cDNA identification and gene expression of two types of acetylcholinesterases in a cultured cell line of *Aedes albopictus*, compared to mosquito whole body extracts

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Abstract

Two cDNA sequences containing open reading frames encoding two types of acetylcholinesterase (AChE) precursors, *Ace*-orthologous AChE (AO-AChE) and *Ace*-paralogous AChE (AP-AChE), with 635 residues and 702 residues, respectively, were determined in *Aedes albopictus* by PCR-based techniques. The partial and whole cDNAs for AO-AChE and AP-AChE, respectively, were also sequenced from a cultured cell line of mosquito, NIAS-AeA1-2, derived from *Ae. albopictus* neonatal larvae. Comparing the sequences of the respective AChEs between cultured cells and mosquito whole body extracts, many nucleotide polymorphisms were found in both AChE cDNAs but only one amino acid substitution. The expression level of AChE genes in the cultured cells was low, 1/2 and 1/7 that of the mosquito body for AO-AChE and AP-AChE, respectively. The expression ratio of AO-AChE/AP-AChE was 1/2 in the cultured cells. AP-AChE genes was more highly expressed than AO-AChE genes in both cultured cells and the mosquito body. In an enzyme assay, the activity of AChE per protein in the cultured cells was 1/8 that of the mosquito body. The function of AChE in cultured cells is discussed based on the present and previous papers.

Key words: *Ace*-orthologous; *Ace*-paralogous; AChE; mosquito cell; real time PCR

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) terminates neural excitement at the postsynaptic membrane by hydrolyzing the neurotransmitter, acetylcholine (ACh). In insects, AChE has mainly been studied in relation to insecticide resistance because the enzyme is the target of organophosphorus and carbamate insecticides and its insensitivity to insecticides is one of the main factors for resistance. The insect AChE gene was first sequenced in *Drosophila melanogaster* (Hall and Spierer, 1986) and several amino acid substitutions conferring insecticide insensitivity in AChE were first elucidated in this species as *Ace* (Mutero et al., 1994) followed by the Brachycera fly species, *Musca domestica* (Huang et al., 1997; Kozaki et al., 2001; Walsh et al., 2001), *Lucilia cuprina* (Chen et al.,

2001), and *Bactrocera oleae* (Vontas et al., 2002). However, no amino acid substitution in the *Ace* homologues (*Ace* orthologous AChE, AO-AChE) was found in other insects in which AChE insensitivity had been well characterized (Fournier and Mutero, 1994). Recently, a second type of AChE less homologous to *Ace* (*Ace* paralogous AChE, AP-AChE) was found in the green bug, *Schizaphis graminum* (Gao et al., 2002) and the mosquito, *Anopheles gambiae* (Weill et al., 2003). Amino acid substitutions at several different positions conferring insecticide insensitivity have been reported in AP-AChEs of *Culex pipiens* (Weill et al., 2003), *An. gambiae* (Weill et al., 2003), *An. albimanus* (Weill et al., 2004), *C. tritaeniorhynchus* (Nabeshima et al., 2004), *M. persicae* (Nabeshima et al., 2003), *Aphis gossypii* (Andrews et al., 2004; Toda et al., 2004) and *Plutella xylostella* (Baek et al.,

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2005). Recent studies of several insect species have shown that the expression level of the AP-AChE gene was much higher than that of the AO-AChE gene, and suggest that AP-AChE plays a main role in terminating synaptic transmission. Therefore, a question as to the functions of AChE except for the primary function mentioned above, arises because of the existence of two types of the enzyme in insects. Some functions of AChE such as cell growth, cell-substrate adhesion and neurite growth have been suggested in vertebrates (Layer et al., 1993; Huby et al., 1999; Johnson and Moore, 1999; Ruben et al., 2004; Silman and Sussman, 2005). On the other hand, several insect cell lines having AChE activity have been reported (Ohbayashi et al., 1997). Cell lines of *Ae. albopictus* (NAIS-AeAl-2; Mitsuhashi, 1981) derived from neonatal larvae seem to be the best material for studying secondary functions because they have high AChE activity and the original insect species have two types of AChE. As the first step, we attempted to isolate cDNAs for two types of AChE, AO-AChE and AP-AChE, from both cultured cells and mosquito body, sequence their cDNAs and determine the expression of these AChE genes.

MATERIALS AND METHODS

Mosquito strain. The insecticide-susceptible strain of *Ae. albopictus* originating from Ikaken strain, Tokyo, Japan, was maintained at the National Institute of Infectious Diseases. Mosquitoes were reared under constant conditions at 25°C and a photoperiod 16L:8D without insecticide exposure.

Cultured cells and culture medium. A cultured cell line, NIAS-AeAl-2, established from *Ae. albopictus* neonate larvae by Mitsuhashi (1981) was used. The cells were cultured at 25°C in MM medium (Mitsuhashi and Maramorosch, 1964) containing 3% fetal bovine serum (FBS). For enzyme assays, cells collected with a scraper (Sumilon) were centrifuged at $1,000\times g$ for 10 min at 4°C. The supernatant of culture media was removed and cells subjected to the AChE assay as follows.

Enzyme assays. Cultured cells and mosquito bodies were homogenized with a polytron in 1 ml of 50 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100 and the homogenates were centrifuged

at $1,000\times g$ for 10 min at 4°C. The supernatant was used as an enzyme source. The activity was measured according to the method of Ellman et al (1961) with minor modifications (Mamiya et al., 1997). Forty microliters of enzyme source were added to each well of a microtiter plate containing 140 μ l of 50 mM Tris-HCl buffer (pH 8.0) and 20 μ l of DTNB solution (0.4 mM DTNB+1.5 mM NaHCO₃) to initiate the reaction (25°C). Then 40 μ l of substrate and 1.0 mM acetylthiocholine (ATCh) were added to each well. Enzyme activity was measured photometrically at 412 nm and 25°C with a Microplate Reader Model 450 (Bio-Rad). Additionally, the amounts of protein in the cultured cells and mosquito whole bodies were determined with a BCA Protein Assay Kit (Pierce). Bovine serum albumin was used as the standard. The Michaelis constant (K_m) was calculated based on the results of the reaction with different concentrations of substrate (100 to 0.001 mM ATCh). K_m values were estimated with a Lineweaver and Burk plot.

RNA extraction and cDNA synthesis. RNA was extracted from 115 mg of adult mosquitoes and 8×10^6 cultured cells with Isogen (Nippon Gene) according to the manufacturer's protocol and directly used for reverse transcription. In both mosquito and cultured cells, first-strand full-length cDNA was synthesized with oligo (dT) primers (QT'), SuperScript II (Invitrogen). The reaction volume was diluted to 40 μ l with distilled water. The first strand cDNA synthesized from 3 μ g total RNA was used in the PCR and 3'- and 5'-RACE analyses.

Isolation of AChE cDNAs from mosquito body. cDNA synthesized from both mosquito and cultured cells was amplified with ExTaq DNA Polymerase (Takara). The reaction volume was 25 μ l including 0.5 μ l of template. For isolating AO-AChE cDNA from mosquito whole bodies, degenerate primers S18ACE and S20''ACE were used to amplify the first cDNA fragment and then a second PCR was performed with S24ACE and AS4ACE gene-specific and degenerate primers, respectively (see Table 1). Both PCR reactions were carried out at 94°C for 2 min as a preheat step, followed by 40 cycles at 94°C for 30 s, 50°C for 1 min, 68°C for 30 s, and 72°C for 5 min as a final extension. The above product was nested with S24ACE and AS30ACE primers in a third PCR re-

Table 1. Primers used in this study

Forward primer		Reverse primer	
RT		QT	Q0-Q1'-(dT) ₁₈
AaA ChE1			
PCR			
S18ACE	GGTGAAGAAATGTGGAAYCCNAATAC	AS20"ACE	CATAAARCCDCCDCRATAATCCA
S24ACE	TATCTGAATATCTGGGTGCCG	AS4ACE	ACNCCCATCCAYTCNCCCCA
		AS30ACE	TGRTTYTGRTAICCRCTRTTICC
3'RACE			
S38ACE	AACGTAATCGTGGCATCCAT	Q0	CCAGTGAGCAGAGTGACG
S30ACE	TCTGAGCCGGAGCGCGAAG	Q1'	GAGGACTCGAGCTCAAGC
5'RACE			
5'Primer	GTCTACCAGGCATTCGCTTCAT	AS41ACE	TGTACACGTCCAGAGTCGATG
Full length			
S55ACE	TGATCAAATCGAGTGTTTTTG	AS55ACE	ATAATATTAAGGTGGGGTGGGACAG
AaA ChE2			
PCR			
F7ACE	ACIATGTGGAAYCCIAAYACICC	R2ACE	TCTTCCGCATATCGCTGGGCAAAC
3'RACE			
F11ACE	AGCGTTCGCTAGCCAGTGGAC	Q0	CCAGTGAGCAGAGTGACG
F10ACE	CAGGCCGTTAGAGAGCTGAA	Q1'	GAGGACTCGAGCTCAAGC
F20ACE	GGTGACGAGATCAACTATGTATTC	Q1'	GAGGACTCGAGCTCAAGC
5'RACE			
		R11ACE	TCTAGGGTAGCAGTACCGGAGT
AUAP	GGCCACGCGTCGACTAGTAC	R15ACE	TCGGATGTGACATGGACCTA
Full length			
F55ACE	ATTGCGGACGAGTAAACCTC	R55ACE	GTAGTATTGATGTTATTTTGAACCA

action. The 3' end of the cDNA was amplified with gene-specific primers (S38ACE and Q0 having the anchor sequence of QT' and nested with S30ACE and Q1') under conditions of 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 53°C for 30 s, 72°C for 2 min, and 72°C for 5 min. To isolate the 5' end, a CapfishingTM Full-length cDNA Premix Kit (Seegene) was used according to the manufacturer's protocol. The first cDNA fragment was amplified with 5'primer and AS41ACE primer at conditions of 95°C for 2 min, followed by 30 cycle at 95°C for 30 s, 58°C for 30 s, 72°C for 2 min, and 72°C for 5 min.

For isolating AP-AChE cDNA from mosquito whole bodies, the thermal cycler program was 95°C for 2 min as a preheat step, followed by 35 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and 72°C for 5 min as a final extension. Degenerate primer and gene-specific primers (F7ACE and R2ACE) were used to amplify the first cDNA fragment. Gene-specific primer R2ACE was designed based on the fragment reported by Weill et al. (2002, GenBank No. AJ438598). The 3' end of

the cDNA was amplified with AP-AChE-specific primers (F11ACE and Q0 for first PCR, then F10ACE and Q1' for second PCR and nested with F20ACE and Q1' for third PCR). For isolating the 5' end, 5' RACE system, version 2.0 (Invitrogen) was used according to the manufacturer's protocol. The first cDNA fragment was amplified with R11ACE, and then this product was amplified with an abridged universal amplification primer (AUAP) and R15ACE.

Each of 0.5 µg total RNA derived from five individual adult mosquitoes was reverse-transcribed and amplified by PCR for gene-specific primers (AO-AChE: S55ACE and AS55ACE; AP-AChE: F55ACE and R55ACE) and their sequences were determined.

Isolation of AChEs cDNA from cultured cells.

For isolating full-length AP-AChE cDNA from cultured cells, PCR was performed with the gene-specific primers F55ACE and R55ACE at 95°C for 2 min as a preheat step, followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 2.5 min, and 72°C for 5 min as a final extension. To isolate AO-

AChE cDNA from cultured cells, degenerate primers (S18ACE and AS4ACE) were used in a first PCR reaction and nested with S18ACE and AS30ACE at 94°C for 2 min as a preheat step, followed by 40 cycles at 94°C for 30 s, 50°C for 1 min, 72°C for 1 min, and 72°C for 5 min as a final extension. Although we successfully isolated full-length AO-AChE cDNA with S55ACE and AS55ACE from mosquito whole bodies, we were unable to isolate full-length AO-AChE cDNA with their primers and other primers (data not shown) from cultured cells.

DNA sequencing. Amplified sequences were determined by direct sequencing using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo) and a Genetic Analyser 3100 System (Applied Biosystems).

Quantitative real time PCR. Total RNAs of cultured cells and mosquito whole bodies extracted with Isogen (Nippon Gene) were purified with a RNase-Free DNase Set and RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. First-strand cDNAs were synthesized from respective 4–5 µg of RNAs in a 20 µl volume using ReverTra Ace (Toyobo) as described in the manufacturer's protocol. To acquire the accurate production of standard curves, 98 and 132 bp of PCR products from respective AO-AChE and AP-AChE were serially diluted to 0.08 amol/µl, 0.40 amol/µl, 2.00 amol/µl and 10.00 amol/µl, and 85 bp cDNA from ribosomal RPS3 was diluted to 2.00 amol/µl, 10.00 amol/µl, 50.00 amol/µl and 250.00 amol/µl. The PCR product of ribosomal RPS3 was used to determinate the relative transcription level of AChE. The PCR products and synthesized first-strand cDNAs with the primers specified in Table 1 were used as template for quantitative real-time PCR (qRT-PCR). The reactions were performed in 12.5 µl of iQ SYBR Green Supermix (Bio-Rad), 1 µl of template, 0.5 µl of each 10 µM primer (Table 1 bottom) and made up to 25 µl with distilled water. PCR was conducted with 1 cycle of 95°C for 3 min, 50 cycles of 95°C for 15 s, 54°C for 30 s, and 72°C for 30 s; 1 cycle of 95°C for 1 min; 1 cycle of 50°C for 1 min; and finally 90 cycles of 50–95°C/10 s, increasing the setpoint temperature by 0.5°C to generate melting curves. The qRT-PCR reaction was repeated three times for each sample. The results were analyzed and their initial copy numbers determined by optical system software

Table 2. K_m value and specific activity of cultured cells and whole body extracts of mosquito larvae

Source	K_m ($\times 10^{-5}$ M)	AChE activity (nmol/h/mg protein)
Cultured cell (NIAS-AeA1-2)	2.27	1,159.9
First instar larvae	1.57	8,161.3

iCycler iQ version 3.0 (Bio-Rad).

RESULTS

Enzyme assay

The specific activity and K_m values of AChE in cultured cells and mosquito whole bodies were determined and are shown in Table 2. AChE activity of the cultured cell was 1/7 of the mosquito body. K_m values of cultured cells and mosquito bodies were similar in value and the ratio of first-instar larvae to cultured cells was 0.69. Both values were close to that of *Culex tritaeniorhynchus* AO-CxT (0.0338 mM) and AP-CxTS (0.0363 mM) (Oh et al., 2006). The ratio of first larva to AO-CxT and AP-CxTS was 0.46 and 0.43, respectively.

AO-AChE and AP-AChE cDNA sequences of mosquito body

Nucleotide sequences of cDNAs for AO-AaAChE (2438 bp) and AP-AaAChE (2182 bp) with open reading frames (ORF) of 635 and 702 amino acid residues, respectively were determined from five Ikaken strain specimens. The cleavage of the signal peptide in AO-AaAChE and AP-AaAChE was predicted to be between Gly₂₆/Ile₂₇ and Cys₃₆/Arg₃₇, respectively, from the first methionine. These protein precursor sequences are shown in Fig. 1, with sequences from two other mosquito species *An. gambiae* and *C. tritaeniorhynchus*, *D. melanogaster* and *Torpedo californica*. Common features of AChE are conserved in both AO-AaAChE and AP-AaAChE: the catalytic triad (AO-AaAChE: Ser₂₅₆, Glu₃₈₅, His₄₉₉, AP-AaAChE: Ser₃₂₇, Glu₄₅₃, His₅₆₇), three pairs of Cys residues (AO-AaAChE: Cys_{92–119}, Cys_{310–325}, Cys_{461–583}, AP-AaAChE: Cys_{195–222}, Cys_{381–394}, Cys_{529–650}) putatively forming intra-subunit disulfide bonds and 9 and 11 aromatic residues lining the active site gorge of *T. californica* in AO-

drome	:	-----	:	-
aedal-o	:	-----	:	-
anoga-o	:	-----	:	-
cult-r-o	:	-----	:	-
aedal-p	:	-----MEIRGLIT-RL-LGPCHIRHLILCSLGIYSILVQS--VHCRHHDIGSSA--HQLG-----SKYAQSSSSLS--	:	61
anoga-p	:	-----MEIRGLLMRLRLGRMVLGLLGTALLLILPFSALVQGRHHILNNGAAGSHQLSAAAGVGLSSQSAQSSGLASGV	:	78
cult-r-p	:	MRPLQAMIRGLIT-RL-LGPCHIRHLILCSLGIYSILVQS--VHCRHHDIGSSAA--HQLG-----SKYSSSSLS--	:	67
torca	:	-----	:	-
drome	:	-----MAISCRQSRVLFMS-LPLPLTIPLEPLVIVLS	:	30
aedal-o	:	-----MSTVRLWCNMISLLLCIT	:	19
anoga-o	:	-----MASAYYHQSVAVG--GNVLVLLGAT	:	24
cult-r-o	:	-----MSSISMV--VGILCIA	:	15
aedal-p	:	-----SSQSSSSL--VEEP--ALNKDSDAFFTPYIGHGDSVRIVDAELGTLEREHVHS--TTTRRRG--	:	121
anoga-p	:	MSSVPAAGASSSSSSSSSSAEDDVARITLSKDAFFTPYIGHGDSVRIVDAELGTLE--HVHSGATPRRRG--	:	154
cult-r-p	:	-----SSQSSSSL--AEEA--TINKDS--GHGDSVRIVDAELGTLEREHHS--TTTRRRG--	:	119
torca	:	-----	:	-
drome	:	LHLGVGVGVIQLVYVSSSPVVRGRTV-CSEVHVYQZIPYANPPVDELRFRRKVPABPNHGVLDATGLSATCVEREYEF	:	113
aedal-o	:	V-IIPAYGIFRLVYVSSSPVVRGRTV-LCREVHVNSVFFAKPPVDCGLRFRRKVPABPNHGVLDATRLPFCICEREYEF	:	101
anoga-o	:	V-ICPAYIIRLVYVSSSPVVRGRTV-CQREVHVNSVFFAKPPVDCGLRFRRKVPABPNHGVLDATRLPFCICEREYEF	:	106
cult-r-o	:	V-IIPVYGIPLRVYVSSSPVVRGRTV-CQREVHVNSVFFAKPPVDCGLRFRRKVPABPNHGVLDATRLPFCICEREYEF	:	97
aedal-p	:	ESSDANDN-DGLITIDKSKIRGILEAPSSKVDANNGIPYACPPGLRFRHRRVPKNGVGNATKPNSSCVIIVDTVFG	:	204
anoga-p	:	ESNDANDN-DGLVNDKSKIRGILEAPSSKVDANNGIPYACPPGLRFRHRRVPKNGVGNATKPNSSCVIIVDTVFG	:	237
cult-r-p	:	ESNDSDTS-DGLVITIDKSKIRGILEAPSSKVDANNGIPYACPPGLRFRHRRVPKNGVGNATKPNSSCVIIVDTVFG	:	202
torca	:	-----DDH-SBLVNDKSKIRGILEAPSSKVDANNGIPYACPPGLRFRHRRVPKNGVGNATKPNSSCVIIVDTVFG	:	76
drome	:	-----S-----S**S	:	197
aedal-o	:	-----S-----S**S	:	180
anoga-o	:	-----S-----S**S	:	185
cult-r-o	:	-----S-----S**S	:	176
aedal-p	:	-----S-----S**S	:	255
anoga-p	:	-----S-----S**S	:	288
cult-r-p	:	-----S-----S**S	:	253
torca	:	-----S-----S**S	:	127
drome	:	-----S-----S**S	:	281
aedal-o	:	-----S-----S**S	:	261
anoga-o	:	-----S-----S**S	:	268
cult-r-o	:	-----S-----S**S	:	259
aedal-p	:	-----S-----S**S	:	332
anoga-p	:	-----S-----S**S	:	365
cult-r-p	:	-----S-----S**S	:	330
torca	:	-----S-----S**S	:	205
drome	:	-----S-----S**S	:	363
aedal-o	:	-----S-----S**S	:	343
anoga-o	:	-----S-----S**S	:	350
cult-r-o	:	-----S-----S**S	:	341
aedal-p	:	-----S-----S**S	:	411
anoga-p	:	-----S-----S**S	:	444
cult-r-p	:	-----S-----S**S	:	409
torca	:	-----S-----S**S	:	285
drome	:	-----S-----S**S	:	447
aedal-o	:	-----S-----S**S	:	427
anoga-o	:	-----S-----S**S	:	434
cult-r-o	:	-----S-----S**S	:	425
aedal-p	:	-----S-----S**S	:	495
anoga-p	:	-----S-----S**S	:	528
cult-r-p	:	-----S-----S**S	:	493
torca	:	-----S-----S**S	:	368
drome	:	-----S-----S**S	:	530
aedal-o	:	-----S-----S**S	:	511
anoga-o	:	-----S-----S**S	:	518
cult-r-o	:	-----S-----S**S	:	509
aedal-p	:	-----S-----S**S	:	579
anoga-p	:	-----S-----S**S	:	612
cult-r-p	:	-----S-----S**S	:	577
torca	:	-----S-----S**S	:	452
drome	:	-----S-----S**S	:	605
aedal-o	:	-----S-----S**S	:	590
anoga-o	:	-----S-----S**S	:	597
cult-r-o	:	-----S-----S**S	:	588
aedal-p	:	-----S-----S**S	:	657
anoga-p	:	-----S-----S**S	:	690
cult-r-p	:	-----S-----S**S	:	655
torca	:	-----S-----S**S	:	528
drome	:	-----S-----S**S	:	649
aedal-o	:	-----S-----S**S	:	635
anoga-o	:	-----S-----S**S	:	645
cult-r-o	:	-----S-----S**S	:	633
aedal-p	:	-----S-----S**S	:	702
anoga-p	:	-----S-----S**S	:	737
cult-r-p	:	-----S-----S**S	:	701
torca	:	-----S-----S**S	:	575

Fig. 1. Alignment of AChE1(-o) and AChE2(-p) proteins of *Ae. albopictus* (aedal), *An. gambiae* (anoga), *C. tritaeniorhynchus* (cult-r), *D. melanogaster* (drome), and *T. californica* (torca). Residues involved in the catalytic triad of *T. californica* AChE are shown with plus signs. Aromatic residues of the active site gorge in *T. californica* AChE that are conserved and not involved in insect AChEs are shown with dollar and sharp symbols, respectively. Slashes show intra-subunit-forming disulfide bonds in *T. californica* AChE.

Table 3. Codons and deduced amino acids of the variable positions in the AO-AChE gene of *Ae. albopictus*

Source	Polymorphic nucleotide positions (codons)											
	454	525	528	567	576	579	651	691	774	891	1071	1137
Mosquito body	TTC	AGY	GGT	GCA	GGA	AAT	GCA	TTG	GGT	GCT	CTG	GTT
Cultured cell	ATC	AGC	GGC	GCG	GGG	AAC	GCG	CTG	GGC	GCC	CTA	GTA
Codon	Phe→Ile	Ser	Gly	Ala	Gly	Asn	Ala	Leu	Gly	Ala	Leu	Val
(position)	(152)	(175)	(176)	(189)	(192)	(193)	(217)	(231)	(258)	(297)	(357)	(379)

Table 4. Codons and deduced amino acids of the variable positions in the AP-AChE gene of *Ae. albopictus*

Source	Polymorphic nucleotide positions (codons)																
	160	405	483	588	702	780	789	801	891	960	975	1053	1068	1530	1767	1854	2093
Mosquito body	GCA	ATC	ATC	GTT	CCC	CAT	CTC	GAG	TTG	CGG	GGT	CTC	CCC	AAY	GAR	TTY	AAY
Cultured cell	TCA	ATM	ATY	GTC	CCA	CAY	CTY	GAR	TTR	CGR	GGC	CTS	CCY	AAT	GAG	TTT	AAC
Codon	Ala→Ser	Ile	Ile	Val	Pro	His	Leu	Glu	Leu	Arg	Gly	Leu	Pro	Asn	Glu	Phe	Asn
(position)	(54)	(135)	(161)	(196)	(234)	(260)	(263)	(267)	(297)	(320)	(325)	(351)	(356)	(510)	(589)	(618)	(731)

AaAChE and AP-AaAChE residues. AO-AaAChE and AP-AaAChE precursors show high identities to the respective types of AChE, 91% and 84% with *An. gambiae* and 93% and 93% with *C. tritaeniorhynchus*, respectively. On the other hand, the identities of AO-AChE to AP-AChE with *Ae. albopictus*, *An. gambiae* and *C. tritaeniorhynchus* are 42%, 39% and 39%, respectively. cDNA sequence polymorphisms were observed at five and four positions of AO-AChE and AP-AChE, respectively.

Comparison of AChE cDNA sequences between mosquito body and cultured cells

Both AO-AChE and AP-AChE cDNAs were isolated from cultured cells. For AO-AChE, a partial region 818 bp isolated in cultured cells was compared with the mosquito body from Asp₁₁₈ to Phe₃₈₉: 12 base pair substitutions and only one amino acid substitution Ile₁₅₂ to Phe₁₅₂ were found (Table 3). The first methionine ATG and stop codon compared in AP-AChE, cDNA sequence polymorphisms were observed at 17 positions (Table 4). Only one amino acid substitution was presented at Ser₅₄ to Ala₅₄.

Expression level of AO-AChE and AP-AChE genes in mosquito body and cultured cells

Expression levels of AP-AChE and AO-AChE

Fig. 2. The expression level per RPS3 of AO-AChE and AP-AChE genes in cultured cells and mosquito larvae.

genes in cultured cells and mosquito whole bodies were determined and compared by qrtPCR. The results are shown in Fig. 2. The expression level of AO-AChE gene was low compared to AP-AChE gene in both the mosquito bodies and cultured cells; 1/7 in the first-instar larvae and 1/2 in the cultured cells. The ratios for mosquito bodies to cultured cells were two and seven in AO-AChE and AP-AChE, respectively. The expression level of the reference gene RPS3 in the cultured cells and mosquito bodies were standardized.

DISCUSSION

Two types of acetylcholinesterases (AO-AChE and AP-AChE) were isolated from the tiger mosquito, *Ae. albopictus*, and complete amino acid sequences of both structural proteins were determined. The structural proteins of the two types had all amino acids for the enzyme function and showed very high homology to the respective proteins in other mosquitoes. We also showed by PCR cloning that cultured cells originating from neonatal larvae of *Ae. albopictus* expressed two types of AChE mRNAs. A complete amino acid sequence of AP-AChE and partial sequence of AO-AChE were determined. When the sequences of the cultured cells were compared with those of the respective AChE type in the mosquito, only one non-synonymous substitution was found among many nucleotide polymorphisms in both AChE cDNAs. The Ala₅₄ to Ser₅₄ substitution in AP-AChE is located at a cleaved region during formation of the mature protein (Haas et al., 1988) and the Ile₁₅₂ to Phe₁₅₂ substitution in AO-AChE is located at the N-terminus. Both are thought not to affect the properties of enzyme activity. These results seem to support the conclusion that both AChEs keep their functions in the cultured cells. As for AO-AChE, however, the cDNA product of the cultured cells could not be amplified from the first-strand cDNA after even more than 80 PCR cycles with gene-specific primers for isolating the full-length cDNA (data not shown). 5' RACE or 3' RACE were also tried with gene-specific primers of the mosquito AChE, but a specific band was not obtained. It has been reported that there are long UTR promoting erroneous fusions occur during PCR in marginal regions of the AChE gene in *P. xylostella* (Ni et al., 2003; Baek et al., 2005).

In cultured cells, only 1/7 AChE activity was detected compared to the first-instar larvae of the mosquito. The activity measured appears to be a mixture of both types of AChEs which is apparently expressed in the mosquito body and cultured cells. The activities of two AChE could not be differentiated by enzyme assay.

To elucidate the exact expression of both types of AChE genes in the mosquito body and cultured cells, qrtPCR was adopted. QrtPCR showed that the expression level of AO-AChE was lower than AP-AChE gene in the mosquito body and cultured

cells. The total AChE gene expression in cultured cells was 1/5 that in the larvae. The gene expression level and enzyme activity of AChE in cultured cells was comparable, taking the fluctuation of the protein quantity in insect tissues and cultured cells into account.

In addition to NIAS-AeAl-2, several insect cell lines, such as NIH-SaPe-4 originating from embryonic tissues of *Sarcophaga peregrina*, NIAS-MB-32 from the ovary of *Mamestra brassicae* (Ohbayashi et al., 1997) and S3 from *D. melanogaster* (Berger and Wyss, 1980) have been reported to have high AChE activity. However, the existence of AChE activity in insect cell lines does not depend on their origins: for example; Kc, SL-3, SES-BoMo-15A, TUAT-BME-11 and 15 cell lines derived from embryonic tissues; NIAS-MB-25 from *M. brassicae* ovary; and SL-3, the mother cell line of S3, showed no AChE activity (Ohbayashi et al., 1997). These examples seem to indicate that the expression of AChE is not essential for cultured cells. On the other hand, AChE is known to promote cell proliferation in vertebrate and nematode cell lines (Huby et al., 1999; Ruben et al., 2004) and to play a role in cell adhesion (Johnson and Moore, 1999). In the NIH-SaPe-4 cell line, inhibition of AChE activity by eserine did not affect cell growth (Ohbayashi et al., 1997), eserine which inhibited 80–90% of AChE activity in NIAS-AeAl-2 cultured cells brought no effect and the recovery of the activity occurred in 5 d (Mizuno, unpublished data). If AChE has functions besides ACh hydrolysis (non-cholinergic function) in these cultured insect cells, AChE protein without catalytic activity must have a role. The 'peripheral' anionic site has been shown to be associated with the non-cholinergic functions of AChE (Johnson and Moore, 1999; Silman and Sussman, 2005). To elucidate the functions of AChE, gene-silencing experiments with RNA interference, for example, seem to be the best procedure for cultured cells.

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