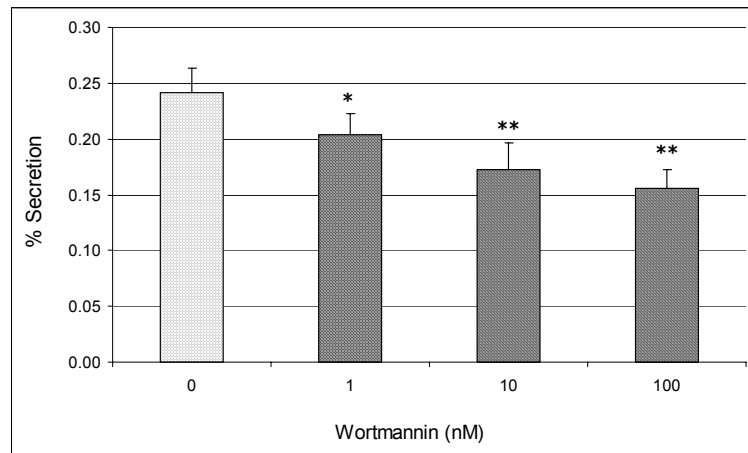

Figure 2.29. Effect of Wortmannin on dopamine-stimulated apyrase secretion. *Salivary glands were permeabilized with digitonin, washed, incubated with HBSS containing various concentrations of Wortmannin and stimulated with 10 μM dopamine in the presence of calcium. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.01, (**) p<0.001.*

2.6.12. Inositol (1, 4, 5) tri-phosphate (IP$_3$)

In the ixodid tick *A. americanum* it has been clearly indicated that IP$_3$ levels are raised in response to PGE$_2$. In response to IP$_3$, intracellular calcium was released from microsomes and exocytosis of salivary gland proteins occurred. In order to investigate the effect of elevated levels of IP$_3$ on the exocytosis of apyrase from argasid salivary glands, permeabilized cells were stimulated with HBSS containing IP$_3$. The results (Figure 2.30) indicate that elevated IP$_3$ levels are insufficient for inducing secretion. This finding indicates that elevated IP$_3$ (and hence elevated intracellular calcium levels) are not sufficient for inducing exocytosis by itself and that other signaling pathways are also required for inducing exocytosis in *O. savignyi*.
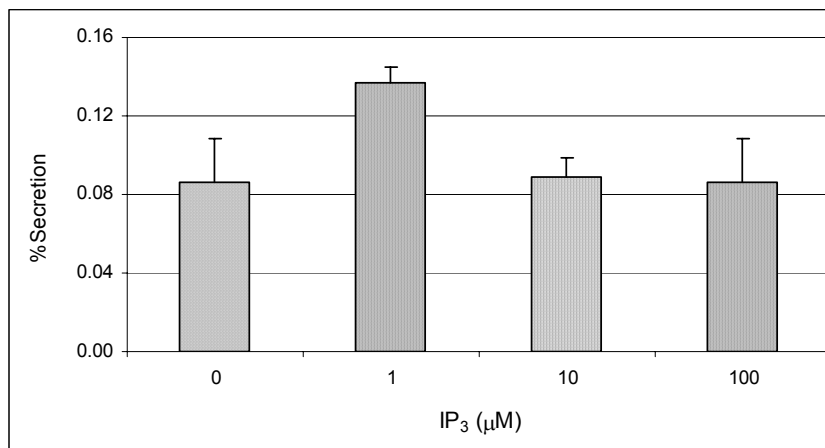

Figure 2.30. Effect of IP$_3$ on apyrase secretion from permeabilized salivary glands of *O. savignyi*. *Salivary glands were permeabilized with digitonin, washed, incubated with HBSS containing various concentrations of IP$_3$. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12.*

2.6.13. PLC INHIBITOR (U73,122)

As described previously, IP$_3$ and increased intracellular calcium are key processes in regulated exocytosis in secretory cells. IP$_3$ is synthesized by phospholipase C (PLC) that is in turn activated by either the α-subunits of the G$_{q/11}$ subfamily or by the G$_{\beta\gamma}$ dimers. Since ixodid ticks contain an EP1-type of PGE$_2$ receptor, it most likely is linked to G$_{q/11}$ and PLC-β. In order to determine whether a PLC-activated pathway is involved in exocytosis in *O. savignyi*, we investigated the effect of the PLC inhibitor U73,122 (1-(6-[([17b]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-1H-pyrrole-2,5-dione) which is a non-specific inhibitor of the PLC-β, PLC-γ and PLC-δ isoforms. The results (Figure 2.31) indicate

that an active PLC is required for exocytosis of apyrase. At concentrations of 10, 50 and 100 μM U73,122 we observed complete inhibition of apyrase secretion when cells were stimulated by dopamine. This indicates that the normal products produced by PLC (i.e. IP_3 and DAG) are required for exocytosis.
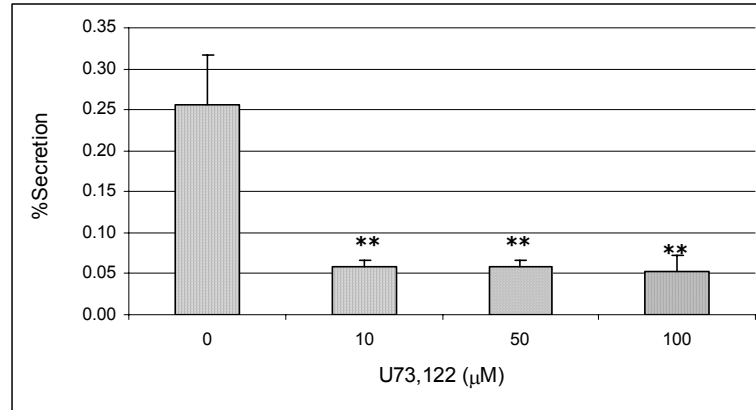

Figure 2.31. Effect of U73,122 on dopamine-stimulated apyrase secretion from permeabilized salivary glands of *O. savignyi*. *Salivary glands were permeabilized with digitonin, incubated with HBSS containing various concentrations of U73122, washed and stimulated with HBSS containing 1.3 mM calcium and 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (**) p<0.001.*

2.6.14. Actin inhibitor (Cytochalasin D)

Early ultrastructural studies of actively secreting cells, such as pancreatic or neuronal cells, revealed an extensive actin network and a dense sub-plasmalemmal actin cortex. It is predicted that this zone creates an obstacle that obstructs exocytosis and endocytosis at the plasma membrane, the so-called actin-physical barrier model (Eitzen 2003). Data from various experiments support this model and imply that depolymerization of F-actin is needed in order for vesicles to gain access to their appropriate docking and fusion sites. However, not all data support this model. Other hypotheses to explain the role of actin in exocytosis include (a) actin remodeling that includes a rearrangement to form structures that spatially restrict fusogenic proteins to active fusion sites and/or (b) the ability to apply a constrictive force on membranes during membrane fusion to overcome the large electrostatic force between two lipid bilayers that opposes their juxtapositioning (Eitzen 2003).

To date the entire mechanism of actin to facilitate membrane fusion and exocytosis is not clearly defined. In numerous cells (adrenal chromaffin-, alveolar type II-, egg-, GH_3-, HIT-

T15 -, anterior pituitary-, mast-, pancreatic β -, parotid acinar-, posterior pituitary terminal-, sperm-, WRK-1 cells and synapses) it has been shown that the actin cytoskeleton function as a barrier and disassembles upon exocytosis stimulus induction (Burgoyne and Morgan 2003). Also, actin is required for the active transport of secretory granules to the plasma membrane (Burgoyne and Morgan 2003).

Cytochalasin D (from *Zygosporium mansonii*) is a cell permeable fungal toxin that disrupts actin microfilaments and inhibits actin polymerization. In a study by Orci *et al.*, the mild application (10 μg/ml) of cytochalasin D significantly increased the secretion of insulin, indicating the presence of an actin-barrier (Orci *et al.* 1972; Eitzen 2003). However, higher concentrations (50 μg/ml) resulted in inhibition of secretion, indicating a dual role for actin (such as a need for actin during the final stages of exocytosis).

During confocal imaging using an anti-actin antibody, we observed an increased sub-plasmalemmal localization of actin (Chapter 3). In order to investigate the possibility of an actin-barrier in the salivary glands of *O. savignyi*, cells were treated with low concentrations (nM) of cytochalasin D and stimulated with dopamine. From the results (Figure 2.32), it is evident that cytochalasin D increased apyrase secretion. At concentrations of 10 and 100 nM, secretion increased with 17% and 49%, respectively. This data indicate that actin plays an important role in apyrase secretion in a manner supporting the actin-physical barrier model. Similar to the work of Orci *et al.*, disassembly of actin was not sufficient for inducing exocytosis. Based on the sub-plasmalemmal localization of actin visible during confocal imaging using an anti-actin antibody (Chapter 3), we propose a barrier function for actin in the salivary glands of *O. savignyi*. In numerous cell types it was shown that actin disassembly is activated by elevation of cytosolic calcium and by activation of PKC (Burgoyne and Morgan 2003). The latter, as well as a function for actin in transport must be investigated in future studies.
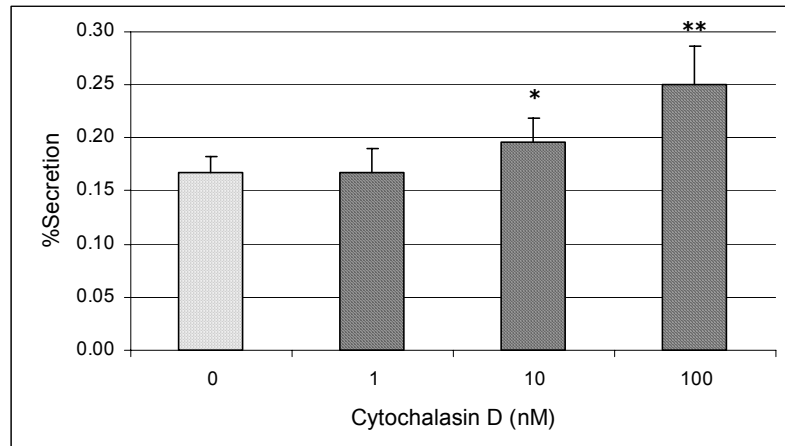
Figure 2.32. The effect of cytochalasin D on dopamine-stimulated apyrase secretion. *Salivary glands were incubated with HBSS containing various concentrations of cytochalasin D for 15 minutes, washed and stimulated with 10μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.05, (**) p<0.001.*

2.6.15.   Tubulin Inhibitor (Colchicine)

Colchicine is an antimitotic agent that disrupts microtubules by binding to tubulin and prevents its polymerization, stimulating the intrinsic GTPase activity of tubulin. Similar to actin, tubules contain various motors such as myosins, kinesins and dyneins required for the transport and delivery of proteins throughout the entire secretory pathway (Apodaca 2001; Neco *et al.* 2003). The effect of disrupting the tubules in salivary glands was investigated by incubating permeabilized glands with colchicine and investigating the effect on dopamine stimulated exocytosis. From the results obtained (Figure 2.34) it is evident that disruption of tubules results in an inhibition of apyrase secretion. At 10 μM colchicine, a 23% decrease in secretion was observed while at higher concentrations such as 50 and 100 μM colchicine a 42% and 44% decrease, respectively, were observed. This indicates that tubules are involved in regulated exocytosis and most likely via the transport of granules to the plasmamembrane where fusion occurs.
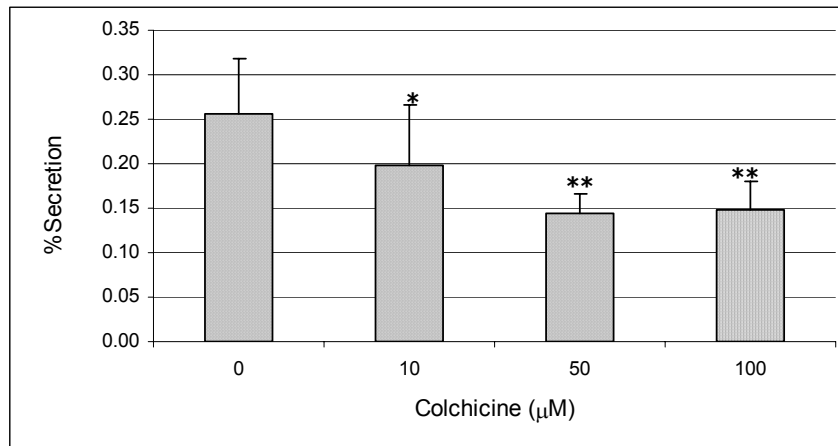
Figure 2.34. The effect of colchicine on dopamine-stimulated apyrase secretion. *Salivary glands were permeabilized with digitonin, incubated with HBSS containing various concentrations of colchicine for 15 minutes, washed and stimulated with 10 μM dopamine. The % apyrase secreted from the salivary glands is indicated. Error bars represent SD with n=12. (*) p<0.05, (**) p<0.001.*

2.7. CONCLUSION

All of the signaling pathways (steps) investigated during this study are summarized and compared with known pathways of the ixodid tick, *A. americanum* in Table 2.11.

Table 2.11. Comparison between the signaling pathways regulating exocytosis from the salivary glands of *A. americanum* (Ixodidae) and *O. savignyi* (Argasidae).

Signaling step	*A. americanum* (Ixodidae)	*O. savignyi* (Argasidae)
Dopamine/ Adrenergic agonists	- Stimulate exocytosis - Dopamine (D1) receptor K_a = 0.4 μM - Isoproterenol K_a= 0.15 mM	- Stimulate exocytosis - Isoproterenol is less sufficient in stimulating exocytosis
Cholinergic agonist	- No effect	- No effect (Carbachol)
Adenylyl cyclase & cAMP	-Activation via dopamine receptor linked to Gs protein -cAMP dependent protein kinase identified - PKA phosphorylate 12 proteins - 3 PKA isoforms identified from cDNA	-Dopamine sensitive adenylyl cyclase -cAMP dependent protein kinase identified -Thr phosphorylation of 6 proteins -No serine phosphorylation
Extracellular Ca^{2+}	- Voltage gated Ca^{2+} channel required. -L-type (Verapamil sensitive)	- Voltage gated Ca^{2+} channel required. -L-type (Verapamil sensitive)
Intracellular Ca^{2+}	- Increase is stimulated by IP_3 in response to PGE_2	- Essential for secretion - Mechanism of release unknown
Prostaglandins	- Cytosolic PLA_2 required (OPC sensitive) -AA increase during feeding -PGE_2 receptor (EP1) K_d ~29 nM, linked to cholera sensitive G protein, activates PLC	- Cytosolic PLA_2 required (OPC sensitive)
Na^+-K^+-ATPase	-Volume saliva (fluid) secreted is decreased in the presence of Ouabain -Function in regulating cell volume	-Ouabain inhibits apyrase secretion, i.e. exocytosis.
Membrane potential	Unknown	-Depolarization is unsufficient for inducing exocytosis
N-ethylmaleimide	Unknown - NSF ATPase has been identified	- Exocytosis is NEM sensitive - Role for NSF ATPase in exocytosis
GTPγS	Unknown	- Exocytosis is G-protein regulated and increase in the presence of GTPγS

PI-3 Kinases	Unknown	-PI-3 kinases are involved in exocytosis (Wortmannin sensitive)
Phospholipase C & IP$_3$	- PLC activated by EP1 receptor - IP$_3$ increase $[Ca^{2+}]_i$	-PLC is required for exocytosis (U73,122 sensitive)
Actin	Unknown	- Actin disassembly is required for exocytosis (Cytochalasin D sensitive)
Microtubules	Unknown	-Intact microtubules are required for exocytosis (Colchicine sensitive).

In order to summarize, we constructed a schematic representation of the known and hypothesized factors regulating apyrase secretion from LDCV (Figure 2.35). Dopamine binds to an extracellular D1 type receptor which activates a L-type voltage gated calcium channel, as well as AC via the Gα subunit to produce cAMP. Elevated cAMP levels activate PKA which phosphorylates threonine residues of various target proteins. We propose that the G$_{\beta\gamma}$ subunits activate PI3-kinases to produce PIns (3,4,5)P3. The possibility of activation of PLC by the G$_{\beta\gamma}$ subunits must be investigated. We propose that the activation of PI3-kinases, and hence the production of PIns(3,4,5)P3 is essential for exocytosis since it acts as a secondary messenger which regulates various kinases, GTPases and protein routing throughout the secretory pathway. Activation of PLC, which results in the production of IP3 and DAG, is essential for exocytosis. We hypothesize that IP$_3$ causes elevated intracellular calcium levels and DAG is involved in the activation of PKC. Since GTPγS stimulation results in exocytosis, one can conclude that GTP binding proteins (GTPases) are involved in exocytosis. Disruption of the actin cytoskeleton resulted in enhanced exocyotosis, indicating the possibilty of an actin web forming a barrier between the granules and plasmamebrane and inhibiting non-specific exocytosis. In contrast, disruption of microtubules inhibited exocytosis. This could be due to tubules regulating granule transport.

Inhibition of exocytosis by N-ethylmaleimide indicates that the ATPase NSF, which forms part of the exocytosis machinery, is involved in driving fusion. A striking difference between ixodid and argasid ticks is the inabilty to stimulate apyrase secretion by exogenous PGE$_2$. Since the PLA$_2$ inhibitor OPC completely inhibited exocytosis, one can conclude that the production of free arachidonic acid is essential. The question still remains what the end product of AA is after metabolism, and it's function is in exocytosis. Also, we cannot comment on the activation of the PLA$_2$ since it was not investigated. We propose in Figure 2.35 that it is activated by an influx of extracellular calcium, similar to ixodid ticks.
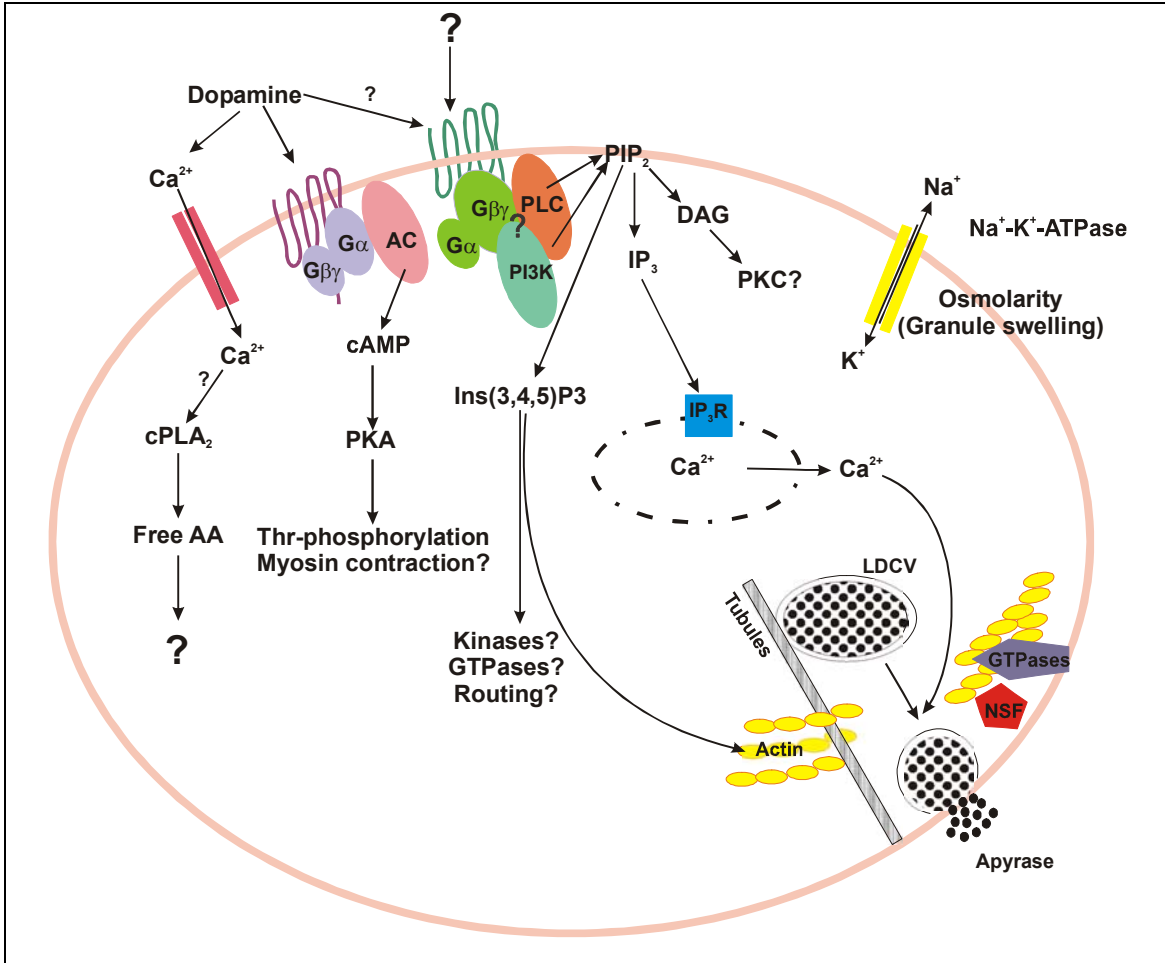
Figure 2.35. Schematic representation of the proposed mechanisms underlying regulated exocytosis of apyrase from LDCVs from the salivary glands of *O. savignyi*.

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