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Molecular biology of tick acetylcholinesterases

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1. ABSTRACT

Ticks vector many pathogens with major health and economic impacts and have developed resistance to most acaricides used for tick control. Organophosphate (OP) acaricides target acetylcholinesterase (AChE) critical to tick central nervous system function. Mutations producing tick AChEs resistant to OPs were characterized; but tick OP-resistance is not fully elucidated, due to remarkable complexity of tick cholinergic systems. Three paralogous tick AChEs exhibiting differences in primary structure and biochemical kinetics are encoded by amplified genes with developmentally regulated expression. Gene silencing data suggest tick AChEs are functional complements *in vivo,* and transcriptomic and genomic data suggest existence of additional tick AChEs. Cholinergic systems are crucial in neural transmission and are also regulators of vertebrate immune function. Ticks exhibit prolonged intimate host contact, suggesting adaptive functions for tick cholinergic system complexity. AChE was recently reported in tick saliva and a role in manipulation of host immune responses was hypothesized. Physiological roles and genetic control of multiple tick AChEs requires further elucidation and may provide unique opportunities to understand and manipulate cholinergic involvement in biological systems.

2. INTRODUCTION

2.1. Economic and health impacts of ticks

Ticks are hematophagous arthropod ectoparasites, of major economic and health importance

worldwide because they are agents of vector-borne disease for a large variety of human and animal pathogens, including bacteria, rickettsia, viruses, and protozoa (1-3). In the United States, the most important tick impacts to humans are considered to be as vectors of Lyme disease and related syndromes (4), and as vectors of bovine babesiosis and anaplasmosis (5). Bovine babesiosis is a world-wide threat to cattle production with annual economic impacts estimated at over 3 billion dollars per year in the United States (6) and 3.2 billion dollars (5) in Brazil (7, 8), as well as major impacts to the economies of many other countries (9, 10). Despite efforts to develop vaccines or other non-chemical based methods to control ticks (11), the use of chemical acaricides is the predominant approach implemented in the field (2). Perhaps the most successful tick control effort to date is the Cattle Fever Tick Eradication Program that succeeded in eradicating *Boophilus microplus* and *Boophilus annulatus* ticks (*Rhipicephalus (Boophilus) microplus* and *R. (B.) annulatus*) from the United States (12). This decades long program, which began in 1906, was jointly administered by federal and state governments and continues to monitor all incoming cattle through inspection, acaricide treatment, and maintenance of an importation barrier (border inspection and quarantine zone) along the U.S.-Mexico border (5, 13). The importation barrier is largely dependent on dip vat treatment of incoming cattle in the organophosphate acaricide, coumaphos, and the likelihood of continuing success of the importation barrier has been questioned (6, 13, 14) due to widespread development of resistance to essentially all available acaricides (15-18).

2.2. Acetylcholinesterase as a target for pesticides

The primary physiological target of organophosphate (OP) and carbamate acaricides is generally considered to be acetylcholinesterase (AChE), the enzyme responsible for degradation of the neurotransmitter acetylcholine at neural synapses in the central nervous systems of both vertebrates and invertebrates (19-21), although there is evidence that some OPs and carbamates may exert substantial or primary metabolic effects in other pathways (21-23). The AChE enzyme in insect and mammalian systems exhibits a very high turnover number, being one of the fastest enzymes known (24), despite having a catalytic triad buried deep within the protein structure (25). A number of studies have indicated that the narrow opening leading to the catalytic site must be flexible, allowing a “breathing” of the enzyme structure (26). The relationship between AChE structure and catalytic function has been reviewed by Silman & Sussman (25). The predominant mechanism of resistance to OP pesticides was characterized in spider mites and other Acari (reviewed in (27)) and insects (28) as the result of one or more mutations in the gene encoding AChE producing a structurally altered enzyme insensitive to OP inhibition. AChE activity extracted from OP-resistant ticks was biochemically characterized in the 1960s-1970s as insensitive to OP inhibition (29-35). Later work utilizing chemical bioassays demonstrated that OP resistance in ticks also has a metabolic component (36, 37).

3. TICK ACETYLCHOLINESTERASES

3.1. Tick cDNAs encoding AChE

Tick cDNAs presumptively encoding AChEs have been reported for a number of tick species, however, *Rhipicephalus (Boophilus) microplus* and *Ixodes scapularis* have perhaps received the most study and serve as the best examples. A cDNA sequence presumptively encoding AChE in *R. (B.) microplus* was reported in 1998 by Baxter and Barker (38), however comparison of cDNA sequences between OP-resistant and OP-susceptible strains failed to reveal the presence of OP-resistance variations in the *AChE* sequence despite clear evidence of AChE activity resistant to inhibition (38-40). An additional cDNA sequence putatively encoding a second tick AChE was reported by Hernandez *et al.* (41), and subsequently confirmed in additional tick species (42), although again, no mutations responsible for OP-insensitive AChE were found. A third *R. (B.) microplus* cDNA presumptively encoding yet another tick AChE (*BmAChE3*) was reported in 2004 by Temeyer *et al* (43). AChE is a member of a large family of structurally related enzymes and the presence of three paralogous genes encoding AChE had not previously been reported in any organism except nematodes (44-48), suggesting the need for confirmation of the biochemical identity of the three possible AChEs of *R. (B.) microplus* (43).

3.2. Expression & biochemical characterization of recombinant tick AChE

Baculoviral expression of recombinant constructs of *R. (B.) microplus BmAChE3* demonstrated its biochemical identity as an AChE (49). Sequencing of cDNAs encoding BmAChE3 in transcripts from OP-susceptible and OP-resistant strains identified a number of sequence polymorphisms presumptively associated with resistance. Baculoviral expression demonstrated that at least one of these mutations from OP-resistant ticks (R86Q) resulted in production of a recombinant BmAChE3 that exhibited increased substrate affinity and insensitivity to inhibition by paraoxon (50). The R86Q mutation was the first mutation identified from an ixodid *AChE* gene biochemically demonstrated to result in OP insensitivity. The mutation, which replaced an arginine with glutamine at amino acid position 86 in BmAChE3, resulted in a 19.2.-fold reduction in the rate of paraoxon inhibition. A PCR-

Table 1. Physical and biochemical properties of recombinant BmAChE1, BmAChE2, and BmAChE3 of *R. (B.) microplus*

Enzyme	Accession[1]	# amino acids	Calculated mw[2]	Calculated pI[2]	AcSCh K_M[3] (μM)	Substrate preference (AcSCh/BuSCh)[4]	Eserine sensitivity[5] (nM, IC_{50})	Malaoxon sensitivity[5] (nM, IC_{50})
BmAChE1	CAA11702	595	65687.66	4.66	4.25	4.2	1	10
BmAChE2	AAC18857	563	62820.34	6.30	52.7	4.3	250	20
BmAChE3	AAP92139	620	69853.97	7.58	90.19	25.6	150	50

[1]Accession at NCBI (http://www.ncbi.nlm.nih.gov/), [2]Calculated molecular mass & pI of entire amino acid sequence (http://web.expasy.org/compute_pi/), [3]The KM is the substrate concentration at which the reaction rate is one-half maximum velocity (55), [4]Relative hydrolysis of acetylthiocholine/butyrylthiocholine (55), [5]Concentration of inhibitor reducing reaction rate to 50% maximum velocity (55)

RFLP assay was developed to identify the presence of the R86Q mutation in BmAChE3 which demonstrated that although the R86Q mutation was present at higher frequency in tick strains characterized as OP-resistant, it was also found in OP-susceptible strains indicating that its presence alone was insufficient to produce phenotypic resistance to organophosphate acaricides as was also true of several other reported polymorphisms in BmAChE3, *i.e.* I54V, V137I and I492M (50-54).

Baculoviral expression of *R. (B.) microplus* recombinant AChEs (*rBmAChE1, rBmAChE2*, and *rBmAChE3*) allowed biochemical confirmation of each of the BmAChEs as AChEs (summary of general properties in Table 1), that (i) preferentially hydrolyzed acetylthiocholine over butyrylthiocholine, (ii) were sensitive to acetylcholinesterase inhibitors (eserine and BW284c51), and the organophosphates (OPs) malaoxon and paraoxon, (iii) were insensitive to inhibition by iso-OMPA, a butyrylcholinesterase inhibitor, and (iv) rapidly hydrolyzed acetyl-β-methyl-thiocholine (55). In addition, recombinant BmAChE3 and BmAChE1 enzymes encoded by transcripts of OP-resistant strains of *R. (B.)* microplus were shown to be insensitive to paraoxon inhibition (52, 55).

3.3. Molecular modeling and tick AChE

Production of recombinant tick AChEs enables direct screening of natural products (56-58) and other compounds to assess inhibitory activity for development of new control technology or as chemical leads for design of synthetic ligands based on structure-activity relationships (15, 59, 60). As summarized in Swale *et al* (60), AChE contains two sites for binding by substrates and inhibitors, one at the catalytic site (CS) and a peripheral site (PS) near the entrance to the catalytic gorge. The CS contains the catalytic triad (S200, H440, E327 (*Torpedo californica* numbering)) and W84, which binds to the acetylcholine trimethylammonium group. The PS (W279, Y70, D72, and Y121 (*T. californica* numbering)) binds substrate and orients it for entry to the catalytic gorge. Novel bivalent synthetic carbamates were designed to simultaneously occupy the PS and CS sites of *Anopheles gambiae* AChE resulting in up to 500-fold selectivity for mosquito versus human AChE (61). A series of these compounds were screened against mammalian and arthropod rAChEs and it was demonstrated that these compounds were capable of specific inhibition of wild-type and OP-insensitive recombinant AChEs of *R. (B.) microplus* and *Phlebotomus papatasi* (60). Further, it was revealed during the course of these studies that recombinant BmAChE1 (rBmAChE1) of *R. (B.) microplus* exhibited a unique insensitivity to tacrine, a cholinesterase inhibitor, compared to all other species studied, suggesting a unique catalytic structure. Molecular homology modeling revealed the presence of two orthologous substitutions in BmAChE1 compared to human AChE at W384/Y337 and T335/W286 (*Rm*/human numbering). Molecular modeling suggested that the tryptophan residue (W384) in BmAChE1 resulted in reduced access to the catalytic site that could not be resolved by reorientation of the residue's bulky side group. Additional biochemical differences unique to the rBmAChE1 enzyme prompted site-directed mutagenesis to substitute phenylalanine for the tryptophan residue at position 384 of BmAChE1 (W384F). Phenylalanine occupies a smaller space than tryptophan and a closer resemblance to tyrosine, which is found in most other AChE enzymes, including human AChE. The W384F mutant of rBmAChE1 exhibited significant increases in inhibition by tacrine and two bivalent inhibitors, BW284c51 and donepezil. Increased potency of tacrine and BW284c51 to the W384F mutant rBmAChE1 was consistent with the hypothesis that W384 restricted access of large ligands to the acyl site but did not restore IC_{50} values to those obtained in other species. This suggested that additional orthologous substitutions in conjunction with the W384/Y337 (*Rm*/human numbering) may also contribute to constriction of the BmAChE1 gorge geometry and access to the catalytic site. Various rBmAChE1 constructs containing a number of additional amino acid substitutions from OP-resistant strains of *R. (B.) microplus* were analyzed for sensitivity to experimental carbamates. Although most of these constructs did not exhibit significant

Table 2. *R. (B.) microplus* mutations associated with OP-resistance

BmAChE1[1]	BmAChE2	BmAChE3
P157S	F9L[2]	I48L[5]
D188G[1]	A26T[2]	I54V[4,5]
E195G	E75G[2]	V71A[5]
E196G[1]	W114R[2]	I77M[5]
Y230C	L141F[2]	S79P[5]
S282G	K208R[2]	R86Q[4,5]
V331A[1]	I210V[2]	I123M[4]
T362A	V297I[3]	I492M[4]
F390S[1]	P343S[2]	T548A[4]
L417P	M349V[2]	T548Y[4]
Q488R	S364T[3]	
I493T	M406V[2]	
N566D[1]	H412Y[3]	
W571R	S364T[3]	
W571F	M406V[2]	
P590A[1]	H412Y[3]	
	R468K[3]	
	K555M[2]	

[1]rBmAChE1 substitutions present in Tux-11 (OP-insensitive) unmatched in Deutch 5 (OP-sensitive) (60), [2]BmAChE2 substitutions associated with resistance (60), [3]BmAChE2 substitutions associated with resistance (127), [4]R86Q mutant exhibits 19.2-fold reduced sensitivity to paraoxon (50, 65) (60), [5]Amino acid substitutions associated with resistance in Indian strains of *R. (B.) microplus* (128)

cross resistance to coumaphos or carbamates, the Tuxpan #11 rBmAChE1 exhibited 5-fold resistance to carbamate, suggesting that this novel carbamate would be subject to potential control failure in the field. The Tuxpan #11 construct carried 4 additional substitutions, of which two (D188G and E196G) were unique. Propoxur (approved by WHO for mosquito control) exhibits greater mammalian toxicity than exhibited or predicted for the synthetic carbamates, suggesting substantially improved safety of the novel synthetic carbamates compared to current methylcarbamates if used at similar application rates with equivalent or better *in vivo* pesticide activity (60).

Molecular modeling approaches have also been used to select candidate derivatives of carvacrol and salicylic acid to evaluate efficacy against larval *R. (B.) microplus* (15). Higher acaricidal activity was observed for derivatives of carvacrol and salicylic acid compared to the parent compounds, providing further evidence of the utility of *in silico* modeling approaches for development of efficacious pesticides. In addition, *in silico* structure-activity modeling evaluating shape-based inhibitor docking has provided major advantages in throughput and evaluation of novel structures than is possible through physical biochemical approaches providing novel leads for chemical synthesis and evaluation (62, 63).

3.4. Tick AChE polymorphisms associated with insensitivity to inhibition

Multiple single nucleotide polymorphisms (SNPs) in each of the three identified *BmAChE*s have been associated with organophosphate resistance by cDNA sequencing or mutation-specific genotyping of OP-resistant or susceptible strains of *R. (B.) microplus* (Table 2). However, genetic association of these polymorphisms does not confirm direct involvement in production of altered AChEs that are insensitive to inhibition. Some of the resistance-associated polymorphisms may reflect a founder effect that persists in the genetic lines that gave rise to the phenotypically resistant ticks, while others may reflect secondary mutations that stabilize the structure of altered, inhibition-insensitive enzymes, or reduce fitness costs associated with the resistance mutations (64). Characterization of SNPs associated with resistance in different tick strains may provide new clues related to the physiological importance and roles of polymorphisms in the multiple tick *AChE*s. Biochemical confirmation of altered sensitivity to inhibition is needed to elucidate the specific contributions of such resistance-associated polymorphisms to the development of resistance. As previously mentioned in sections 3.2. and 3.3., baculoviral expression of *BmAChE3* transcripts containing the R86Q substitution and the

BmAChE1 Tuxpan #11 transcript were each shown to produce recombinant AChEs with significantly reduced sensitivity to biochemical inhibition by certain organophosphate or carbamate inhibitors. The vast majority of polymorphisms that have been associated with phenotypically resistant tick strains have not been biochemically confirmed to result in reduced sensitivity to inhibitors, and await biochemical demonstration of altered AChE inhibition.

3.5. Genomic amplification and functional complementation of tick *AChE*s

Developmental regulation of *BmAChE* expression as well as amplification of *BmAChE1, BmAChE2*, and *BmAChE3* is evident, and multiple transcripts are expressed for each of the three paralogous AChEs within synganglia from individual ticks (14, 52, 54, 65). Genomic segments of *BmAChE1* display the presence or absence of an intron within allelic PCR amplicons, suggesting gene duplication associated with gain or loss of an intron (14, 52, 65). Further, all of the *BmAChE* genes appear to be present in multiple copies within the *R. B. microplus* genome (14, 52, 54, 65, 66). Interestingly, gene silencing of any one or of any two of the *BmAChE*s does not result in significant phenotypic effects, but silencing of all three *BmAChE*s resulted in ≥50% mortality of ticks receiving dsRNA by microinjection strongly suggesting that the BmAChEs functionally complement one another *in vivo* (14, 52, 54, 65, 66).

3.6. Tick AChE and acaricide resistance

Development of phenotypic resistance to OPs in ticks is complex and multigenic. A number of point mutations in insects result in OP resistance (28), and some of the same amino acid substitutions account for resistance in spider mites (Acari), including F331W (*Torpedo californica* numbering), G119S, G328A, A201S, and A280T (summarized in (27)). Although none of these mutations have been reported in ticks to date, targeted mutagenesis to produce and express rBmAChE1 containing the G119S substitution resulted in greater than 1000-fold insensitivity to paraoxon compared to the wild type enzyme (unpublished data). This very high inhibition insensitivity resulting from an SNP indicates that there is no structural peculiarity of tick AChE preventing expression of high level inhibitor insensitivity. Given that tick resistance to OPs is complex and multigenic, it is plausible that individual SNPs may be present at low frequency, as might be expected if they have an intrinsic fitness cost, yet remain undetected by the methods employed thus far. It has been estimated that retention of approx. 2.5% of normal AChE activity is metabolically sufficient to maintain life (67), suggesting that possession and low-level expression of a highly resistant AChE allele among a background of much more highly expressed, but OP-susceptible AChE forms, could result in phenotypic resistance. The deleterious effects of high fitness cost *BmAChE* mutations may be mitigated by gene duplication and expression of allelic diversity, including both OP-resistant and OP-susceptible alleles capable of functional complementation (52, 54, 65). Gene duplication or increased expression may expand the quantity of enzyme being produced, thereby requiring a larger number of inhibitor molecules to bind to the larger target pool resulting in functional phenotypic resistance even without altered enzyme kinetics. Similarly, phenotypic resistance may occur through increased sequestration of inhibitor molecules by binding to scavenger proteins such as additional AChEs, butyrylcholinesterase, carboxylesterases, or other cellular components (68-70). Gene duplication has been reported as a mechanism of OP resistance in spider mites (70, 71) and mosquitoes (72, 73) resulting in expression of functionally complementary paralogous AChEs that reduce or eliminate the fitness cost associated with the primary resistance mutation (71, 74, 75). The presence of multiple paralogous tick *AChE*s, together with gene duplication and expression of multiple allelic transcripts from each of the paralogous *AChE*s greatly complicates the design of novel inhibitors by target-guided synthesis or structure-based drug design (76).

3.7. Additional tick AChE genes

Genomic and transcriptomic studies of *Dermacentor variabilis, R. (B.) microplus,* and *Ixodes scapularis* strongly support the presence of additional genes encoding AChE in ticks (43, 66, 77, 78); however, biochemical identity has been verified for only a few acarine AChEs (55, 79). Cholinesterases belong to a very large enzyme family with similar amino acid sequences comprising their primary structures (80-82). In addition, there are numerous reports that substitution of a single amino acid in the primary structure of many of these enzymes can result in significant alteration of their biochemical properties, perhaps best exemplified by certain resistance mutations (69, 82-85).

As can be seen in Figure 1, biochemically characterized tick AChEs (BmAChE1, BmAChE2, and BmAChE3) are represented in only 3 branches of the *Ixodes scapularis* AChE phylogram (66), leaving representatives from several putative branches to be biochemically identified and characterized. Some of the branches may be unique to *I. scapularis* and may be absent in ticks that exhibit high host specificity such as *R. (B.) microplus* which completes its development from larval stage to engorged adult on a single host; however, incomplete genomic data available for *R. (B.) microplus* strongly indicates the presence of multiple transcript sequences that are tentatively identified as AChEs by BLAST searches of available databases (77). The physiological roles and genetic control of the

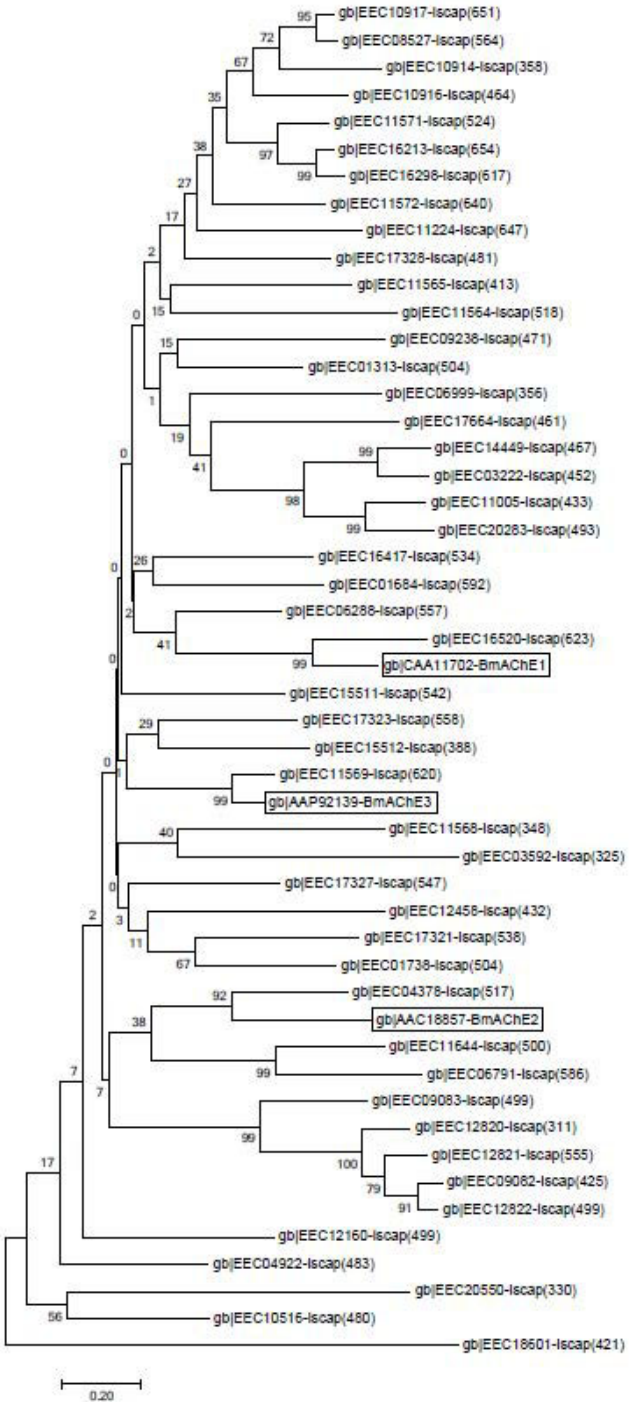

Figure 1. Phylogram of *Ixodes scapularis* putative AChEs. An unrooted phylogram demonstrating diversity of selected amino acid sequences putatively identified as AChEs of *Ixodes scapularis* was constructed using MUSCLE multiple sequence alignment (129). *Ixodes scapularis* amino acid sequences putatively annotated as AChEs in GenBank (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov) were retrieved and duplicate sequences eliminated. Putative Iscap AChE sequences ≥300 amino acids were subjected to BLASTp searches against the GenBank database, eliminating any that indicated highest sequence identity to non-cholinesterases, and retaining only those Iscap sequences putatively identified by BLASTp results as tentative AChEs. Protein sequences (≥300 amino acids) of *Ixodes scapularis* putative AChEs were used together with BmAChE1, BmAChE2, and BmAChE3 of *Rhipicephalus microplus* (boxed, included in the analysis as reference points) to infer an evolutionary history (unrooted phylogram) using the Neighbor-Joining method (130). The optimal tree with the sum of branch length = 20.97993606 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches (131). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (132) and are in the units of the number of amino acid substitutions per site. The analysis involved 50 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 65 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (133).

multiple AChEs in ticks remain open questions, but may provide unique opportunities to further elucidate cholinergic involvement in multiple important biological systems.

4. NON-NEURONAL CHOLINERGIC ROLES

4.1. Non-neuronal cholinergic control of physiology and metabolism

Acetylcholine (AcCh) is an ancient molecule involved in cholinergic signaling and physiological control throughout the animal kingdom, and has also been found in plants, algae, protozoa, fungi and bacteria (86-88), suggesting that it has been present from the beginning of life (89, 90). In addition to the classical function of AChE hydrolyzing the neurotransmitter acetylcholine at neural and neuromuscular synapses, AChE and its variant forms have been proposed to function in a number of physiological interactions and processes in an autocrine or paracrine-like manner. Non-neuronal acetylcholine (AcCh) regulates basic cell functions, including differentiation, migration, ciliary activity, signaling, immune function, angiogenesis, secretion, cell proliferation, cytoskeletal organization and apoptosis (82). Some of these interactions appear to be mediated by the peripheral binding site of AChE. The adhesion-mediating Leu-Arg-Glu (LRE) motif is present in neuroligins, neurotactin, glutactin as well as mammalian AChEs and invertebrate AChE2. Enrichment of this motif and its structural positioning suggests that it functions in cell adhesion at the neuromuscular junction and in maintenance of cytoskeletal integrity (91). Indeed, octopus AChE has been proposed to play an important role in arm regeneration (92). Complete or partial cholinergic systems, including AcCh synthesis (choline acyltransferase), hydrolysis (AChE), and muscarinic and nicotinic AcCh receptors (mAcChR and nAcChR), are expressed in most types of human cells (93, 94). Activated lymphocytes, dendritic cells, macroe phages, mast cells and other immune cells produce and release AcCh (94, 95). Cholinergic systems exert significant control over a wide variety of metabolic, cellular, and tissue physiological functions, including neural function, cellular proliferation, apoptosis, and tumorigenesis (96, 97). Many, if not all of these multi-tissue effects are mediated through the differential activation of a variety of muscarinic and nicotinic acetylcholine receptors and subsequent effects exerted through the balance of activation of the multiplicity of different muscarinic and nicotinic receptors. In general, muscarinic receptors are members of the G-protein family coupled to second messenger transduction processes (metabotropic) and are considered to exert pro-inflammatory effects while nicotinic receptors exert their predominantly anti-inflammatory effects through ionotropic processes, with multiple receptor subtypes within each group (94). Different receptors exhibit differential sensitivity to various OPs and/or acetylcholine concentrations and selective changes in activation of receptor subsets may alter expression or sensitivity of cells and other receptors, producing a wide variety of inter-related cascade reactions (21). In addition, cholinergic regulation of invertebrate immune function in Mollusca was found to be similar to that occurring in mammals (98). MicroRNA manipulation of the invertebrate cholinergic function was implicated in altered expression of tumor necrosis factor-like proteins and bacteriostatic activity of oyster haemocytes during bacterial infection, suggesting potential manipulation of cholinergic involvement in pathogen infection of invertebrates (99). Further, AChE exhibits deoxyribonuclease activity as a mechanism in the induction of apoptosis (100). Although the functional roles performed by tick AChEs in addition to hydrolysis of AcCh at neural synapses remain to be elucidated, the large number of putative tick AChEs represented in the phylogram (Figure 1) may serve similar functions to those described above in tick development, physiology and metabolism, with either functional separation in different AChEs, or functional redundancy.

4.2. Tick salivary AChE: potential immune interaction at the tick host interface

Increased understanding of the molecular interactions between tick vectors, their vertebrate hosts and the pathogens that they transmit is critical to development of effective measures for control of tick-borne diseases (9, 101). Parasites have been selected through coevolution with their hosts to successfully manipulate host immune systems, thereby promoting parasite survival (102). Ticks and some other Acari exhibit prolonged and intimate contact with their hosts (103, 104), suggesting a potential adaptive function for such unusual complexity in their cholinergic systems (105). AChE has recently been reported to be present in tick saliva (105-107) and a potential role in manipulation of the host immune response was hypothesized (105). AChE has also been reported in the saliva of the bed bug, *Cimex lectularius* (108). One plausible function of AChE in tick saliva would be to reduce the local tissue concentration of acetylcholine (89) altering the balance between activation of muscarinic and nicotinic acetylcholine receptors, resulting in principal activation of muscarinic receptors, which have been shown to be sensitive to much lower concentrations of AcCh than nicotinic receptors (21). Indeed, cholinergic activation of muscarinic receptors in the microvasculature of the mouse results in production of nitric oxide, producing vasodilation (109) and thereby would promote tick blood feeding. In addition, recent studies indicate cholinergic involvement of nicotinic AcChRs in modulation of inflammatory processes and nociception (acute and chronic pain) in mice (110, 111), which are also likely to be directly affected by alteration of tissue concentrations of AcCh. Tick saliva is complex in

composition and known to modulate inflammatory and local host immune responses as well as to facilitate pathogen transmission and establishment within the host, but the specific salivary factors responsible have not been fully elucidated (104, 112-114).

There has been mounting evidence in recent years for a significant cholinergic role in activation and regulation of vertebrate immune functions, including immune cell proliferation, production of cytokines, and T helper cell differentiation and presentation of antigens (115). The cholinergic system modulates inflammation, acting on both the innate and adaptive immune systems (116, 117). Activated immune cells increase acetylcholine synthesis and secretion (118), and activation of CD4+ or CD8+ T cells alters their expression of specific acetylcholine receptor subsets toward specific developmental lineages (119). Similarly, macrophages, microglia and B cells also alter their expression of specific subsets of acetylcholine receptors (120-122). Acetylcholine has been demonstrated to decrease production of pro-inflammatory cytokines by activated macrophages (123). Cholinergic stimulation of muscarinic or nicotinic receptor activation is able to mediate either pro or anti-inflammatory effects and the balance affects every aspect of immune function (94, 115-117, 124).

Based on the reported cholinergic involvement in every aspect of vertebrate immune function, secretion of AChE in tick saliva would be expected to exert profound effects on innate and adaptive immune response of the host to tissue damage at the tick attachment site and the presence of tick salivary antigens. The skin is one of the primary protective barriers and immune sentinel cells residing in the skin (dendritic cells) mediate immune responses resulting from wound, tissue damage, pathogen invasion or other insult. The dendritic cells respond to tissue or epithelial cell insult by production of cytokines, interferons, chemokines, tumor necrosis factors and other immune mediators based on the type and magnitude of alarm signal they detect, and they recruit and interact directly with other immune cells to regulate and direct the innate and acquired immune responses (125, 126). In the case of *R. (B.) microplus*, the BmAChE1 present in the tick saliva (105, 107) has a much higher affinity for AcCh than the mammalian AChE (105) which should result in localized depletion of AcCh in host tissues near the tick attachment site, altering homeostatic balance of AcCh receptor stimulation resulting in vasodilation, alteration of immune cell function and directed progression of immune cell developmental lineages. The ensuing host inflammatory and immune response to tick attachment and feeding would be further modulated by interaction with the other complex pharmacologically-active components of tick saliva (104). Figure 2 summarizes some of the additional functions that tick AChEs may perform to partially account for the unusual complexity of tick cholinergic systems.

5. PERSPECTIVES

A critical regulatory role for cholinergic involvement in a wide variety of physiological and developmental processes, as well as a characterized role in neural and neuromuscular transmission, is supported by a substantial body of literature. A brief list of hypothetical predictions that are based on a potentially transformative role for tick AChEs in host-ectoparasite interactions is presented below. Results from the suggested lines of work would expand our understanding of tick physiology and host-vector-parasite relationships.

5.1. Potential effects of tick salivary AChE on response to tick exposure in vaccinated animals

Tick salivary AChE likely alters the balance between activation of specific nicotinic and muscarinic AcCh receptors in sentinel immune cells responding to tissue injury or presence of foreign antigens thereby changing direction or magnitude of subsequent immune response. Recombinant BmAChE1 produced in enzymatically active and inactive forms could be utilized in experiments challenging the responses of vaccinated animals to subsequent antigen exposure in the presence or absence of enzymatically active tick AChE. Evidence supporting modulation of immune response in vaccinated animals would suggest the need to include epitopes directing the production of tick AChE-neutralizing antibodies in anti-tick vaccines.

5.2. Investigation of host-parasite interactions manipulating host response to ticks and pathogens

Tick salivary assisted transmission (SAT) of pathogens has been widely demonstrated, but the salivary components responsible for this phenomenon have not previously been identified. This paper lightly reviews a substantial body of literature providing support for the hypothesis that tick salivary AChE has broad effects on host innate and acquired immune responses, with effects on vasodilation, inflammatory processes, and differential activation of muscarinic and nicotinic AcCh receptors in immune sentinel cells that initiate and direct subsequent systemic immune responses. As noted in section 5.1., production of rBmAChE1 in enzymatically active and inactive forms could be utilized in experiments challenging the responses of animals or animal cells to pathogen exposure in the presence of either form to investigate the effect on responses affecting pathogen survival and establishment.

5.3. Effects of tick cholinergic systems on ability to harbor and vector pathogens

Extensive cholinergic regulatory systems within the tick may reduce deleterious effects of pathogen presence within the ticks, possibly reducing

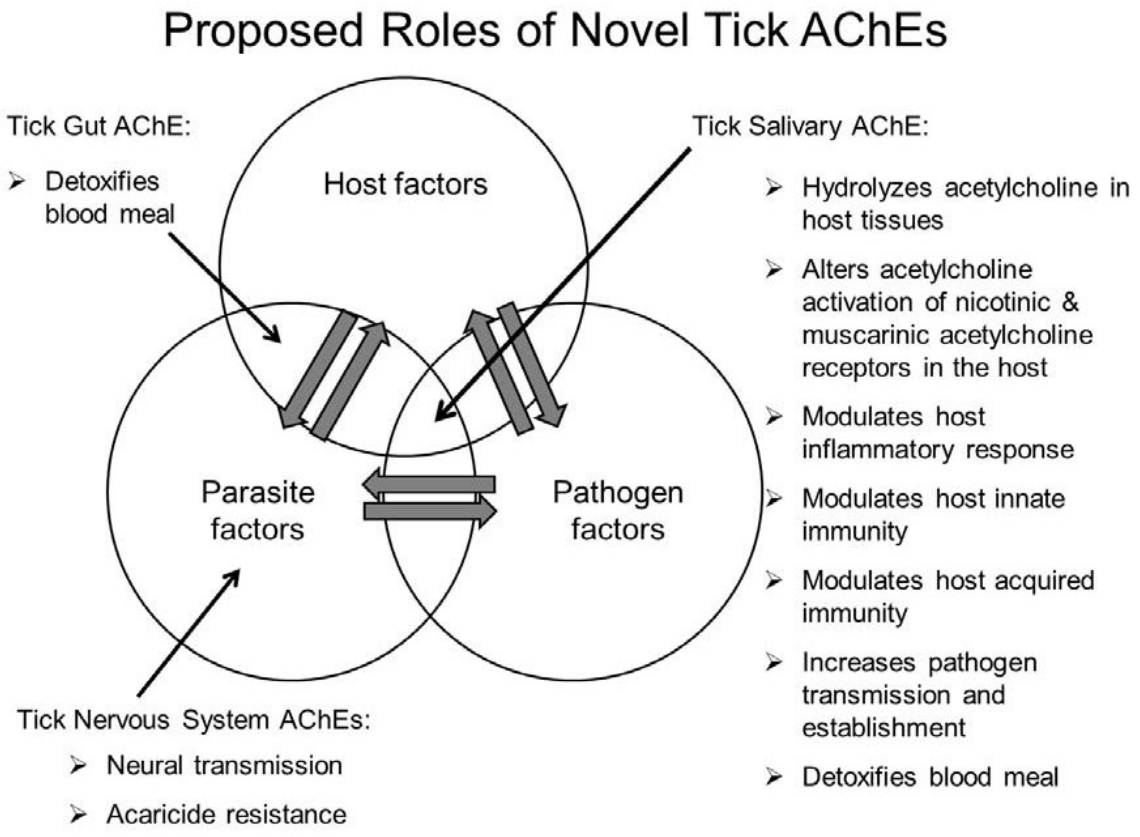

Figure 2. A Ven diagram was constructed illustrating proposed roles of AChE in saliva, nervous system, and possibly gut of ticks and their presumptive interactions between host animals, ticks, and vectored pathogens.

virulence effects within the tick either altering the tick response to pathogen presence by controlling tissue damaging inflammatory processes within the ticks, or by reducing production or release of toxins by the pathogens.

5.4. Tick physiology and metabolic control exerted by cholinergic processes

Investigations designed to study the regulation of potential autocrine & paracrine function of AcCh on physiological and developmental pathways within ticks should be undertaken to further elucidate metabolic and developmental control pathways. Information obtained through such studies would be expected to identify targets for potential development of new acaricides, vaccines or other novel tick control technologies.

5.5. Biochemical identity and function of multiple tick AChEs

Biochemically confirmed tick or other acarine AChEs map in only a few of the branches of a phylogram listing *Ixodes scapularis* presumptively identified AChE sequences, leaving many branches to be biochemically characterized and studied for developmental and tissue-specific expression. Identification of specific tick AChEs demonstrated to perform critical or unique essential functions could enable design of ligands or other effector molecules that specifically target these activities producing highly targeted and novel tick control technology.

5.6. Tick AChEs as tools to investigate cholinergic involvement in vertebrate systems

As noted in section 4, there is a substantial body of evidence demonstrating cholinergic involvement in a variety of mammalian and other vertebrate systems. Among such systems are many autoimmune dysfunctions and inflammatory syndromes that may benefit from cholinergic manipulation, *e.g.,* by development and administration of drugs targeting specific components of cholinergic systems. Examples of known cholinergic involvement that have benefited somewhat by development of specific cholinergic drugs are Alzheimer's disease and asthma. Cholinergic components derived from ticks could be developed and utilized for *in vitro* or *in vivo* studies to model cholinergic roles in vertebrate systems. A more complete understanding of cholinergic roles in cellular

and developmental physiology could lead to improved treatment or control of malfunctions of metabolic and genetic disorders.

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