

CHAPTER 6

CONCLUDING DISCUSSION

The mechanisms underlying regulated exocytosis of large dense core vesicles/granules (LDCVs) from the salivary glands of *O. savignyi*, and also from other argasid ticks, have not been addressed before. Knowledge regarding the secretion of compounds essential for tick feeding is vital since both the signaling mechanisms, as well as the components involved in transport, docking and fusion of the granules are all possible targets for designing an anti-tick feeding strategy.

During this study, the release of the enzyme apyrase, which has been localized to LDCVs of the 'a' cells, was investigated. In all cases, the amount of enzyme secreted from the glands was extremely low. This is consistent with the findings of Mans, who indicated that after feeding or stimulation with dopamine, acini revealed no detectable secretion of granules (Mans 2002a). Further studies also indicated that during feeding of ticks (until fully engorged), only ~17% of the total apyrase activity and 37% of the total salivary gland proteins were secreted, compared to unfed ticks. This indicates that fusion in *O. savignyi* most likely occurs via the kiss-and-run hypothesis and that the granule membrane does not get fully incorporated into the plasma membrane.

Extracellular stimuli indicated that the adrenergic receptor is involved in controlling exocytosis, and not cholinergic receptors, supporting the observations of Mans in *O. savignyi* (Mans 2002a) and McSwain *et al.* for *A. americanum* (McSwain *et al.* 1992a). The effect of other external stimuli, such as GABA and brain extracts which affected ixodid tick exocytosis, must still be investigated (Lindsay and Kaufman 1986; McSwain 1989). The similarity of the response between argasid and ixodid ticks with dopamine is remarkable. In both cases, cAMP levels are increased and PKA is activated, while elevated cAMP levels are inadequate for stimulating exocytosis. In argasid ticks, elevated cAMP levels were found to inhibit exocytosis. We therefore hypothesize that cAMP acts as an "off switch", regulating exocytosis from salivary glands of *O. savignyi*. The possible downstream effects of PKA regulated protein (described in Chapter 2) must be investigated in order to completely understand the role of cAMP during tick feeding.

Prostaglandins, especially PGE₂, have been shown to be critical for activating exocytosis in ixodid ticks. In *O. savignyi*, stimulation of glands with PGE₂ did however not result in activation of exocytosis. We did however indicate the role of an active phospholipase. In order to address this phenomenon, the generation of free arachidonic acid and the conversion thereof must be investigated during future studies. In *A. americanum*, the presence of an EP1-receptor, linked to a G_q-protein (which increase IP₃ and DAG) explains the positive effect on exocytosis. One possibility of the non-excitatory effect of PGE₂ in *O. savignyi*, could be the possibility of an EP2, EP3 or EP4 receptor (Table 2.7). These do not result in activation of PLC, but rather modulate cAMP levels. The latter has been proposed for the salivary glands of the blowfly, *Calliphora erythrocephala*, where PGs act by attenuating the 5-hydroxytryptamine effects by down regulating adenylyl cyclase, or by up-regulating cAMP degradation via interaction with phosphodiesterase (Stanley-Samuelson and Pedibhotla 1996). In insects, prostaglandins (PGs) are known for regulating ion and water transport similarly to that of other animals e.g. frog skin glands, toad urinary bladder, bivalve gill tissue, tick salivary glands, insect Malpighian tubules and insect rectal tissue (Stanley-Samuelson and Pedibhotla 1996). Therefore, the effect of PGs on fluid secretion and not protein secretion in *O. savignyi* must be investigated. Finally, the possibility of increased levels of other prostaglandins, such as PGF_{2a} and PGD₂ which are elevated in ixodid ticks upon feeding, must also be addressed (Bowman 1995d).

In most stimulatory cells, intracellular calcium is usually stored in ER, and released upon stimulation e.g. via activation of the IP₃ receptor. In *O. savignyi* we were able to show that an active PLC as well as an increase in intracellular calcium is essential for secretion of apyrase. Previous studies indicated that in granule containing cells of *O. savignyi* the ER and Golgi is not readily visible. This raises an interesting question as to the storage of intracellular calcium. In some cells, such as gonadotrophs, the poorly visible ER was found to locate close to secretory granules and are attached to the plasma membrane (Burgoyne and Morgan 2003).

During this study, we were the first to indicate the essential role of the cytoskeleton during exocytosis from tick salivary glands. We propose that the actin barrier is an important regulator of exocytosis and that upstream signaling pathways, such as PKA and PKC, regulate its assembly and disassembly. Interestingly, microtubules are also required for

exocytosis, and therefore one can hypothesize that the LDCVs still need to be transported to the site of fusion.

We identified homologues of the SNAREs syntaxin, VAMP and SNAP25 as well as the GTPase Rab3a, in the salivary glands of *O. savignyi*. Similar to secretory cells, studies performed in *A. americanum* indicated these proteins to be essential for exocytosis. RNA-interference directed against synaptobrevin (VAMP) and nSec1 were found to decrease expression of the transcript, levels of the protein and also inhibited PGE₂ stimulated anticoagulant secretion (Karim *et al.* 2004a; Karim *et al.* 2004b).

RNA isolation from salivary glands of unfed *O. savignyi* was found to contain only the 18S rRNA subunit. In contrast, RNA isolated from the entire fed *O. savignyi* tick (excluding the salivary glands) as well as from the fed argasid tick larvae of *Argas (P.) walkerae*, contained the 28S, 18S and 5S rRNA subunits. Universally, rRNA genes are organized in tandem repeats, separated by short transcribed spacers to form a single transcript unit of ~7500 bp, the 45S primary transcript (Voet and Voet 1995). These genes are transcribed by RNA polymerase I and post-transcriptionally processed and assembled with ribosomal proteins. Although not evident from the RNA gels in this study, a high molecular mass band has been observed during denaturing agarose electrophoresis of RNA from unfed *O. savignyi* salivary glands (Mans 2002). Therefore, one possible explanation is that rRNA is not post-transcriptionally processed in unfed *O. savignyi*. Secondly, similarly to the stringent response of prokaryotes (where the rate of rRNA synthesis is proportional to the rate of protein synthesis), signaling mechanisms could be involved in controlling rRNA synthesis and hence protein synthesis in *O. savignyi*. During non-feeding stages, which could be as long as 5-6 years, energy saving is essential and once feeding starts, signaling mechanisms could "switch on" the pathways regulating rRNA synthesis, processing and hence protein synthesis.

Although probes of SNAREs have been used with great success in the identification of SNARE homologues in other organisms or tissues (Table 3.6), they were unsuccessful during this study, as well as for identifying SNAREs in *A. americanum* (personal communication with Dr. S. Karim, Oklahoma State University, Stillwater, USA). Similarly, degenerate primers for SNAREs were also unsuccessful, most likely due to the difference in nucleotide sequence between known SNAREs and those of *O. savignyi*. This is supported by the data obtained during this study, where syntaxin was identified and found to share little sequence homology

with known syntaxins in the regions where primers were designed. In *A. americanum*, gene specific primers were used successfully in order to clone the SNARE synaptobrevin/VAMP (Karim *et al.* 2004a) as well as the regulatory protein nSec1 (Karim *et al.* 2004b). In both cases, the identified sequences shared identity to those of human and mouse only on amino acid sequence level. VAMP from *A. americanum* shared 65% identity to human VAMP, while nSec shared 74% identity to nSec1 from *Mus musculus*.

During two-hybrid studies, the syntaxin bait identified two novel domains that interact with syntaxin. Since these domains were repeatedly identified, we regard them as significant. Future studies are however needed to confirm their identity. By using α SNAP as bait as well as during functional complementation, putative syntaxin homologues were identified in *O. savignyi*. In both cases, the N-terminal domains of the syntaxin homologues were identified. This is noteworthy since it has been described that the N-terminal domains are involved in controlling membrane fusion in eukaryotes (Dietrich *et al.* 2003).

Pull-down assays with α -SNAP isolated all SNAREs indicating that the protein-protein interactions between SNAREs in *O. savignyi* resemble those of eukaryotes. Amino acid sequencing of these identified SNAREs will be valuable in designing *O. savignyi* sequence specific primers for cloning of these proteins.

In conclusion, this is the first study describing the mechanism underlying exocytosis from an argasid tick. Although their feeding pattern differs greatly from those of ixodid ticks (Chapter 1), the mechanism regulating exocytosis is remarkably similar. Similar to all eukaryotes, from yeast to man, argasid ticks seem to use the same conserved core machinery in a preserved mechanism in order to control exocytosis from LDCVs.

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