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Effects of methamidophos and deltamethrin on *in vitro* protein phosphorylation in *Monochamus alternatus*

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Abstract *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is not only a serious pest insect to pine trees but also the main vector of pine wood nematode *Bursaphelenchus xylophilus*, which causes pine wilt disease. To explore the insecticidal mechanism of insecticides to *M. alternatus*, we chose methamidophos and deltamethrin as the representatives of two groups of insecticides (organophosphates and pyrethroids), which are widely used for pest control in China and investigated their effects on phosphorylation of proteins from the insect. Phosphorylation of proteins from the insect fat body and head was determined by *in vitro* ^{32}P-labelling. In the fat body, deltamethrin obviously reduced basal phosphorylation levels of proteins at 111, 95, 77, and 44 kDa, but enhanced the basal phosphorylation level of a protein at 138 kDa. However, in the presence of calmodulin but not cyclic adenosine monophosphate (cAMP), deltamethrin increased phosphorylation of the protein at 111 kDa. In the head, deltamethrin inhibited basal phosphorylation levels of proteins at 113, 98, and 51 kDa, but potentiated phosphorylation of a protein at 167 kDa activated by cAMP. Methamidophos inhibited phosphorylation of a protein at 44 kDa in the fat body. Although methamidophos did not impact basal phosphorylation levels of any proteins in the head, it inhibited calcium/calmodulin (Ca^{2+}/CaM)-stimulated phosphorylation of a protein at 51 kDa. Together, our data indicate that methamidophos and deltamethrin altered phosphorylation levels of various proteins in the head and fat body of the pine insect and these two kinds of insecticides acted on the proteins that can be phosphorylated in the tissues respectively, which is possibly related to their toxicity.

Key words methamidophos, deltamethrin, *Monochamus alternatus*, protein phosphorylation

Introduction

Monochamus alternatus Hope (Coleoptera: Cerambycidae) is not only a pest insect that can cause serious damage to *Pinus* but also the main vector of pine wood nematode *Bursaphelenchus xylophilus*, which causes pine wilt disease, a devastating disease of pine forests (Morimoto & Iwasaki, 1972; Kobayashi *et al*., 1984; Yoshimura *et al*., 1999). This could lead to huge economic loss (Fan & Sun, 2006). Studies have been focusing on its biology (Shibata, 1984; Kawai, 2006; Zhou & Togashi, 2006; Li & Zhang, 2006), ecology (Yamasaki *et al*., 1989; Anbutsu & Togashi, 1997; 2001) and control methodology (Sakai & Yamasaki, 1990; Aikawa & Togashi, 2000; Maehara & Futai, 2001). However, little is known about the toxicology of insecticides in this insect.

Organophosphorus and pyrethroid insecticides can kill the insect by inhibiting acetylcholinesterase and interfering functions of ion channel of cell membrane, respectively (Narhashi *et al*., 1998, 2000; Narhashi, 2002). However,

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potential targets of the insecticides in the insect are still not well understood. Protein phosphorylation is critical in signal transduction. Most of the cell physiological processes are regulated by protein phosphorylation (Greengard, 1978). Studies on protein phosphorylation have provided solid evidence to elucidate the mechanisms of many events at the molecular level (Miyazawa & Matsumura, 1990; Enan & Matsumura, 1991; Schuh *et al*., 2002; Patel *et al*., 2004). The effects of insecticides on protein phosphorylation in fly and fruit fly have been documented (Leng & Xiao, 1995). However, it is unclear how insecticides affect protein phosphorylation in *M. alternatus*.

Methamidophos and deltamethrin, respectively two kinds of chemical insecticides, organophosphorus compounds and pyrethroids, are widely used for pest control in many countries. They can kill the insects instantly and the mechanism for acute toxicity is well known; however, the underlying mechanism for non-acute toxicity remains to be determined. In rodents and other mammals, methamidophos and deltamethrin not only inhibit acetylcholinesterase (AChE) and affect ion channels respectively, but also impact other targets (Enan & Matsumura, 1991, 1992; Enan *et al*., 1996; Schuh *et al*., 2002). The effects of these two insecticides on protein phosphorylation are beginning to be recognized in crustaceans and invertebrates (Greengard, 1978; Leng & Xiao, 1995; Patel *et al*., 2004). To find out their toxicity to non-cholinesterase and non-ion channel targets, we studied the effects of the insecticides on protein phosphorylation of *M. alternatus*.

Materials and methods

Chemicals

[Gamma-^{32}P] adenosine-5'-triphosphate (ATP: 3000 Ci/mmol) was the product of Furui Company of Biotechnology (Beijing, China). Methamidophos was the product of Shandong Pesticide Factory (Jinan, China). Deltamethrin was the product of Roussel Uclaf Company (Romanville, France). Coomassie brilliant blue G-250 and R-250, dithiothreitol (DTT), adenosine-5'-triphosphate disodium salt (Na_2-ATP), and 2-mercaptoethanol were purchased from Fluka Chemika (Buchs, Switzerland). Tris, Triton X-100, N, N, N', N'-methylene bisacrylamide, bovine serum albumin (BSA), and piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES), cyclic adenosine monophosphate (cAMP) were purchased from Sigma (St. Louis, MO, USA). N, N, N', N'-tetram-ethylethylenediamine (TEMED) and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Acrylamide and sodium dodecyl sulfate (SDS) were the products of Serva Fine Chemicals Company (Heidelberg, Germany). Protein molecular weight (MW) marker used in electrophoresis was obtained from Promega (Madison, WI, USA). Calmodulin (CaM) was obtained as a gift from Professor Juan Bai of the Department of Biology, Hebei Normal University, Shijiazhuang, China.

Insects and the preparation of samples

Monochamus alternatus was collected from Ma'anshan, China. Healthy larvae at 3−4 instars was selected for the experiments. After being starved for 24 h, the larvae were anatomized on an ice plate. The head and the fat body were dissected and then washed with ice-cold homogenizing buffer (10 mmol/L Tris, pH 7.4, 0.1% Triton X-100). The tissues from three or four insects as one sample were homogenized with 30 up-and-down strokes in a motor-driven Teflon-glass homogenizer at 4°C. The homogenates were transferred into a centrifuge tube and then centrifuged at 1 000 *g* for 5 min. The supernatants were immediately used for assay. All experiments were carried out in triplicate.

Protein determination

Protein concentrations were determined by the method of Bradford (1976) using Coomassie brilliant blue G-250 using BSA as a standard. The amount of 100 μL of the homogenate supernatant prepared as described above was used to assay the protein concentration for each sample.

Protein phosphorylation assay

The phosphorylation reaction was undertaken according to Arif *et al*. (2003) with a slight modification. The standard assay mixture, in a final volume of 100 μL, contained 35 μg protein, 10 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 10 μmol/L [γ - ^{32}P] ATP, pH 6.5. Additionally, proteins were incubated in the absence or presence of either 300 μmol/L $CaCl_2$ plus 1 μg CaM or 10 μmol/L cAMP. Proteins were incubated with 100 μmol/L deltamethrin or methamidophos for 10 min at 35°C. The phosphorylation reaction was then initiated by the addition of [γ - ^{32}P] ATP. The reaction was terminated after 1 min by adding 100 μL of SDS-PAGE (polyacrylamide gel electrophoresis) sample buffer (containing 0.125 mol/L Tris-HCl, pH 6.8, 4.5% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) bromophenol blue), followed by heating for 3 min in a water bath at 90°C.

Six groups were set up in the experiment; the group assignment is listed in Table 1.

Table 1 Group assignments of protein phosphorylation assay.

Groups	Control			Insecticide-treated		
	None	cAMP	Ca^{2+}/CaM	None	cAMP	Ca^{2+}/CaM
Insecticides	−	−	−	+	+	+
cAMP	−	+	−	−	+	−
Ca^{2+}/CaM	−	−	+	−	−	+

"+" means the component was added to the reaction system and "−" means the component was absent from the reaction system. cAMP, cyclic adenosine monophosphate; Ca^{2+}/CaM, calcium/calmodulin.

Phosphoprotein separation and detection

The proteins were resolved on SDS-polyacrylamide gels. Electrophoresis was performed in the buffers described by Laemmli (1970) with 4% (w/v) and 10% (w/v) acrylamide in the stacking and resolving gels, respectively. Aliquots of the samples (6.5 μg/well) were subjected to electrophoresis under conditions of constant voltage (200 V) until the dye front reached the bottom of the gels. The apparent electrophoretic mobilities of the resolved proteins were determined from molecular standards of MW-70−2 that had been subjected to electrophoresis under conditions identical to the head and fat body samples and were presented in kilo-Daltons (kDa).

Following the electrophoresis, the gels were fixed and stained for proteins with 0.1% Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid for 4 h. The gels were then destained in 40% methanol and 10% acetic acid for 2−4 h and dried naturally between two sheets of dialysis membrane for 18−24 h. Autoradiographs of ^{32}P-labeled proteins were obtained by the Typhoon 9400 imaging system (Amersham Biosciences, Arlington Heights, IL, USA). The amount of phosphorylated proteins in each autoradiographic band was quantified by determining the area under the corresponding peak in the densitometric scan obtained by using ImageQuant software (Nonlinear Dynamics, Amersham Biosciences, Arlington Heights, IL, USA).

Statistical analysis

The quantitative data from autoradiography of protein phosphorylation are expressed as means ± SE. Control and experimental groups of data were compared by one-way analysis of variance (ANOVA). Student's *t*-test was used to compare differences between two groups. A difference between means was considered significant if $P < 0.05$.

Results

Effect of deltamethrin on phosphorylation of proteins from the fat body of M. alternatus

Six bands could be detected on the gel (Fig. 1A). Deltamethrin (100 μmol/L) inhibited the basal levels of phosphorylation of the proteins at 111, 95, 77, and 44 kDa. However, the insecticide enhanced the basal level of phos-

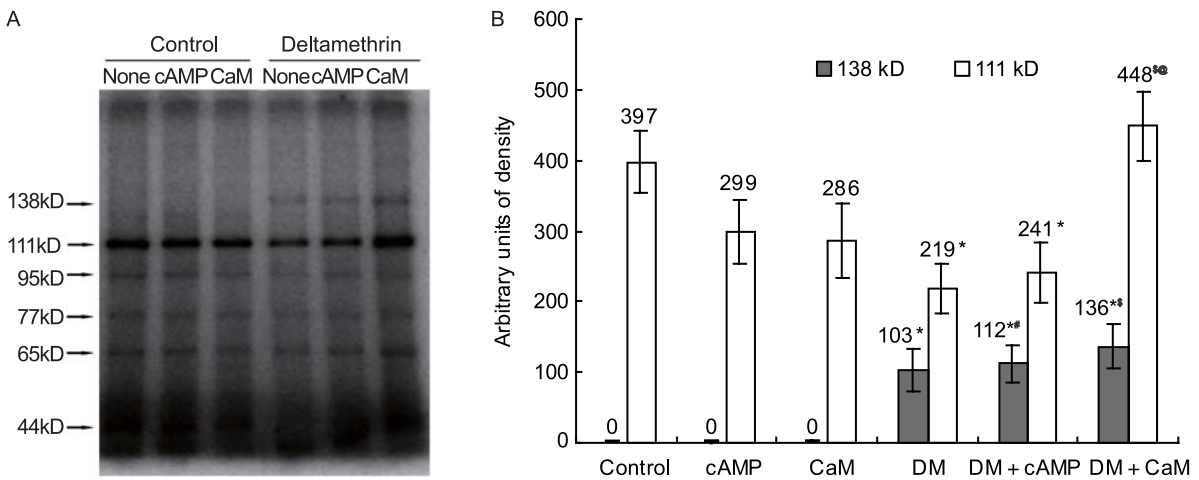

Fig. 1 Phosphorylation of proteins from the fat body of *Monochamus alternatus* larvae *in vitro*. Autoradiography (A) of phosphorylation of the fat body proteins obtained from the insects and quantitative histograms (B) of the 138 and 114 kDa proteins. From left to right: lanes 1, 2 and 3 represent control samples; lanes 4, 5 and 6 are samples treated with deltamethrin (DM). The data are represented as mean ± SE ($n = 3$). $^{*}P < 0.05$, compared with control; $^{@}P < 0.05$, compared with DM with no addition; $^{\#}P < 0.05$, compared with control with cyclic adenosine monophosphate (cAMP) addition; $^{\$}P < 0.05$, compared with control with calcium/calmodulin (CaM) addition.

phorylation of a protein at 138 kDa (Fig. 1A,B). The band of 138 kDa protein could not be detected in the fat body sample without deltamethrin treatment. However, it became obvious when treated with deltamethrin (Fig. 1A), indicating that the phosphorylation reaction was activated by the insecticide. The phosphorylation level of protein at 111 kDa was inhibited by deltamethrin, but the level was increased when the phosphorylation reaction occurred in the presence of Ca^{2+}/CaM but not cAMP (Fig. 1A,B). The results suggest that calmodulin may activate a kinase to increase the phosphorylation at 111 kDa.

Effect of deltamethrin on phosphorylation of proteins from the head of M. alternatus

Eight bands were visualized on the gel. Clearly deltamethrin inhibited basal levels of phosphorylation of proteins at 113, 98, and 51 kDa (Fig. 2A,B). These bands were very weak in the sample treated with deltamethrin alone. In particular, there was no detectable band at the position of 167 kDa. However, deltamethrin potentiated cAMP-activated phosphorylation of several bands, including one at 167 kDa (Fig. 2A). We also observed that CaM increased phosphorylation of proteins at 59 and 64 kDa, which was not influenced by deltamethrin (Fig. 2A).

Effect of methamidophos on phosphorylation of proteins from M. alternatus

As shown in Figure 3, methamidophos (100 μmol/L) did not affect phosphorylation of proteins from the fat body of *M. alternatus* larvae except for a protein at 44 kDa, which was decreased when the sample was treated with methamidophos. However, Ca^{2+}/CaM completely reversed the inhibitory effect of methamidophos on the phosphorylation of this protein (Fig. 3A,B).

For the proteins from the head of *M. alternatus*, at least eight phosphorylation bands were detectable by autoradiography. Methamidophos did not alter the basal levels of phosphorylation of these proteins (Fig. 4A). cAMP- or Ca^{2+}/CaM-treatment dramatically increased phosphorylation of a protein at 51 kDa (Fig. 4A). Methamidophos did not affect cAMP-stimulated phosphorylation of the 51 kDa protein, but significantly inhibited Ca^{2+}/CaM-stimulated phosphorylation of the protein. Actually, methamidophos inhibited Ca^{2+}/CaM-stimulated phosphorylation of all eight proteins (Fig. 4A).

Discussion

Phosphorylation reaction is a very important regulation process for physiological function in the body (Greengard, 1978). The key factors of the phosphorylation system include substrate proteins, kinases and phosphatases. Kinases phosphorylate substrate proteins, whereas phosphatases catalyze dephosphorylation of the phosphorylated proteins (Cohen, 1992; Hubbard & Cohen, 1993; Hanks & Hunter, 1995; Faux & Scott, 1996). Some kinases, such as protein kinase A (PKA) and protein kinase C

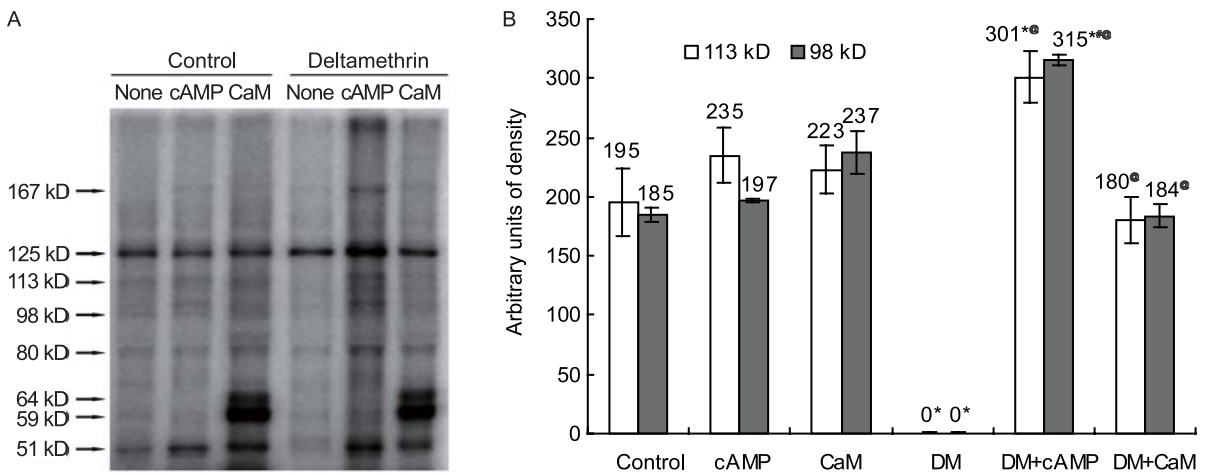

Fig. 2 Phosphorylation of proteins from the head of *Monochamus alternatus in vitro*. Autoradiography (A) of phosphorylation of the head proteins obtained from the insects and quantitative histograms (B) of the 113 and 98 kDa proteins. From left to right: lanes 1, 2 and 3 represent control samples; lanes 4, 5 and 6 are samples treated with deltamethrin (DM). The data are represented as mean ± SE ($n = 3$). $^{*}P < 0.05$, compared with control; $^{@}P < 0.05$, compared with DM with no addition; $^{\#}P < 0.05$, compared with control with cyclic adenosine monophosphate (cAMP) addition.

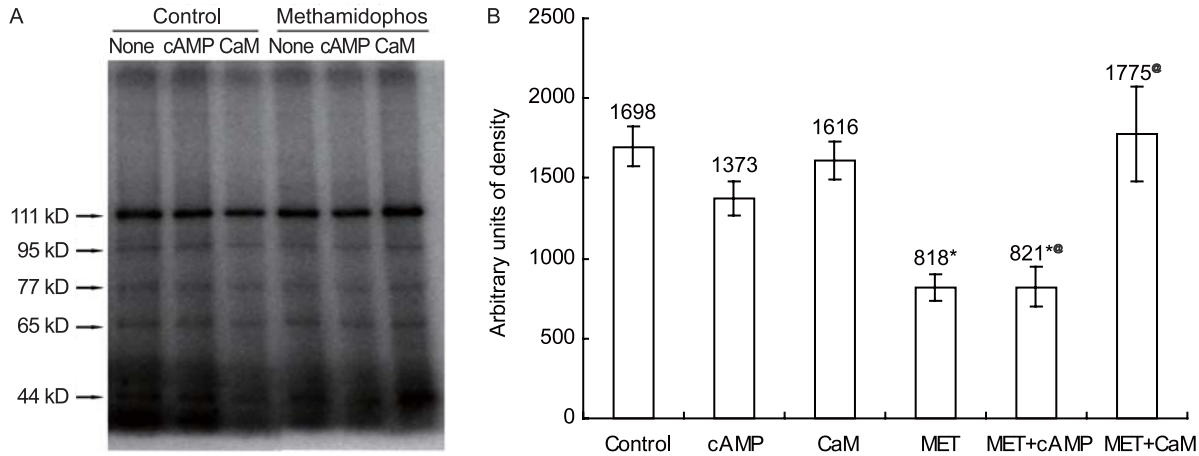

Fig. 3 Phosphorylation of proteins from the fat body of *Monochamus alternatus in vitro*. Autoradiography (A) of phosphorylation of the fat body proteins obtained from *M. alternatus* larvae and quantitative histograms (B) of the 44 kDa protein. From left to right: lanes 1, 2 and 3 represent control samples; lanes 4, 5 and 6 are samples treated with methamidophos (MET). The data are represented as mean ± SE ($n = 3$). $^{*}P < 0.05$, compared with control; $^{\#}P < 0.05$, compared with control with cyclic adenosine monophosphate (cAMP) addition; $^{@}P < 0.05$, compared with MET with no addition.

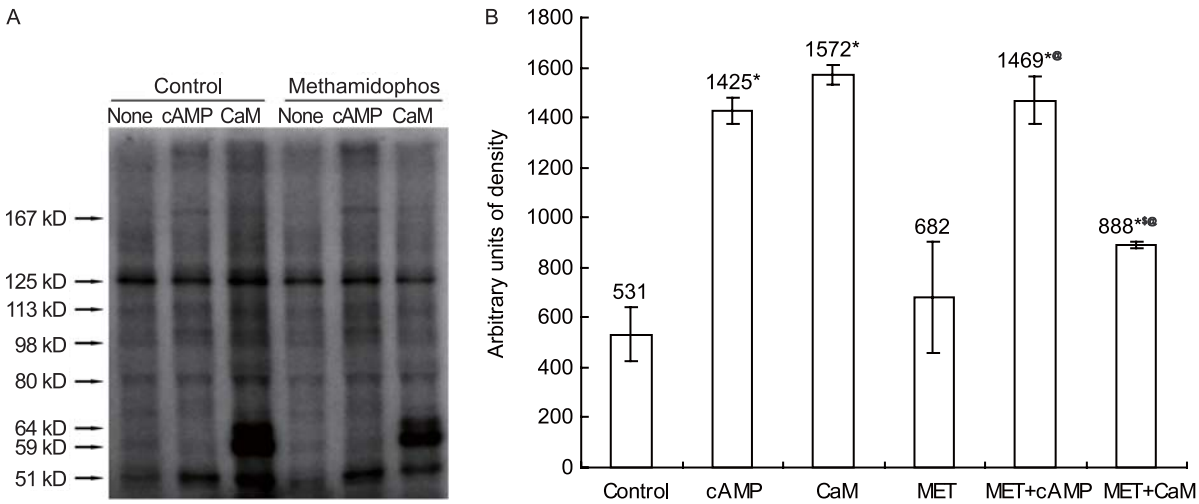

Fig. 4 Phosphorylation of proteins from the head of *Monochamus alternatus in vitro*. Autoradiography (A) of phosphorylation of head proteins obtained from the insects and quantitative histograms (B) of the 51 kDa protein. From left to right: lanes 1, 2 and 3 represent control samples; lanes 4, 5 and 6 are samples treated with methamidophos (MET). The data are represented as mean ± SE ($n = 3$). $^{*}P < 0.05$, compared with control; $^{\$}P < 0.05$, compared with control with calcium/calmodulin (CaM) addition; $^{@}P < 0.05$, compared with MET with no addition.

(PKC), can be stimulated by cAMP and Ca^{2+}/CaM, and inhibited by insecticides (Miyazawa & Matsumura, 1990; Schuh *et al*., 2002; Biondi, 2004). Therefore, cAMP and Ca^{2+}/CaM were used in this study as controls. Our results indicate that methamidophos and deltamethrin have significant effects on the phosphorylation of proteins from *Monochamus alternatus*. These two kinds of insecticides have different effects on the phosphorylation level of the proteins, possibly related to their different effects on the corresponding kinases and phosphatases in the reaction system.

We found that deltamethrin could significantly inhibit phosphorylation of most proteins from the fat body and head of *Monochamus alternatus*. However, it also acti-

vated phosphorylation of some proteins. Deltamethrin completely inhibited phosphorylation of proteins at 113, 98, and 51 kDa from the head of the insect, but significantly increased phosphorylation of 138 kDa protein from the fat body. cAMP or Ca^{2+}/CaM reversed the inhibitory effect of deltamethrin on phosphorylation of proteins at 113, 98, and 51 kDa from the head, suggesting that deltamethrin may activate some phosphatases to dephosphorylate these proteins, whereas cAMP or Ca^{2+}/CaM may activate some kinases to counteract the dephosphorylation induced by deltamethrin. Clearly, more studies are needed to address this question. cAMP or Ca^{2+}/CaM did not alter the phosphorylation of the 138 kDa protein, suggesting a mechanism independent of cAMP or calmodulin.

Our findings also reveal that the effects of methamidophos on cAMP and Ca^{2+}/CaM-stimulated phosphorylation of different proteins are complicated. Methamidophos inhibited phosphorylation of a 44 kDa protein from the fat body of the insect, which was completely reversed by Ca^{2+}/CaM, but not by cAMP. In contrast, methamidophos did not affect the basal phosphorylation level of a 51 kDa protein from the head. Both cAMP and Ca^{2+}/CaM increased the phosphorylation of the 51 kDa protein. It appears that methamidophos did not affect cAMP-activated phosphorylation of the proteins, but potently inhibited Ca^{2+}/CaM-stimulated phosphorylation of the 51 kDa protein. The data suggest that methamidophos may inhibit a Ca^{2+}/CaM-activated kinase, which is responsible for phosphorylation of the 51 kDa protein.

It is familiar to us that insecticides can act on the target proteins in insects to cause death of the insects exposed after a short time. However, protein phosphorylation is an important event in cell metabolism regulation, which may be involved in the targets of these insecticides. Our results indicated that some kinds of kinases and phosphatases could be affected by the insecticides deltamethrin and methamidophos and the levels of phosphorylation of some proteins were affected correspondingly. These effects of the insecticides on protein phosphorylation may be one of the toxicity mechanisms of the insecticides to Japanese pine sawyer, and possibly other insects. The proteins changed at phosphorylation levels in the insect may be the non-classical targets of the insecticides (deltamethrin and methamidophos). As such, the proteins detected on the gel could be interesting points. It is necessary to identify these proteins affected by deltamethrin and methamidophos, in order to further understand the mechanisms of the two insecticides. Tryptic mapping and sequencing of peptides may provide useful data.

Together, we studied the effects of deltamethrin and methamidophos on the protein phosphorylation in *M. alternatus*, and gained some interesting data. However, how the insecticides affect phosphorylation of various proteins in the insect remains unknown. Further work is needed to identify the phosphorylated proteins and the possible mechanism of the insecticides.

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