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Characterizing the Physiological Role of Inward Rectifier Potassium (Kir) Channels in Tick Salivary Gland Function

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CHARACTERIZING THE PHYSIOLOGICAL ROLE OF INWARD RECTIFIER POTASSIUM (KIR) CHANNELS IN TICK SALIVARY GLAND FUNCTION

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Abstract

The tick salivary gland is a critical tissue that enables blood feeding, maintenance of the ionic gradients, and facilitates pathogen transmission. Therefore, the broad objective of this investigation was to leverage pharmacological approaches to investigate the role of potassium ion channels to salivary gland function of *Amblyomma americanum* in an effort to identify a tractable target site for therapeutic development.

Data collected in Chapter 2 clearly illustrated that the *A. americanum* salivary gland is reliant upon inward rectifier potassium (Kir) channels for fluid secretion, which was similar to previous work performed by our laboratory on *Drosophila* salivary gland. Therefore, the physiological role of Kir channels was further characterized in fluid secretion, osmoregulation, and feeding capability in live ticks.

Further characterization showed that a subtype of Kir channels, termed ATP-gated Kir channels (K_{ATP} channels), are critical for proper salivation because the agonists VU0071063 and pinacidil inhibited salivation with an IC_{50} of 2 µM and 200 µM, respectively. Importantly, the inhibitory effect was negated by pre-treatment with ATP, which is known to inhibit K_{ATP} channels and provides support that VU0071063 is indeed reducing salivation through modulation of K_{ATP} channels.

In Chapter 3, we tested the hypothesis that K_{ATP} channels are critical for ion secretion and absorption, thus osmoregulation. K_{ATP} channel agonists were found to increase the concentration of Na^+, K^+, and Cl^- ions in the secreted saliva by 10-15 fold when compared to control. These data suggest that K_{ATP} channels are likely maintaining, at least in part, the intracellular loop currents that drive ion secretion and/or reabsorption during salivary gland activity.

In Chapter 4, we aimed to translate the in vitro data collected in chapters 2 and 3 into a functional study that tested the utility of K_{ATP} modulators to alter feeding and survivability to a live tick. Data show that both K_{ATP} agonists, pinacidil and VU0071063, increased the rate of detachments during blood feeding, reduced the volume of ingested blood, and lead to mortality. The data collected in this thesis provide significant support for targeting Kir/K_{ATP} channels in future therapeutic development campaigns to reduce the burden of tick vectored pathogens.

Chapter 1. Literature Review

1.1. Ticks

Ticks are hematophagous ectoparasites of vertebrates that are classified into subclass Acari, class Arachnida, phylum Arthropoda. There are over 800 species of ticks that are grouped into three families: Argasidae (soft tick), Ixodidae (hard tick) and Nuttalliellidae (monospecific family) (Horak et al., 2003; Guglielmone et al., 2010). The body of ticks can be divided into two parts, gnathosoma and idiosoma. The gnathosoma, or capitulum, bears the mouthparts that are comprised of two palps and a pair of chelicerae and the hypostome. The idiosoma describes the anatomical region caudal to the head and is comprised of legs, spiracles, reproductive and digestive openings.

Ticks have four developmental stages: egg, six-legged larva, eight-legged nymph, and eight-legged adult. Ticks are obligate hematophagous arthropods, and all life stages, except the egg, requires a blood meal to transit to the subsequent life stage. For hard ticks, after having a blood meal, they drop off the host and molt into the next life stage. When adult female ticks get fully engorged, they lay thousands of eggs and then die. Although both, ixodid and argasid ticks require blood meals prior to molt, these families differ in the time required for feeding. Ixodid ticks are known to have a single feeding period that lasts for an extensive length of time (i.e. 2-3 days) whereas argasid ticks have multiple partial feeding events that last for minutes to hours (Klompen et al., 1996). Ixodid ticks can be further grouped into three categories, one-host, two-host and three-host ticks, based on the number of hosts that are needed to complete the life cycle from larvae to adults. One-host ticks, such as *Rhipicephalus annulatus*, feed and mate on a single host to finish the three life stages (larva, nymph and adult) (Ziapour et al., 2017). Two-host

ticks, such as *Hyalomma dromedarii*, larvae and nymphs feed on one host, fall off the host, and then adults will obtain the final blood meal for reproduction on a second host (Apanaskevich et al., 2008). Lastly, three-host ticks, such as *Dermacentor variabilis* (Anderson and Magnarelli, 1980) are most common among ixodid ticks and life cycle requires the ticks to feed on three different hosts for each molt.

Ixodid adult feeding process can be divided into three stages, attachment, slow feeding, and rapid engorgement (Sauer et al., 1995). Once mated, females feed much faster and the body weight can increase 200 times to get fully engorged. Then females drop off the host and find a place to lay eggs until death.

1.2. Medical and Veterinary Relevance of Ticks

Recent intensification of human and animal movements, combined with socioeconomic and environmental changes, as well as the expanding geographical distribution of several tick species, have all contributed to the growing global threat of emerging or re-emerging tick-borne diseases (Dantas-Torres et al., 2012). Ticks are distributed worldwide and are of enormous medical and veterinary relevance owing to the direct damage they cause to their hosts and because they transmit a large variety of human and animal pathogens.

Tick-borne infectious diseases represent a serious world health concern and a major obstacle for animal health and production (Rajput et al., 2006). Ticks have been reported to transmit a variety of infectious microorganisms, e.g. bacteria, viruses, protozoa, fungi, and helminthes and are second only to mosquitoes as vectors of human and animal pathogens (Sonenshine and Hynes 2008, Peter et al., 2005). In addition to transmission of pathogens, some species of ticks produce a toxin that causes paralysis, termed tick paralysis (Grattan-Smith et al.,

1997). From a veterinary perspective, ticks are responsible for significant economic losses that stem from severe blood loss that leads to anemia, reducing growth rate and milk production, and mortality from pathogen infection (Rajput et al., 2006).

Soft ticks are competent vectors for a variety of vertebrate pathogens that cause severe diseases in humans and animals. These pathogens are thoroughly reviewed in detail by Manzano-Roman et al (2012) and will not be detailed here. Briefly, the majority of the pathogens that are transmitted by argasid ticks are viruses, such as African swine fever virus, but are also documented to transmit borreliae bacteria that causes relapsing fevers in humans (Manzano-Roman et al., 2012). Furthermore, laboratory studies have shown that various species of argasid ticks can transmit pathogens that are not usually associated with ticks, such as West Nile virus, various RNA flaviviruses, bluetongue virus, and hepatitis B (Lawrie et al., 2004; Charrel et al., 2007; Bouwknegt et al., 2010; Jupp et al., 1987). Tick-mediated transmission of these pathogens has the potential to significantly impact human health and the economy tied to veterinary health, and substantiates the need to identify mechanisms that can non-discriminately halt the transmission of pathogens.

Similar to soft ticks, the genera that comprise the taxonomic family Ixodidae, or hard ticks, are major vectors of human and animal pathogens in the United States and throughout the world. The Ixodidae family includes the genera *Ixodes*, *Amblyomma*, *Dermacentor* and *Rhipicephalus* which comprise approximately 70% of the world's tick fauna and are responsible for the majority of diseases stemming from ticks. Hard ticks are documented to transmit dozens of microbial disease causing agents, including protozoans, bacteria, and viruses to animals, including humans, pets, livestock, and wildlife. Although the agents of disease transmitted by hard ticks are diverse, the most commonly observed pathogens affecting human health are

Borrelia burgdorferi (causative agent of Lyme disease), *Ehrlichia chaffeensis* (causative agent of Human monocytic ehrlichiosis), and *Rickettsia* spp (the causative agents of tick-borne rickettsioses). In addition to these pathogens, ixodid ticks are known to transmit a variety of different viruses that cause extreme morbidity and mortality to vertebrates, particularly humans. In addition to human health, ticks represent a major pest of animals and are a major concern for the health of domestic animals and livestock. The most relevant parasite to livestock is the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, which is a vector of the protozoan and bacterial pathogens that cause babesiosis and anaplasmosis, respectively (Andreotti et al., 2011). The impact of these pathogens to the livestock community is significant due to the high rates of mortality and economic losses are furthered substantially as increased concentrations of tick infestations lead to reduction in milk production and weight gain, as well as overall declines in cattle health (Jonsson et al., 2008). To this point, it is estimated that the global cost by tick and tick-borne disease in cattle was between 13.9 and 18.7 billion USD annually (de Castro, 1997), indicating the significance of proper tick management. Unfortunately, the significant advancements in the fields of tick genomics, tick saliva proteins, and vaccine technology have translated poorly into successful prevention efforts and has resulted in a continued increase in tick-borne disease cases (Piesman and Eisen, 2008).

1.3. Examples of Tick-Borne Diseases

Lyme disease, which is caused by the spirochete, *Borrelia burgdorferi*, is the most commonly reported vector-borne disease and is transmitted by the blacklegged tick, *Ixodes scapularis* Say, in the United States. By virtue of its wide distribution and high infection prevalence in vector ticks, Lyme disease was recognized as a public health problem decades ago and became a nationally recognizable disease in 1991 (Schwartz et al., 2017). Since then both the

number and geographic distribution of Lyme disease cases have continued to increase, as has the range of its main vector. CDC reports shows that just over 25,000 individuals per year (2005-2014) were afflicted with Lyme borreliosis. However, the number of cases reported to the CDC has nearly tripled over the past 20 years, with more than 28,000 confirmed cases for the year 2015, despite extensive research efforts aimed at controlling this disease. Additionally, Lyme disease has a large geographical distribution along the eastern coast of the United States, and is steadily spreading into the Midwest, highlighting the need for the development of novel methods to reduce the burden of this disease (Piesman, 2006).

Human monocytic ehrlichiosis is caused by the obligatory intracellular bacteria, *Ehrlichia chaffeensis*, which is transmitted by the lone star tick, *Amblyomma americanum* (Ewing et al., 1995). This species of tick has a large geographic distribution, spanning nearly the entire eastern half of the United States, posing a real risk to the residents of numerous southeastern states of being exposed to ehrlichiosis. Similarly, tick-borne rickettsioses have been reported in this tick vector (Cohen et al., 2009; Jiang et al., 2010) and are caused by members of the spotted fever group (SFG) *Rickettsia* classified in the genus *Rickettsia* (Rickettsiales: Rickettsiaceae). *Rickettsia* is a gram negative, obligate intracellular bacterium. Generally, the clinical symptoms of all tick-borne rickettsioses are similar and include fever, headache, rash, myalgia, and nausea (Walker and Ismail 2008; Parola et al. 2005). However, in severe cases, endothelial cells become infected and results in hypotensive shock that leads to acute renal failure and is potentially fatal.

Tick-borne viruses are established in six different virus families: Asfarviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae, Bunyaviridae, Flaviviridae (Labuda and Nuttall, 2004). The viruses are obligate parasites and therefore, require the transcription, translation, and

post-translation processing machinery of their hosts to propagate. Powassan virus (POWV) is a RNA virus in the genus *Flavivirus* and is a fatal, neurotropic virus (Kazimírová et al., 2017) that represents the only member of tick-borne encephalitis serogroup present in North America (Hermance and Thangamani, 2017). The number of POWV disease cases has increased from only one case in 2004 to 31 cases in 2017 and highlights the significant impact that POWV could have to human health worldwide (Hinten et al., 2008; U.S. Geological Survey 2016). POWV disease was first reported in 1958 in Powassan, Ontario (McLean et al., 1962) and is endemic to the northeast and upper midwest of the United States, yet cases have been reported in Far-Eastern Russia (Anderson et al., 2012). POWV is transmitted by a variety of tick species including, *D. andersoni, D. variabilis, Ixodes cookie* and *I. scapularis* (Ebel, 2010). The incubation period for Powassan virus ranges from 8 to 34 days (Ebel, 2010) and symptoms observed in humans are characterized by fever, headache, disorientation, vomiting, prostration, respiratory distress, and spastic paresis. The infection can lead to meningoencephalitis that carries a human fatality rate of approximately 10%, yet approximately 50% of those people who develop neurological symptoms end up with long-term sequelae (Dhama et al., 2014).

Crimean–Congo hemorrhagic fever is present in Africa, western China through southern Asia, the Middle East, and down to southeastern Europe. And this fever induces extreme morbidity and oftentimes result in high mortality (Ergönül, 2006). The etiological agent is a RNA virus, genus *Nairovirus* that is transmitted by a variety of tick species, including *Amblyomma variegatum*, *Hyalomma anatolicum*, *Hyalomma marginatum*, *H. marginatum rufipes, Hyalomma truncatum, Hyalomma lusitanicum, Hyalomma plumbeum, Rhipicephalus bursa, Rhipicephalus rossicus* and *D. marginatus* (Bente et al., 2013). The average incubation period in humans lasts 3.2 days from the tick bite (Swanepoel et al., 1987). Hemorrhagic

manifestations include epistaxis, gingival bleeding, bleeding gastric mucosa and hematuria, with death due to shock from blood loss, neurological complications, pulmonary hemorrhage or infections resulting in a rate of approximately 30% lethality (Mertens et al., 2013).

1.4. Current Methods to Control Tick-Borne Diseases

Tick control has historically been based on the use of neurotoxic acaricides, but the indiscriminate use of the same or related chemistry has resulted in development of acaricide resistant tick populations that limits the effectiveness of control (Ghosh et al., 2007; Wharton & Roulston, 1970; Graf et al., 2004; Abbas et al., 2014). For example, the cattle tick, *Rhipicephalus (Boophilus) microplus* has been documented to possess target site and/or metabolic resistance to multiple classes of acaricides, including arsenic, chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids (George et al., 2004; Perez-Cogollo et al., 2010; Klafke et al., 2006). The emergence of multi-acaricide resistant ticks has led to unprecedented level of acaricide failure among multiple species of ticks and is presumed to be the underlying reason for thereemergence of tick borne diseases worldwide (Vudriko et al., 2017). To circumvent the development of insecticide resistance, many farmers and governmental agencies are increasing the deployment rates and concentration of acaricides, which will continue to reduce the efficacy of synthetic molecules and raises the concern for environmental contamination (Graf et al., 2004; Willadsen, 2004; Piesman, 2006). Currently, methods are being explored that have the potential to minimize the amount of acaricides applied, such as self-treatment devices (Pound et al., 2000) and combining the pheromone and acaricides (Sonenshine, 2006), but the development of acaricide resistance and poorly managed control programs will likely remain an issue. To mitigate the dependency to synthetic acaricides, studies have tested the efficacy of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* as potential biocontrol

agents for ticks (Coutinho Rodrigues et al., 2016; Ren et al., 2016; Camargo et al., 2016). However, the efficacy of these control agents were shown to vary significantly among tick species, tick stage and fungus strain, limiting the utility of these agents (Wassermann et al., 2016; Merino et al., 2013). Due to the spread of tick-borne diseases, the increase in tick population ranges, and the reducing efficacy of control programs, there is a need to develop novel approaches for tick control that focus on interrupting pathogen transmission by the vector.

Vaccination of reservoir hosts or humans is an attractive alternative for the control of tick infestations and pathogen transmission as it is a more environmentally friendly method when compared to neurotoxic acaricides (Tsuda et al., 2001; de la Fuente et al., 2007; Parizi et al., 2012). It stands to reason that several tick-borne diseases can be controlled simultaneously by targeting a common vector versus targeting immunomodulatory antigens specific for a pathogen (de la Fuente et al., 2007) and furthermore, targeting a conserved physiological mechanism within all ticks will prevent transmission of a pathogen irrespective of the pathogen type (i.e. bacterial and viral). Commercially available vaccines against cattle fever ticks are approved for use outside of the United States, including Gavac® (Heber Biotec; Havana, Cuba), TickGARD (Hoechst Animal Health; Australia), and TickGARD^{PLUS} (Intervet Australia; Australia). These vaccines are based on the recombinant form of the concealed antigen, Bm86, obtained from the mid gut of *R. microplus* (Freeman et al., 2010). Studies have indicated that vaccination with Bm86 vaccines can reduce tick feeding and reproduction ability, resulting in decreased transmission of pathogens and increased mortality (Merino et al., 2013). For instance, Gavac was shown to reduce cattle tick populations by 55–100%, which resulted in 60% reduction of the acaricide application and reduced the financial burden of the farmer by $23.4 USD per animal

each year (De la Fuente et al., 1999). Recent work is providing new indication that the target antigen of Bm86 derived vaccines is conserved in a number of tick species, resulting in efficacy against other related tick species (Pipano et al., 2003; Popara et al., 2013; De Vos et al., 2001; Rodríguez-Valle et al., 2012; McKenna et al., 1998; Olds et al., 2012). In the field, promising results against *R. microplus* have been obtained by using vaccines alone (De La Fuente et al., 1998; Shahein et al., 2013) or in combination with acaricides (Redondo et al., 1999; Cunha et al., 2012). However, the high specificity of these vaccines, specifically Tick-GARD, has limited effective control of field populations, presumably due to strain variations among differing populations across different geographic locations.

Although the data collected with Bm86 derived vaccines suggest that vaccination is a viable approach to control tick-borne diseases, the development of vaccines that exploit a novel target is bottlenecked by the discovery of effective antigens. To date, tick antigen targets are restricted to certain functional classes, such as structural proteins from salivary glands, hydrolytic enzymes and membrane-associated proteins. Interestingly, little work has been performed to determine the potential to exploit other functional proteins as a vaccine target, such as transmembrane receptors and ion channels, which are proven target proteins as they are targets for acaricides (Willadsen, 2006). In 2014, Guerrero and colleagues cloned out an aquaporin gene RmAQP1 from gut tissue of fully engorged female *Rhipicephalus microplus* and employed it as an antigen to conduct vaccine trials. Data clearly show that targeting the aquaporin protein with antigenic methods was effective in reducing the functional capacity of the tick since approximately 75% of the larvae did not develop into adults (Guerrero et al., 2014). These data also support the notion that pathways critical for osmoregulation are putative target sites for vaccine discovery to control tick populations. Considering this, it is essential to define

how ion transport underlies tick salivary gland function, which ultimately facilitates blood feeding and pathogen transmission, to enable the identification of novel pathways for vaccine and/or chemical development.

1.5. Relevance of Tick Salivary Glands in Blood Feeding and Osmoregulation

Tick salivary glands are a paired structure composed of two branches of grape-shaped acini that are located laterally and extend from hypostome to the spiracles, usually intertwined with trachea (Figure 1.1 A). The two salivary glands have individual duct that finally attaches to the hypostome, which is responsible for depositing the saliva into the mouth (Figure 1.1 B). Ixodid female salivary glands consist of three types of acini, type I, II, III (Figure 1.2 A), while male ixodid ticks employ a fourth acini type (type IV) that is associated with sperm transmission (Bowman and Sauer, 2004). On the contrary, argasid ticks only have two types of acini, I and II that have a similar function when compared to the like acini of hard ticks (Coons and Roshdy, 1981). For hard ticks, the differing types of acini are immediately recognizable based on the physical location within the salivary gland anatomy. Type I acini are located on the anterior portion of the main salivary duct (Figure 1.2 B), type II acini are on the secondary branches, and type III acini are located on the most distal end tertiary branches of the salivary glands (Figure 1.2 C).

The cellular structure of the acinus has been described in detail in the literature and therefore, will only be briefly described here (Needham et al., 1990; Coons and Roshdy, 1973; Fawcett et al., 1981). Acinus I, attaches directly to the main duct that originates from the salivarium and is composed of agranular cells that include one large central cell surrounded by a number of peripheral cells. Type I acini consist of four cell types: central lamellate, peripheral lamellate, peritubular, and circumluminal cells.

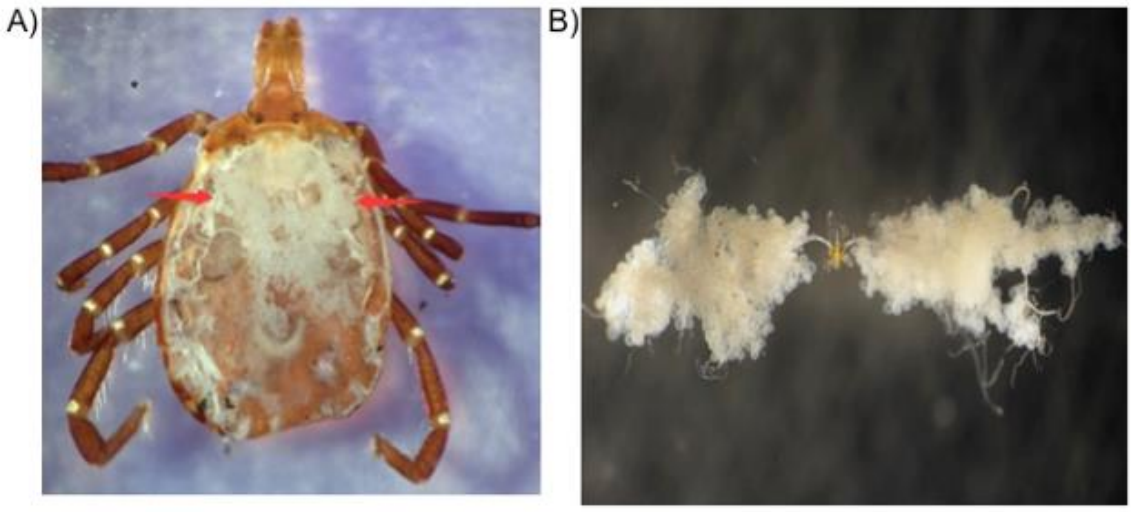


Figure 1.1. Anatomical orientation of *Amblyomma americanum* salivary glands in the tick (A) and dissected out of the tick (B).

During the feeding process, type I acini do not present significant morphological changes in terms of size and shape (Nunes et al., 2008; Binnington, 1978), but significant changes in the lipid phases, mitochondrial dissolution, and autophagic structures have been documents in the Type I acini of in *Amblyomma americanum* (Barker et al., 1984). Changes were also reported in the same species by Needham et al 1990, showing that changes in the mitochondria configuration occurs in dehydration/rehydrating conditions, leading to the speculation that Type I acini are responsible for water vapor uptake from unsaturated air, allowing for survival for months during off-host life. Indeed, this hypothesis was recently confirmed by the work of Park and colleagues that showed type I acini are the primary structures responsible for water and ion absorption in the off-host phase, as determined by the absorption function of type I acini via Na/K-ATPase in the off-host phase of female *I. scapularis* (Kim et al., 2016).

Type II acini attach to the main duct via a short lobular duct or directly. It is made up of granular cells of six types: A, B, C_1, C_2, C_3 and C_4, ablumenal and adlumenal interstitial cells.

The characteristics that distinguish different granular cells is based on the reactions with specific stains and the morphology of the granules (Binnington, 1978). The size of Cell B and C increases during cell activity (e.g. saliva generation), but Cell A has a reduced size when compared to types B and C (Binnington, 1978; Nunes et al., 2008), indicating that different cell types may have distinct roles for fluid generation, fluid secretion, reabsorption, and water retention.

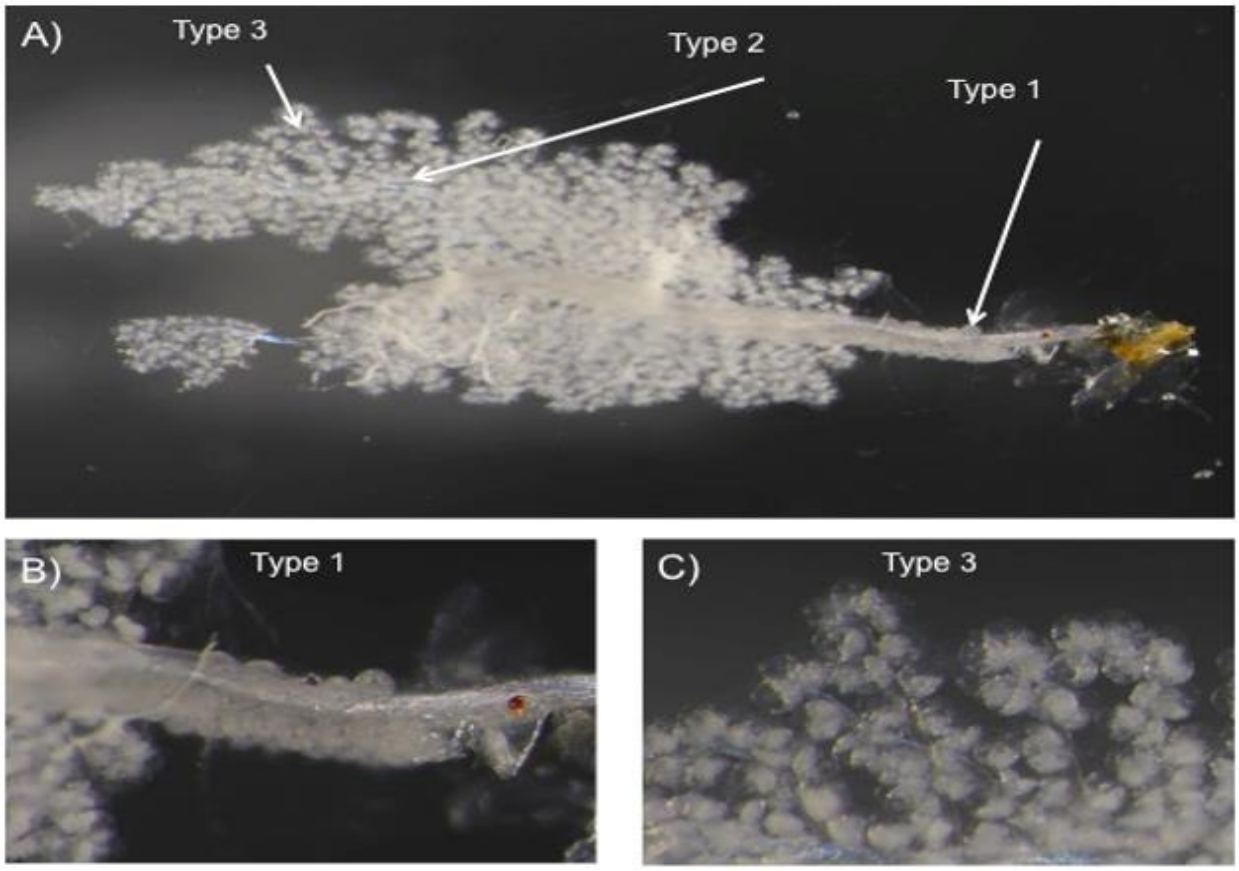


Figure 1.2. Types of acini in the *Amblyomma americanum* salivary gland. (A) individual salivary gland at 80x magnification providing orientation for type 1, type 2, and type 3 acini. (B) magnification of the main salivary duct that harbors Type 1 acini. (C) magnification of the distal branches that harbor Type 3 acini. Type 2 acini are difficult to distinguish from Type 3.

Acinus III is larger in size than Acinus II and is distributed abundantly at the distal part of the gland. Type III acini are composed of three types of granular cells, D, E, F, numerous ablumenal interstitial cells and a single adlumenal interstitial cell. During tick feeding, acinus III experiences a significant size change in cell volume (Sauer et al., 1995; Bowman and Sauer, 2004; Kim et al., 2014) and it has been shown that both acinus II and III swell and contract to secret fluid that was accumulated in the lumen and into the duct (Coons et al., 1994; Sauer et al., 1995;). The significant transformation of the F cells and proliferating ablumenal interstitial cells are believed to be the foundational measure that is responsible for secretion of fluid from the salivary gland cells, to the ducts, and out of the mouth (Bowman and Sauer, 2004). Additionally, acinus III are believed to play a major role in fluid uptake from the hemolymph and excretion to the host, thus acini are likely to represent the principal cell type responsible for salivation, ingestion, osmoregulation, and ultimately, pathogen transmission.

1.6. Tick Salivary Gland Function

The multifunctional and morphologically complex salivary gland is critical to the biological success of ticks because it performs a key role in two events during blood feeding (Sauer et al., 1995; Bowman & Sauer, 2004). First, the salivary gland is the primary tissue responsible for maintaining a proper salt and water balance during blood feeding. Mammalian blood contains high concentrations of sodium and potassium salts that would be toxic to the tick without coordinated osmoregulatory mechanisms during feeding, which is performed through the salivary gland by returning about 65-70% of the fluid and ion content of the blood meal back into the host during salivation (Binnington & Kemp, 1980; Bowman & Sauer, 2004). Considering this function, a failure to osmoregulation is likely to make the blood meal toxic and may represent a novel mechanism of acaricides that can be employed for tick population control.

Secondly, the tick salivary gland is critical to blood feeding as it secretes many bioactive proteins, such as anticoagulants and immunomodulatory proteins, that are critical for complete acquisition of the blood meal, suggesting that inhibition of salivary gland function will prevent protein secretion and blood ingestion. For instance, prostaglandins (PGs), generated in salivary glands, constitute a high amount in the tick saliva, which enhances tick feeding ability in three ways: immunosuppression, anti-haemostasis and anti-inflammatory (Bowman et al., 1996). During tick feeding, IgG-binding proteins were found in salivary gland and host immunoglobulin was detected in tick saliva, indicating that salivary gland is involved in the removal of foreign protein and overcome the host immune system (Wang & Nuttall, 1994). The hypothesis that interruption of normal salivary gland function can reduce the blood feeding capabilities of ticks has been test using an RNAi (RNA-mediated interference) approach to reduce mRNA expression of the aquaporin gene, IrAQP1, in *Ixodes ricinus.* The mRNA of this gene is upregulated in the salivary gland of partially fed ticks and genetic depletion of this gene resulted in a 50% reduction in tick body weight when feeding on artificial feeder and 21% reduction on a vertebrate host (Campbell et al., 2010).

Considering that the salivary glands are of great importance for blood feeding success, osmoregulation, and pathogen transmission (Binnington & Kemp, 1980; Labuda et al., 1993; Sukumaran et al., 2006), we hypothesize that disruption of salivary glands function represents a putative mechanism to reduce tick-borne diseases by inducing tick mortality and/or reducing pathogen transmission.

1.7. Neuroendocrinology of Tick Salivary Glands

A complete understanding of the steps and primary machinery required for proper salivary gland function within ticks is lacking, but significant amounts of work has been

performed to characterize the neuroendocrinology and neural innervations of tick salivary glands. Tick salivary glands are controlled by the central nervous system (Kaufman, 1978) and specifically, the glands are innervated from the pedipalpal nerve of the synganglion, which travels along the duct and connect to each single acinus (Megaw, 1977). Pharmacological studies have implicated several components involved in the process of salivary secretion: dopamine (DA), octopamine, γ-aminobutyric acid (GABA), ergot alkaloids, and the muscarinic acetylcholine receptor agonist pilocarpine (PC) (Kaufman, 1983; Lindsay and Kaufman, 1986; Kaufman and Wong, 1983; Kaufman, 1978). DA receptors have been identified on within the salivary gland acini as well as the nerves directly innervating the gland (Binnington and Stone, 1977), suggesting that salivary gland function is at least partially dependent upon DA. Indeed, DA is one of most potent stimulator of saliva secretion against multiple tick species (Sauer et al., 2000; Kim et al., 2016). A more comprehensive model of DA action on the tick salivary gland has been proposed (Sauer et al., 2000) in which DA activates two independent signaling pathways: cAMP-dependent signal transduction, which leads to fluid secretion, and a calcium-dependent signaling pathway, which stimulates prostaglandin E2 (PGE2) release through protein kinases. Furthermore, Park and colleagues have shown that two dopamine receptors, InvD1L and D1, are present in the salivary gland of the blacklegged tick that control inward fluid transport on the epithelial cells of the acini and expulsion of saliva from the acini to the salivary ducts (Kim et al., 2014; Šimo et al, 2014; Šimo et al., 2011; Šimo et al., 2012).

In addition to dopamine, other neuromodulators are involved in tick salivary secretion. In *A. americanum*, octopamine induced salivary secretion of isolated salivary glands, although it required 100 times higher concentration than dopamine (Needham and Pannabecker, 1983; Pannabecker and Needham, 1985). In *A. hebraeum*, gamma-aminobutyric acid (GABA)

potentiated dopamine-induced salivary secretion as a synergist, while GABA itself had no effects on salivary secretion (Lindsay and Kaufman, 1986). Pilocarpine, an agonist of the muscarinic acetylcholine receptors, induced salivary secretion by injection into the tick, while salivary glands without synganglion (tick brain) were not activated for the fluid secretion from *A. hebraeum* (Kaufman, 1978).

Although the neuroendocrinology and neural innervation of tick salivary glands is well documented, relatively little is known regarding the ion channels and transporters responsible for maintaining the membrane potential of the acini, maintenance of ionic balance, function of the salivary glands, and concentration of the saliva for osmoregulatory purposes. Earlier studies showed that extracellular calcium and cAMP were involved in dopamine-mediated salivation (Kaufman, 1976; Hume et al., 1984; Krolak et al., 1983; Needham and Sauer, 1979), suggesting that calcium and cAMP are likely serving as secondary messengers for downstream ion channels and transporters, such as calcium activated K^+ channels or GPCR-dependent K^+ channels (Albsoul-Younes et al., 2001). These downstream targets may contribute to salivary gland function and represent prospective targets to induce salivary gland failure, which is evidenced by the work performed Park and Colleagues that illustrate the importance of the Na^+-K^+-ATPase pump for saliva and ion secretion. However, little additional literature exists on other K^+ or Cl^- ion channels responsible for maintaining these ionic gradients in arthropod (Kim et al., 2016). In mammalian salivary glands, the stimulation of gland secretion is associated with a pronounced efflux of K^+ ions from the acinar cells into the blood, which has been shown to result from an increase in basolateral membrane permeability to K^+ ions. These data suggest that potassium ion transport is critical for proper salivary gland function in vertebrates and raises the intriguing possibility that they are essential in arthropod glands as well (Cook et al., 1994). Determining

the role and ‘druggability’ of these proteins in the tick salivary gland may provide targets for ceasing tick feeding through preventing salivation or hindering osmoregulation during feeding and thus, provide a platform for mitigation of tick-borne pathogen transmission.

1.8. Background of Potassium Transport Pathways

Potassium ion channels and transporters are a foundational component of physiological mechanisms since they are responsible for establishing and maintaining the membrane potential of animal cells and serve crucial roles in cellular regulation. Studies focused on the physiology of tick salivary gland have advanced our understanding into the mechanistic underpinnings of saliva secretion, but the ion transport pathways required for tick salivation remain understudied and unclear. Proper function of any polarized epithelial tissue, such as the salivary gland, requires strict regulation and maintenance of the membrane potential and resistance to enable an intracellular current that drives ion transport. In other polarized tissues, such as mosquito Malpighian tubules, transcellular and paracellular pathways are electrically coupled to form an intraepithelial current loop that enables electrogenic ion and water transport across membranes. Potassium channels have been shown to be crucial to maintaining cell membrane potential and resistance in mosquito Malpighian tubules and mammalian salivary glands (Herok et al., 1998; Nakamoto et al., 2008; Romanenko et al., 2007; Melvin et al., 2005). This suggests that alterations of the K^+ ion conductance pathways in the apical and/or basolateral membranes will alter the membrane potential and resistance, leading to a decrease in the loop current and inhibiting the transepithelial NaCl and KCl secretion across the salivary gland membranes. In the Malpighian tubules and vertebrate salivary glands, altering this conductance pathway prevents the secretion of urine or saliva, respectively. Currently the Na^+-K^+-ATPase is the only K^+ transport pathway to be studied in the tick salivary gland (Kim et al., 2016), which limits the

inferences that can be made for the mechanisms ticks use to maintain the membrane physiology of salivary glands and drive the generation of saliva. To address this gap in knowledge, this thesis research aimed to characterize the role of various K^+ pathways in the feeding cascade and salivary gland function of the tick, *Amblyomma americanum*. Below are brief summaries of various potassium transport pathways speculated to serve a role in tick salivary gland function.

1.9. Inward Rectifier Potassium (Kir) Channels

Inward rectifier potassium (Kir) channels belong to a large 'superfamily' of K^+ ion channels that includes the voltage-gated, two-pore, calcium-gated, and cyclic nucleotide-gated channels (Miller, 2000). Kir channels are a superfamily of potassium channels expressed in a variety of excitable and non-excitable cells and serve essential roles in the regulation of various physiological processes ranging from maintaining the resting membrane potential, cardiac and neuronal excitability, epithelial transport, metabolic homeostasis, muscle contraction, and cellular signaling. Over the course of the previous 10-15 years, Kir channels have emerged as an important therapeutic target in mammals as is evidenced by the fact that genetic mutations of Kir channels result in numerous diseases, such as Andersen's syndrome (Plaster et al., 2001; Scholl et al., 2009). Further, arthropod Kir channels have been proposed to represent an important ion channel in arthropods as they have recently been shown to represent a putative insecticide target in mosquitoes (Swale et al., 2016), critical for *Drosophila* salivary gland function (Swale et al., 2017), essential for proper function of *Aedes aegypti* and *Drosopihla* Malpighian tubules (Raphemot et al., 2013a; Wu et al., 2015), innate antiviral immunity (O'Neal et al., 2017a; Eleftherianos et al., 2011), and honey bee cardiac function (O'Neal et al., 2017b).

Although information pertaining to arthropod Kir channels is growing, nearly all of the structural and biophysical properties that are known about Kir channels stems from mammalian

Kir channels. In mammals, Kir channels are encoded by the *KCNJx* genes, which are comprised of sixteen members that play essential physiological roles in modulating the functions of most organ systems (Hibino et al., 2010). The *KCNJx* family is further subdivided in seven groups (Kir1.x to Kir7.x) based on amino acid sequence homology (Swale et al., 2014; Raphemot et al., 2014b). Further, Kir channels are classified depending on the regulatory and gating mechanisms of the channel with the majority of the groups being constitutively active Kir channels (Kir1.x, Kir2.x Kir4.x, Kir5.x, and Kir7.x), an individual group being activated by G-protein coupled receptors (Kir3.x), and an individual group being regulated by nucleotides, such as ATP. For arthropods, *Aedes aegypti* has been shown to possess 5 genes encoding Kir channels (Kir1, Kir2A, Kir2B, Kir2B', and Kir3) with splice variants existing for the genes Kir2A and Kir2B (Rouhier and Piermarini, 2014). *Drosophila melanogaster* is known to possess 3 Kir channel encoding genes, termed *ir*, *irk2*, and *irk3* (Luan and Li, 2012). Tissue expression patterns of *Drosophila* Kir channels are highly variable and are described in Luan and Li (2012), but it is predicted that the gating of the Kir channel is dependent upon the tissue expression versus the encoding gene as it is in mammals.

High-resolution X-ray crystal structures reveal that Kir channels exhibit a simple structural topology characterized by N- and C-terminus cytoplasmic domains, two transmembrane-spanning domains (TM1 and TM2), and a pore-forming loop containing the conserved K^+ selectivity filter sequence, termed a GYG motif (Figure 1.3A; Dibb et al., 2003; Bichet et al., 2003; Hibino, 2010). Kir channels assemble as tetrameric complexes that are comprised of identical (homomers) or different (heteromers) Kir subunits (Figure 1.3B). This tetrameric assembly of Kir channels creates a water-filled pore permitting the movement of K^+

ions down their electrochemical gradient and across the cell membrane (Fig 1.3C**),** allowing Kir channels to serve as biological diodes for various cell types.

Unlike voltage-gated potassium (K_v) channels, Kir channels do not possess a voltage-sensing domain, but alter the current amplitude based on voltage. By convention, the movement of K^+ ions inside a cell generates an inward current, whereas K^+ ions efflux generates an outward current. The voltage-dependent decrease in outward current is called "rectification" and is resultant of an intracellular blockade of the Kir channel pore divalent cations, such as magnesium and polyamines (e.g. putrescine, spermine and spermidine). The blockade of the pore that leads to a change in current is termed "inward rectification" (Fig. 1.3D). Because of the physical property of inward rectification, Kir channels exhibit typically larger inward currents at hyperpolarized potentials (more negative than the Nerst potential for potassium, E_K) as compared to outward currents (Hibino, 2010). Furthermore, Kir channels display various degrees of inward rectification between family members, which are broadly classified as "strong" or "weak" rectification, which is a unique property that contributes to potassium homeostasis in different tissues and organ systems (Figure 1.3D). The strength of rectification has been shown to be dependent upon the charge of the 'rectification residue' (N171, Kir1.1, human nomenclature) with negative charged amino acids enabling strong rectification and neutral or positive charged amino acids leading to weak rectification (Swale et al., 2014).

The pharmacology of classically gated Kir channels (i.e. non ATP-sensitive Kirs) is underdeveloped, but quickly growing due to the development of high-throughput screening platforms that enable the interrogation of large chemical repositories (Weaver et al., 2004; Raphemot et al., 2013b). Swale and colleagues thoroughly reviewed the current state of the field and highlighted recent developments of the pharmacology for mammalian Kir channels (Swale et

al., 2014) and therefore, will not be reiterated here. In regards to insect specific Kir channel inhibitors, the field was completely unexplored prior to the interest of targeting Kir channels as a novel target site for insecticides.

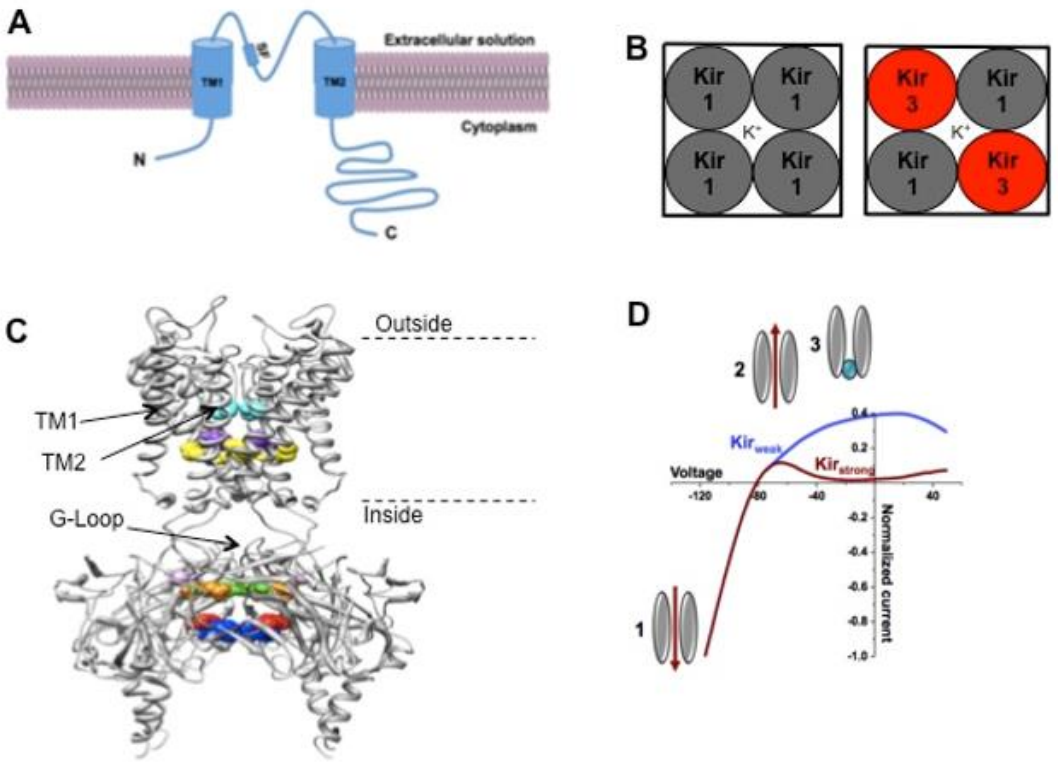
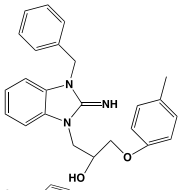
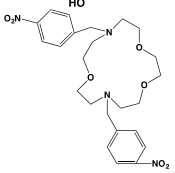
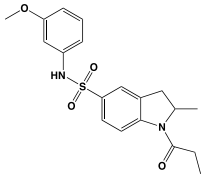
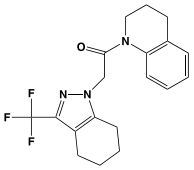
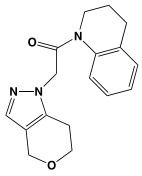


Figure 1.3. Overall Kir channel structure and characteristic currents. (A). Schematic of a Kir channel subunit indicating the cytoplasmic N- and C-terminus domains, two transmembrane-spanning domains (TM1 and TM2) and the pore-forming loop with selectivity filter (SF). (B) Schematic of the tetrameric complex formed between homomeric and heteromeric Kir channel subunits. Potassium ions passing through the pore is indicated. (C) Homology model of the Kir1.1 channel. The colored regions are the rectification residues of mammalian Kir channel families and small-molecule binding sites (Swale et al., 2014). Key structures are indicated as TM1 and TM2, SF and the G-loop. Adapted from Bhave et al., *Future Med Chem*. 2010 May; **2**(5):757-74. (D) Representative current-voltage relationships of Kir channel depicting currents generated by strong (red) and weak (blue) rectifiers. A schematic illustrating various stages of Kir channel current is shown as (1) inward current, (2) outward current and (3) pore block by divalent cations or polyamines. Panel D was modified from Raphemot et al 2013a.

However, the lack of specific pharmacological probes mired efforts to explore the integrative physiology and toxicological potential of insect Kir channels. To remedy this issue, mosquito Kir channels were the focus of two large drug discovery campaigns in an effort to identify molecules capable of modulating insect Kir channels and the leading Kir channel inhibitors are shown in Table 1.1. and 1.2. The first campaign focused on the identification of modulators targeting the *Aedes aegypti* Kir1 channel, which is the principal K^+ conductance pathway on the basolateral side of the Malpighian tubule principal cells and is required for proper tubule function (Scott et al., 2004; Beyenbach et al., 2011). The investigators screened 75,000 structurally diverse small molecules from the Vanderbilt Institute of Chemical Biology library against the *Ae. aegypti* Kir1 channel and identified over 300 potential inhibitors. Three small-molecule inhibitors of *Ae*Kir1, VU573, VU590, and VU625, have been thoroughly tested to determine the potency, selectivity, and activity in a living cell (Raphemot et al., 2014a; Rouhier et al., 2014). The molecules have inherent downfalls that limit marketability of the compounds as insecticides, but are well suited for probing the physiological function of Kir channels in vivo. For instance, VU573 is potent but inhibits an array of Kir channels (Raphemot et al., 2013a), VU590 is a weak inhibitor of mosquito Kir1 channels and is more potent against human Kir1.1 (Lewis et al., 2009), and VU625 is metabolized to prevent in vivo studies (Raphemot et al., 2014a). However, it is important to note that VU625 is the most potent inhibitor of insect Kir channels identified to date. In an effort to mitigate the inherent downfalls of VU573, VU590, and VU625, a second drug discovery campaign was performed and targeted the *Anopheles gambiae* Kir1 channel. A total of 25,000 structurally diverse scaffolds were screened and a total of 17 sub-micromolar inhibitors of *An. gambiae* Kir1 was identified (Swale et al., 2016). The leading molecule, VU041, was shown to have an IC_{50} value of approximately

500 nM in voltage clamp electrophysiology studies using a stable cell line expressing the Kir1 channel and importantly, was determined to be one of the most selective insect Kir channel modulators developed as it had no detectible activity against other insect Kir channels (e.g. Kir2x, Kir3).

Table 1.1. Pharmacological modulators of described insect Kir channels.

Compound	Structure	Mammalian Kir IC_{50} (μM)	Insect Kir1 IC_{50} (μM)	References
Inhibitors				
VU0160573 (VU573)		Kir1.1= 10±3 Kir2 = 4±0.3 Kir3 = 2±0.4 Kir7.1 = 5±3	5 ± 1.2	Raphemot et al., 2013a
VU590		Kir1.1 = 0.1 ±0.05 Kir7.1 = 10±3	10 ± 2	Lewis et al., 2009; Rouhier et al., 2014
VU625		Kir1.1 > 30 Kir2 > 30 Kir3 = 8.6 Kir6.2/SUR1 > 30 Kir7.1 > 30	0.31 ± 0.1	Raphemot et al., 2014a
VU041		Kir1.1 > 30 Kir2 = 2.5 Kir3 >30 Kir6.2/SUR1 > 30 Kir7.1 > 30	0.5 ± 0.1	Swale et al., 2016
VU730		Kir1.1 > 30 Kir2 > 30 Kir3 >30 Kir6.2/SUR1 > 30 Kir7.1 > 30	0.7 ± 0.2	Swale et al., 2016

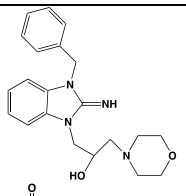
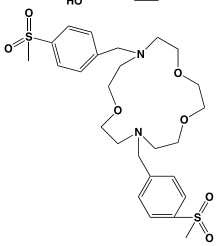
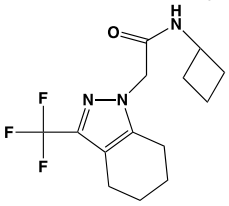
Similarly, *in vitro* screening assays for VU041 show that this compound has a relatively clean ancillary pharmacology against a panel of mammalian Kir channels with no activity against Kir1.1, Kir4.1, Kir7.1, and Kir6.2/SUR1. However, VU041 moderately inhibits Kir2.1,

which is highly expressed in the human heart, thus inhibition of this channel may have deleterious consequences on heart function. To remedy this potential issue, analogs of VU041 were developed to determine if any structural changes led to increased selectivity for *An*Kir1 vs. Kir2.1. Addition of a carbonyl in the 4- position of the tetrahydroindazole led, but the deletion of the nitrogen moiety in conjunction with the carbonyl group retained the activity toward *An*Kir1, but lost activity toward Kir2.1 (IC_{50}>30 mM). This molecule was named VU730 and is highly potent with clean ancillary pharmacology against other insect and human Kir channels. Finally, moving the nitrogen outside of the ring system led to an inactive compound and similarly, other smaller ring systems were not tolerated. Considering this, we synthesized molecules with a smaller ring system to generate VU937, which inhibited the *An*Kir1 channel activity by 60-fold less than VU041 (IC_{50}: 29,670 nM) and was deemed to be 'inactive'. The selectivity combined with the development of an inactive analog of VU041 highlights the potential of VU041 to be an effective probe of the physiological function of insect Kir1 channels.

1.10. ATP-Sensitive Kir (K_{ATP}) Channels

ATP sensitive Kir channels (K_{ATP}) are one of the subgroups of Kir channels that are not yet characterized in arthropods, but have been thoroughly investigated and reviewed in mammals (Alejandro et al., 2009; Ballanyi, 2004; Nichols, 2006; Babenko et al., 1998). In mammals, K_{ATP} channels are assembled by the combination of Kir6.x channels and sulfonylurea receptors (SUR) at a stoichiometric ratio of 4:4 (Kir:SUR) (Hibino et al., 2010). In this heteromeric channel complex, Kir6.x is responsible for pore formation and the obligate heteromer, SUR, is required for activation and regulation of the K^+ conductance (Aguilar-Bryan et al., 1995).

Table 1.2. Inactive analogs of insect Kir channel inhibitors.

Inactive Analogs (active)	Structure	Potency Reduction to mosquito Kir1	References
VU342 (VU573)		> 10x	Raphemot et al., 2013a
VU608 (VU590)		> 20x	Rouhier et al., 2014
VU937 (VU041)		> 60x	Swale et al., 2016

The SUR complex is a member of the ATP-binding cassette (ABC) transporter family and is predicted to possess two intracellular nucleotide binding domains (NBD1 and NBD2) and three transmembrane domains (TMD0, TMDl, and TMD2) that contains five, six, and six transmembrane helices (TMs), respectively. Two genes for SUR has been identified, encoding the proteins SURl and SUR2, which are expressed in different tissues (Aguilar-Bryan et al., 1995; Isomoto et al., 1996). SURl is predominantly expressed in pancreatic β-cells and neurons. Alternative splicing of SUR2 produces a cardiac/skeletal muscle isoform (SUR2A) and a smooth muscle isoform (SUR2B) (Isomoto et al., 1996). SUR1 and SUR2 proteins are encoded by the *ABCC8* and *ABCC9* genes, respectively. The different assembly of the different Kir6 subtypes with different SUR subtypes results in distinct channel biophysics and conductance properties (Hibino et al., 2010).

The opening of K_{ATP} channels is inhibited by ATP (Cook et al., 1984) whereas the channel is activated by magnesium bound nucleotides and ADP (Terzic et al., 1995) and therefore, K_{ATP} channels sit at the crossroads of cell metabolism and membrane excitability (Nichols, 2006; Lee et al., 2017). Thus, K_{ATP} channels are open during states of low metabolic activity, resulting in hyperpolarization of the membrane, which has cytoprotective effects (Cole et al., 1991; Wind et al., 1997). Conversely, during times of high metabolism, K_{ATP} channel activity decreases and the resulting membrane depolarization triggers cellular responses specific to that cell type, such as insulin secretion in the case of the pancreatic β-cells (Ashcroft, 2005).

The pharmacology of K_{ATP} channels is the most developed of all Kir channels and consists of both, activators and inhibitors. However, as is the case with expression patterns of K_{ATP} channels, all of the available modulators have been designed to treat human pathologies and no studies have been performed to identify specific modulators of arthropod K_{ATP} channels. The molecular structure, potency, and selectivity of drugs targeting the major vertebrate K_{ATP} channel subtypes are summarized in Table 1.3. and are thoroughly reviewed in Kharade et al (2016).

Kir6.2/SUR1 channels are the molecular targets for inhibitory sulfonylureas and related drugs that are used to treat Type 2 diabetes by exciting pancreatic β-cells and inducing insulin secretion (Aguilar-Bryan et al., 1995; Loubatières-Mariani, 2007). Between 1956 and 1966, the first-generation ‘sulfonylureas’ (tolbutamide, chlorpropamide, acetohexamide and tolazamide) were introduced into clinical practice for the treatment of diabetes mellitus. Lead optimization efforts to improve the hypoglycemic efficacy led to the development of second-generation sulfonylureas, such as glibenclamide (glyburide), and gliclazide. The third-generation sulfonylurea antagonist, glimepiride, was introduced in the late 1980s.

Opposite to the K_{ATP} inhibitors, pharmacological activation, or opening, of K_{ATP} channels tend to hyperpolarize the cell membrane potential that dampens cell excitability and reduces calcium entry into the cells. This physiological ramification enables K_{ATP} activators to be used clinically to inhibit insulin secretion, reduce blood pressure through inhibition of vascular smooth muscle to control vascular vasodilation, reverse male pattern baldness, and alleviate angina pectoris. Pharmacological activators of K_{ATP} channels are structurally diverse and consist of two generations of structures that differ in activity and potency. The first-generation of K_{ATP} activators consist of the benzopyrans, benzothiadiazines, cyanoguanidines, pyridylnitrates and thioformamide while the second-generation molecules consist of the cyclobutenediones, dihydropyridines and tertiarycarbinols. The two most common K_{ATP} activators are diazoxide and pinacidil, which preferentially target SUR1 and SUR2 containing channels, respectively. Although not as commonly used as pharmacological probes, nicorandil and minoxidil are commercialized activators of K_{ATP} channels that preferentially target SUR2 subunits and are used in the clinic to treat human pathologies associated with K_{ATP} channel mutations (Ashcroft and Gribble, 2000). An additional K_{ATP} activator, termed VU063, was serendipitously discovered during a high-throughput screen against mosquito Kir1 channels (Raphemot et al., 2014b). Subsequent characterization with fluorescent biochemical assays and voltage clamp electrophysiology demonstrated that VU063 is approximately 12-fold more potent than diazoxide, activates Kir6.2/SUR1 with faster kinetics than diazoxide, acts directly on Kir6.2/SUR1 to open the channels, and is specific for K_{ATP} channels containing SUR1 (Raphemot et al., 2014b).

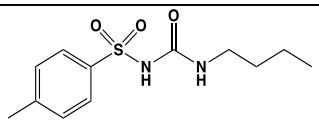
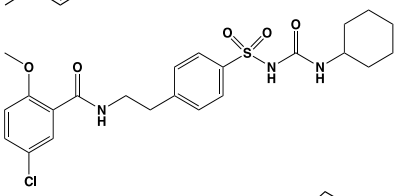
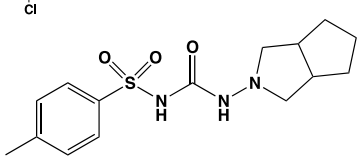
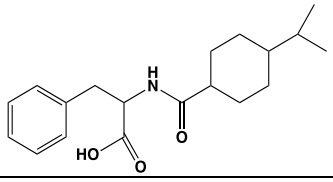
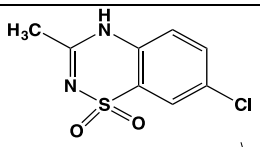
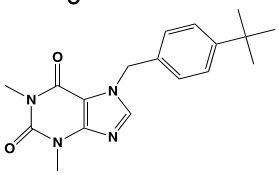
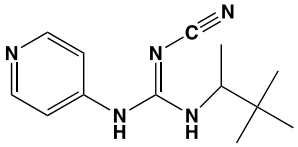
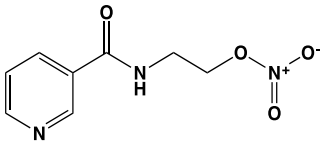
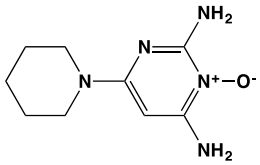
As with inhibitors of SURs, the potency and selectivity of the K_{ATP} activators differ based on varying interactions with Mg-nucleotides at the binding domains (Koster et al., 1999).

The varying interactions with the binding sites of the different heteromeric proteins limit the ability to develop a highly specific modulator of K_{ATP} channels. For example, the two activators, diazoxide and pinacidil, exhibit preferential activity toward SUR1- and SUR2-dependent channels, respectively, but diazoxide exhibits equivalent activity toward SUR2-containing channels in the presence of intracellular magnesium and nucleotides (D'hahan et al., 1999).

1.11. Two Pore Domain Potassium (K2P) Channels

K2P channels have been identified to be a critical K^+ conductance pathway within the Kingdoms Animalia and Plantae (Renjigunta et al., 2015; González et al., 2015). The molecular structure of K2P channels is simple when compared to other potassium ion channels because they assemble as dimeric versus tetrameric proteins. Each subunit has four transmembrane domains (M1–M4), two-pore domains (P1 and P2) and two extracellular cap helices (C1 and C2). Another unique character is that there is an extracellular loop between M1 and P1, which forms a 'cap' structure in the channel, which is stabilized by a disulfide bridge (Renigunta et al., 2015; Kuang et al., 2015; Feliciangeli et al., 2015). In 2012, two crystal structures of K2P channels, TWIK-1 (Miller and Long, 2012) and TRAAK (Brohawn et al., 2012), have been solved, shedding a light on the gating properties of this channel enabling additional studies rooted in drug discovery and genetic manipulation targeting specific biophysical features of K2P channels. Although the biophysical properties, physiological importance, and evolutionary history of K2P channels is not fully understood, genetic evidence has now validated that these channels are functionally important in the central nervous system, the heart, blood vessels, the kidneys, endocrine and exocrine glands of mammals.

Table 1.3. Described small-molecule modulators of human K_{ATP} channels

Compound	Structure	Selectivity	IC_{50}	References
Inhibitors				
Tolbutamide		SUR1>SUR2	2-7 μM	Gribble et al., 1998
Glibenclamide (glyburide)		SUR1>SUR2	SUR1: 4 nM SUR2A: 27 nM	Gribble et al., 1998
Gliclazide		SUR1	50 nM	Gribble and Ashcroft., 1999
Nateglinide		SUR1	100 nM	Sunaga et al., 2001
Activators				
Diazoxide		SUR1>SUR2	60-80 μM	Dabrowski et al., 2003
VU0071063		SUR1	7 μM	Raphemot et al., 2014b
Pinacidil		SUR2	1 μM	Lefebvre and Horacek ,1992
Nicorandil		SUR2	100 μM	Sato et al., 2000
Monoxidil		SUR2	182 μM	Sato et al., 2004

In mammals, a total of 15 K2P subunits have been identified and in accordance with the sequence homology they can be divided into six major groups: 1) TWIK clade (weakly inwardly rectifying K2P channels; 2) TREK clade (lipid and mechanosensitive K2P channels); 3) TASK clade (acid pH-sensitive K2P channels); 4) TALK clade (alkaline pH-activated K2P channels); 5) THIK clade (halothane-inhibited K2P channels); 6) TRESK clade (spinal cord K2P channels) (Honoré, 2007; Renjigunta et al., 2015; Ryoo and Park, 2016). Nearly every subunit has distinct regulatory mechanisms, which is one of the most unique characteristics that differ them from other ion channels.

K2P channels are often described as 'leak channels' (Kim, 2005), 'background channels' (Feliciangeli et al., 2015), 'open rectifier' channels (Goldstein et al., 2001) or 'K^+-selective holes' (Duprat et al., 1997). In their quantitative analysis of the mechanisms underlying the action potential in squid axons, Hodgkin and Huxley introduced a nonselective leak conductance to make the total ionic current zero at the resting potential and it was hypothesized that K2P channels are the leak current that Hodgkin and Huxley initially identified. However, this notion is currently being challenged as the families of K2P expands toward channels that have both, inward and outward conductances, which indicates the properties of K2P are not compatible with a leaky pore. In contrast to K_v, Na_v and Ca_v channels, K2P -channels do not possess a specialized voltage sensing domain and most K2P channels do not show any inactivation during depolarizing voltage steps. Therefore, K2P channels are sometimes described as voltage insensitive, and in studies of the function of K2P channels in native cells, their activation by depolarization is often not appreciated.

In mammals, K2P channels have been attributed to shaping the firing frequency of neurons and for determining the action potential configuration (Renigunta et al., 2015) and have

been shown to play key roles in the proper function of the human thalamus (Bista et al., 2015), adrenal gland (Kim and Kang, 2015) and digestive tract (Heitzmann and Warth, 2008). Unfortunately, little is known regarding the expression or role of K2P channels in the salivary glands and specifically no information pertaining to K2P channels in arthropods exists. However, due to the importance of K^+ ion transport in the function of polarized epithelial cells and that K2P channels constitute a unique transport pathway capable of translocating K^+ ions in both, inward and outward, directions, we speculate that K2P channels may be critical for proper functioning of the tick salivary gland.

1.12. Big Potassium (BK) Conductance Channels

BK channels are relatively unexplored and the scientific community has only recently identified their importance in tissue function. BK channels are oftentimes calcium (Ca^{++}) or voltage-activated channels and have a large unitary conductance of ~100–300 pS. These channels have been shown to activate in response to membrane depolarization and binding of intracellular Ca^{++} and/or Mg^{++} that results in potassium efflux (Latorre et al., 1989; Schreiber and Salkoff, 1997). BK channels are formed by four pore-forming subunits that are encoded by a single *Slo1* gene. BK channels achieve functional diversity primarily through alternative splicing of *Slo1* mRNA and modulation by accessory β subunits (Rosenblatt et al., 1997; Tseng-Crank et al., 1994). There are four types of β subunits (β1–4); each type displays a distinct tissue-specific expression pattern and uniquely modifies gating properties of the channel /react-text (Orio et al., 2002; Behrens et al., 2000; Brenner et al., 2000). In mammalian neurons, BK channels are localized to the axons and presynaptic terminals and functionally coupled to voltage-gated Ca^{++} channels. During an action potential, membrane depolarization and Ca^{++} entry into the cell

activates BK channels that causes an efflux of K+ ions and terminates the action potential and close Ca^{++} channels (Orio et al., 2002; Behrens et al., 2000; Brenner et al., 2000). Through this negative feedback mechanism, BK channels regulate membrane excitability and intracellular Ca^{++} signaling.

Currently, the full-length nucleotide sequence of a calcium activated potassium channel from mammalian salivary glands have been identified, cloned, and expressed (Nehrke et al., 2003). Importantly, electrophysiological methods have validated the presence of a functional calcium activated potassium channel with large conductance that is localized at the basalolateral membrane of acinar cells in mouse and rat salivary glands (Maruyama et al., 1983). In current model of mammalian salivation, potassium efflux into interstitial fluid is proposed to be involved in maintaining the electrochemical driving force for Cl^- efflux into acinar lumen (Melvin et al., 2005), which is supported by the reduction of intracellular potassium concentration in mouse submandibular gland when stimulated by cholinergic agonist bethanechol (Poulsen and Oakley, 1979). Moreover, Ca^{++} and cAMP are transmitters in acinar cells that regulate protein and fluid secretion in tick salivary gland and therefore, we speculate that calcium activated potassium channels are involved in the efflux of potassium ions (Petersen and Maruyama, 1984). Due to the role of BK channels in vertebrate salivary glands combined with the critical role of calcium mobilization in human ad tick salivary glands, we speculate that BK channels represent a critical conductance pathway within the physiological cascade of events necessary for saliva secretion.

1.13. Cation-Chloride Cotransporters

In absorptive and secretory epithelia, such as the salivary gland, transcellular ion transport depends on specific plasma membrane proteins for mediating ion entry into and exit from cells. In basolateral membrane of almost all epithelia (with exception of choroidal plexus),

sodium exit and potassium entrance occur through Na^+−K^+−ATPase, generating electrochemical gradients that constitute a driving force for Na^+ influx and K^+ efflux. Transport of these ions following their gradients can be accomplished by specific ion channels, allowing membrane passage of ions alone or by transporters in which Na^+ or K^+ transport is accompanied by other ions or solutes by means of several different solute transporters. These membrane proteins are known as secondary transporters because ion or molecule translocation is not dependent on ATP hydrolysis but rather on gradients generated by primary transporters (Boudker and Verdon, 2010). A secondary transport mechanism that is very active in transcellular ion transport in epithelial cells is one in which cations (Na^+ or K^+) are coupled with chloride (Cl^-), with a stoichiometry of 1:1; therefore, ion translocation produces no change in transmembrane potential. For this reason, these transporters are known as electroneutral cation coupled cotransporters. In addition to being heavily implicated in ion absorptive and secretory mechanisms, electroneutral cation coupled cotransporters play a key role in maintenance and regulation of cell volume in both epithelial and nonepithelial cells. Importantly, the net effect of Cl^- movement inside or outside cells is negligible since Na^+−K^+−ATPase pumps are capable of correcting the Na^+ influx and K^+ efflux that is driven by electroneutral cotransporters.

In mammalian salivary glands, the primary driving force for generating fluid secretion is generated by Cl^- uptake via $Na^+/K^+/2Cl^-$ cotransporters (Melvin et al., 2005). Considering this, we speculate that two cation chloride cotransporters, sodium-potassium-2 chloride cotransporter (NKCC) and potassium-chloride cotransporter (KCC), are responsible, in part, for salivary gland function.

1.14. Overarching Hypothesis to be Tested

The premise of this thesis is that blood feeding is critical for tick survivability and the physiological systems enabling blood feeding, such as the ion transport pathways in the salivary gland, represent putative target sites for prevent feeding and survivorship. Therefore, we will test the overarching hypothesis that potassium ion channels and transporters are critical for salivary gland function and pharmacological modulation of these proteins will prevent fluid secretion, ion secretion, ingestion of blood, and survivorship.

To test this hypothesis, we examined the following objectives.

1.15. Objectives of the Study

1. Determine the influence of K^+ transport pathways to the fluid secretory activity of the isolated salivary gland of *Amblyomma americanum*
2. Determine if the osmoregulatory function and ion secretory activity of the isolated salivary gland is dependent upon Kir channels
3. Determine the impact pharmacological modulation of Kir channels has to the blood feeding behavior, blood ingestion, and survivability of *Amblyomma americanum* as a proof-of-concept for targeting Kir channels to control tick-borne pathogens.

Chapter 2. Test the Hypothesis that Pharmacological Modulators of K^+ Ion Channels and Transporters Will Reduce the Fluid Secretion of the Isolated Salivary Glands from *Amblyomma americanum*

2.1. Introduction

During blood feeding, tick salivary glands are the organ responsible for secreting bioactive proteins and lipids to overcome the host immune system and the saliva is the vehicle that carries nearly all pathogens from the tick to the host. Due to the critical roles tick salivary glands and saliva have in various biological events critical to the biological success of ticks and their associated pathogens, the function of the salivary gland has been the target of copious amounts of research in an effort to describe the foundational physiology of gland function and develop products to reduce tick-borne diseases. Currently, the majority of research performed on the tick salivary gland has aimed to characterize the neural innervation of the glands and the neuroendocrinology responsible for saliva generation and subsequent fluid secretion.

Tick salivary glands are innervated by nerves that originate from the synganglion (Bowman & Sauer, 2004) and dopamine has been shown to be a primary neurotransmitter responsible for fluid secretion when applied to an isolated salivary gland (Kim et al., 2014) or injected into hemolymph (Kaufman, 1978). Treated with dopamine, acinar cells of the salivary gland increase concentrations of intracellular cAMP and calcium, which implies the physiological systems responsible for tick salivary gland function relies on these molecules to serve as secondary messengers and activate downstream pathways that regulate fluid and protein secretion (Needham and Sauer 1979; Sauer et al., 2000). Often, the downstream pathways are ion channels and transporters that, in mammals, have been shown to establish the driving force for fluid secretion and tissue function (Almassy et al., 2012). Specifically, potassium transport

pathways are critical for the proper function of mammalian salivary glands and are commonly expressed by various cell types to control tightly regulated epithelia, fluid secretion, and membrane polarization. However, little information exists regarding the functional role of K^+ transport pathways in tick salivary glands. Considering this, understanding the physiological role of ion transport in salivary gland function is important.

In mammals, parotid acinar cells are responsible for the production of saliva (Cook et al., 1994) and the driving force for fluid and electrolyte secretion is the movement of chloride (Cl^-) ions across the epithelia. Chloride ions are significantly more concentrated intracellularly via the concerted effort of several transporters, such as sodium-potassium-chloride (NKCC) cotransporter and stimulation of select sensory systems induces flux of Cl^- ions into the lumen of the gland (Cook et al., 1994). Similar to the mechanism that has been proposed for tick salivary glands, stimulation of the sensory system induces release of acetylcholine from parasympathetic nerves and ultimately causes an increase in the intracellular free calcium concentration $(Ca^{2+})_i$ (Putney, 1986). The widely accepted model explaining the molecular mechanism underlying the secretion of the primary acinar cell fluid theorizes that Ca^{2+} plays a pivotal role in the activation of K^+ ion channels that are members of the large-conductance "maxi-K" (BK; KCa1.1) and intermediate K^+ (IK; KCa3.1) families (Maruyama et al., 1983; Wegman et al., 1992; Nehrke et al., 2003). Ultimately, fluid secretion occurs as cations, primarily Na^+, are drawn paracellularly through tight junctions into the lumen due to the negative membrane potential established by Cl^- efflux (Wegman et al., 1992). Water follows the osmotic potential and forms the primary acinar cell secretion in form of saliva. This fluid is thought to reflect the Na^+, K^+, and Cl^- composition of the interstitial fluid bathing the basolateral surface of the acinar cells. The final composition of

saliva is substantially modified in the duct and results in a hypotonic solution, relatively low in Na^+ and Cl^- and conversely high in K^+ and HCO_3^- (Cook et al., 1994; Melvin et al., 2005).

Although the mechanism of salivation and salivary gland function is relatively clear in mammals, the role of K^+ ion conductance pathways in the tick salivary gland are underexplored. Recently, Kim et al (2016) described the expression patterns and functional role of *Ixodes scapularis* Na^+-K^+-ATPase pumps. The data provide clear evidence that Na^+-K^+-ATPase, and in a broader sense K^+ transport pathways, are critical for fluid secretion from the tick salivary gland, yet few studies have characterized the role these various pathways serve to saliva secretion. The large gap in knowledge pertaining to the foundational mechanisms that regulate the function of the tick salivary gland has limited the development of products to control ticks and the pathogens they vector.

Considering 1) multiple K^+ ion channels represent a critical ion conductance pathway for saliva generation and secretion in mammals, 2) Na^+-K^+-ATPase pumps in tick salivary glands are required for osmoregulation and saliva secretion, and 3) the importance of K^+ ion channels in maintaining membrane physiology of polarized epithelial cells, we hypothesized that the functionality of tick salivary glands depends on K^+ ion transport and further, pharmacological modulation of these pathways will reduce the volume and rate of saliva secretion. Therefore, the goals of Chapter 2 were to employ physiological assays (e.g. Fluid secretion assay) combined with pharmacological techniques to determine the physiological importance of potassium ion channels and transporters in the tick salivary gland. The data presented in this chapter will aid in bridging the fundamental knowledge gap regarding the unexplored physiological pathways in the tick salivary gland, which will provide a more holistic understanding to gland function and blood

feeding biology that can be used to develop products for halting tick feeding and pathogen transmission.

2.2. Materials and Methods

2.2.1. Pharmacological Modulators and Reagents

The Kir channel inhibitor VU041 and the inactive analog VU937 were originally discovered in HTS against the *Anopheles gambiae* Kir1 channel (Swale et al., 2016). Both compounds were synthesized by Dr. Corey Hopkins at the Vanderbilt Center for Neuroscience Drug Discovery using methods described in Swale et al (2016). Pinacidil, glybenclamide, tolbutamide, diazoxide, VU063, fluoxetine, furosemide, bumetinide, paxilline, and VU271 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were designated to be >98% pure. Chemical structures of the modulators used in this study are shown in Tables 1.2 and 1.3.

Hanks Balanced Salt Solution (HBSS) with calcium chloride and magnesium chloride was purchased from Life Technologies (14025-092) and was used in all Fluid secretion (Ramsay) assays (Ramsay, 1954). Dimethyl sulfoxide (DMSO) served was the solvent that was used to dissolve all small-molecule chemicals prior to dilution into HBSS and was purchased from Sigma-Aldrich (St. Louis, MO, USA). Clear 100% silicon II Chaulk (General Electric, M90050) was purchased from Ace Hardware and was used for the construction of the feeding membrane.

2.2.2. Ticks

Non-blood fed adult Lone Star ticks (*Amblyomma americanum*) were purchased from the Tick Rearing Center at Oklahoma State University (Stillwater, OK, USA).

2.2.3. Artificial Feeding System

We performed all of the physiology studies on partially fed ticks since the physiology and gene expression profile is different between an unfed, feeding, and fed tick. To obtain partially fed ticks, we adopted an artificial host system that enables blood feeding of multiple ticks on a silicone membrane in lieu of an animal host. This method has been previously described (Tajeri et al., 2016; Kröber and Guerin, 2007; Trentelman et al., 2017; Kim et al., 2016), however the reproducibility of this method is difficult and a common complaint among laboratories is the lack of feeding. A modified artificial membrane was made to get partially engorged ticks and conduct mortality and ingestion experiments. 10 g silicone glue (Silicone II) was mixed evenly with 3g silicone oil AR 20 (Sigma-Aldrich; St. Louis, MO, USA) and 2g Hexane (Sigma-Aldrich; St. Louis, MO, USA). Silicone mixture (the volume is about first segment of index finger) was applied onto the lens paper that was purchased from Tiffen Company LLC (Hauppauge, NY, USA). The silicone was spread evenly using a glass spreader to an approximate thickness of 60-80 μm (Krober and Guerin, 2007; Trentelman et al., 2017). The membrane was placed at room temperature for 14-21 days prior to use. The constructed feeding chamber is shown in Figure 2.1A.

Glass chambers (28 mm outer diameter, 2 mm wall thickness, 45 mm cylinder with a 35 mm OD bead that is 12 mm up from the bottom of cylinder) were ordered from Greatglass (Wilmington, DE, USA) and were used to construct the tick-feeding chamber. A membrane that was dried for >14 days were glued on the chamber with the same silicone glue at least one day before loading ticks. Glued chambers were placed into six-well cell culture plate with water to ensure the membrane did not leak. A total of 10 females and 5 males were loaded into one chamber with freshly shaved heifer hair. Cotton balls were inserted into the top of the chamber

and approximately 1 cm of head space was maintained for tick feeding (Figure 2.1B). We identified this to be key for increasing the feeding rates. After testing for leaks, the artificial membrane was immersed in 5 ml defibrinated bovine blood that was poured into a 6-well microassay plate and tick feeding was allowed to commence immediately upon placement into the chamber (Figure 2.1C-D). The blood was purchased from Hemostat Laboratories (Dixon, CA, USA). Gentamycin and ATP were added to the blood at a concentration of 5 µg/ml and 1µM, respectively. Tick feeding chambers were maintained in water bath at 38°C with a 16 h:8 h light:dark photoperiod. The blood was changed twice a day at 12 h interval for the duration of the experiment.

2.2.4. Ex Vivo Fluid Secretion Assays

The Ramsay assay is an assay that was developed to characterize the physiology of insect Malpighian tubules and the foundational role of various membrane bound proteins to urine formation, urine secretion, and osmoregulation (Ramsay, 1954). Modifications to enable measurements of secreted saliva of ticks have been previously described and used in this study (Ramsay, 1954; Kim et al., 2014; Simo et al., 2014). Partially engorged female ticks (weighing 10-20 mg, 3-4 days blood-fed) were prepared from the artificial feeding system described above (Kröber and Guerin, 2007). Salivary glands and the corresponding ductwork were dissected from the partially fed tick and then incubated in HBSS buffer for 1 h before initiating the Ramsay assay. After incubation, a single female gland was immersed in 15 µl HBSS buffer containing a HBSS + DMSO (vehicle control) or compound solubilized in HBSS. The main duct was pulled out of the HBSS and drawn across a narrow grease dam made of high vacuum grease (Dow Corning Corporation, Midland, MI, USA) that was approximately 1 mm in height and immobilized on the surface of a petri dish. After drawing the main duct across the grease dam,

the entire gland was submerged in heavy mineral oil to collect the secreted saliva. The preparation is shown in Figure 2.1E.

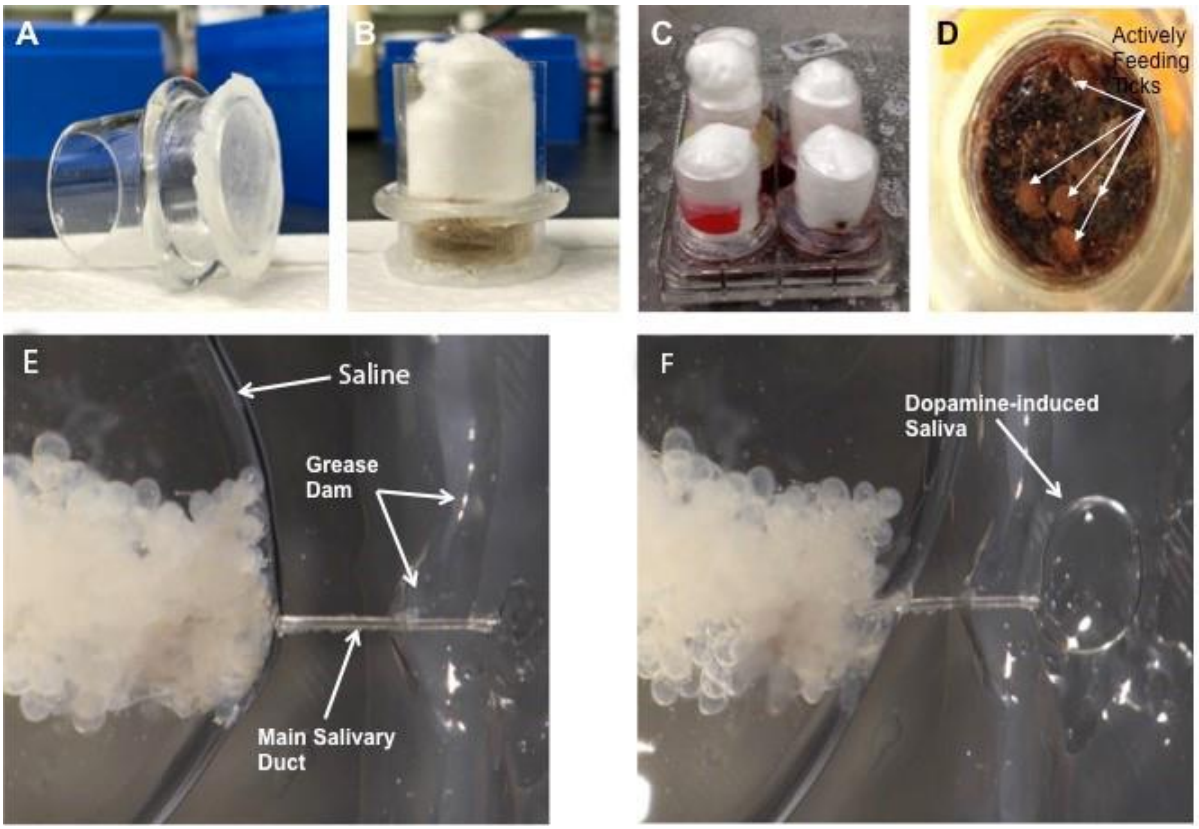


Figure 2.1. Artificial host feeding system used for tick blood feeding. The construction of the feeding chamber and images describing their construction are shown in panels A-D. The Ramsay assay method is shown with freshly isolated glands before (E) and after (F) dopamine application.

Saliva secretion was stimulated by applying 15 µL of 200 µM dopamine hydrochloride dissolved in HBSS and basal levels of dopamine mediated excretion measurements were taken in the first 5 minutes after preparation of the fluid secretion assay (Figure 2.1E). A micro-injector (Nanoliter 2010, World Precision Instrument, Inc., Sarasota, FL, USA) controlled by a micro-syringe pump controller (Micro4, World Precision Instrument, Inc., Sarasota, FL, USA) was

used for withdrawing the secretion formed at the tip of the duct in the heavy mineral oil, and the volume withdrawn was recorded in every 5 min for 30 min. To study the influence of K^+ channel modulators on saliva secretion, the gland was incubated in the compound solution for 30 minutes prior to exposure to dopamine that initiates salivation. Concentration-response curves were performed with one gland of the tick exposed to dopamine only (control) and the second gland treated with the small-molecule modulator prior to dopamine exposure, which increased the rigor of the experimental design through paired analysis. Total salivation for each time point of the treated glands was compared to the volume secreted at the same time point for dopamine only treated glands to obtain percent saliva secreted when compared to control. For the ATP challenge experiment, glands were incubated in HBSS buffer containing both 500 μM ATP and 300 μM Pinacidil/1 μM VU063 for 30 min before dopamine exposure.

2.2.5. Data Analysis

Dissection of the salivary glands and mounting the glands for the Ramsay assay was performed under a Nikon SMZ1270 steromicroscope equipped with a DS-Ri2 camera. Videos and still images were captured with Nikon Elements Professional imaging software connected to the Nikon SMZ1270 microscope.

The salivation time course data presented for CRC in this chapter was all collected with paired salivary glands where one salivary gland was treated with dopamine only and the other salivary gland was treated with the small-molecule modulator. The ability to pair the glands negated the variability due to factors that influenced individual ticks, such as blood meal size and age. The data points for each time point represent an average where $n > 3$ and the means for each time point of the treated glands was statistically compared to the same time point of the

control (dopamine only treatment) by a paired t-test. Statistical significance was denoted by $P<0.05$.

IC_{50} values were determined through the generation of concentration-response curves that were constructed with 5-6 concentrations. The percent salivation for each concentration of pinacidil/VU063 was determined by the formula: (secreted volume of gland treated with chemical + dopamine / secreted volume of gland treated with dopamine only) * 100. Each comparison was made from paired glands and the data points for each concentration represent the average % salivation of 3-5 paired glands. IC_{50} values were calculated by nonlinear regression (variable slope) using a Hill equation in GraphPad Prism™ (GraphPad Software, San Diego, CA, USA).

For the ATP challenge experiment, bars represent mean (n=3-5) volume of secreted saliva over a 5 min period from 15-20 minutes while the error bars represent SEM. A one-way ANOVA with a multiple comparisons post-test was performed to determine statistical significance compared to dopamine only treatment.

2.3. Results

2.3.1. Influence of Pharmacological Inhibitors of K^+ Channels and Transporters to Secretory Activity of the Isolated Salivary Gland

To test the relevance of specific potassium transport pathways to tick salivation, we applied representative modulators to the isolated salivary gland to determine the influence to dopamine-induced saliva secretion. Barium chloride ($BaCl_2$) is an indiscriminate blocker of all Kir channels at low- to mid-micromolar concentrations and therefore, we employed $BaCl_2$ as a probe of Kir channels to infer the role of Kir channels to the secretory activity of the tick salivary gland. Exposure to 500 μM $BaCl_2$ was shown to be statistically significant ($P<0.05$) as each time

point was approximately 60% reduced at 5-, 10-, 15-, and 20-minutes when compared to the dopamine only treated (control) glands (Figure 2.2A). The pharmacological library for K2P channels is extremely underdeveloped which limits the ability to specifically probe the physiological role of K2P channels. Fluoxetine has been shown to be a potent inhibitor of K2P channels and was used to determine the role of K2P channels to the secretory activity of the tick salivary gland. A dramatic reduction and, at most time points, a complete block of saliva secretion was observed in glands exposed to fluoxetine (700 μM). All of the analyzed time points were significantly ($P<0.01$) reduced when compared to the dopamine-only treated glands. Of all time points, the greatest mean (n=3) volume secreted volume was at 10-minutes and was found to be 14.3 ± 5 nL/5min whereas the mean volume secreted of paired control glands was 189 ± 34 nL/5 min (Figure 2.2B).

Transporters have been shown to be important for establishing Cl^- and K^+ ion gradients in mammalian salivary glands and therefore, we used two specific inhibitors of the sodium-potassium-chloride (NKCC) and potassium-chloride cotransporters to probe their function in isolated salivary glands. NKCC is an exploited drug target and therefore, furosemide is well characterized and known to be highly specific and potent against human NKCC proteins. The secreted volume of saliva from furosemide (1 mM) exposed glands was shown to be reduced by 50-65% when compared to the control (dopamine only) glands at 10-, 15-, 20-, 25-, and 30-minutes, which was a statistically significant ($P<0.05$) reduction. However, although secreted volume at the 5-minute time point was 40% reduced when compared to control, it was not statistically significant (Figure 2.2C). A similar pattern of reduction was observed after exposure of the isolated gland to VU271, which is a potent inhibitor of human KCC and importantly, has no activity against the human NKCC protein. This selectivity is critical for clear interpretation

of the data by ensuring any alteration of saliva secretion is indeed due to KCC inhibition. A statistically significant ($P<0.01$) reduction of volume secreted was observed at all time points with VU271 treated glands secreting 50-70% less saliva when compared to control glands (Figure 2.2D). The greatest inhibition of salivation was observed at 25 minutes with VU271-treated glands secreting a mean (n=3) volume of 5 ±5 nL whereas the control glands secreted a mean of 145 ± 6 nL / 5 min at the same time point, which is a 65% reduction. The least amount of inhibition to saliva secretion was observed at 10-minutes with a mean secreted volume of 65 ± 4 nL / 5 min whereas paired control glands were shown to secrete an average of 192 ±11 nL/5 min.

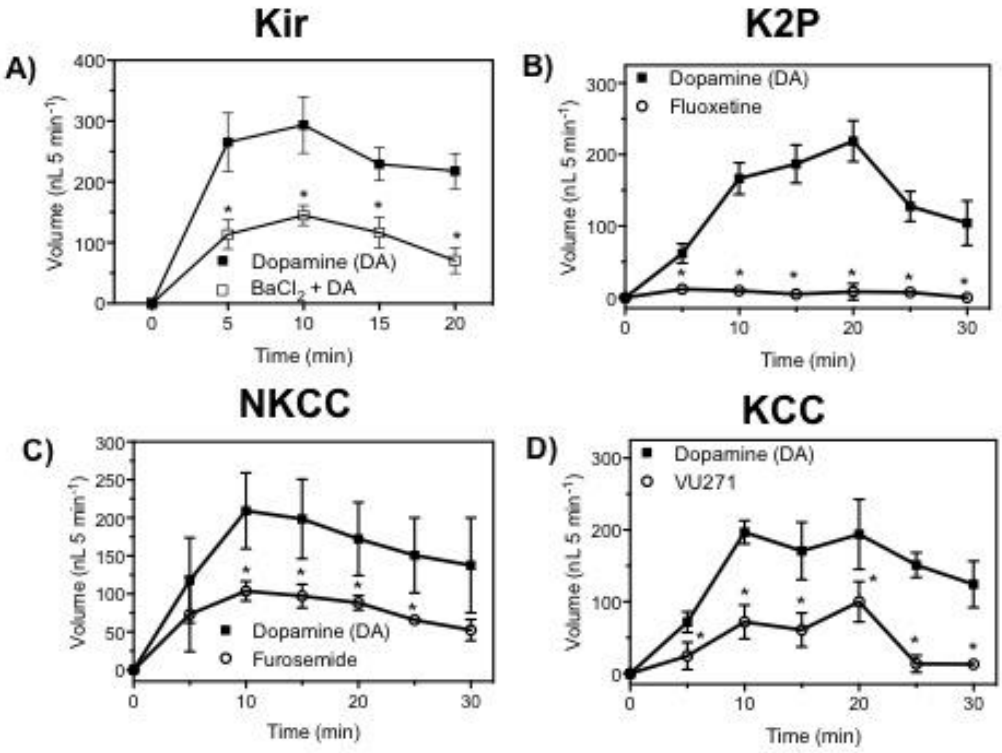


Figure 2.2. Fluid secretion assay using a whole isolated salivary gland from *Amblyomma americanum*. The dopamine-mediated saliva secretion pattern over a 30-minute duration after treatments of barium chloride (A), fluoxetine (B), furosemide (C), and VU271 (D). Asterisks represent statistical significance at $P<0.05$ as determined by a paired t-test for each time point.

Select pharmacological activators of K_{ATP} channels were shown to have dramatic effects to the secretory activity of the isolated salivary gland (Figure 2.3C). Pinacidil (1 mM) was shown to prevent nearly all secretory activity at time points ranging from 10 to 30 minutes with the greatest mean (n=6) volume secreted being 10 ± 3 nL at 20-minute time point. This is a dramatic and statistically significant ($P<0.0001$) reduction in secreted volume when compared to paired dopamine only control glands that secreted 178 ± 40 nL at the same time point (Figure 2.3C). The pinacidil treated glands were found to secrete an average of 30 ± 12 nL at the 5-minute time point, which was the highest mean volume secreted by pinacidil treated glands but was still significantly ($P<0.05$) reduced when compared to control glands that secreted an average of 81 ±11 nL at the same 5-minute time point (Figure 2.3C). The second activator studied, VU063, was shown to have a near identical pattern of inhibition when compared to pinacidil, but at a 3-fold less concentration. VU063 treated glands secreted less than 3 nL of saliva at the 10-30 minute time points, but displayed marginal secretion at the 5-minute time point.From 0-5 minutes, the VU063-treated glands secreted an average (n=3) of 23 ± 9 nL of saliva whereas the paired control glands secreted 107 ± 21 nL during the same time point, a statistically significant ($P<0.001$) reduction in salivation (Figure 2.3C). Diazoxide, nicorandil, and minoxidil are three structurally diverse activators of human K_{ATP} channels, but were found to not influence the secretory activity of the isolated tick salivary gland (Figure 2.3C).

2.3.3. Pinacidil- and VU063-mediated Inhibition of Secretory Activity in Isolated Tick Salivary Glands

Pinacidil and VU063 were shown to reduce the secretory activity of the isolated salivary gland presumably through modulation of K_{ATP} channels (Figure 2.4A). In mammalian systems, the off-target effects of pinacidil and VU063 are minimal because both molecules have been

shown to be highly specific for K_{ATP} channels with clean ancillary pharmacology. But, the possibility of modulating the activity of off-target proteins that may alter tick salivary gland activity remains present and therefore, we aimed to ensure the reduced secretory activity was indeed due to modulation of K_{ATP} channels. First, we generated concentration response curves to determine the concentration required to inhibit 50% of gland activity (IC_{50}) to describe the potency of each molecule. Pinacidil was found to be moderately potent at reducing the secretory activity of the *Amblyomma americanum* salivary gland with IC_{50} values in the mid-micromolar range. At 10 minutes, pinacidil was shown to have an IC_{50} value of 389 μM (95% CI: 218-692 μM; Hillslope: -2.4; r^2: 0.89) whereas the 20- and 30-minute IC_{50} values were found to be 1.56- and 1.50-fold reduced, respectively Figure 2.4B. The structurally different activator, VU063, was shown to be 169-fold more potent than pinacidil with an IC_{50} value of 2.3 μM (95% CI: 1.1-4.7 μM; Hillslope: -1.7; r^2: 0.85) at 10 minutes. A dramatically increase in potency of VU063 was also observed at 20 and 30 minutes with IC_{50} values of 4.6 μM (95% CI 2.8-7.4 μM; Hillslope: -1.9; r^2: 0.88) and 2.2 μM (95% CI: 1.1-4.7 μM; Hillslope: -1.0; r^2: 0.9), respectively. The VU063-mediated inhibition of fluid secretion plateaued at 10 minutes since the change in potency from 10 to 30 minutes was near unity, which is different than the time course of inhibition for pinacidil and indicates modification of the receptors with VU063 is significantly faster than pinacidil (Figure 2.4C).

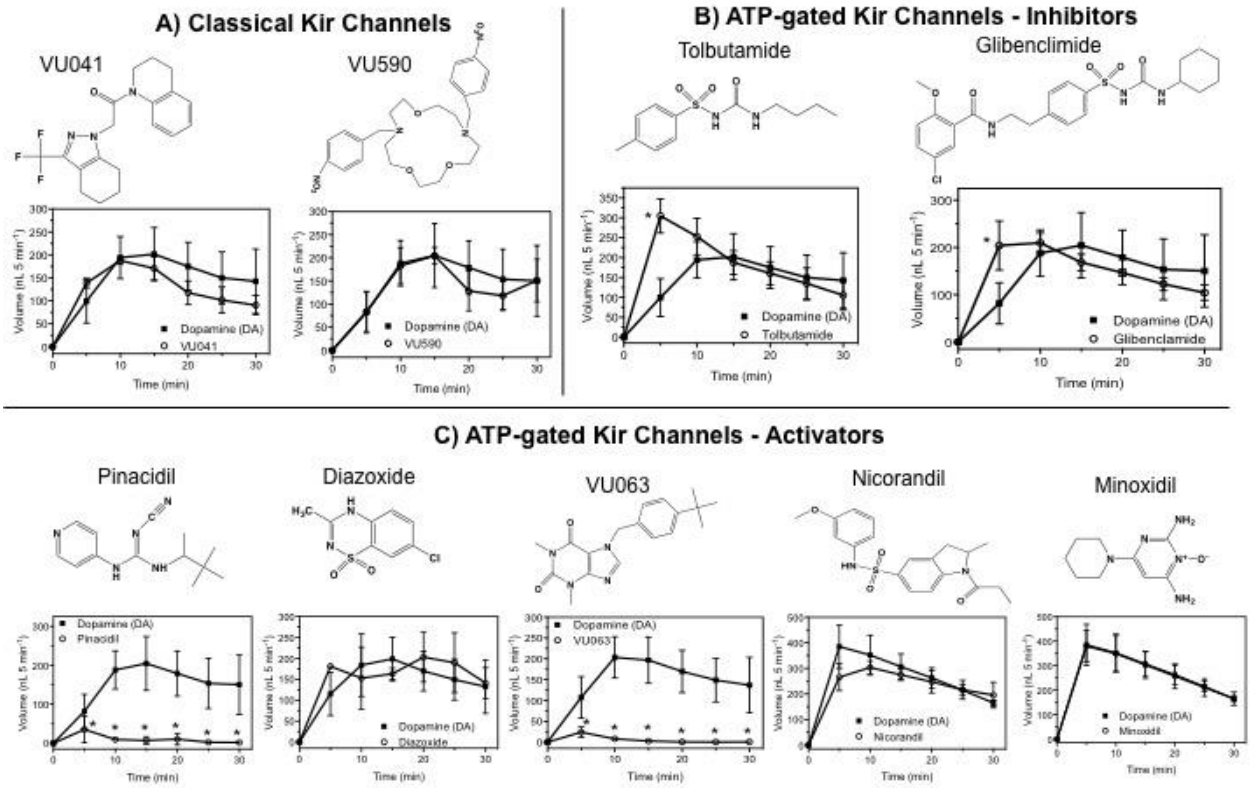


Figure 2.3. Influence of various Kir channel modulators to the secretory activity of the *Amblyomma americanu*m isolated salivary gland. The influence of classical Kir channel modulators (A), K_{ATP} channel inhibitors (B), and K_{ATP} channel activators (C) were studied to determine the influence to saliva secretion. Each data point represents a mean ($n > 5$) volume of saliva secreted after 30-minute exposure to modulator followed by stimulation with 100 μM dopamine. Each treatment group was paired to a dopamine control from the same tick. Asterisks represent statistical significance at $P<0.05$ as determined by a paired t-test for each time point.

K_{ATP} channels are inhibited by the presence of extracellular ATP and provides opportunities to ensure that the pharmacological activators are modulating K_{ATP} channels to induce a physiological response through co-applications of ATP and the activator. In theory, the presence of ATP should irreversibly close the K_{ATP} channel and prevent pharmacological activation of the channel, thus reducing the potency of pinacidil and/or VU063 against the tick salivary gland. To test this hypothesis, we exposed the salivary gland to 300 μM pinacidil,

which was shown to reduce the mean (n=3) secretion volume by 3.3-fold (Dopamine: 157 ± 15 nL/5 min to 43 ± 8 nL/5 min), which was a statistically significant reduction ($P<0.01$). Exposure to 500 μM ATP did not influence the secretory activity of the isolated gland (Figure 2.4D) when compared to dopamine controls, whereas higher concentrations were shown to reduce the secretory activity. Importantly, the mean (n=3) volume of secreted saliva after the isolated salivary gland was treated with 500 μM ATP and 300 μM pinacidil was found to be 127 ±9 nL / 5min, which was not statistically different from control secretion volumes (Figure 2.4D). Similarly, the volume of the secreted saliva after the isolated salivary gland was treated with 500 μM ATP and 1 μM VU063 was found to be 111 ± 17 nL / 5min, which was significantly increased from the volume secreted after 1 μM VU063 but not statistically different from control secretion volumes (Figure 2.4D).

2.4. Discussion

The salivary gland represents a critical tissue for the biological success of ticks and is an essential organ for the transmission of pathogens that induce extreme morbidity and mortality in humans and animals. Despite the importance of the gland, little information exists regarding the physiological pathways that are essential for maintaining the cellular milleu of the gland, generation of osmotic gradients for saliva production, and overall salivary gland function. Recently, the Na^+-K^+-ATPase was characterized in the tick salivary gland and highlights large gaps in knowledge pertaining to the foundational role ion channels and transporters serve in tick salivary gland function (Kim et al., 2016). The studies described in this chapter aim to fill this critical knowledge gap by employing pharmacological and physiological methodologies to probe the functional role various K^+ ion conductance pathways have to the secretory activity of the *Amblyomma americanum* salivary gland.

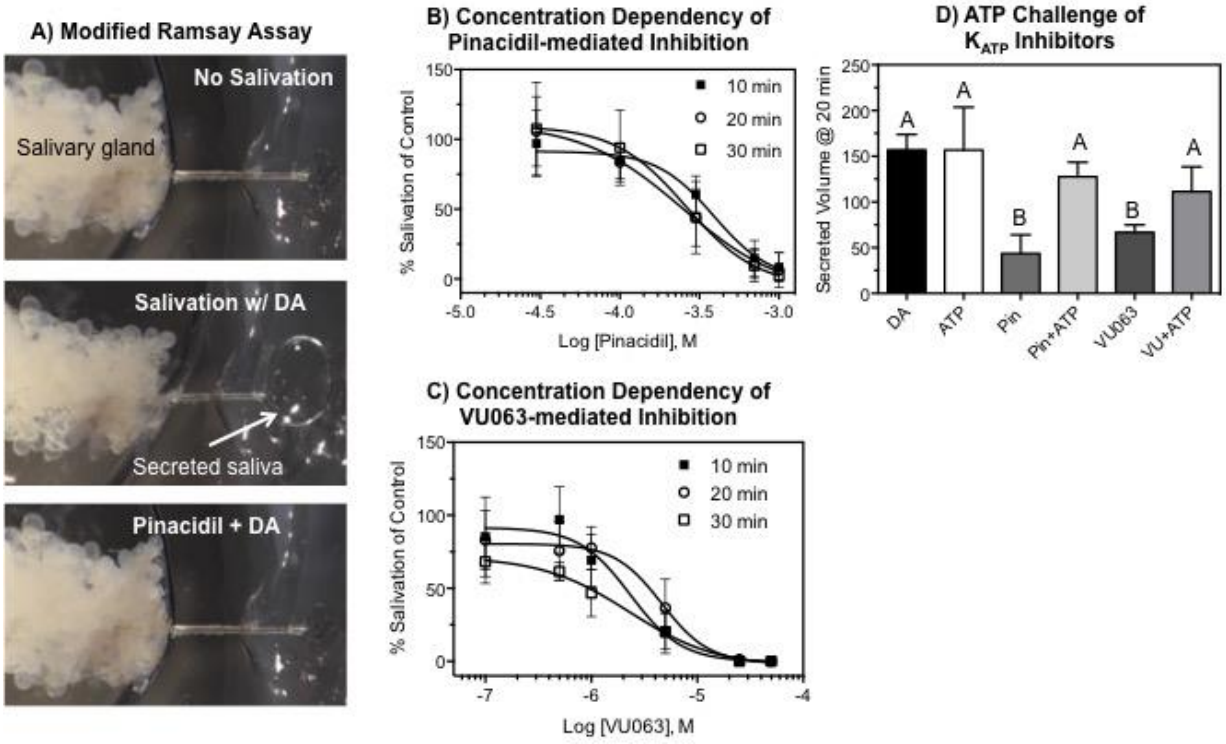


Figure 2.4. Validation and potency determination of pinacidil- and VU063-mediated inhibition of saliva secretion. Still photographs highlighting the reduced secretory activity after pinacidil exposure (A). Concentration-response curves to determine potency of pinacidil (B) and VU063 (C) at 10 min (closed square), 20 min (open circle), and 30 min (open square) after dopamine stimulation. Each data point represents a mean (n>3) percent salivation of control where each treated gland was compared to a paired dopamine only treated gland. Error bars represent SEM. Validation that the reduced secretory activity was determine by application of ATP (D). Bars represent mean (n=3) salivation volume at 20 minutes with error bars representing SEM. Bars not labeled by the same letter represents statistical significance at $P<0.05$ as determined by a one-way ANOVA compared to DA treated (control) glands.

The data presented in this chapter provide clear evidence that K^+ ion transport pathways are critical for proper function of the tick salivary gland as measured by total saliva secretion. Various pharmacological probes were employed to test the influence each pathway had to saliva secretion and identified that inhibition of Kir channels, K2P channels, NKCC, and KCC resulted in reduced saliva secretion and thus, reduced functional capacity of the tick salivary gland.

However, due to the relatively poor selectivity of fluoxetine to K2P channels and the unknown selectivity of the other modulators to arthropod proteins, a conservative inference from these data are that K^+ conductance pathways are fundamental for proper gland function of ticks and are likely 'druggable' targets to induce feeding cessation. However, additional studies are required to fully probe the physiological role of each of these proteins and requires the development of pharmacological libraries specific for the tick, which is outside the scope of this thesis. Fortunately, barium chloride ($BaCl_2$) is a cation that is an indiscriminate Kir channel blocker and was not designed to target a specific mammalian protein, which allows $BaCl_2$ to be used as a physiological probe in a variety of organisms.

Recently, Beyenbach and colleagues employed $BaCl_2$ in a Ramsay assay to explore the physiological role of Kir channels in fluid secretion by mosquito Malpighian tubules (Scott et al., 2004). The use of $BaCl_2$ showed that Kir1 channel represents a principal conductance within the Malpighian tubules and provides a transport pathway for K^+ ions from the hemolymph to the Malpighian tubule lumen (Scott et al., 2004; Beyenbach et al., 2011). Further, inclusion of $BaCl_2$ in the Ringer solution of the Ramsay assay reduced the fluid secretion rates from the Malpighian tubules by approximately 60% when compared to control tubules, which provided significant evidence that Kir channels are a critical pathway for proper function of the Malpighian tubules. Interestingly, the tissue physiology of the arthropod salivary gland is very similar to that of the Malpighian tubules, as they both are a polarized epithelial membrane that rely on potassium ion transport across membranes to generate isosmotic fluid to ultimately form of urine or saliva. The physiological similarities led us to speculate that Kir channels may serve a similar function in the tick salivary gland as it does in the mosquito Malpighian tubule. Considering 1) the physiological similarities between the Malpighian tubules and salivary

glands, 2) the importance of Kir channels in the function of various insect tissues (Scott et al., 2004; Swale et al 2016; O'Neal et al., 2017b), 3) the established role of Kir channels in mammalian salivary glands (Hayashi et al., 2003; Zhou et al., 2010), and 4) our data validating the critical role of K^+ ion conductances in tick salivary glands (Figure 2.2), we hypothesized that Kir channels also serve a critical role in the function of tick salivary glands and aimed to highlight the general influence of Kir/K_{ATP} channel modulation to fluid secretion through pharmacological manipulations.

To begin testing the physiological role of Kir channels to the secretory function of tick salivary glands, ex vivo physiological assays were performed using the voltage dependent Kir blocker, barium chloride ($BaCl_2$). $BaCl_2$ is useful pharmacological tool to test the physiological role of Kir channels since, at physiological membrane potentials, Kir channels are up to 1000-fold more sensitive to $BaCl_2$ than other K^+ ion channels (Robertson et al, 1996) and does not modulate K_v, Ca^{++}, or other ion channel families until concentrations approach the millimolar range. Our data showed that exposure of the *A. americanum* salivary gland to 500 μM $BaCl_2$ reduced the secretory volume by approximately 60% (Figure 2.2), which is near identical to the reduction observed in the mosquito Malpighian tubules (Scott et al., 2004). These data support the notion that an inward conductance of potassium ion is indeed important for tick salivary gland function.

To validate this notion, structurally diverse insect specific Kir channel modulators and well-validated K_{ATP} channel inhibitors and activators were employed using the Ramsay assay. Interestingly, inhibition of Kir channels with VU041 and VU590 did not have any impact on the secretory activity of the *A. americanum* salivary gland. This is potentially due to two reasons. First, constitutively active Kir channels, which are the target of these two molecules, are not

required for tick salivary gland function or secondly, that the two insect specific Kir channel modulators are not able to inhibit tick Kir channels due to unknown differences in the structural makeup of the tick Kir channel or an altered small-molecule binding site. The lack of activity with VU041 and VU590 was surprising since Kir2.1 was identified to be responsible for setting the resting membrane potential and involved in spontaneous fluid secretion in bovine salivary glands (Hayashi et al., 2003). However, we speculated the lack of activity was due to differences between ruminant and tick feeding biology, most notably the requirement for ATP to be present for successful feeding in hematophagous arthropods (Langley, 1976).

Considering ATP serves as a phagostimulant and is required for feeding of hematophagous arthropods, we employed structurally and mechanistically diverse small-molecule modulators of K_{ATP} channels to test the hypothesis that tick salivary glands have employed K_{ATP} channels to control the membrane physiology and secretory activity of the tick salivary gland. Indeed, activators of K_{ATP} channels were shown to dramatically reduce, or eliminate at high concentrations, the secretory activity of the *A. americanum* salivary gland whereas inhibitors of K_{ATP} channels stimulated saliva secretions at 5 minutes that was reduced to control levels at subsequent time points. Importantly, opposite patterns of salivation were observed with activators and inhibitors, the inhibition of salivation was concentration dependent, and the reduction of salivation after exposure to pinacidil/VU063 was negated with the application of ATP. These data provide substantial evidence that the reduced salivation is indeed due to modulation of K_{ATP} channels and supports the notion that K_{ATP} channels are required for salivary gland function and saliva secretion of ticks. This finding is the first to describe the presence and importance of K_{ATP} channels in tick salivary glands, but is not unprecedented in the

literature since multiple K_{ATP} channels were shown to play a significant role in fluid secretion from the submandibular gland in rats (Zhou et al., 2010).

In mammals, there are different types of K_{ATP} channels constituting different combinations of Kir6.x and SURs in native tissues, e.g., Kir6.2 with SUR1 forms an insulin secretion K_{ATP} channel (Sakura et al. 1995), Kir6.2 with SUR2A forms a cardiac-type K_{ATP} channel (Inagaki et al. 1996), Kir6.2 with SUR2B forms a smooth-muscle–type K_{ATP} channel (Isomoto et al. 1996), and Kir6.1 with SUR2B forms a vascular smooth-muscle–type K_{ATP} channel (Yamada et al. 1997). Importantly, different combinations of Kir6.x and SUR result in different pharmacological sensitivities that reflect the various K_{ATP} channels in native tissues (Seino and Miki 2003). For instance, glibenclamide blocks the Kir6.2/SUR1 channel, and only slightly inhibits the Kir6.2/SUR2A channel (Inagaki et al. 1995, 1996; Gribble et al. 1998), whereas tolbutamide inhibits Kir6.2/SUR1 currents with high affinity, but does not inhibit Kir6.2/SUR2A (Gribble et al. 1998). Similarly, VU063 selectively activates Kir6.2/SUR1 channel whereas pinacidil activates Kir6.1/SUR2 channels. Our data suggest that the pharmacological profile of tick K_{ATP} channels are different when compared to human K_{ATP} channels since pinacidil and VU063 both prevented salivation, but target different SUR proteins in humans. Further, diazoxide did not influence salivation whereas VU063 completely inhibited salivation, but both activate the same K_{ATP} heteromer in humans (Raphemot et al., 2014b).

Determining the specific functional role of K_{ATP} channels in the tick salivary gland acini is of obvious interest and important for the subsequent development of products aiming to prevent tick salivation and feeding. However, it presently impossible to describe with any certainty the specific role of the channel without electrophysiological data describing how these channels interface with other ion channels and neuroendocrine systems. At the present time, we

speculate K_{ATP} channels are responsible for maintenance of the acinar membrane potential, presumably the basolateral membrane, which maintains the loop current that drives the electrogenic transport of Cl^- and Na^+ absorption and K^+ secretion. Further, we speculate that K_{ATP} channels are employed in this case over constitutively active Kir channels because as the saliva secretion speed increases, the reabsorption activity for Na^+, Cl^-, and HCO_3^2 would also increase. Consequently, the ATP consumption would increase, reducing the concentration of intracellular ATP, and subsequently opening K_{ATP} channels that would enable maintenance of the membrane potential.

2.5. Conclusion

We found that exposure to potent and selective modulators of five potassium transport pathways (Kir, K_{ATP}, K2P, KCC, and NKCC) significantly reduced the volume secreted from the isolated tick salivary gland. Further characterization showed that K_{ATP} channels are critical for proper salivation and activation of the channel with pinacidil or VU063 inhibited fluid secretion in a concentration dependent manner. Importantly, the reduction in fluid secretion after exposure to pinacidil or VU063 was reduced after pretreatment with ATP, providing significant evidence that pinacidil and VU063 altered fluid secretion through activation of K_{ATP} channels. Therefore, the data collected in this chapter support the hypothesis that pharmacological modulation of K^+ ion channels and transporters alter the fluid secretion from the isolated tick salivary gland.

Chapter 3. Test the Hypothesis that Kir Channels Are Critical for the Osmoregulatory Role of the Tick Salivary Gland

3.1. Introduction

Mammalian blood contains high concentrations of sodium and potassium salts that would be toxic to hematophagous arthropods if they were not quickly dealt with. Considering this, a blood-feeding lifestyle imposes an interesting set of water balance challenges for hematophagous arthropods and therefore, maintaining a balance of salt and water concentrations is essential to overcome the potentially fatal dose of salts derived from the host blood. In most insects, diuresis occurs via the Malpighian tubules and altering the Malpighian tubule function could be especially debilitating after engorging on blood. In mosquitoes, in response to engorgement of blood during feeding, the Malpighian tubules mediate a pronounced diuresis that eliminates approximately 40% of the water, 40% of the Na^+ ions, approximately 145% of the K^+ ions, and ~60% of the Cl^- from the ingested blood plasma (Williams et al., 1983). This diuretic action is controlled by numerous neuropeptide hormones that act on the membrane receptors to trigger the intracellular signaling cascades, resulting in active fluid transport by generating electrochemical gradients (Beyenbach, 2003; Coast et al., 2007; Gäde and Goldsworthy, 2003; Davies et al., 1995; Gäde, 2004; MacPherson et al., 2001; Pollock et al., 2004; Pollock et al., 2003; Rosay et al., 1997). In addition to a host of hormonal factors, several ion transport mechanisms are essential between the primary and stellate cells of the Malpighian tubules. In brief, electrochemical gradients are established by the concerted action of V-type H^+-ATPase (V-ATPase) and Na^+-K^+-ATPase pumps to generate a negative voltage across the basolateral membranes of the epithelia. This negative potential, in concert with electroneutral transporters, drive the movement of NaCl and KCl from the hemolymph, across the basolateral membranes of

the tubule cells through ion channels, and into the tubule lumen to facilitate ion excretion. The reverse mechanism occurs for ion reabsorption.

Several lines of evidence strongly support the hypothesis that Kir channels are major routes of K^+ ion uptake in the mosquito Malpighian tubules. Pharmacological inhibition of basolateral Kir channels reduces the fluid secretion of the isolated tubule by approximately 50% and reduced the urinary K^+ excretion by approximately 60%, which alters the hemolymph K^+ homeostasis (Scott et al., 2004). Moreover, limiting the capacity of Malpighian tubules to produce urine after a blood meal could dampen their capacity to excrete metabolic wastes associated with blood digestion (e.g., heme, nitrogenous wastes), which may lead to hemolymph poisoning and lethality (Raphemot et al., 2013a). Indeed, inhibitors designed to be specific for Kir1, which are primary K^+ conductance pathway in the mosquito Malpighian tubules, were shown to reduce the excretory capacity in the whole mosquito and induce mortality after a K^+ challenge or blood meal (Raphemot et al., 2014a; Swale et al., 2016). These data suggest that targeting the osmoregulatory function of blood feeding arthropods through altering the ion conductance pathways of the osmoregulatory organ will lead to toxicity via a completely novel mode of action when compared to conventional insecticides. Considering this, we aimed to determine the ability to alter the osmoregulatory capacity of the tick *A. americanum* through inhibition of salivary gland K^+ ion channels.

Interestingly, the tissue physiology of the arthropod salivary gland is very similar to that of the Malpighian tubules, as they both are a polarized epithelial membrane that relies on ion transport across membranes to generate isosmotic fluid in the form of urine or saliva. These physiological similarities led us to speculate that Kir channels are likely to serve a similar function in the tick salivary gland as they do in the Malpighian tubules. The multifunctional and

morphologically complex salivary gland is critical to the biological success of ticks as it performs key roles in the events during blood feeding. The salivary gland is the primary tissue responsible for maintaining a proper salt and water balance during blood feeding by returning about 65-70% of the fluid and ion content of the blood meal back into the host to alleviate the burden of the increased derived from the blood meal (Bowman and Sauer, 2004). For example, of the ingested blood constituents the saliva of *Dermacentor andersoni* contains 74% of its total secreted water, 96% of its total secreted sodium, and 16% of the total secreted potassium, while the hindgut is responsible for processing the remainder of the water and ions (Kaufman and Phillips, 1973a).

Considering 1) the importance of Kir channels in the mosquito Malpighian tubules, 2) the similarities in function and physiology of the tubules and salivary gland cells, and 3) previous work highlighting the importance of Na^+-K^+-ATPase to the osmoregulatory function of tick salivary glands, we hypothesize that pharmacological modulation of Kir and/or K_{ATP} channels in will alter the concentrations and rate of Na^+, K^+, and Cl^- ion secretion and thus, the osmoregulatory capacity of the organ. We speculate that altering the ability to osmoregulate during blood feeding will have deleterious consequences to the tick and could represent a novel process and pathway to exploit for therapeutic development.

3.2. Materials and Methods

3.2.1. Fluid Collection

Unfed female ticks were fed on the artificial host system (Figure 2.1) and ticks that weighed 10-20 mg were detached and saliva was collected using the Ramsay assay and quantified via Nanoliter2010 (WPI) every 5 minutes for 30 minutes. Secreted saliva for each time point was spotted on a slide glass and stored at -80°C for the further analysis. Dopamine

HCl, pinacidil, and VU063 were purchased from Sigma Aldrich (St. Louis, MO, USA). The methodology for saliva collection, chemical treatment of the glands, and modulator preparation was the same as described in sections 2.2.1 and 2.2.4. Importantly, all pinacidil or VU063 treated glands were compared to a dopamine treated gland that was extracted from the same tick, which enabled a paired analysis.

3.2.2. Preparation of the Saliva for Elemental Analysis

Elemental analysis for Na^+, K^+, and Cl^- ions in the secreted saliva was modified based on the methods described in Kim et al (2016). The secreted saliva from each time point and each treatment group was thawed from -80°C storage and the solutes from the saliva droplet were reconstituted in 200-300 nL of ultra-distilled water and mixed thoroughly to ensure even distribution of solutes within the 200-300 nL droplet. The water and dissolved salts were then spotted onto a clean silicon wafer substrate (Pelcotec^{TM}) by 'dripping' 1 nL of the reconstituted solution. A total of 9-12 drips, or spots, for each time point of each treatment group were deposited onto the silicon wafer using a modified Nanoliter2010 (World Precision Instruments, Sarasota, FL, USA) for subsequent analysis.

The Nanoliter2010 required slight modifications to the equipment to reduce the drop volume to be 1 nL that was required for analysis of the elemental composition. The bottom volume limit for the Nanoliter2010 is advertised to be 2.3 nL, which caused spreading of the salt in the 2.3 nL spot area to approximately 250 microns in diameter. The spreading of the spot area is potentially due to the sample preparation and nucleation of the material that prevented the formation of a single crystal. To obtain an appropriate resolution of the K^+ salt crystals, we had to magnify the droplet to 1200X , which was too high magnification to image the entire 2.3 nL

spot. Therefore, we modified the wire plunger to a size 180 μM that enabled the drop size to be reduced to 1 nL.

3.2.3. Elemental composition of tick saliva

The secreted saliva was analyzed via scanning electron microscopy (SEM)/energy-dispersive X-ray spectroscopy (EDS) with a silicon drift detector (SDD) to obtain qualitative elemental composition and concentration of dopamine only-, pinacidil-, and VU063- treated glands. A Dual-Beam Focused Ion Beam (FIB) SEM equipped with EDS (EDAX) at Shared Instruments Facility (SIF) was used to image and analyze each dried saliva spot. The parameters of the image was set to be 10 kV and 5.7 nA current. We used EDAX TEAM software to acquire spectra to identify the Na^+, K^+, and Cl^- ions based on the characteristic X-ray. Due to the use of a silicon background, silicon was excluded from the analysis and only Na^+, K^+, and Cl^- were used for concentration calculations, but the value of silicon was used as an internal control.

3.2.4. Standard Curve for Na^+, Cl^-, and K^+ Ions

The concentration of each element was determined by the construction of a standard curve that was developed by spotting NaCl (Fisher Scientific) and KCl (Fisher Scientific) onto the silicon substrate with differing concentrations: NaCl (10, 50, 100, 200, 400, 600 and 800 mmol l^{-1}) and KCl (2, 4, 8, 16, 32, 64, and 100 mmol l^{-1}). The standard curves for each ion were generated based on atomic percentage values of each concentration. The atomic percentages of the treatment groups were compared to the standard curves to enable calculation of the concentration of the ions. SEM-EDS images for the K+ and Na+ standard curve are shown in figure 3.1A and B, respectively.

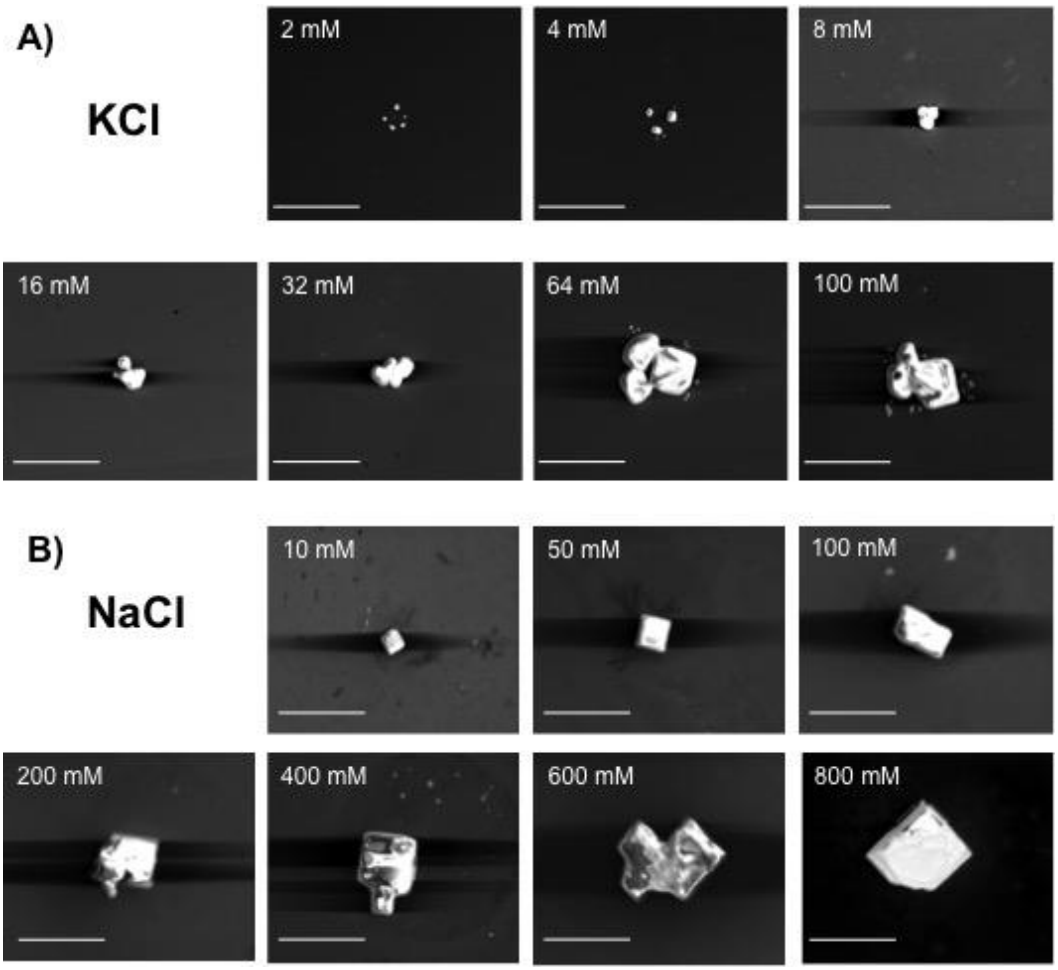


Figure 3.1. SEM-EDS images of the standard curves for elemental analsysis. **(**A) KCl standard curve with concentrations ranging from 2 to 100 mM. (B) NaCl standard curve with concentrations ranging from 10 to 800 mM. The sale bar represents 40 uM.

3.2.5. Data Analysis for the Elemental Composition of Tick Saliva

The percent atomic of nine saliva droplets were determined using the SEM-EDS equipment and averaged to obtain a mean percent atomic for each individual time point and treatment group. compared to the percent atomic of the standard curve that was generated for each element (Na^+, Cl^-, and K^+) and the concentration of total Na^+, Cl^-, and K^+ in the whole saliva droplet was determined with the formula for a linear regression ($y = mx + b$). The molar

concentration of the element was multiplied by the dilution factor that stemmed from the reconstitution of the dried saliva and then divided the product by the total volume of saliva secreted. This resulted in the total concentration of each element per nL of secreted saliva.

3.3. Results

3.3.1. Influence of VU063 to Elemental Composition of Saliva

Exposure of *A. americanum* salivary glands to VU063 (5 μM) yielded a significant increase in the Na^+, K^+, and Cl^- ions secreted per nL of saliva at all time points with the exception of 5-minute time point. For Na^+, a mean increase of 0.9-, 8.5-, 8.6-, 14.5-, 6.2-, and 4.7-fold was observed for 5-, 10-, 15-, 20-, 25-, and 30-minute time points when compared to the dopamine only treated glands, respectively. For Cl^- ions, a mean increase of 1.3-, 5.3-, 9.7-, 15.4-, 5.5-, and 5.3-fold was observed 5-, 10-, 15-, 20-, 25-, and 30-minute time points when compared to the dopamine only treated glands, respectively. For K^+ ions, a mean increase of 0.8-, 10.4-, 7.5-, 12.8-, 11.9, and 10.5-fold was observed for 5-, 10-, 15-, 20-, 25-, and 30-minute time points when compared to the dopamine only treated glands, respectively (Figure 4.2A). All time points for each element was found to be statistically significant with the exception of the 5-minute time point, which showed no change in elemental composition compared to control glands (Figure 3.2A)

3.3.2. Influence of Pinacidil to Elemental Composition of Saliva

Exposure of *Amblyomma americanum* salivary glands to pinacidil (700μM) yielded a significant increase in the Na^+, K^+, and Cl^- ions secreted per nL of saliva, which was similar to the observed effect of VU063. For Na^+, a mean increase of 6.9-, 5.4-, 9.2- 7.5-, 9.9-, and 27-fold was observed for 5-, 10-, 15-, 20-, 25-, and 30-minute time points when compared to the

dopamine only treated glands, respectively. For Cl^- ions, a mean increase of 8.7-, 7.0-, 10.6-, 11.5-, 9.0-, and 16.5-fold was observed 5-, 10-, 15-, 20-, 25-, and 30-minute time points when compared to the dopamine only treated glands, respectively. For K^+ ions, a mean increase of 3.5-, 6.6-, 14.3-, 8.5-, 20.9, and 9.2-fold was observed for 5-, 10-, 15-, 20-, 25-, and 30-minute time points when compared to the dopamine only treated glands, respectively (Figure 3.2B). The increase observed for each ion at each time point was found to be statistically significant ($P<0.05$) when compared to control concentrations.

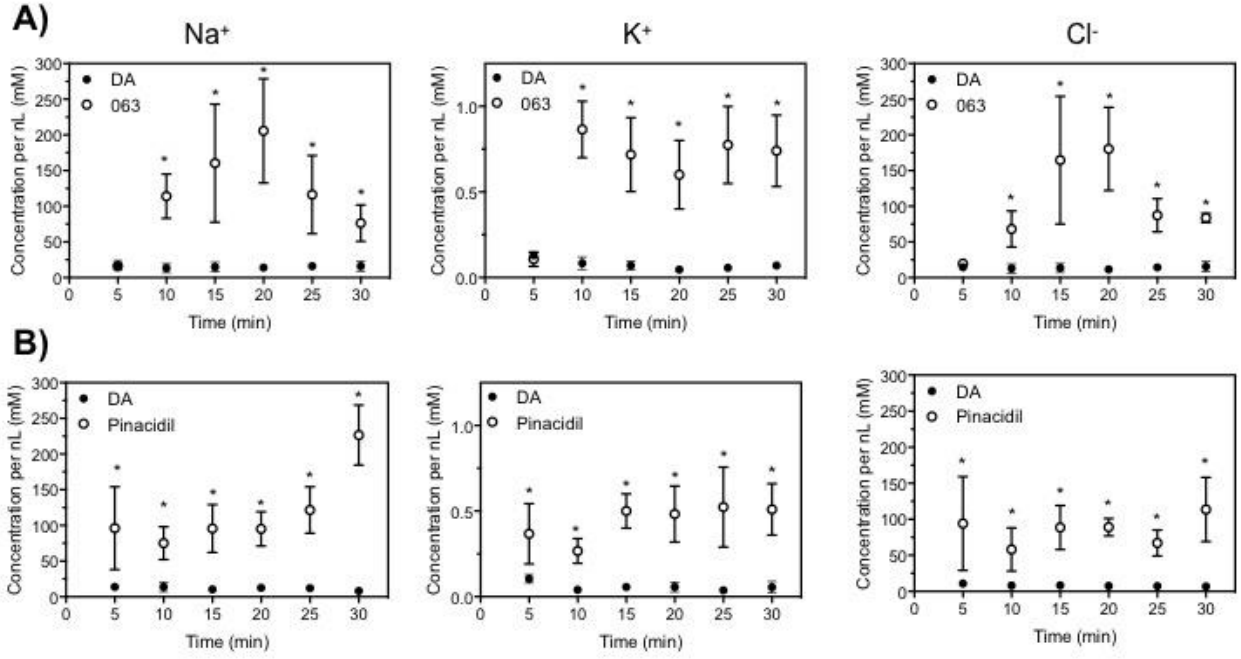


Figure 3.2. Ion composition and osmotic concentration of secreted saliva from isolated salivary glands. Qualitative determination of the concentration of Na^+, K^+, and Cl^- after VU063 (A) and pinacidil (B) treated salivaty glands (open circles) compared to dopamine (DA; 100 µM) treated glands (closed circles). SEM/EDS was used to analyze each ion concentration from each time point. Standard curves for each ion (Na^+, K^+, and Cl^-) were generated with NaCl and KCl (see figure 3.1). Asterisks represent statistical significance at $P<0.05$ as determined by an ANOVA-Tukey-Kramer HSD test.

3.4. Discussion

Salivary secretions were subjected to measurement of their ion compositions to determine if K_{ATP} channels influence the osmoregulatory capabilities of the *A. americanum* salivary gland. The initial analysis of saliva using scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM-EDS) identified Na^+, K^+, and Cl^- as the three major ions in the secreted saliva, which is identical in previous work (Kim et al., 2016). Sodium rich saliva has been described in a number of tick species, including *A. americanum* (Hsu and Sauer, 1975) and *D. andersoni* (Kaufman and Phillips, 1973a), yet the concentration of other ions, such as K^+ and Cl^-, were not determined and further, the mechanisms of ion secretion and absorption were not determined. Further, studies in *Ixodes scapularis* have shown that inhibition of Na^+-K^+-ATPase with ouabain resulted in hyperosmolar dopamine-induced salivary secretions with increased concentrations of Na^+, K^+, and Cl^- in the saliva compared to that of the dopamine- only treatment (Kim et al., 2016).

Kim et al., (2016) provided the first study that highlighted the influence of K^+ ion transport pathways to the fluid and ion secretion in the tick salivary gland and showed a clear influence of pharmacological inhibition to both, fluid and ion secretion.

Kim et al., (2016) analyzed the role of the Na^+-K^+-ATPase in fluid and ion secretion and showed that ouabain significantly increased the total ion concentration in the secreted saliva while modestly decreasing saliva secretion. Interestingly, Na^+-K^+-ATPase is differentially expressed between the acini types and was localized to the apical membrane of type III acini and to the basolateral membrane of type I cells. The ouabain influence to salt and saliva secretions, the expression patterns, and the morphological changes observed to type III acini lead the authors to speculate that Na^+-K^+-ATPase carries two opposite physiological roles: ion and water

influx into the lumen of type III acini that forms the primary saliva and reabsorption of ions through type I acini located on the main duct (Kim et al., 2016).

The data collected in current study were similar to that observed after inhibition of Na^+-K^+-ATPase as we observed dramatic increases in ion accompanied by large reductions in dopamine-induced fluid secretion (chapter 2) after activation of K_{ATP} channels. These data demand further explanation for the role of K_{ATP} channels to maintain the balance between ion and fluid secretion. Based on the data collected in Malpighian tubules of mosquitoes and the similarity of tubule cell physiology when compared to the physiology of salivary gland acini, we hypothesize that the cations Na^+ and K^+ take a transcellular pathway that mediates active transport of Na^+ and K^+ whereas Cl^- takes a paracellular shunt pathway that enables passive transport (Masia et al., 2000; Scott et al. 2004). Transcellular and paracellular pathways are electrically coupled, forming an intraepithelial current loop that enables electrogenic transport across membranes. Based on previous localization of Kir channels in exocrine tissues (Piermarini et al., 2015; Ishikawa et al., 1993; Wegman et al., 1992; Zhou et al., 2010), we anticipate that Kir/K_{ATP} channels are expressed at the basolateral membrane of tick salivary gland type III acini and therefore, inhibition of these K^+ channels would increase the resistance of the basolateral membrane and decrease the loop current, functionally inhibiting the transepithelial NaCl and KCl secretion across the salivary gland membranes. Conversely, activation of Kir/K_{ATP} channels would likely increase the NaCl and KCl secretion across the acini membranes to continuously generate hyperosmotic saliva. Although membrane electrophysiology experiments need to be performed to validate this hypothesis, our data support this notion since we observed exposure of the *A. americanum* salivary gland to two structurally distinct K_{ATP} agonists dramatically increased the concentration of all three major ions (Na^+, K^+,

Cl^-) in the secreted saliva. These data suggest that there is a tight regulation and functional dependency between the ion transport proteins expressed in the apical and basolateral membranes that establishes the electrogenic properties of the acini that ultimately facilitates ion secretion and/or reabsorption during salivary gland function of *A. americanum*.

In addition to an increase in salt concentrations in the secreted saliva, it was clearly illustrated that pharmacological activation of K_{ATP} channels dramatically reduced the fluid secretion that is likely tangentially connected to the change in salt secretion. We hypothesize that K_{ATP} channels establish a favorable electrochemical gradient for the accompanying transport of anions (Cl^-) that generates an osmotic gradient that forces water to be osmotically obligated to follow. This coupling of osmotic coupling of water to anions ultimately generates isosmotic fluid that is known as saliva. Based on our data, we hypothesize that altering the flux of K^+ ions through K_{ATP} channels alters the electrochemical gradients and intracellular loop currents that reduce the flow of anions and thus, reduce the osmotic gradient that drives the formation of saliva.

It is important to note that it is possible that K_{ATP} channels are expressed in type I, II, and III acini. Therefore, the reduction in saliva secretion may be due to altered loop currents within the Type II and III acini whereas the increase in salt concentration is due to altered physiology of the type I acini, which are responsible for fluid secretion and reabsorption, respectively.

The data collected in this chapter highlight the fact that K_{ATP} channels are indeed critical for proper ion secretion and thus, the osmoregulatory function of the tick salivary glands. However, no conclusions regarding the mechanism by which K_{ATP} channels control ion secretion and reabsorption or fluid secretion can be drawn solely from the data collected here. Therefore, future studies are required to validate the data collected in this chapter and provide a model of

transepithelial NaCl and KCl secretion. These studies include 1) confirmation of increased osmolarity of the saliva after K_{ATP} channel activation, 2) the individual influence each acini type has to the elemental composition of the saliva before and after K_{ATP} channel modulation, 3) immunohistochemistry to determine spatial expression patterns of K_{ATP} channels, 4) membrane electrophysiology to couple the changes in ion secretion with polarization changes to the basolateral membrane of the acini after modulation of K_{ATP} channels, and 5) the functional coupling of K_{ATP} channels to additional K^+ ion channels and transporters.

3.5. Conclusion

The data collected in this chapter show that the total concentration of sodium, potassium, and chloride ions are significantly increased (10x-15x) after salivary gland exposure to pinacidil and VU063. Because KATP channels are a subclass of Kir channels, the data support the hypothesis that Kir channels are critical for proper osmoregulation that occurs through the tick salivary gland.

Chapter 4. Test the Hypothesis that Pharmacological Modulation of Kir Channels Will Alter the Feeding Behavior and Survivability of *Amblyomma americanum*

4.1. Introduction

Human diseases stemming from tick-borne pathogens have been researched extensively in an effort to identify novel targets that can be exploited for the development of therapeutics to alleviate the burden of these diseases. Unfortunately, the significant advancements in knowledge relative to tick genomics, tick saliva proteins, and vaccine technologies have translated poorly into successful control efforts. To substantiate this point, the number of cases of Lyme borreliosis reported to the Centers for Disease Control and Prevention (CDC) has nearly doubled over the past 20 years, with more than 28,000 confirmed cases for the year 2015, despite extensive research efforts aimed at controlling this disease (Piesman, 2006). Similarly, rickettsial disease cases are steadily increasing within the United States and some strains of *Rickettsia rickettsii*, the causative agent of Rocky Mountain Spotted Fever, can cause significant mortality in humans (Parola et al., 2005). The primary arthropod vectors of *R. rickettsii* are *Dermacentor andersoni* and *D. variabilis*. However, recent studies have shown that the most predominant human biting tick, *Amblyomma americanum*, is capable of acquiring, maintaining, and transmitting *R. rickettsii* isolates originating from two different geographical regions of the US (Levin et al., 2017). The shift in vector suggests increases in human rickettsial cases are possible without intervention. The steady increase in tick populations and pathogens (Johnson et al., 2017), increased recognition of vector competency of human biting ticks for rickettsial diseases (Levin et al., 2017), and the movement toward an epidemic of tick-transmitted pathogens

(Hanincová et al., 2006) highlights the significance of research aimed to identify 'druggable' targets to curb the health and economic burden of ticks.

The use of synthetic molecules for tick control is a proven method for reducing transmission of tick-borne pathogens since the majority of tick control programs are reliant on the use of neurotoxic acaricides. Unfortunately, consistent use of acaricides that share similar mechanisms of toxicity have increased the selection pressure and driven the evolution acaricide resistant tick populations (Miller et al., 2005; George et al., 2004). Thus, there is a need to develop novel approaches for tick control that 1) exploit a novel target site to induce failure to a system or lethality, 2) a novel physiological process that is critical for survival but outside of the nervous system, and 3) disrupts pathogen transmission or acquisition by the vector. There are at least two conceivable approaches to reduce survivorship and/or pathogen transmission without neurotoxic insecticides: 1) prevent salivation into the host during feeding, and 2) prevent osmoregulation during feeding to destabilize the ionic balance in the animal that will lead to toxicity.

Importantly, ATP-sensitive Kir channels were shown to be a critical conductance pathway in the tick salivary gland that are required for salivation (Chapter 2) and modulation of these channels dramatically altered osmoregulatory capabilities of the isolated tick salivary gland (Chapter 3). Therefore, the ultimate objective of chapter 4 is to translate the *ex vivo* data of chapters 2 and 3 into a functional study to test the hypothesis that if salivary gland failure through dysregulation of K^+ ion channels during blood feeding occurs, then altered blood feeding biology or mortality will be observed.

4.2. Materials and Methods

4.2.1. Pharmacological Modulators and Reagents

The K_{ATP} activators, pinacidil and VU0071063, were purchased from Sigma-Aldrich (St. Louis, MO, USA and was dissolved in dimethyl sulfoxide purchased from Fisher Scientific. The inactive analog to VU0071063, termed VU063-I, was initially designed during a medicinal chemistry campaign performed by Dr. Jerod Denton and colleagues (Vanderbilt University, Nashville, TN, USA) in an effort to increase the potency and selectivity of the parent molecule (Raphemot et al., 2014b).

Defibrinated bovine blood was purchased from Hemostat Laboratories (Dixon, CA, USA) and gentamycin and ATP were purchased from Fisher Scientific (were added to the blood at a concentration of 5 µg/ml and 1µM, respectively. All other materials and reagents used for the artificial feeding system are described in Chapter 2.

4.2.2. Ticks

Non-blood fed adult Lone Star ticks (*Amblyomma americanum*) were purchased from the Tick Rearing Center at Oklahoma State University (Stillwater, OK, USA). Prior to allowing the ticks to blood feed, the ticks were stored for 14 days in an incubator at 28° C and 60% RH.

4.2.3. Artificial Host Feeding System

The design of the membrane feeding system is described in Chapter 2. However, Chapter 4 used a modified the assay where the small-molecule modulators were dissolved in the blood to mimic a realistic method for exposing the feeding tick to a therapeutic agent. All chambers included the fluorescent tracer, Rhodamine B, at a concentration of 100 ppm to ensure that all ticks included in data analysis had fed on the blood. The salivary glands of all ticks that were dead or removed from the membrane for quantification of imbibed volume were placed under a

fluorescence stereomicroscope (SteREO Lumar.V12, Carl Zeiss, Gottingen, Germany) and initially observed using incandescent illumination. Non-fluorescent digital images were captured with AxioVision version 4.6 (Carl Zeiss) by using an 800-ms exposure time. The specimens then were observed under fluorescence microscopy using a rhodamine filter cube (excitation wave-length, 540 nm; emission wavelength, 625 nm) and were captured at an exposure time of 300 milliseconds. Minimal to no auto-fluorescence of the negative control negated the need to optimize the fluorescence exposure time.

4.2.4. Changes to Tick Blood Feeding Biology

Ten unfed female and five unfed male *Amblyomma americanum* adults were labeled by attaching a small (0.5 cm x 0.5 cm) square of paper that denoted a number 1-10 and added into the feeding chamber (Figure 4.1A). The labels were attached to the dorsal side of the tick with double-sided sticky scotch tape and label tape. Ticks were monitored every 12 hours and the time of attachment, the time of detachment, the time of reattachment was recorded for each tick. The treatment groups were control (solvent only), pinacidil (1 mM), and VU063 (300 μM) and all treatment groups included Rhodamine B at 100 ppm. Prior to analysis individual ticks were imaged to identify fluorescence on the mouthparts and/or salivary glands to ensure all ticks included in the data analysis contacted the blood meal. Blood was changed every 12 hours and the chemicals were prepared fresh daily.

The changes in feeding biology was determined by quantifying the number of detachments per tick and the time until the first detachment for the treated groups compared to the non-treated control group. The average detachments per tick was determined by counting the number of detachments for each female tick in the feeding chamber until mortality or complete detachment due to the termination of feeding. Mortality was not considered a detachment. The

total number of detachment for each tick was summed in each chamber and the detachment rate for 3 feeding chambers were averaged (n: 30-40 individuals). The mean was used for comparison of pinacidil and VU063 treatment groups compared to control.

To determine the time to the first detachment, each tick was monitored and the "attachment status" of the individual was recorded. At the end of the feeding period or upon death, the time point from the initial attachment to the time of first detachment was summed for each tick and averaged amongst the chamber. Mortality was not considered in the data analysis and the time from attachment to first detachment was only determined for live ticks.

The average number of detachments and the average time to the first detachment for pinacidil and VU063 treated groups was compared to control groups by an unpaired t-test where statistical significance was denoted as $P<0.05$.

4.2.5. Tick Mortality

Ten unfed female and five unfed male *A. americanum* adults were placed into the feeding chamber and mortality was quantified by monitoring individual ticks that were labeled as described in section 4.2.4 (Figure 4.1A). Importantly, the ticks used for measuring the changes in blood feeding behavior (section 4.2.4) were not included in the data analysis for mortality. Ticks were monitored every 12 hours and the live or dead status of each individual tick was denoted. Mortality was defined as a tick completely non-responsive to mechanical stimuli and only ticks that were previously attached were included in this measurement. To ensure mortality was indeed due to ingestion of the chemical modulators, the fluorescent tracer, Rhodamine B, was included in the blood meal at a concentration of 100 ppm and each tick that was considered "dead" was analyzed for fluorescence to ensure the mouthparts had contacted the blood meal (Figure 4.1B). Data points represent the percent mortality for each chamber at the defined time

point. A total of 3-5 chambers were used for each treatment group and a control chamber was performed alongside each replicate of the treatment groups to ensure the mortality was not an artifact of an extraneous factor, such as blood or temporal differences.

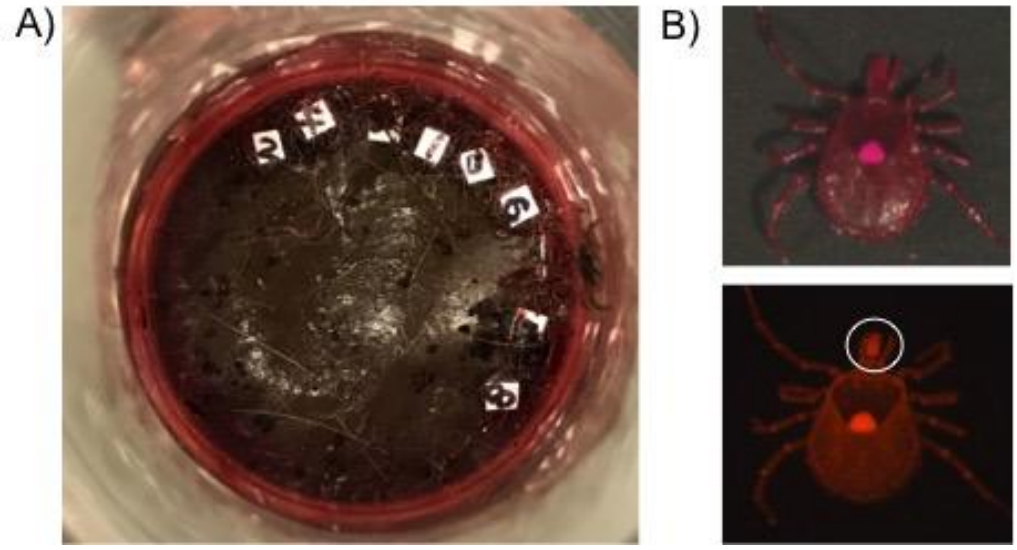


Figure 4.1. Methodology for time course in vivo studies. Individual ticks were labeled with a numerical value and various parameters were assessed every 12 hours (A). Fluorescent images were captured of ticks presumed to have fed to ensure the mouthparts (white circle) and/or salivary glands were fluorescent, indicating blood feeding (B).

The treatment groups were control (solvent only), pinacidil (1 mM), and VU063 (300 μM). Blood was changed every 12 hours and the chemicals were prepared fresh daily.

Statistical analysis was performed on the average mortality for each time point and was compared to the same time point of the control through an unpaired students t-test with significance being represented as $P<0.05$.

4.2.6. Measuring Total Imbibed Volume of Blood

Individual unfed ticks were labeled as described before (section 4.2.4) and were weighed to the nearest 0.1 mg. Ten female and five male ticks were inserted into the feeding chamber and the point of attachment for each tick was recorded. Ticks were subsequently removed after 1-, 2-, 3-, 4-, 5-, and 6-days of feeding. Ticks that detached or died prior to the required feeding time was discarded from data analysis. At the predetermined time point, the attached tick was removed from the membrane and immediately weighed to determine the change in mass. The blood was found to have a mass of 1 mg / 1 μL and therefore, an increase of 10 mg corresponded to an ingestion of 10 μL of blood. Due to the high mortality rate of ticks exposed to VU063, we were unable to determine the ingestion of VU063 treated ticks past 2 days of feeding and therefore, did not include it in this study.

The treatment groups were control (solvent only), pinacidil (1 mM), and VU063 (300 μM) and all treatment groups included Rhodamine B at 100 ppm to ensure all ticks included in the data analysis contacted the blood meal. Blood was changed every 12 hours and the chemicals were prepared fresh daily.

Statistical differences in blood ingestion were determined for each day by comparing the mean ($n>3$) volume ingested to the control group with an unpaired students t-test. Statistical relevance was denoted to be $P<0.05$. Bars represent mean ingestion volume and error bars represent SEM.

4.3. Results

4.3.1. Pharmacological Modulation of K_{ATP} Channels Alters Blood Feeding Biology of *Amblyomma americanum*

To start blood feeding, ticks must first attach to the host and remain attached until they are fully engorged with blood. To assess the impact modulation of K_{ATP} channels have to altering the blood feeding behavior, we first measured the number of times individual ticks detached from the artificial membrane after the initial attachment. Individual ticks in the control group were found to detach from the membrane an average (n=60 individuals) of 0.23 ± 0.1 times during the course of a complete feeding event. Inclusion of pinacidil into the blood meal was shown to dramatically increase the number of detachment events per tick with an average (n=60 individuals) detachment rate of 2.1 ± 0.4 times per blood feeding event (Figure 4.2A). This increase in detachment rate for pinacidil treated ticks was shown to be a statistically significant ($P < 0.0001$) increase when compared to control groups. Similarly, ticks exposed to VU063 during blood feeding displayed a higher rate of total detachments during the blood feeding period with an average of 1.9 ± 0.2 detachments, which was significantly reduced when compared to control ticks (Figure 4.2A).

In addition to the total number of detachments, we analyzed the average time from initial attachment onto the membrane to the first detachment. Of the small percentage of control ticks that detached, the average time between initial attachment onto the membrane to the first detachment was found to be 112 ± 18 hours (Figure 4.2B). Importantly, the time until first detachment was approximately 50-70% of the feeding time required to obtain a complete blood meal (168-200 hours or 7-9 days) with the artificial host membrane feeding system. On the contrary, pinacidil exposed ticks were found to feed for an average (n=22) of 28 ± 3 hours prior

to the first detachment, which was a statistically significant (P<0.001) reduction (Figure 4.2B). Similarly, the average (n=36) time from attachment to first detachment with VU063 exposed ticks was 15 ± 1.5 hours, which is statistically significant (P<0.001) when compared to control but not significantly different when compared to pinacidil (Figure 4.2B).

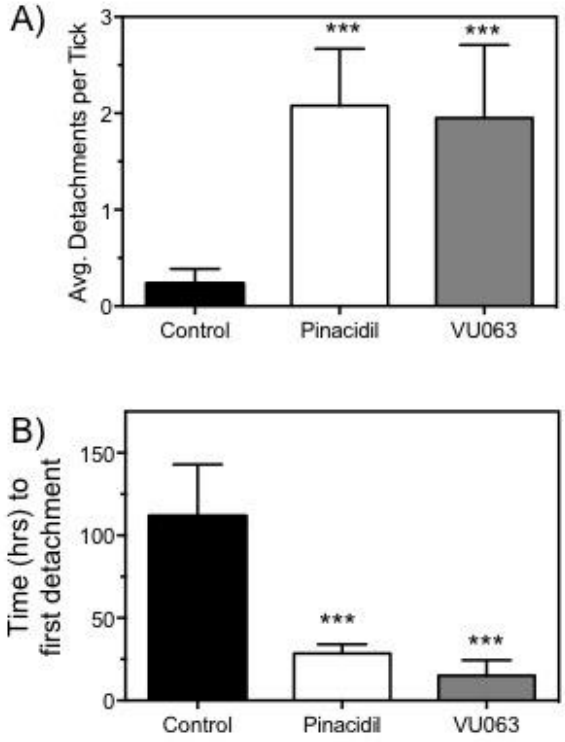


Figure 4.2. Changes in blood feeding behavior after exposure to K_{ATP} activators. Changes to the behavior were analyzed by determining the average number of detachments per tick during a feeding cycle (A) and the time from the point of attachment to the first detachment (B). Bars represent mean (n>30) and error bars represent SEM. Asterisks (***) represent statistical significance at P<0.001.

4.3.2. Exposure to K_{ATP} Channel Agonists during Blood Feeding Induces Mortality

We measured the toxicity of pinacidil and VU063 to *Amblyomma americanum* when the small-molecule modulators were added to the blood meal. Fluorescence of the salivary gland

from Rhodamine B was determined for all dead ticks to ensure the tick died after ingestion of blood. Control ticks displayed higher than expected mortality when feeding on the artificial host system but the percent mortality was significantly ($P<0.05$) reduced for days one through nine when compared to ticks continuously exposed to pinacidil (1 mM) during feeding.

The time to effectively kill 50% of ticks (ET_{50}) was found to be significantly different between pinacidil and control treatments with pinacidil ET_{50} being 1 day whereas the ET_{50} for control groups was 5 days. At day one, we observed a mean 13 ± 1.0% mortality in control groups and a 50 ± 8% mortality at day 1 of pinacidil fed ticks (Figure 4.3A). Further, mortality exceeded 90% at day 5 in pinacidil treatment groups whereas the same percent mortality was not reached in control groups until 13 days of feeding. No statistical difference in mortality between the paired control groups was observed at 10+ days.

Exposure to VU063 (300 μM) yielded mortality that was significantly greater than the mortality observed in control groups and at a faster rate when compared to pinacidil even though it was at a 2.5-fold lower concentration than pinacidil (Figure 4.3B). Statistically significant ($P<0.05$) differences in mortality was observed for each time point. The ET_{50} for VU063 was found to be less than 12 hours with 71 ± 4% mortality at the 12-hour time point. The ET_{50} for the control group was found to be 4.75 days, which is greater than 9.5-fold when compared to the ET_{50} of VU063 treated animals. Mortality for VU063 treated groups reached 100% 3-days after the start of feeding, whereas control ticks that did not reach 75% mortality 8-days (Figure 4.3B).

4.3.3. Signs of Intoxication

Ticks were found to display lethargy and uncoordinated movements after exposure to K_{ATP} activators during blood feeding. The speed of movement and ambulatory rates were significantly reduced in chemically exposed ticks when compared to control ticks and, although

the forelegs and chelicerae occasionally twitched, the tick would maintain a lethargic and quiescent state when placed on their dorsal side.

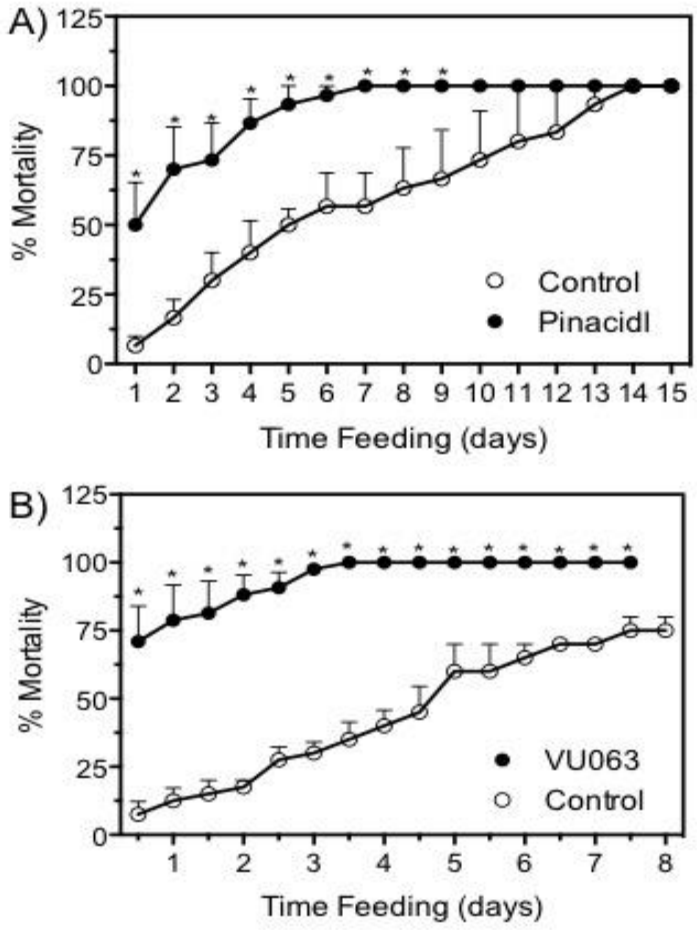


Figure 4.3. Time course of mortality after exposure to K_{ATP} activators during blood feeding. Pinacidil (A) and VU063 (B) mediated mortality compared to control treated tick populations. Data points represent mean (n>30) mortality and error bars represent SEM. Asterisks (*) represent statistical significance at $P<0.05$.

Further, the exposed ticks were not able to walk on all eight legs and would slowly raise their heads and forelegs upward and attempt to walk on their hindlegs, which would oftentimes result in falling onto their back. When the ticks were successful in ambulatory motions, they

were not capable of walking in a straight line and would veer left or right. This was in complete contrast to control ticks, which would walk quickly in a straight line to the edge of the holding chamber and climb the sides of the chamber with no tremors or abnormal behavior.

4.3.4. Pharmacological Activation of K_{ATP} Channels with Pinacidil Dramatically Reduces Blood Ingestion

To determine if the reduced salivary gland function described in Chapters 2 and 3 resulted in a reduced ability to imbibe blood during active blood feeding, we quantified the daily intake volume of blood from active feeding *Amblyomma americanum* when pinacidil (1 mM) was included into the blood meal. With the artificial host system, control and pinacidil treated ticks were shown to imbibe nearly the same volume of blood at days 1 and 2 with control treated ticks averaging (n=9) approximately 0.9 ± 0.2 μL and 1.5 ± 0.4, respectively (Figure 4.4A). Statistically significant differences ($P<0.05$) in the total volume of ingested blood were observed at days 3, 4, 5, and 6. At day 3, control ticks were shown to intake 1.7-fold more blood than pinacidil treated ticks, a statistically significant difference ($P<0.05$), with control ticks averaging a total blood intake volume of 3.7 ± 0.9 μL during the 3-day feeding period. At day 4, control ticks imbibed 2.3-fold greater volumes of blood over the 4-day feeding period when compared to pinacidil exposed ticks, which was a statistically significant difference ($P<0.05$), with control ticks averaging 9.1 ± 2.6 μL (Figure 4.4A). Day 5 and day 6 were the most dramatic differences between control and pinacidil treated ticks with control ticks ingesting 14.3- and 12.2-fold more blood than pinacidil treated ticks throughout the entire feeding period, respectively, which was a highly significant difference between the two treatment groups ($P<0.01$ and $P<0.001$, respectively). Control ticks were shown to ingest an average of 20.1 ± 8 μL and 45 ± 12 μL

during the 5-day, 6-day feeding period, respectfully (Figure 4.4A). Fluorescent images showing the reduced ingestion of blood are shown in (Figure 4.4B)

We presumed that VU063 would reduce the ability of actively feeding ticks to ingest blood with a greater efficacy than pinacidil since VU063 was found to be significantly more potent at reducing the fluid secretion from the isolated salivary gland. Unfortunately, the increased mortality rate of VU063 exposed ticks (ca. 90% at day 2) prevented the ability to accurately determine the ingestion volume beyond day 1.

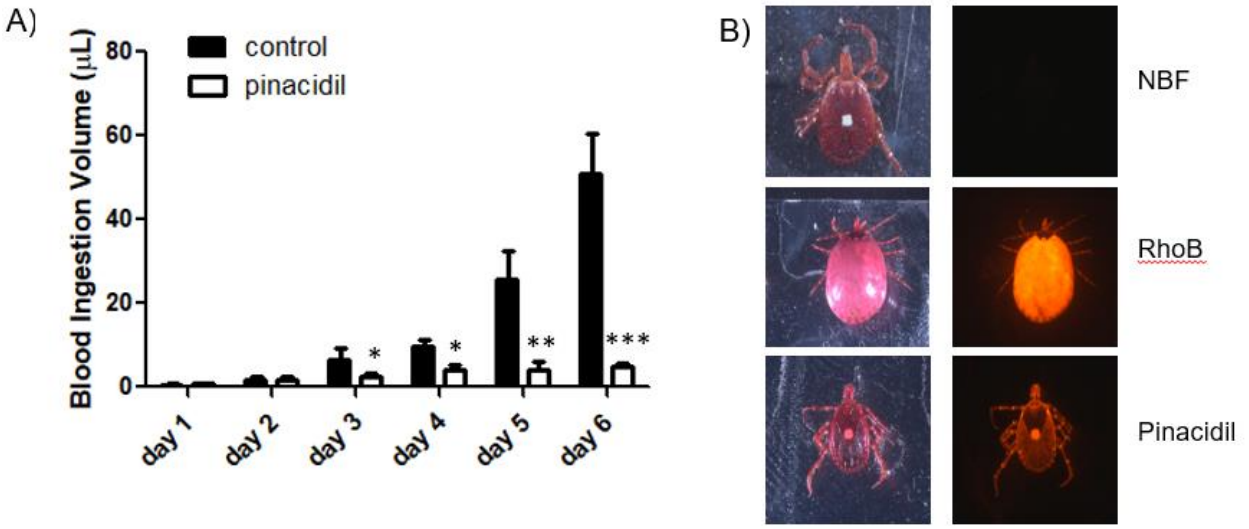


Figure 4.4. Time course of the average blood ingestion. Total blood ingestion was determined based on weight change from day 0 (unfed) to day 6 of ticks that were attached and presumably actively feeding (A). Bars represent mean ingestion volume and error bars represent SEM. Asterisks represent statistical significance with * $P<0.05$, ** $P<0.01$, and *** $P<0.0001$. Fluorescent images showing the ticks without ingestion (NBF), and ticks in control and pinacidil treated groups after six-day feeding (B).

4.4. Discussion

The use of synthetic acaricides is the primary method of tick control to protect from tick-borne pathogens in livestock and humans, yet acaricide resistance is dramatically reducing the

efficacy of current chemical classes. Therefore, it is imperative to develop strategies to continue deploying synthetic acaricides or identify novel methods of control, such as vaccine development. To achieve this, novel target sites are needed that prevent feeding and ideally, induce lethality prior to transmission of pathogens. Considering this, the ultimate objective of Chapter 4 is to bridge the promising in vitro data suggesting Kir channels are critical for proper tick salivary gland function to in vivo data to determine the applicability of targeting these proteins for tick population control and reduced pathogen transmission.

It is commonly stated in health agency guidelines and the general media that the risk of tick-borne pathogen infection increases with length of time a tick is attached. Studies have been performed to determine the different dynamics of pathogen transmission for each pathogen, but multiple studies from different groups have aimed to characterize the temporal pattern of transmission for *B. bugdorferi* sensu stricto, which is the causative agent of Lyme disease. In a 1987 paper, the published data from an animal model study of the time for *B. burgdorferi* spirochetes to be transferred to rodents and observed an increased risk of infection with longer tick attachment times (Piesman et al., 1987). In this study, 7% of the test rodents were infected in <24 hours, 35% of the rodents were infects in <48 hours, and 93% of the rodents were infected within 72 hours of blood feeding. Based on the confidence intervals from this data set, these data suggest that the probability of transmission within 24 hours could be as high as 20% and up to 57% within a 48 hour time period (Piesman et al., 1987). Similar to this study, Shih and Spielman used a xenodiagnostic method to determine that transmission of *B. bugdorferi* spirochetes occurs mainly after 2 days of feeding (Shih and Spielman., 1993). Importantly, this study also noted that partially fed ticks efficiently re-attach to a new host and in these new hosts, transmission occurred in 83% of cases within 24 hours (Shih and Spielman, 1993). The

implication of shorter transmission times in the second host after partial feeding on the first host has significant relevance to human and veterinary infection dynamics, since partial feeding behavior in ticks has been observed in the natural environment (Balashov, 1972).

More recent work has aimed to characterize the temporal pattern of transmission for *Rickettsia rickettsii* to vertebrate hosts and these data show a significantly reduced timeline for transmission when compared to *B. bugdorferi*. For instance, *Amblyomma aureolatum* was found to successfully transmit a virulent strain of *R. rickettsii* to a vertebrate host after a feeding period of 16 hours, but one animal was found to have scrotal lesions during the febrile period after 12 hours of tick feeding (Saraiva et al., 2014). Similarly, *D. andersoni* was shown to transmit *R. rickettsii* to vertebrate hosts after 10 hours of feeding in two studies (Ricketts, 1909; Moore, 1911). There is evidence that the risk of bacterial pathogen infection increases with longer tick attachment time, and the common advice to remove a tick quickly is sound. However, the ability to quickly identify ticks on the human or animal is oftentimes not practical. For instance, the small size of nymphal ticks makes them difficult to be detected even on exposed skin and nearly impossible in areas where there is limited visibility and within the hairline. Further, the painless bite of the tick often prevents the host from knowing they are being parasitized. Therefore, since the time required for transmission of *B. burgdorferi* and *R. rickettsii*, and likely other bacterial pathogens, is approximately 12 hours, it is imperative to alter the blood feeding biology of the ticks to detach from the host or induce mortality prior to 12-hours of feeding.

Here, we show that inclusion of K_{ATP} modulators into the blood meal dramatically alters the blood feeding biology of *Amblyomma americanum* by increasing the number of detachments per feeding cycle and decreased the time of the first detachment from 112 hours to 28- and 15 hours for pinacidil and VU063, respectively. Although detached ticks have been shown to

reattach in the lab and the field (Gregson, 1967; Piesman, 1991), the relatively quick time to detachment when exposed to K_{ATP} modulators has the potential to mitigate or reduce acquisition of the pathogen. Importantly, only 50% of the ticks that detached were shown to reattach, which should reduce the potential for pathogen transmission by 50%. In addition to an altered feeding behavior, inclusion of pinacidil into the blood meal dramatically reduced the volume of blood imbibed through the 6-day feeding period, which is consistent with an inability to salivate that was described in Chapter 2. This is of relevance to reducing pathogen transmission as it has been shown that ticks that have received a partial blood meal are able to reattach to another host and transmission of *B. bugdorferi* is more rapid when compared to a continuously feeding tick on a single host (Shih and Spielman, 1993). Therefore, since pinacidil exposed ticks imbibe dramatically less blood, it is possible that they will not acquire enough spirochetes to become an infected vector. Further, we speculate that the reduced volume of spit returned to the host, which correlates to the reduced blood intake, is likely to be a low enough volume to not vector the pathogen to a second host if the tick was previously infected.

It would be of great benefit to induce mortality with the same intervention method that is used to interrupt the dynamics of pathogen transmission. Therefore, we assessed the mortality rate of ticks that were exposed to pinacidil and VU063 during blood feeding. When compared to control ticks, both K_{ATP} activators were shown to dramatically increase the rate of mortality with approximately 50% and 75% mortality after 24 and 12 hours of attachment for pinacidil and VU063, respectively. We speculate that the observed mortality is due to an altered osmoregulatory capacity since we have shown that pinacidil and VU063 significantly increase the total elemental composition of the secreted saliva, which induces hypokalemia and hyponatremia that reduced functionality of the nervous system and other critical organ systems.

To the contrary, inhibition of K_{ATP} channels is likely to prevent the ability to concentrate the saliva with Na^+ and K^+, leading to a buildup of ions in the hemocele to induce mortality. Vertebrate blood is enriched in K^+ and Na^+ salts that must be quickly excreted to prevent altering the ionic composition of the arthropod, which will have deleterious consequences to tissue physiology and survivability. To substantiate this notion, previous work has shown that Kir channels represent a critical conductance pathway for the Malpighian tubules, which is the osmoregulatory organ of mosquitoes and inhibition of Kir channels induces Malpighian tubule failure (Scott et al., 2004). Further, challenging the mosquito with a bolus Na^+/K^+ ions or vertebrate blood after pharmacological modulation of Malpighian tubule Kir channels induces mortality (Swale et al., 2016; Raphemot et al., 2014a).

4.5. Conclusion

The ultimate objective of Chapter 4 was to translate the in vitro data collected in previous chapters to an applied objective that would test the hypothesis that pharmacological modulation of KATP channels will alter the blood feeding behavior, feeding success, and survivorship. Data collected in this chapter show that pinacidil and VU063 dramatically altered the feeding behavior of ticks actively feeding on the artificial host system, pinacidil significantly reduced the amount of blood imbibed when compared to control, and increased mortality to where 50-75% of ticks were dead within the first 12 hours of feeding. Therefore, the data support our initial hypothesis.

Summary and Conclusions

Ticks are hematophagous arthropod vectors that transmit pathogens causing numerous diseases that induce extreme morbidity and mortality to humans and animals worldwide. To successfully blood feed for long durations, the tick must be able to overcome a number of biological challenges, such as a host defensive response and an enrichment of K^+, Na^+, and Cl^- ions derived from the host blood. The salivary gland is the organ responsible for enabling blood feeding since it secretes various bioactive components to negate the host response and removes excess water and ions through saliva secretion. Considering this, tick salivary gland function is critical for successful feeding and survival and suggests the salivary gland represents a putative target tissue to induce feeding cessation and mortality. Due to this, previous work has aimed to characterize the neuroendocrine control of the tick salivary gland in an effort to identify pathways that could be exploited as a novel target site. Interestingly, little to no work has focused on the ion transport pathways of the tick salivary gland, which are likely to be equally as important as the neuroendocrine systems. Therefore, the overarching goal of this thesis research was to characterize the influence K+ ion channels and transporters have to fluid secretion (objective 1), osmoregulatory function (objective 2), and blood feeding behavior and success (objective 3).

In objective 1, we aimed to determine if pharmacological modulation of various K+ ion transport pathways would reduce the fluid secretion rates from the isolated salivary gland of *Amblyomma americanum*. The poorly developed pharmacological libraries limited data interpretation of the BK, K2P, KCC, and NKCC transporters, but the dramatic reduction of fluid secretion after exposure to barium chloride suggested that Kir channels are critical for proper

gland function. Indeed, exposure to activators of ATP-gated Kir channels reduced the secretory activity of the isolated salivary gland in a concentration dependent manner. Thus, the data presented in this chapter provide clear evidence that K^+ ion transport pathways, and specifically K_{ATP} channels, are critical for proper function of the tick salivary gland as measured by total saliva secretion.

In objective 2, we aimed to determine if the osmoregulatory function of the tick salivary gland was dependent upon K_{ATP} channels since these channels were shown to serve a key role in gland function for fluid secretion. Indeed, exposure to both K_{ATP} modulators, pinacidil and VU063, dramatically altered the elemental composition of the secreted saliva and suggests that the ability to regulate the ionic composition of the hemoceal during blood feeding will be hindered. We observed a dramatic increase of Na^+, K^+, Cl^- ions in the secreted saliva after activation of K_{ATP} channels, indicating a significant decrease in the cation concentration within the hemoceal that likely hinders neural function. Thus, these data suggests that tight regulation of the K_{ATP} channels is critical for proper ion flux that drives the secretion and absorption of ions into and from the saliva during blood feeding.

Considering the altered osmoregulatory function of the salivary gland, we hypothesized that the inability to maintain ionic homeostasis will alter the blood feeding behavior and lead to mortality from a depletion of Na^+ and/or K^+ ions that are required for neural transmission. This hypothesis was tested in objective 3 and indeed, we observed a significant alteration of the blood feeding behavior by an increased rate of detachment during blood feeding when compared to control. Importantly, exposure to K_{ATP} activators during blood feeding dramatically increased the mortality when compared to control ticks with the signs of intoxication reminiscent of neural failure. We anticiapate that the neural poisoining is due to an decreased hemoceal concentration

of Na^+ and/or K^+ ions that depolarize the nerve cells. Intuitively, inhibitors will likely reduce the ability to secrete cations into the saliva and cause a build up of Na^+ and K^+ in the hemoceal that is dreived from the mammalian blood that is likely to lead to mortality as well

Considering Kir channels are a critical pathway that enables proper osmoregulation of mosquitoes and ticks and that we have shown pharmacological modulators of Kir channels induces mortality, we conclude that Kir channels represent a putative target site that should be thoroughly vetted for future development of therapeutics that can reduce tick feeding, induce mortality, and reduce the burden of tick-borne pathogens.

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