Insect Biochemistry and Molecular Biology 39 (2009) 913–919

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

RNAi of *ace1* and *ace2* in *Blattella germanica* reveals their differential contribution to acetylcholinesterase activity and sensitivity to insecticides

L. Revuelta [a], M.D. Piulachs [b], X. Bellés [b], P. Castañera [a], F. Ortego [a], J.R. Díaz-Ruíz [a], P. Hernández-Crespo [a], F. Tenllado [a,*]

[a] Centro de Investigaciones Biológicas, CSIC, Departamento de Biología Medioambiental, Ramiro de Maeztu 9, 28006 Madrid, Spain
[b] Institut de Biología Evolutiva, CSIC-UPF, Passeig Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain

ARTICLE INFO

Article history:
Received 26 August 2009
Received in revised form
23 October 2009
Accepted 3 November 2009

Keywords:
AChE
RNA interference
ace genes
Cockroach
Insecticides

ABSTRACT

Cyclorrhapha insect genomes contain a single acetylcholinesterase (AChE) gene while other insects contain at least two *ace* genes (*ace1* and *ace2*). In this study we tested the hypothesis that the two *ace* paralogous from *Blattella germanica* have different contributions to AChE activity, using RNA interference (RNAi) to knockdown each one individually. Paralogous-specific depletion of *Bgace* transcripts was evident in ganglia of injected cockroaches, although the effects at the protein level were less pronounced. Using spectrophotometric and zymogram measurements, we obtained evidence that BgAChE1 represents 65–75% of the total AChE activity in nerve tissue demonstrating that *ace1* encodes a predominant AChE. A significant increase in sensitivity of *Bgace1*-interfered cockroaches was observed after 48 h of exposure to chlorpyrifos. In contrast, *Bgace2* knockdown had a negligible effect on mortality to this organophosphate. These results point out a key role, qualitative and/or quantitative, of AChE1 as target of organophosphate insecticides in this species. Silencing the expression of *Bgace1* but not *Bgace2* also produced an increased mortality in insects when synergized with lambda-cyhalothrin, a situation which resembles the synergistic effects observed between organophosphates and pyrethroids. Gene silencing of *ace* genes by RNAi offers an exciting approach for examining a possible functional differentiation in *ace* paralogous.

1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is an essential enzyme at cholinergic synapses in all animals, as well as at neuromuscular junctions in nematodes and vertebrates (Soreq and Seidman, 2001). AChE terminates neurotransmission by rapidly hydrolyzing the neurotransmitter acetylcholine (ACh) in the synaptic cleft. In insects, AChE has mainly been studied in relation to insecticide resistance because the enzyme is the target of organophosphate and carbamate insecticides and its insensitivity to insecticides is one of the main factors accounting for resistance (Matsumura, 1985; Weill et al., 2003).

Many insects have more than one copy of the AChE gene. A subfamily of *Drosophila melanogaster ace*-orthologous genes (*ace2*) is common to all insect species, whereas a subsequently identified additional subfamily of *ace*-paralogous genes (*ace1*) is present in insects other than the Cyclorrhapha flies (Huchard et al., 2006). The functional sense of these two AChEs is still unclear. *ace1*-type AChE appears to encode major AChE responsible for the physiological hydrolysis of ACh in non-Cyclorrhapha insects as judged by its much higher transcription level compared to *ace2* in *Plutella xylostella* (Baek et al., 2005), *Helicoverpa assulta* (Lee et al., 2006) and *Aedes albopictus* (Mizuno et al., 2006). Other evidences for the predominant role of *ace1* have been reported, such as the AChE activity curves in the presence of inhibitors (Bourguet et al., 1996; Huchard et al., 2006). In addition, several amino acid substitutions conferring insensitivity to organophosphate and carbamate insecticides have been reported for *ace1*-type AChE genes, further highlighting a greater significance of AChE1 in synaptic transmission in non-Cyclorrhapha insects (Nabeshima et al., 2003; Weill et al., 2003; Cui et al., 2006; Kono and Tomita, 2006; Alout and Weill, 2008). Besides its classical function in cholinergic synapses, non-synaptic functions for cholinesterases have been described in vertebrates (Paraoanu and Layer, 2008). Recently, it has been reported that silencing AChE genes in *Helicoverpa armigera* affected larval growth and development (Kumar et al., 2009). Whether these non-classical functions of AChE in insects are encoded by *ace2* or even rely on hydrolysis of ACh is a matter of controversy (Cousin et al., 2005).

* Corresponding author. Tel.: +34 1 8373112; fax: +34 1 5360432.
E-mail address: tenllado@cib.csic.es (F. Tenllado).

0965-1748/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2009.11.001

The German cockroach, *Blattella germanica* (L.) (Dictyoptera, Blattellidae), is an important household insect pest worldwide. It can act as a mechanical vector and reservoir for pathogens. Different organoclorine and organophosphate insecticides, as well as carbamate and pyrethroid compounds, have been used as control agents. However, due to extensive but undesigned application of insecticides, resistance to different organophosphates and pyrethroids has been developed by this species (Spencer et al., 1998; Wei et al., 2001). Therefore, there is a need for finding alternative pest management strategies, which should also conform to health regulations.

Two AChE genes, *Bgace1* and *Bgace2*, have been cloned from *B. germanica* (Kim et al., 2006). Based on the transcription pattern and fluorescence *in situ* hybridization, *Bgace1* appears to be the primary AChE responsible for the physiological hydrolysis of ACh, whereas *Bgace2* may have supplementary functions. While it was obvious that the two types of AChE were expressed at the mRNA level in ganglia and other tissues, AChE activity was measured as a mixture of the two types (Kim et al., 2006; Mizuno et al., 2007). Thus, the respective contribution of both *ace1* and *ace2* to catalytic activity of AChE in *B. germanica* and insects in general remains to be elucidated.

Post-transcriptional gene silencing by RNA interference (RNAi) has become a powerful tool in functional genomics. RNAi is mediated by double-stranded RNA (dsRNA) that is cleaved into 21–23 nucleotide small interfering RNAs (siRNAs) by an RNase III-type enzyme known as Dicer (Fire et al., 1998). Introduction of dsRNA or siRNAs into a cell triggers the destruction of the cognate mRNA, which often leads to loss-of-function phenotypes. Thus, RNAi provides an alternative to traditional genetics when transgenic animal models are difficult to generate (Bellés, 2010). Here we used RNAi to knockdown expression of each paralogous, *ace1* and *ace2*, separately, using dsRNAs synthesized from divergent regions of *Bgace1* and *Bgace2*. Thereafter, we attempted to assay the AChE activity and insecticidal sensitivity in these gene-specific knockdown cockroaches. We provide direct evidence at the protein level for differential contributions of *ace1* and *ace2* to AChE activity, and their relationship with organophosphate sensitivity.

2. Material and methods

2.1. Insects

Adult females of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% relative humidity. Thoracic ganglia were dissected under Ringer solution, frozen with liquid N_2, and preserved at −80 °C until use. All dissections, injections and insecticide application were carried out on carbon dioxide-anaesthetized specimens.

2.2. Synthesis of dsRNAs and injection

To produce the dsRNAs for *Bgace1* (dsBgace1) and *Bgace2* (dsBgace2), PCR fragments for each transcript were cloned into pGEM-T Easy vector (Promega). For dsBgace1, a 390-bp DNA fragment spanning positions 437–826 of *Bgace1* (GenBank accession number: DQ288249) was used. For dsBgace2, a 481-bp DNA fragment spanning positions 224–704 of *Bgace2* (GenBank accession number: DQ288847) was used. As a control, a non-coding sequence of 92 bp from the pSTBlue-1 vector was used (dsMock).

Sense and antisense RNAs were synthesized in vitro from the corresponding DNA plasmids by using either SP6 or T7 RNA polymerases. To generate the dsRNAs, equimolar amounts of sense and antisense RNAs were mixed, heated at 95 °C for 10 min, cooled slowly to room temperature and stored at −20 °C until use. Formation of dsRNA was confirmed by testing a shift in gel mobility of the annealed material compared to each single-stranded RNA. The obtained dsRNAs were resuspended in diethyl pyrocarbonate-treated water. Freshly ecdysed adult females were injected into the abdominal cavity with a 5 μg dose of dsRNA in a volume of 1 μl.

2.3. RT-PCR/Southern blot analyses

To check the transcript titers of the AChE genes, RT-PCR followed by Southern blotting with specific probes was performed as described in Aguilar et al. (2003). Total RNA was isolated from the thoracic ganglia of individual insects 3 or 7 days after injection with dsRNA, using the GenElute Mammalian Total RNA kit (Sigma). One microgram of each RNA extraction was DNase treated (Promega) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) and random hexamers (Promega). To estimate mRNA levels, cDNA samples were subjected to PCR with a number of cycles within the linear range of amplification for each transcript; with 24 cycles for Bgace1 and with 25 cycles for Bgace2 at 94 °C (30 s), 54 °C (30 s) and 72 °C (45 s). Primers used for amplifying Bgace1 transcript were: forward (Bgace1F), 5′-CACTGATCCCTTGAGAGATATTC and reverse (Bgace1R), 5′-CCAGGAAAATCACCGAATACG (positions 302–826). Primers used for amplifying Bgace2 transcript were: forward (Bgace2F), 5′-ACCGTGTGAGTAGCAGGTCG and reverse (Bgace2R), 5′-ACGTACTCTTTGCGGCACCCA (positions 167–704). Bgace1F and Bgace2F anneal outside the region targeted for silencing to ensure that the endogenous transcripts are tested. As a reference, the same cDNAs were subjected to RT-PCR/Southern blotting with a primer pair specific for *B. germanica actin5C*, as described by Maestro et al. (2005). cDNA probes for Southern blot analyses were generated by PCR with the same primer pairs using plasmid DNAs containing the corresponding cDNA clones as templates. The probes were labeled with fluorescein using the Gene Images random prime-labeling module (Amersham Biosciences). Densitometry analyses of blots were performed in a calibrated densitometer GS-800 (Bio-Rad), using the Quantity One (Bio-Rad) software.

2.4. AChE assays

For protein extraction, pools of three thoracic ganglia from *B. germanica* 7 days after treatment with dsRNA were homogenized in 100 μl of 0.1 M sodium phosphate buffer (pH 7) containing 0.5% (v/v) Triton X-100. The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was decanted and used as enzyme preparation. Protein concentration of each enzyme preparation was determined by the Bradford method using bovine serum albumin as a standard protein (Bradford, 1976).

AChE activity was determined by the spectrophotometric method of Ellman et al. (1961), using acetylthiocholine iodide as substrate (ATChI, Sigma). Three biological replicates were carried out in triplicate and blanks were used to account for spontaneous breakdown of the substrate. The reaction mixture consisted of 1 mM ATChI, 1 mM DTNB (5′5 dithio-bis (2-nitrobenzoic acid), Sigma), and 10 μl AChE preparation in 200 μl of 0.1 M potassium phosphate buffer (pH 7.2). The increase in absorbance at 412 nm was recorded every 20 s over a 10 min interval with a SpectraMax plate reader (Molecular Dynamics) at 30 °C. Means and standard deviations were determined for all RNAi treatments. Analyses of variance (ANOVA) were performed to determine whether there were statistically significant differences among treatments, using the Student–Newman–Keuls multiple comparison test (SNK test). All data were analyzed with a significance level of 5%.

For AChE inhibition experiments, the enzyme preparation was preincubated with chlorpyrifos-oxon (Dr. Ehrenstorfer GmbH) at a concentration of 5×10^{-10} M for 5 min before the substrate was added to start the reaction. Insecticide concentration was set at the approximate molarity which gives ca. 20% inhibition of AChE activity, as determined in preliminary assays with untreated cockroaches. Residual AChE activity was determined against the control that was performed in parallel but lacked chlorpyrifos-oxon.

2.5. Native electrophoresis and activity staining

Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was performed in a vertical electrophoresis unit (Protean III mini cell, Bio-Rad) using 12% polyacrylamide gels, with a discontinuous Tris–glycine buffer system. Gels and running buffer contained 0.5% Triton X-100. The same volume of each AChE preparation from *B. germanica* (5 μg) and *Ceratitis capitata* (2.5 μg), a reference insect species expected to have one *ace* locus, was loaded into each well, and run at 200 V for 180 min in cold chamber. Following electrophoresis, the gel was activity-stained to visualize AChE bands according to the method of Karnovsky and Roots (1964) using ATChI or propionythiocholine iodide (PrTChI) as substrate. Densitometry analyses of zymograms were performed as described above. The staining intensity of each band is a function of the enzyme activity, and the enzyme activity was measured by the area delimited by the corresponding band. The activities of the individual bands in dsBgace-injected cockroaches were given as percentages of the levels measured in specimens injected with dsMock.

AChE preparations were digested with purified phosphatidylinositol-specific phospholipase C (PIPLC; EC 3.1.4.10) from *Bacillus cereus* (Sigma–Aldrich) to cleave glycolipid anchors. Digestions (5 μg of sample) were performed for 1 h at 37 °C with 2 μl of PIPLC (0.065 U/μl).

2.6. Insect bioassays

The injected cockroaches were kept in Petri dishes (150 × 15 mm) to observe cockroach survival for up to 30 days after treatment with either dsBgace1 ($n = 52$), dsBgace2 ($n = 56$), or dsMock ($n = 88$). To measure fecundity, we counted the number of (live) nymphs hatched from the oothecae produced by the female cockroaches during the 30 days after the treatment with dsRNA.

The insecticides used in bioassays were chlorpyrifos (Sigma–Aldrich) and lambda-cyhalothrin (Syngenta) dissolved in acetone at 270 and 20 ng/μl, respectively. Doses were chosen based on preliminary tests to give a low rate of mortality in non-injected cockroaches. Control mortality never exceeded 15 and 35% for chlorpyrifos and lambda-cyhalothrin, respectively. Topical application was performed by delivering a 1 μl-drop of insecticide to the abdomen of cockroaches seven days after injection with dsRNA, using a 10 μl-Hamilton syringe (Fischer Scientific). Control cockroaches were treated with acetone alone. Each test consisted of 9–11 cockroaches per treatment, and three to four replicates were performed. Specimens were held at a density of 9–11 cockroaches into a Petri dish. Mortality was registered at 12 h interval after insecticide application. Cockroaches that did not move at all when prodded with forceps were scored as dead. Bioassay data were adjusted to Normal distribution using an arcsin square-root transformation and differences between means were evaluated using an ANOVA followed by the post-hoc Dunnet test, using a 5% significance level.

3. Results

3.1. BgAChE knockdown and specificity

To obtain a direct measurement of differential contributions of *ace* genes to AChE activity in *B. germanica*, we used dsRNA-mediated specific depletion of either *Bgace1* or *Bgace2* transcripts. The RNAi approach has been demonstrated to be very efficient for other genes in *B. germanica* (Maestro et al., 2005). Gene regions were selected for dsRNA production based on the greatest sequence divergence between BgAChE genes. The nucleotide sequence similarities between *Bgace1* and *Bgace2* in the regions targeted by dsBgace1 and dsBgace2 were 41.3% and 48.8%, respectively, and did not include identical stretches of 18 nts or more. Therefore, the dsRNAs were expected to specifically knockdown their respective transcripts.

dsRNAs were injected into the abdomen of freshly ecdysed adult females and mRNA levels were examined in the thoracic ganglia 3 and 7 days after the treatment. Steady state levels of *Bgace* mRNAs were estimated by densitometric analysis of RT-PCR products, with the level measured in specimens treated with dsMock arbitrarily set to 1.0. Administration of dsRNA for *Bgace1* caused about 90% reduction of the corresponding transcript on day 3 without affecting that of *Bgace2*. On day 7, *Bgace1* mRNA was almost undetectable (95% of reduction) (Fig. 1). dsRNA for *Bgace2* was similarly selective in its action, and the level of *Bgace2* transcript was decreased up to 97% compared to the control 7 days after injection of dsBgace2. In no case did dsBgace injections result in significant changes in levels of *actin5C* transcripts that were used for monitoring nonspecific effects of dsRNA and as a control for equal sample loading. Thus, depletion of both *Bgace1* and *Bgace2* mRNAs in ganglia suggests that the dsRNA might have diffused through the hemolymph and have been transported to other tissues.

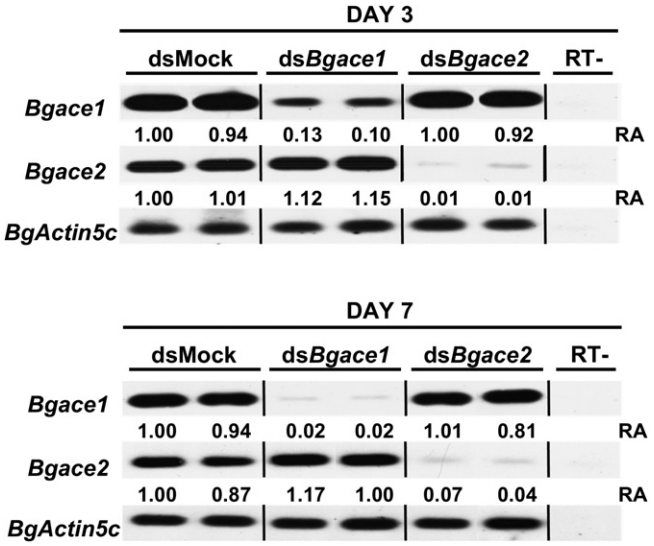

Fig. 1. dsRNA-mediated knockdown of *Bgace1* and *Bgace2* mRNAs in *B. germanica*. dsRNA complementary to pSTBlue-1 (dsMock), *ace1* (dsBgace1), and *ace2* (dsBgace2) was injected into the abdomen of adult females. Total RNA isolated from duplicate samples of thoracic ganglia was analyzed at day 3 and 7, using RT-PCR followed by Southern blot with probes specific to *Bgace1* or *Bgace2*. *Actin5C* levels were used as a reference. The mRNA levels were estimated by Quantity One scanning and normalized adjusting dsMock levels arbitrarily to 1.0. RT-: Negative controls without the reverse transcriptase step. Representative gel of three independent experiments is shown.

3.2. Effects of BgAChE1 or BgAChE2 knockdown on AChE activity

We evaluated the effects of RNAi on AChE activity in thoracic ganglia 7 days after the injection. The enzymatic activity of AChE was reduced 47.5% in dsBgace1-injected specimens (1336.61 ± 29.64 and 701.57 ± 68.54 nmol min^{-1} mg^{-1} protein for dsMock and dsBgace1, respectively), whereas injecting dsRNA targeting *Bgace2* caused about 16% reduction in AChE activity (1120.65 ± 61.06 nmol min^{-1} mg^{-1} protein) (Fig. 2). Both reductions were statistically significant (SNK test, $p < 0.05$). We checked that all ATChI-splitting activity in enzyme preparations derived from dsMock-injected specimens was abolished by 10^{-5} M eserine, a usual condition required for defining AChE activity (data not shown).

Enzyme preparations derived from pools of three dsRNA-injected specimens were separated by native PAGE and their AChE activities visualized by activity staining using ATChI or PrTChI as substrate. Four bands of AChE activity were identified in extracts derived from *B. germanica*, whereas two bands were seen in *C. capitata*, a reference insect species expected to have one *ace* locus (Fig. 3). This AChE banding pattern partially resembles that observed in ganglia of *Blattella* in previous work, where two different molecular forms, i.e., hydrophilic form and amphiphilic form, for both BgAChE1 and BgAChE2 were proposed to account for the presence of the two fast and the two slow migrating bands, respectively (Kim et al., 2006). However, annotation of bands as the putative BgAChE1 and BgAChE2 was merely based on apparent similarity to the banding pattern of two AChEs from *Culex pipiens* (Bourguet et al., 1996). In order to identify quantitatively the effect of *Bgace1* or *Bgace2* mRNA depletion on the four-banded pattern, densitometry analyses were applied to the corresponding electrophoretic bands from each dsRNA-injected group. In agreement with spectrophotometric measurements, an overall reduction in band intensities was observed in either dsBgace1- (ca. 40% reduction with ATChI and 30% with PrTChI) or dsBgace2-injected cockroaches (ca. 19% reduction with ATChI and 10% with PrTChI) compared with the levels measured in specimens injected with dsMock (Table 1). However, contrary to what has been hypothesized, there was no band-specific reduction pattern associated with depletion of either *Bgace1* or *Bgace2* mRNAs, even when extracts were treated with PIPLC which converts the amphiphilic forms of AChE into the corresponding hydrophilic forms.

3.3. Effects of BgAChE1 or BgAChE2 knockdown on insect performance

The dsBgace1- and dsBgace2-injected specimens were compared with dsMock-treated controls daily, but no difference in cockroach survival was observed among the RNAi groups and controls, despite the fact that AChE activities in ganglia were significantly reduced. Furthermore, depletion of either *Bgace1* or *Bgace2* mRNA did not affect fecundity ($p > 0.05$) as compared with

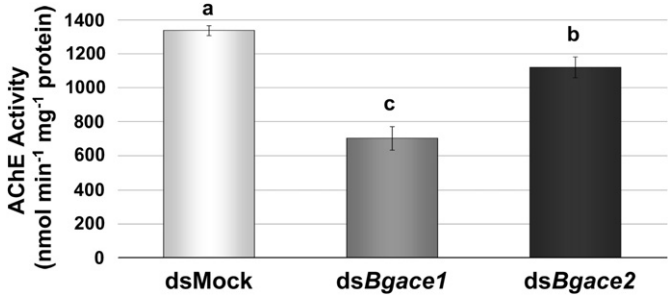

Fig. 2. Effects of BgAChE1 or BgAChE2 knockdown on AChE activity in *B. germanica*. AChE activities present in ganglia extracts derived from dsMock-, dsBgace1-, and dsBgace2-injected animals were determined at day 7 by Ellman reaction using ATChI as substrate ($\epsilon_{412} = 1.36 \times 10^4$ M^{-1} cm^{-1}). Specific activity was measured as nmol of acetylthiocholine iodide hydrolyzed/min/mg protein. Bars represent the means and standard deviations of three biological replicates of each treatment. Different letters above bars indicate statistical significance (SNK, $p < 0.05$).

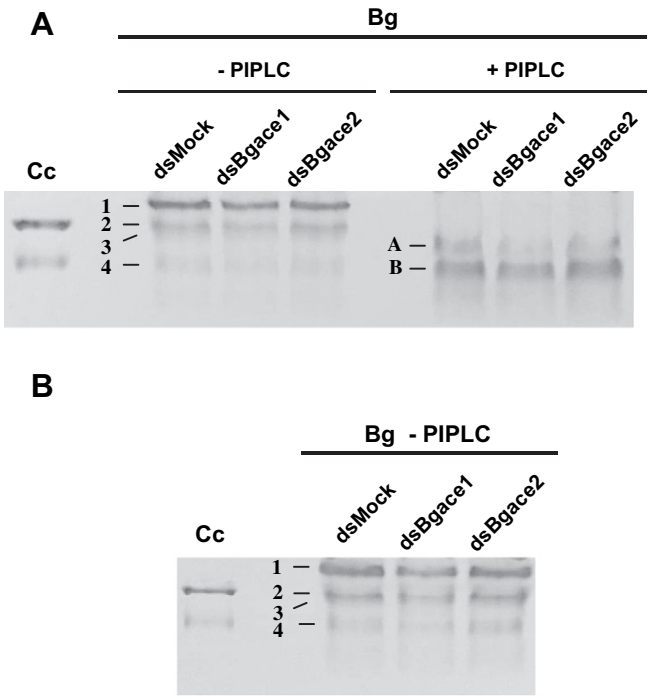

Fig. 3. Native polyacrylamide gel electrophoresis of protein extracts derived from ganglia of *B. germanica* injected with dsMock, dsBgace1, and dsBgace2. Gels were activity-stained to visualize AChE bands according to the method of Karnovsky and Roots using ATChI (A) or PrTChI (B) as substrate. + PIPLC corresponds to protein extracts treated with phosphatidylinositol-specific phospholipase C prior to electrophoresis. Bands 1–4 and A–B correspond to different AChE isoforms in the absence or presence of PIPLC, respectively. Protein extract derived from heads of *C. capitata* (Cc) was used as a control.

Table 1
Densitometry analysis of AChE bands in zymograms of *B. germanica*.[a]

ATChI		
	ds*Bgace1*	**ds*Bgace2***
Total pattern[b]	59.7 ± 4.0	81.5 ± 10.1
Band 1	59.72 ± 13.35	76.03 ± 20.25
Band 2	74.93 ± 8.33	91.40 ± 11.61
Band 3	67.15 ± 10.07	91.27 ± 15.52
Band 4	59.32 ± 13.27	94.91 ± 38.62
ATChI + PIPLC		
	ds*Bgace1*	**ds*Bgace2***
Total pattern	59.9 ± 14.2	87.6 ± 14.5
Band A	51.26 ± 12.23	83.47 ± 6.60
Band B	80.98 ± 13.03	103.21 ± 12.92
PrTChI		
	ds*Bgace1*	**ds*Bgace2***
Total pattern	69.49 ± 1.81	89.71 ± 25.97
Band 1	69.75 ± 18.87	95.51 ± 6.96
Band 2	87.36 ± 10.86	97.17 ± 37.72
Band 3	69.56 ± 6.89	84.89 ± 49.27
Band 4	71.10 ± 11.14	92.54 ± 39.44

[a] Densitometry analyses of gels were performed in a calibrated densitometer GS-800 (Bio-Rad), using the Quantity One (Bio-Rad) software.

[b] Global or individual band intensities (measured in arbitrary units) in dsBgace1- and dsBgace2-injected animals are given as percent abundance relative to intensities of the corresponding band measured in specimens injected with dsMock. Values represent the means and standard deviations of four (ATChI), three (ATChI + PIPLC) and two (PrTChI) experimental replicates.

controls (38.38 ± 9.73, 37.17 ± 11.05 and 38.96 ± 8.04 nymphs per oothecae for dsBgace1, dsBgace2 and dsMock, respectively).

3.4. Effects of BgAChE1 or BgAChE2 knockdown on sensitivity to insecticides

AChE is the primary target of organophosphate insecticides which act by blocking this enzyme through essentially irreversible inhibition, thus preventing repolarization of the nerve cell. To assess whether reduction in AChE activity by RNAi lead to increased sensitivity to organophosphates, dsBgace-injected cockroaches were treated with chlorpyrifos by topical application at 7 days after injection using 50 specimens from each RNAi treatment. The toxicity of chlorpyrifos to *Bgace1*-silenced cockroaches increased progressively from 12 h onwards, reaching at 48 h after treatment an increase of 3.3-fold in mortality compared to specimens injected with dsMock (Fig. 4A). The differences between treatments were statistically significant (Dunnet test, $p < 0.05$). There was also a moderate tendency to increase (1.9-fold) in mortality towards chlorpyrifos in *Bgace2*-silenced cockroaches, but differences were not statistically significant ($p > 0.05$).

Based on three-dimensional structure predictions, it has been hypothesized that structural differences between BgAChE1 and BgAChE2 exists near the catalytic triad which could affect their inhibition properties (Kim et al., 2006). To assess whether differential sensitivities of BgAChE1 and BgAChE2 to chlorpyrifos could have influenced the insecticide bioassay, AChE inhibition experiments were conducted on enzyme preparations extracted from thoracic ganglia of specimens injected with dsRNA 7 days after treatment. As shown in Fig. 4B, AChE activity from *Bgace1*-silenced cockroaches was of similar sensitivity to chlorpyrifos-oxon, the active form of chlorpyrifos, than activity from specimens injected with either ds*Bgace2* or dsMock (normalized values were not significantly different, SNK test).

We further determined the effect of AChE silencing on sensitivity to the pyrethroid lambda-cyhalothrin, whose primary mode of action involves the modification of voltage-sensitive sodium channels. The results of bioassay for lambda-cyhalothrin toxicity in three independent experiments involving 30 specimens from each RNAi treatment showed a significant increase (1.9-fold; $p < 0.05$) in mortality in dsBgace1-injected cockroaches compared to specimens injected with dsMock 48 h after treatment (Fig. 4C). Again, specimens injected with dsBgace2 did not show a significant increase in mortality after treatment with lambda-cyhalothrin.

4. Discussion

In this study we tested the hypothesis that *ace1* and *ace2* contribute differentially to AChE activity, using RNAi to knockdown each one individually by dsRNAs synthesized from divergent regions of *Bgace1* and *Bgace2*. Previous studies on AChE silencing in insects were based on siRNAs targeting a conserved region of AChE1 and AChE2, which may have silenced both AChE paralogous simultaneously (Kumar et al., 2009; Zhou and Xia, 2009). Our approach has proven to be an effective and reliable method to specifically knockdown the expression of *Bgace1* and *Bgace2* mRNAs in ganglia (95% reduction in both cases), a tissue which is not in close proximity with the site of entry of dsRNA. Whether the nature of RNA interference observed here could be regarded as a case of systemic RNAi, as reported in *Tribolium castaneum* (Tomoyasu et al., 2008), needs further investigation.

A significant reduction in total AChE activity was displayed by ganglia proteins prepared from individuals treated with dsBgace1 or dsBgace2 (47–40 and 19–16% reduction, respectively), as revealed by spectrophotometric and zymogram analyses. The fact

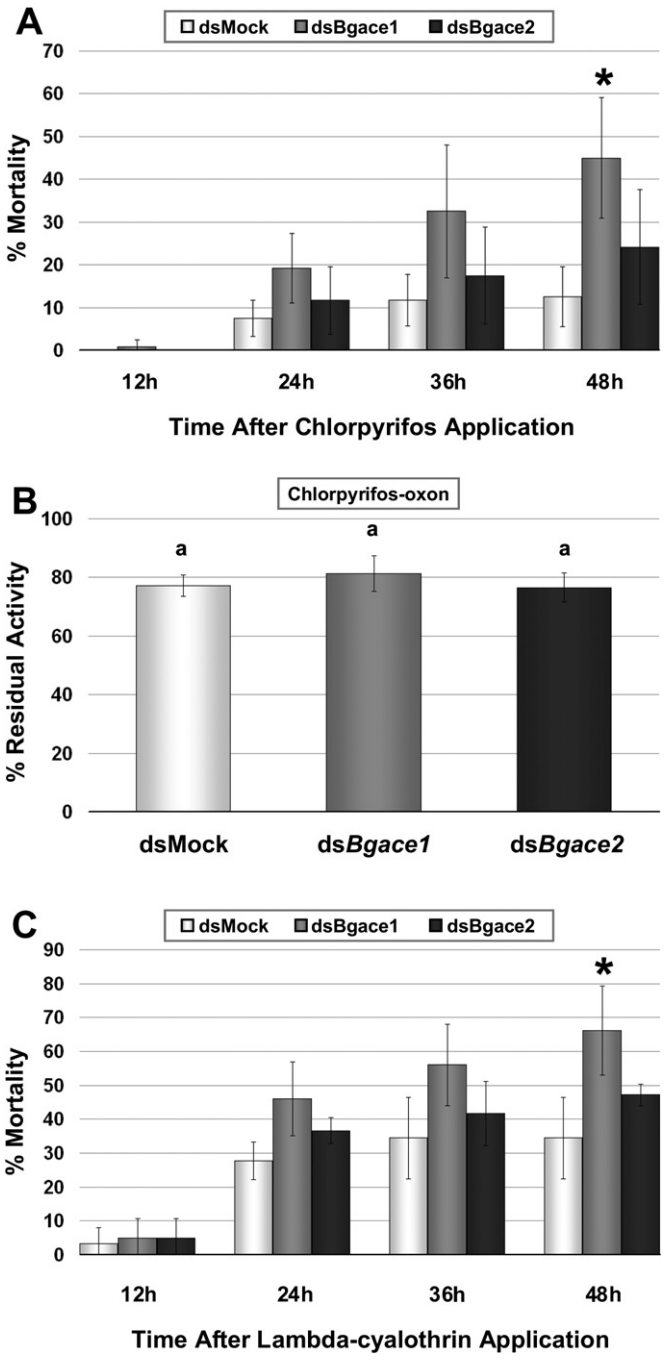

Fig. 4. Toxicity of insecticides to *Bgace1*- and *Bgace2*-silenced *B. germanica*. (A) Chlorpyrifos toxicity to dsMock-, dsBgace1-, and dsBgace2-injected animals at 12, 24, 36 and 48 h after topical application of 270 ng/insect of insecticide. Mortality significantly increased in dsBgace1-injected animals after 48 h exposure (*) to chlorpyrifos (Dunnet test, $p < 0.05$). Bars represent the means and standard deviations of four experimental replicates. (B) Comparative inhibition of AChE activity by chlorpyrifos-oxon in ganglia extracts derived from specimens injected with dsMock, dsBgace1, and dsBgace2. Residual activity is expressed as a percentage of activity without inhibitor. This was quantified by dividing the rate of thiocholine accumulation in a reaction containing insecticide by the rate of the solvent (ethanol)–control reaction. Bars with the same letter are not significantly different (SNK, $p < 0.05$). (C) Lambda-cyhalothrin toxicity to dsMock-, dsBgace1-, and dsBgace2-injected animals at 12, 24, 36 and 48 h after topical application of 20 ng/insect of insecticide. Mortality significantly increased in dsBgace1-injected animals after 48 h exposure (*) to lambda-cyhalothrin (Dunnet test, $p < 0.05$). Bars represent the means and standard deviations of three experimental replicates.

that we see only partial knockdown in AChE activity even when the sum of reduction values is considered (ca. 60% reduction) indicate that the RNAi effects on *ace* proteins are less pronounced than those on mRNAs. This may be explained by the long half-life of AChE, which ranged from 2.5 to 20 days in different mammalian models (Kasprzak and Salpeter, 1985). Thus, complete depletion of AChE activity by RNAi could be difficult to achieve in adult insect, where most of AChE enzymes are already expressed and functional. Consistent with this notion, partial knockdown in AChE activity have been previously reported in RNAi experiments targeting a common region of both *ace1* and *ace2* in *H. armigera* and *Locusta migratoria* (Kumar et al., 2009; Zhou and Xia, 2009).

A greater reduction in AChE activity was achieved by injection with dsBgace1 compared to dsBgace2. Altogether, the similar depletion of *Bgace1* and *Bgace2* mRNAs (95%) and the differential reduction in AChE activity by either dsBgace1 or dsBgace2, suggest that AChE1 is responsible for approximately 65–75% of total AChE activity. This confirms that AChE1 is mainly responsible for AChE activity in ganglia of *B. germanica*, as suggested by previous findings that showed predominant transcription of *Bgace1* in nerve tissues (Kim et al., 2006; Mizuno et al., 2007). A moderate, but significant contribution to ACh hydrolysis is accomplished by AChE2, which suggests either a low or localized enzymatic activity of the *ace2* protein. In agreement with these findings, *Bgace2* mRNA was found only in a subset of neurons in the ganglion (Kim et al., 2006). It is noteworthy that the knockdown of *Bgace2* mRNA did not alter *Bgace1* expression and vice versa, which might suggest non-coordinated transcription and thus, a functional differentiation of *ace* genes. Thus, RNAi appears as a valuable tool to discriminate the activities of both AChE1 and AChE2 in non-Cyclorrhapha insects, besides the inhibitor sensitivity studies previously reported (Bourguet et al., 1996; Huchard et al., 2006; Mizuno et al., 2007). In this sense, our data agree with previous data that indicate a minor, but measurable contribution (ca. 40%) of AChE2 to the total AChE activity in *C. pipiens* (Bourguet et al., 1996).

In previous work it was assumed that the nature of BgAChE bands identified by activity staining in nondenaturating gels was similar to those described in the mosquito *C. pipiens* (Kim et al., 2006). Under this assumption, each of the four bands were assigned to one of the two AChEs, AChE1 and AChE2, each having both hydrophilic and amphiphilic forms. Nonetheless, there is no clear evidence in this regard in cockroaches, as BgAChEs exhibited different multiforms depending on the type of tissue examined (Kim et al., 2006). Results from RNAi experiments are not consistent in some aspects with this interpretation, since a general reduction in each of the four bands was evident upon treatment with either dsBgace1 or dsBgace2, the amplitude of which depended mostly on the dsRNA used. Thus, caution should be taken in assigning banding patterns to any of the *ace* genes in *B. germanica*.

It is not surprising that, despite the vital function played by AChE in insects, reduction of up to nearly 50% in BgAChE1 activity has no dramatic effects on cockroach survival or fecundity under laboratory conditions. For instance, *C. pipiens* collected in insecticide-treated areas had a reduced AChE activity (60%) which may have an associated fitness cost in the field, but no straight effect on mortality (Lenormand and Raymond, 2000; Berticat et al., 2002, 2004). Similarly, over 80% inhibition of AChE by organophosphates is required to observe signs of intoxication in mammals. A reduction in AChE activity of up to 64% by RNAi in *L. migratoria* fails to show any phenotype, albeit treated locusts were more susceptible to malathion (Zhou and Xia, 2009). In this scenario of compromised fitness, our bioassays on dsRNA-injected cockroaches indicated that partial reduction of BgAChE1 led to increased sensitivity to chlorpyrifos whereas BgAChE2 knockdown had a slight effect on mortality. Although we cannot discard differential sensitivities of BgAChE1 and BgAChE2 to chlorpyrifos, similar inhibition of both enzymes to chlorpyrifos-oxon at the dose tested *in vitro* (5×10^{-10} M) suggest that AChE1 is the primary target of organophosphate insecticide in *B. germanica* and the quantity of it is related to chlorpyrifos sensitivity. Alternatively, because a three-fold greater reduction in AChE activity was observed in dsBgace1-injected cockroaches compared to injection with dsBgace2, our results could be explained by a lower concentration of total AChE protein remaining in AChE1-silenced insects. Most mutations conferring AChE insensitivity to organophosphates in non-Cyclorrhapha insects have been associated to *ace1* (Cui et al., 2006; Kono and Tomita, 2006; Alout and Weill, 2008), whereas a few studies have suggested that resistance-associated amino acid substitutions seem to be associated to *ace2* in *Leptinotarsa decemlineata* (Zhu and Clark, 1997) and *Rhopalosiphum padi* (Chen et al., 2007). Our data agree with most studies that point out a key role, qualitative and/or quantitative, of AChE1 as target of organophosphate insecticides in non-Cyclorrhapha insects.

Silencing of AChE1 produced an increased mortality in insects when synergized with lambda-cyhalothrin, a pyrethroid which does not target AChE but instead alters the nerve action potential by modifying the kinetics of voltage-sensitive sodium channels (Soderlund et al., 2002). It has been suggested that pyrethroids may desensitize or delay the closing of the postsynaptic ACh-gated ion channel by modification of the nicotinic receptor, in addition to their known action on the sodium channel (Abbassy et al., 1982). In AChE1 knockdown cockroaches, pyrethroids would enhance the stimulating effect of ACh in the postsynaptic membranes for longer periods which lead to collapse of nervous system, causing eventually death. Since AChE1 knockdown by RNAi resembles in some aspects (reduction in AChE activity) inhibition of AChE by organophosphates, our results could help to explain some of the synergistic effects observed between organophosphates and pyrethroids (Bielza et al., 2007). Nonetheless, an undetected but probable fitness cost associated with alteration in AChE1 activity may also influence lambda-cyhalothrin susceptibility in insects treated with dsBgace1.

The RNAi approach described here has enabled the targeted disruption of the two paralogous genes, *Bgace1* and *Bgace2*, separately. This strategy extents the usefulness of RNAi to study the functional differentiation of the two insect AChEs and may provide insights into the evolutionary and biological significance of an old duplication event present in most insects and arthropods (Huchard et al., 2006). Knockdown of either *ace1* or *ace2* at earlier development stage and in specific tissues may lead to a more potent AChE silencing and then provide a better understanding of each *ace* gene function. Further, a combinatorial pest management strategy, involving the RNAi approach and insecticides, could find applications in control of cockroaches.

Acknowledgements

L.R. was recipient of a predoctoral FPI fellowship from Ministerio de Ciencia e Innovación in Spain (MICINN). This work was supported by Grants BIO2003-03428, BIO2006-10944, AGL2007-63388/AGR, BFU2008-00484 and CGL2008-03517/BOS from MICINN.

References

Abbassy, M.A., Eldefrawi, M.E., Eldefrawi, A.T., 1982. Allethrin interactions with the nicotinic acetylcholine receptor channel. Life Sci. 31, 1547–1552.

Aguilar, R., Maestro, J.L., Vilaplana, L., Pascual, N., Piulachs, M.D., Bellés, X., 2003. Allatostatin gene expression in brain and midgut, and activity of synthetic allatostatins on feeding related processes in the cockroach *Blattella germanica*. Regul. Pept. 115, 171–177.

Alout, H., Weill, M., 2008. Amino-acid substitutions in acetylcholinesterase 1 involved in insecticide resistance in mosquitoes. Chem. Biol. Interact. 175, 138–141.

Baek, J.H., Kim, J.I., Lee, D., Chung, B.K., Miyata, T., Lee, S.H., 2005. Identification and characterization of ace1-type acetylcholinesterase likely associated with organophosphate resistance in *Plutella xylostella*. Pest. Biochem. Physiol. 81, 164–175.

Bellés, X., 2010. Beyond Drosophila. RNAi in vivo and functional genomics in insects. Annu. Rev. Entomol. 55, 111–128. doi:10.1146/annurev-ento-112408-085301.

Berticat, C., Boquien, G., Raymond, M., Chevillon, C., 2002. Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. Genet. Res. 79, 41–47.

Berticat, C., Duron, O., Heyse, D., Raymond, M., 2004. Insecticide resistance genes confer a predation cost on mosquitoes, *Culex pipiens*. Genet. Res. 83, 189–196.

Bielza, P., Espinosa, P., Quinto, V., Abellán, J., Contreras, J., 2007. Synergism studies with binary mixtures of pyrethroid, carbamate and organophosphate insecticides on *Frankliniella occidentalis* (Pergande). Pest. Manag. Sci. 63, 84–89.

Bourguet, D., Capela, R., Raymond, M., 1996. An insensitive acetylcholinesterase in *Culex pipiens* (Diptera: Culicidae) from Portugal. J. Econ. Entomol. 89, 1060–1066.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Chen, M.-H., Han, Z.-J., Qiao, X.-F., Qu, M.-J., 2007. Mutations in acetylcholinesterase genes of *Rhopalosiphum padi* resistant to organophosphate and carbamate insecticides. Genome 50, 172–179.

Cousin, X., Strähle, U., Chatonnet, A., 2005. Are there noncatalytic functions of acetylcholinesterases? Lessons from mutant animal models. BioEssays 27, 189–200.

Cui, F., Raymond, M., Berthomieu, A., Alout, H., Weill, M., Qiao, C.-L., 2006. Recent emergence of insensitive acetylcholinesterase in Chinese populations of the mosquito *Culex pipiens* (Diptera: Culicidae). J. Med. Entomol. 43, 878–883.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, B.C., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *C. elegans*. Nature 391, 806–811.

Huchard, E., Martinez, M., Alout, H., Douzery, E.J.P., Lutfalla, G., Berthomieu, A., Berticat, C., Raymond, M., Weill, M., 2006. Acetylcholinesterase genes within the Diptera: takeover and loss in true flies. Proc. R. Soc. B. 273, 2595–2604.

Karnovsky, M.J., Roots, L.J., 1964. A 'direct-coloring' thiocholine method for cholinesterases. J. Histochem. Cytochem. 12, 219–221.

Kasprzak, H., Salpeter, M.M., 1985. Recovery of acetylcholinesterase at intact neuromuscular junctions after in vivo inactivation with di-isopropylfluorophosphate. J. Neurosci. 5, 951–955.

Kim, J.I., Jung, C.S., Koh, Y.H., Lee, S.H., 2006. Molecular, biochemical and histochemical characterization of two acetylcholinesterase cDNAs from the German cockroach *Blattella germanica*. Insect Mol. Biol. 15, 513–522.

Kono, Y., Tomita, T., 2006. Amino acid substitutions conferring insecticide insensitivity in *Ace*-paralogous acetylcholinesterase. Pest. Biochem. Physiol. 85, 123–132.

Kumar, M., Gupta, G.P., Rajam, M.V., 2009. Silencing of acetylcholinesterase gene of *Helicoverpa armigera* by siRNA affects larval growth and its life cycle. J. Insect Physiol. 55, 273–278.

Lee, D.W., Kim, S.S., Shin, S.W., Kim, W.T., Boo, K.S., 2006. Molecular characterization of two acetylcholine sterase genes from the oriental tobacco budworm, *Helicoverpa assulta* (Guenee). Biochim. Biophys. Acta 1760, 125–133.

Lenormand, T., Raymond, M., 2000. Clines with variable selection and variable migration: model and field studies. Am. Nat. 155, 70–82.

Maestro, O., Cruz, J., Pascual, N., Martin, D., Bellés, X., 2005. Differential expression of two RXR/ultraspiracle isoforms during the life cycle of the hemimetabolous insect *Blattella germanica* (Dictyoptera, Blattellidae). Mol. Cell. Endocrinol. 238, 27–37.

Matsumura, F., 1985. Toxicology of Insecticides, second ed. Plenum Press, New York.

Mizuno, H., Tomita, T., Kasai, S., Komagata, O., Imanishi, S., Kono, Y., 2006. Identification and expression of two types of acetylcholinesterases in a cultured cell line of *Aedes albopictus*, compared to mosquito whole body extracts. Appl. Entomol. Zool. 41, 445–453.

Mizuno, H., Oh, S., Komagata, O., Kasai, S., Honda, H., Kono, Y., Tomita, T., 2007. Differential tissue distribution of two acetylcholinesterase transcripts in the German cockroach, Blattella germanica. Appl. Entomol. Zool. 42, 643–650.

Nabeshima, T., Kozaki, T., Tomita, T., Kono, Y., 2003. An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus persicae*. Biochem. Biophys. Res. Commun. 307, 15–22.

Paraoanu, L.E., Layer, P.G., 2008. Acetylcholinesterase in cell adhesion, neurite growth and network formation. FEBS J. 275, 618–624.

Soderlund, D.M., Clark, J.M., Sheets, L.P., Mullin, L.S., Piccirillo, V.J., Sargent, D., Stevens, J.T., Weiner, M.L., 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. Toxicology 171, 3–59.

Soreq, H., Seidman, S., 2001. Acetylcholinesterase - new roles for an old actor. Nat. Rev. Neurosci. 2, 294–302.

Spencer, A.G., Kristensen, M., Vagn-Jensen, K.M., 1998. The biochemical detection of insecticide resistance in Danish held populations of the German cockroach *Blattella germanica* (Blattellidae). Pest Sci. 52, 196–198.

Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossman, D., Bucher, G., 2008. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome. Biol. 9, R10.

Wei, Y., Appel, A.G., Moar, W.J., Liu, N., 2001. Pyrethroid resistance and cross-resistance in the German cockroach, *Blattella germanica* (L). Pest Manag. Sci. 57, 1055–1059.

Weill, M., Lutfalla, G., Mogensen, K., Chandre, F., Berthomieu, A., Berticat, C., Pasteur, N., Philips, A., Fort, P., Raymond, M., 2003. Comparative genomics: insecticide resistance in mosquito vectors. Nature 423, 136–137.

Zhou, X., Xia, Y., 2009. Cloning of an acetylcholinesterase gene in *Locusta migratoria manilensis* related to organophosphate insecticide resistance. Pest. Biochem. Physiol. 93, 77–84.

Zhu, K.Y., Clark, J.M., 1997. Validation of a point mutation of acetylcholinesterase in Colorado potato beetle by polymerase chain reaction coupled to enzyme inhibition assay. Pest. Biochem. Physiol. 57, 28–35.