J Neurophysiol 90: 259–270, 2003;
10.1152/jn.01096.2002.

Dimethyl Disulfide Exerts Insecticidal Neurotoxicity Through Mitochondrial Dysfunction and Activation of Insect K_{ATP} Channels

Sébastien Dugravot,[1] Françoise Grolleau,[2] David Macherel,[3] Annie Rochetaing,[4] Bernard Hue,[2] Maria Stankiewicz,[5] Jacques Huignard,[1] and Bruno Lapied[2]

[1]*Institut de Recherche sur la Biologie de l'Insecte, UMR Centre National de la Recherche Scientifique 6035, Université de Tours, F-37200 Tours;* [2]*Laboratoire de Neurophysiologie, Unité Propre de Recherche de l'Enseignement Supérieur Equipe d'Accueil 2647, Université d'Angers, Unité de Formation et de Recherche Sciences, F-49045 Angers Cedex 01;* [3]*Unité Mixte de Recherche Physiologie Moléculaire des Semences, F-49045 Angers Cedex 01;* [4]*Laboratoire de Préconditionnement et remodelage du myocarde, Université d'Angers, Unité de Formation et de Recherche Sciences médicales, F-49045 Angers Cedex 01, France; and* [5]*Laboratory of Biophysics, N. Copernicus University, 87–100 Torun, Poland*

Submitted 6 December 2002; accepted in final form 2 February 2003

Dugravot, Sébastien, Françoise Grolleau, David Macherel, Annie Rochetaing, Bernard Hue, Maria Stankiewicz, Jacques Huignard, and Bruno Lapied. Dimethyl disulfide exerts insecticidal neurotoxicity through mitochondrial dysfunction and activation of insect K_{ATP} channels. *J Neurophysiol* 90: 259–270, 2003; 10.1152/jn.01096.2002. The plant-derived insecticides have introduced a new concept in insecticide research. In response to insect attacks, some plants can release volatile sulfur compounds such as dimethyl disulfide (DMDS) in the atmosphere, which are lethal for the generalist insects. We demonstrate that DMDS induced an uncommon complex neurotoxic activity. The studies of *in vivo* toxicity of DMDS in three insect species and mice indicated a highest bioactivity for insects. Although DMDS did not alter the electrophysiological properties of the cockroach *Periplaneta americana* giant axon, it affected the synaptic transmission at the presynaptic level resulting in an inhibition of the neurotransmitter release. Whole cell patch-clamp experiments performed on cockroach cultured dorsal unpaired median (DUM) neurons revealed a dose-dependent hyperpolarization induced by DMDS associated with a decrease in the input resistance and the disappearance of action potentials. The hyperpolarization was inhibited by glibenclamide and tolbutamide, and was dependent on intracellular ATP concentration, demonstrating a neurotoxicity via the activation of K_{ATP} channels. Finally, the same effects observed with oligomycin, 2,4-dinitrophenol, and KCN together with the studies of DMDS toxicity on isolated mitochondria confirmed an unusual action occurring through an inhibition of the mitochondrial respiratory chain complex IV (cytochrome oxydase). This DMDS-induced inhibition of complex IV subsequently decreased the intracellular ATP concentration, which thereby activated neuronal K_{ATP} channels mediating membrane hyperpolarization and reduction of neuronal activity.

INTRODUCTION

Numerous emerging environmental evidence seems to indicate that the use of botanical insecticide represents an exciting alternative way in the biological crop protection (Carlini and Grossi-de-Sa 2002). Some of plants can produce, in response to insect attacks, volatile secondary compounds, also known as chemical defensives, which alter insect metabolism and nervous system activity (Rauscher 1992). By contrast, these substances are ineffective against specialist insects (Brattsen 1992), in which the consumption of these compounds induces the induction of detoxification enzymes, such as P450 enzymes, glutathione *S*-transferase enzymatic system, and β-glucosidases (Feyereisen 1999; Lindroth 1988; Sogorb and Vilanova 2002) Among plants producing the volatile secondary compounds, *Allium* plant species, and particularly the leek *Allium porrum*, can release in the atmosphere, when they are damaged, sulfur volatile compounds such as thiosulfinates, which can lead to the formation of disulfides [e.g., dimethyl disulfide (DMDS), tested in this study] (Auger et al. 1989). Because they are lethal (via a hypothetical neurotoxic activity) for many insect species, nonspecialists of these plants (Dugravot et al. 2002) they could be used in plant protection and particularly in the seed storage systems as fumigant (Arthur 1996; Dugravot et al. 2002). Up to date, the most commonly used fumigant, methyl bromide (CH_3Br), acting in the gaseous state, diffuses through and into large quantities of seeds. It is particularly effective on insect eggs and adults. However, despite its high toxicity to pests, many disadvantages are attributed to CH_3Br, including for instance a reduction of seed germination and a major contribution to a depletion of ozone in the upper atmosphere (Watson 1992). Elimination of all uses and production of CH_3Br is expected by 2005 in industrialized countries and 2015 in developing countries (Bell 2000; Taylor 1996). Consequently, sulfur compounds such as DMDS could represent a new alternative way to fight insect predators while avoiding the use of environmentally aggressive chemicals. However, to date, the precise insecticidal neurotoxic mode of action of DMDS still remains unknown. Consequently, the susceptibility to DMDS of two insect species developing in seed storage system, the Coleoptera Bruchidae *Callosobruchus maculatus* and its parasitoid hymenoptera *Dinarmus basalis*, were analyzed in the present study. We also evaluated the susceptibility to DMDS of *1*) the coackroach *Periplaneta americana*, known to cause many inconveniences in urban entomology, and *2*) adult mice, to examine if DMDS could induce toxicity in mammals. Finally, to characterize the insec-

Address for reprint requests: B. Lapied, Laboratoire de Neurophysiologie UPRES EA 2647—RCIM, Université d'Angers, UFR Sciences, 2 boulevard Lavoisier, F-49045 Angers Cedex 01, France (E-mail: bruno.lapied@univ-angers.fr).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ticide target site of DMDS at cellular and molecular levels, electrophysiological and pharmacological experiments were carried out on the CNS of *Periplaneta americana*, which is known to be a suitable model for better understanding the effects of insecticides (Buckingham et al. 1997; Lapied et al. 2001; Pelhate et al. 1990). Different preparations including isolated giant axon, synaptic transmission, and short-term cultured cockroach neurosecretory cells identified as dorsal unpaired median (DUM) neurons were used to investigate this neurotoxic action. These experiments, associated with complementary approaches such as oximetry and spectrophotometry adapted to mitochondria purified from the pea *Pisum sativum* seeds, have allowed demonstration, for the first time, that DMDS exerts an uncommon complex mode of action through mitochondrial dysfunction, like the complex IV inhibitor that induces an activation of neuronal ATP-sensitive potassium channels, never identified, until now, in insect neuronal preparations.

METHODS

Insect rearing

The Coleoptera Bruchidae *C. maculatus* and its parasitoid hymenoptera *D. basalis* originated from West Africa were mass reared under the following conditions: 33–23°C, 12-h light/12-h dark, 70% relative humidity, and synchronous photo- and thermoperiods as described by (Ouedraogo et al. 1996). The adults emerging from the seeds were placed in petri dishes and were fed with 10% sucrose solution, renewed every 2 days before the experiments were carried out. Adult male cockroaches *P. americana* were obtained from our laboratory stock colony and maintained at 29°C with a photoperiod of 12-hour light/12-hour dark.

Susceptibility of adult C. maculatus, D. basalis, and P. americana to DMDS

To analyze the toxicity of DMDS, 20 pairs of 2-day-old adults of *C. maculatus* and *D. basalis* and 10 adult male *P. americana* were placed for 24 h in 3-l hermetically sealed glass jars containing variable concentrations of this volatile compound (0.33, 0.66, 1, 1.33, and 1.66 μl/l). At the end of the exposure, adults still alive were removed from the jar and isolated in petri dishes for 24 h (*C. maculatus* and *D. basalis*) or for 48 h (*P. americana*). During this isolation period, some individuals ambulated weakly became moribund and died. The other individuals progressively recovered their mobility. The mortality rates were determined at the end of this isolation period. Under each experimental condition, three replicates were made. A dose-response curve was established for each species. The lethal concentration causing 50% of mortality (LC_{50}) was determined by Probit analysis (Finney 1971). Maximum likelihood program software was employed for analysis of the dose-mortality response. ANOVA and χ^2 tests were used for the comparison of the data.

Susceptibility of adult mice to DMDS

Male Swiss mice weighing 35 ± 5 g were used for this study. All procedures were performed in accordance with the regulations of the French ministry of Agriculture for the care and use of laboratory animals. The determination of the LC_{50} of the mice population exposed to DMDS was performed as followed. Each increasing concentration of DMDS, between 1 and 10 μl/l air, was tested on a 30-mouse group subdivided into 6 subgroups of 5 mice. A 30-mouse control group was exposed to confined air. After a 24-h exposure, the number of dead mice was noted, and the results were expressed as percentages in relation to the different concentrations used. During exposure to DMDS, each mice subgroup was placed in a 40-l experimental chamber during 24 h. At first, mice were placed in a small plastic transparent box with water and food. Then, each box perforated on two sides was heightened in an experimental chamber to inhibit all CO_2 effects. In fact, CO_2 and H_2O were respectively captured with potassium hydroxide and calcium dichloride. The temperature was controlled with a thermometer and maintained to 21 ± 1°C. Just before airtight closing the chamber, DMDS was quickly applied, on an absorbent paper (Whatman paper) placed above a glass pot, for a swift and complete spraying of product.

Electrophysiology

SYNAPTIC TRANSMISSION—SINGLE FIBER OIL-GAP RECORDINGS. All experiments were performed on adult male cockroaches (*P. americana*). Briefly, the single fiber oil-gap technique (Hue and Callec 1990) was used to record composite excitatory postsynaptic potentials (cEPSPs) in response to electrical presynaptic stimulation applied at a frequency of 0.1 Hz to the ipsilateral cercal nerve. Direct activation of cholinergic postsynaptic receptors located on dendritic membranes of the isolated giant interneuron (GI) was achieved by means of ionophoretic micro-injection of carbamylcholine (CCh) within the neuropile of the terminal abdominal ganglion (TAG). To assess the physiological properties of the axonal membrane, action potentials were evoked in the intraganglionic part of the TAG by passing suprathreshold square current pulses using a Wheastone bridge circuit. During the experiments, resting potential and unitary EPSPs were continuously monitored on a pen chart recorder. The cEPSPs were recorded as the average of three sweeps. The desheathed TAG was superfused with a saline of the following composition (in mM): 208 NaCl, 3.1 KCl, 10 $CaCl_2$, 2 $NaHCO_3$, and 26 sucrose, pH 7.4. DMDS (100 μM) was bath-applied directly onto the TAG during periods of 30–60 min. Quantitative effects of DMDS were expressed as mean ± SE ($n = 3$).

ISOLATED DORSAL UNPAIRED MEDIAN NEURONS—WHOLE CELL PATCH-CLAMP RECORDINGS. Experiments were performed on dorsal unpaired median (DUM) neuron cell bodies isolated from the midline of the TAG of the nerve cord of adult male cockroaches, *P. americana*, as previously described (Lapied et al. 1989). DUM neuron cell bodies used were maintained at 29°C for 24 h before electrophysiological experiments were carried out. The whole cell patch-clamp recording configuration was used to record ionic currents (voltage-clamp mode) and action potentials (current-clamp mode). Signals were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled from borosilicate glass capillary tubes (Clark Electromedical Instruments, Harvard Apparatus, Edenbridge, UK) with a PP-83 electrode puller (Narishige, Japan) and had resistances of 0.9–1.3 MΩ when filled with the pipette solution (see composition below). The liquid junction potential between bath and internal pipette solution was compensated before the formation of a gigaohm seal (>3 GΩ).

For current-clamp experiments, depolarizing current pulses were elicited at 0.5 Hz with a programmable stimulator (SMP 310, Biologic, Claix, France) Evoked action potentials and membrane potential were displayed and stored on the hard disk of an IBM pentium 100 computer with the pClamp software control (pClamp version 6.03, Axon Instruments). The computer was connected to a 125-kHz labmaster DMA data acquisition system (TL-1–125 interface, Axon Instruments). The bathing solution contained (in mM) 200 NaCl, 3.1 KCl, 5 $CaCl_2$, 4 $MgCl_2$, 10 HEPES buffer, and the pH was adjusted to 7.4 with NaOH. The recording electrode was filled with (in mM) 150 K-aspartate, 10 KF, 10 NaCl, 1 $MgCl_2$, 3 ATP-Mg, 0.5 $CaCl_2$, 10 EGTA, and 10 HEPES buffer, pH 7.4. For voltage-clamp studies of the inward sodium current, step voltage pulses were generated by the computer using pClamp software. Cells were clamped at a holding

potential of –90 mV, and test pulses of 30 ms in duration were applied at 0.3 Hz. The procedure used to record the DUM neuron inward sodium currents is described elsewhere (Lapied et al. 1990, 2001).

In all electrophysiological studies, DMDS stock solution (100 mM) was prepared in dimethylsulphoxide (DMSO). Final dilution contained at most 0.1% DMSO. These concentrations of solvent were found to be without effect on both axonal and neuronal electrophysiological properties. All compounds were purchased from Sigma Chemicals (L'isle d'Abeau Chesnes, France). Experiments were carried out at room temperature (21°C). Data were expressed as mean ± SE when quantified.

Effect of DMDS on cellular respiration

D. MELANOGASTER S2 CELL LINE. The S2 cell line was cultured in Shields and Sang M3 insect medium. For respiratory measurements, cells in the exponential phase of growth were collected with culture medium, concentrated by centrifugation (1000 rpm), and finally suspended in a small volume of medium and kept on ice until experiment. Oxygen consumption was monitored with an oxygen electrode (Oxytherm, Hansatech, King's Lynn, UK) at 30°C in 1 ml of culture medium. Cells (100 μl; 6.8×10^6 cells) were added to the reaction chamber previously equilibrated with 0.9 ml of culture medium. Inhibitors were added as required and indicated in the corresponding figures and legends.

ISOLATED PLANT MITOCHONDRIA. Mitochondria were isolated from 22 h imbibed pea (*Pisum sativum*) seeds and purified using Percoll (Amersham Pharmacia Biotech) gradients using general method for plant mitochondria (Douce et al. 1987). The mitochondria were highly purified according to marker enzyme analysis for cytosol, plastid, peroxysome, and electron microscopy (results not shown). Oxygen consumption was monitored with the oxygen electrode at 25°C in 1 ml of reaction medium containing 0.6 M mannitol, 20 mM MOPS, pH 7.5, 10 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$, and 0.1% (wt/vol) BSA. Mitochondria, substrates, cofactors, and inhibitors were added as required and indicated in the corresponding figures and legends. The analysis of electron transfer from exogenous NADH to cytochrome c was followed spectrophotometrically with a microplate reader (Spectramax plus, Molecular Devices). Reactions were carried out at 25°C in a volume of 250 μl of reaction medium supplemented with 0.04% Triton X100 (vol/vol), 1 mM KCN, 80 μM horse cytochrome c (Sigma), and mitochondria (0.8 mg protein/ml). The reaction was initiated by the addition of 2 mM NADH, and reduction of cytochrome c was followed through its absorbance at 550 nm. A cytochrome oxidase assay based on a classical protocol (Trounce et al. 1996) was adapted to the microplate format that allows the simultaneous recording of multiple absorbance and spectrum. The reaction mixture (270 μl) contained 50 μM MOPS, pH 7.4, 0.05% (vol/vol) Triton X100, 40 μM reduced cytochrome c (stock solution reduced by a crystal of sodium dithionite), and mitochondria (15 μg protein/ml). The reaction was incubated at 25°C inside the microplate reader and absorbance at 550 nm was recorded every 5 s. After 3 min of reaction, cytochrome c spectra were recorded simultaneously on all samples. Protein concentrations were determined by Bradford bioassay (Biorad, UK) using BSA as a standard.

RESULTS

Susceptibility of insect species and mice to DMDS

For the three insect species, the rates of mortality increased with the DMDS concentrations, but their susceptibility to this sulfur compound was different (Fig. 1*A*). Adults *D. basalis* were very susceptible to DMDS since the LC_{50} was 0.31 μl/l air. A dose of 0.66 μl/l air was enough to cause 100% mortality. DMDS was also tested on adult *C. maculatus* (Fig. 1*A*). They were less susceptible than adults *D. basalis* since a dose of 0.65 μl/l air was required for LC_{50}. All insects were killed after a 24-h exposure to a dose of 1.33 μl/l air. For comparison, the LC_{50} estimated for adults *P. americana* was 1.01 μl/l air. To obtain 100% mortality 1.66 μl/l air DMDS was needed. These results indicated that after a 24-h DMDS exposure, the cockroaches were 3.3- and 1.5-fold less susceptible to DMDS than *D. basalis* and *C. maculatus*, respectively. In parallel, we performed experiments to determine if mammals were also susceptible to DMDS vapors. Figure 1*B* illustrates that all adult mice were dead when they were exposed to DMDS doses ranging from 5 to 10 μl/l air. The mice were not killed for lower doses (from 0.1 to 1 μl/l air). For concentrations ranging from 1.5 to 1.9 μl/l air (in 0.1 μl/l concentration increment), the rate of mortality was strongly increased from 43.5 ± 2.0% to 78.6 ± 1.7%, respectively. The 24-h LC_{50} value was estimated at 1.5 μl/l air and a concentration of 5 μl/l air DMDS was needed to achieve 100% kill.

Because DMDS exhibited highest bioactivity against insects, we examined further the effectiveness of DMDS on the insect CNS as a potent insect neurotoxic agent.

Effects of DMDS on the cockroach CNS

Because one of the most attractive aspects of using DMDS is its ability to exhibit insecticide neurotoxicity, we performed a new series of experiments on the cockroach CNS to obtain more insight into its insecticide mode of action. As already indicated above, the cockroach CNS possesses a number of

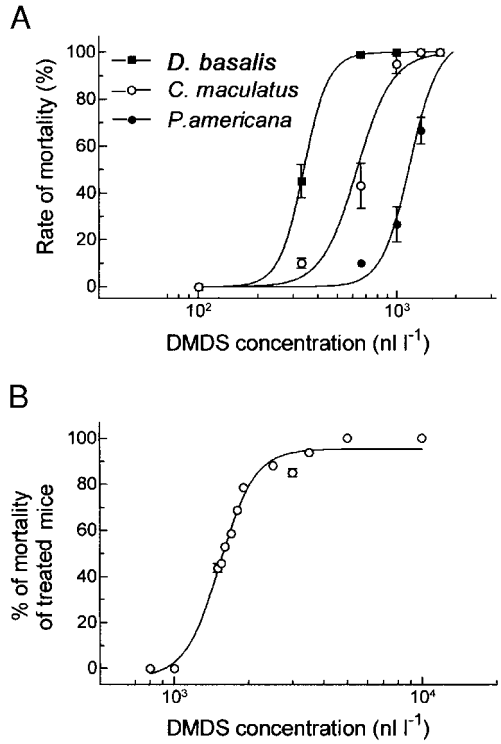

FIG. 1. Toxic activity of dimethyl disulfide (DMDS) against 3 insect species including *D. basalis*, *C. maculatus*, *P. americana*, and mice. *A*: comparative curves illustrating susceptibility of *D. basalis*, *C. maculatus*, and *P. americana* to different doses of DMDS. *B*: toxic effect of DMDS on mice represented by the percentage of mortality in response to different concentration of DMDS. Values are means ± SE.

interesting features which make it suitable for studying neurotoxicity of insecticides. The neurotoxic effect of DMDS were studied on three distinct preparations, including *1*) isolated giant axon (Pelhate et al. 1990), *2*) cercal afferent/giant interneuron synapses in the terminal abdominal ganglion (Hue and Callec 1990), and *3*) short-term cultured neurosecretory cells identified as DUM neurons (Grolleau and Lapied 2000; Wicher et al. 2001). The effects of DMDS was first studied on the isolated axon. Both action potentials and ionic currents (i.e., inward sodium and outward potassium currents) recorded under current- and voltage-clamp conditions, respectively (Pelhate et al. 1990) were not affected at 100 μM DMDS (data not shown). This indicated that the voltage-dependent ionic channels underlying cockroach axonal electrical activity could not be proposed as responsible for the toxic effect of DMDS.

Consequently, we wanted to determine whether DMDS could affect the cockroach synaptic transmission between sensory fibers which originate from mechanoreceptors of the cerci and GIs (Hue and Callec 1990). Previous findings have reported that the electrical stimulation delivered on cercal nerve XI evokes composite excitatory postsynaptic potentials (cEPSP) mainly due to the activation of postsynaptic nicotinic acetylcholine receptors (nAChRs) (Hue and Callec 1990). In this study, the superfusion of the experimental chamber with saline containing DMDS (100 μM) induced postsynaptic depolarization (about 5 mV, not shown) together with a gradual decrease (15% and 90% within a period of time of 10–60 min, respectively) of both cEPSP (Fig. 2*A*, *a* and *b*) and random unitary EPSPs (Fig. 2*B*, *a* and *b*). The synaptic transmission did not recover after DMDS application. Although it was not possible to obtain a subthreshold cEPSP by increasing the presynaptic cercal nerve stimulation during DMDS treatment, ionophoretic microapplication (300 ms in duration, 300 μA) of CCh could evoke postsynaptic potential with an amplitude very similar to that of recorded in control (Fig. 2*C*, *a* and *b*). It is interesting to mention that the DMDS effects were mimicked by 10 μM 2,4-dinitrophenol (2,4-DNP, a nonspecific mitochondrial uncoupler, data not shown). These results suggested that postsynaptic AChRs located on the GI dendritic tree cannot be considered as a synaptic target for DMDS molecules. By contrast, these experiments led us to conclude that DMDS could interfere with presynaptic processes depending on ATP known to be involved in synthesis and/or release of the neurotransmitter as it was previously demonstrated with threonine-6-bradykinin (Hue and Piek 1989). Finally, the lack of effects of 100 μM DMDS (Fig. 2*D*, *a* and *b*) observed on action potential elicited in the GI axonal membrane by passing depolarizing square current pulse (5 ms in duration, 8 nA in amplitude) confirmed the results obtained in both current- and voltage-clamp conditions on isolated giant axon. From these results, it is tempting to suggest, among other possibilities, that the alteration of the neurotransmitter release depending on intracellular ATP observed at presynaptic level might reflect an alteration in the oxydative phosphorylation process induced by DMDS.

To substantiate this hypothesis, isolated short-term cultured DUM neurons were used to facilitate the study of the neurotoxic effect of DMDS on the electrophysiological properties of individual cells (Fig. 3). The somata of DUM neurons generate overshooting sodium-dependent action potentials and are characterized by a membrane potential depending on the external

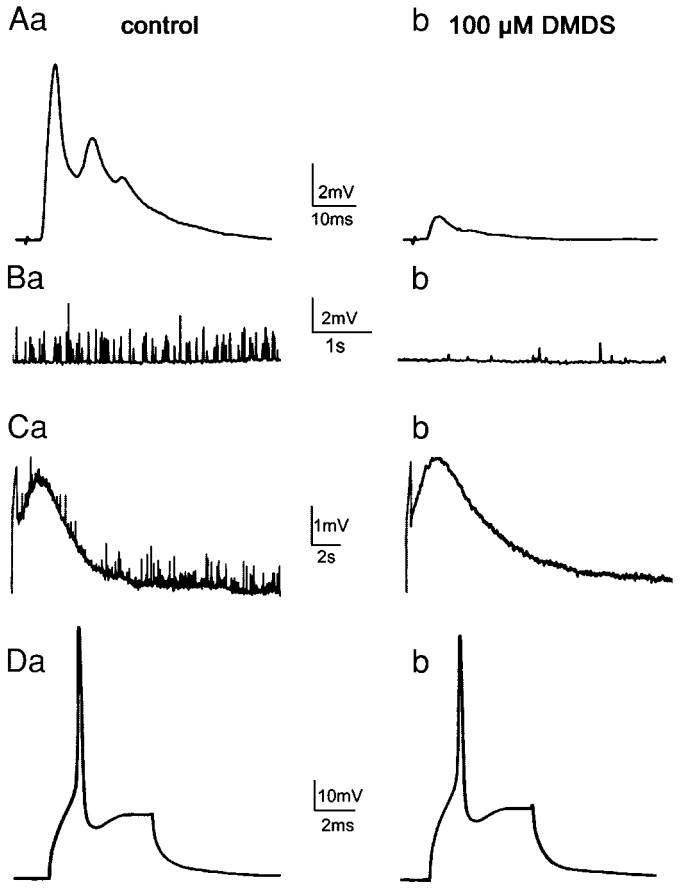

FIG. 2. Effects of DMDS on cholinergic synaptic transmission at the cercal-nerve giant interneuron synapse of the cockroach. *A*: subthreshold composite excitatory postsynaptic potentials (cEPSPs) triggered by presynaptic (cercal nerve) electrical stimulation (*Aa*) was irreversibly reduced following application of 100 μM DMDS (*Ab*). *B*: in the same way, the random unitary EPSPs were also strongly reduced in amplitude after DMDS treatment (*Bb*) compared with control (*Ba*). By contrast, the postsynaptic potential evoked by iontophoretic microapplication (300 ms in duration, 300 μA) of carbamylcholine (CCh) (*Ca*) was not affected by superfusion of DMDS (*Cb*). Similar results were observed when 100 μM DMDS was tested on the action potential (*D*, *a* and *b*) elicited in the giant interneuron (GI) axonal membrane by passing a 5-ms depolarizing square pulse (8 nA in amplitude).

concentration of both sodium and potassium (Grolleau and Lapied 2000). When isolated DUM neuron cell body was superfused with 100 μM DMDS, two distinct effects depending on time of application were observed. As illustrated in Fig. 3*Aa*, the amplitude of the action potentials, triggered by a depolarizing current pulse (0.7 nA, 50 ms in duration) slightly decreased 10 min after applying 100 μM DMDS. This effect was associated with a hyperpolarization of the membrane potential (23.6 $\pm$ 2.8 mV, $n = 6$) obtained 16 min after DMDS treatment (Fig. 3*Aa*). Because it is known that the sodium inward current is responsible for the depolarizing phase of action potentials (Lapied et al. 1990), we tested DMDS (100 μM) on the inward sodium current under voltage-clamp condition (Fig. 3*B*). As expected from current-clamp experiments, the amplitude of the inward sodium current elicited by a 30-ms depolarizing pulse to 0 mV from a holding potential of –90 mV was also reduced (21.7 $\pm$ 2.4%, $n = 7$). This effect correlated well with the reduction of the action potential amplitude. To ensure that the hyperpolarization observed was due to an activation or an inhibition of the resting conductances (Grol-

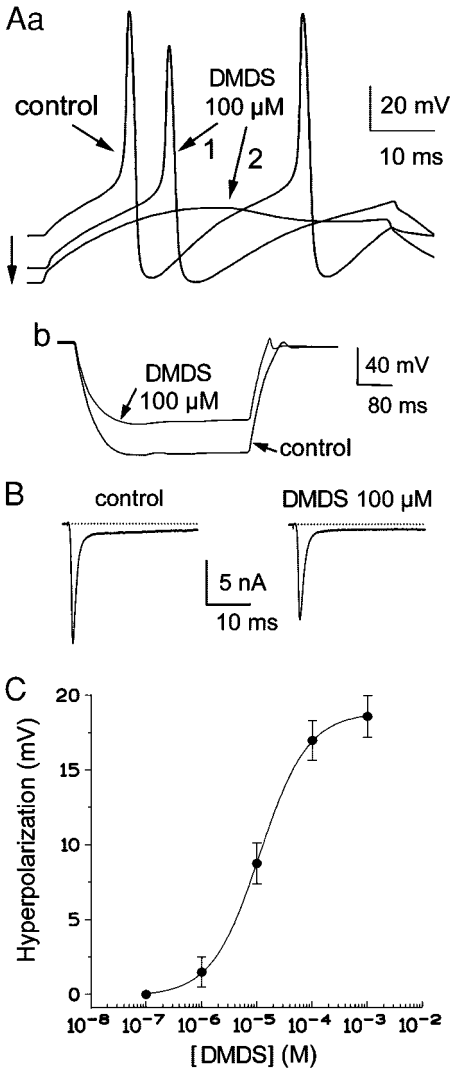

FIG. 3. Action of DMDS on the electrophysiological properties of isolated dorsal unpaired median (DUM) neurons recorded with the whole cell patch-clamp technique. *Aa*: superimposed action potentials elicited by a 50-ms depolarizing current pulse (0.7 nA) recorded from an isolated cell body held at –51 mV prior to (control) and after 10 (*1*) and 16 min (*2*) of application of 100 μM DMDS. The arrow indicated the important hyperpolarization of the membrane potential observed under DMDS treatment. *Ab*: DMDS-induced reduction of the input membrane resistance studied in response to a 400-ms hyperpolarizing current pulse. *B*: inward sodium current traces obtained by a 30-ms depolarizing pulse to 0 mV from a holding potential of –90 mV in the absence (control) and presence of 100 μM DMDS. Ionic currents were leak- and capacity-corrected. *C*: semi-logarithmic dose-response curve for the hyperpolarization of the membrane potential produced by DMDS. Smooth line represents the best fit to the mean data according to the Hill equation (r = 0.998). Data are mean values ± SE.

leau and Lapied 2000), we tested the effect of DMDS on the DUM neuron input resistance. As shown in Fig. 3*Ab*, 100 μM DMDS produced a decrease in the input resistance (by 32 ± 4%, n = 6) in response to a hyperpolarizing current pulse (400 ms in duration). This indicated that the hyperpolarization was due to the activation of a hyperpolarizing conductance and not to the loss of a depolarizing resting conductance (Grolleau and Lapied 2000). In other words, the activation of potassium channels involved in the maintenance of the membrane potential could accounted for the membrane hyperpolarization observed in the presence of DMDS. Because this last effect represents an unusual neurotoxic effect for such compounds exhibiting insecticide activity, the remaining part of this study will mainly be focused on the mode of action of DMDS on the membrane potential. To express more quantitatively the effect of DMDS on the membrane potential, isolated DUM neuron cell bodies were exposed to various concentrations of DMDS (Fig. 3*C*). Mean values of hyperpolarization were plotted against the logarithm of the noncumulative concentration of DMDS. The threshold concentration inducing hyperpolarization was about 500 nM, and this hyperpolarization became more important with increasing DMDS concentration (Fig. 3*C*). The sigmoid curve corresponded to the best fit (r = 0.998) according to the Hill equation. The EC_{50} value estimated for DMDS (i.e., the concentration of DMDS that produces 50% increase of the hyperpolarization) was 8.7 μM. For comparison, the corresponding concentration calculated from the LC_{50} (1.01 μl/l air) estimated from the study of the in vivo toxicity performed with adult cockroaches was 11.2 μM (DMDS density 1.046). The maximum hyperpolarization was obtained at a concentration of 1 mM. As indicated above, because it appeared that the potassium channels could be suspected to be involved in the hyperpolarization produced by DMDS, different potassium channel blockers were tested (Fig. 4*A*). We first examined the effect of the most commonly used blocker, TEA-Cl, known to inhibit potassium channels in DUM neurons (Grolleau and Lapied 2000). As illustrated in Fig. 4*A*, TEA-Cl (5 mM) was a weak inhibitor of the hyperpolarization observed after application of 100 μM DMDS. Similar effects were obtained with higher TEA-Cl concentrations (i.e., 10 mM, data not shown). By contrast, sulfonylurea drugs glibenclamide and tolbutamide, known to block ATP-sensitive potassium (K_{ATP}) channels (Inagaki et al. 1996) strongly reduced the effect of DMDS. As indicated in Fig. 4*A*, bath application of DMDS in the presence of 100 μM glibenclamide and 100 μM tolbutamide only induced a small hyperpolarization (17.5 ± 2.3% and 12.4 ± 4.5%, respectively, n = 4) compared with control (100%). This suggested that K_{ATP} channels could be involved in the hyperpolarization induced by DMDS. The following experiments were designed to reinforce this hypothesis. In all the DUM neuron tested, glibenclamide (100 μM) alone caused an important depolarization (more than 25 mV), suggesting that these K_{ATP} channels were activated under resting condition and contributed to the membrane potential of DUM neurons. Interestingly, under this experimental condition, DMDS only produced a small conductance change (i.e., 2–4 mV of hyperpolarization, Fig. 4*B*). As illustrated in the inset of Fig. 4*B*, the decrease in input resistance provoked by 100 μM DMDS (see Fig. 3*Ab*) can be counteracted by glibenclamide (100 μM). Finally, for comparison, it should be noted that 100 μM diazoxide, a well-known K_{ATP} channel opener (Ashcroft and Gribble 2000), induced a similar DUM neuron hyperpolarization to that of DMDS (Fig. 4*A*). Because K_{ATP} channels are closed for high concentration of cytoplasmic ATP and are open when the ATP concentration decreases below a threshold (e.g., Ashcroft and Gribble 1998), DUM neuron K_{ATP} channels can also be identified by their sensitivity to various intracellular ATP concentrations. Using the conventional whole cell recording configuration, we examined the DMDS sensitivity of the DUM neuron K_{ATP} channels by combining incubation of isolated cell bodies with 100 μM DMDS with dialysis of different ATP concentration pipette solutions. After 4 min of dialysis,

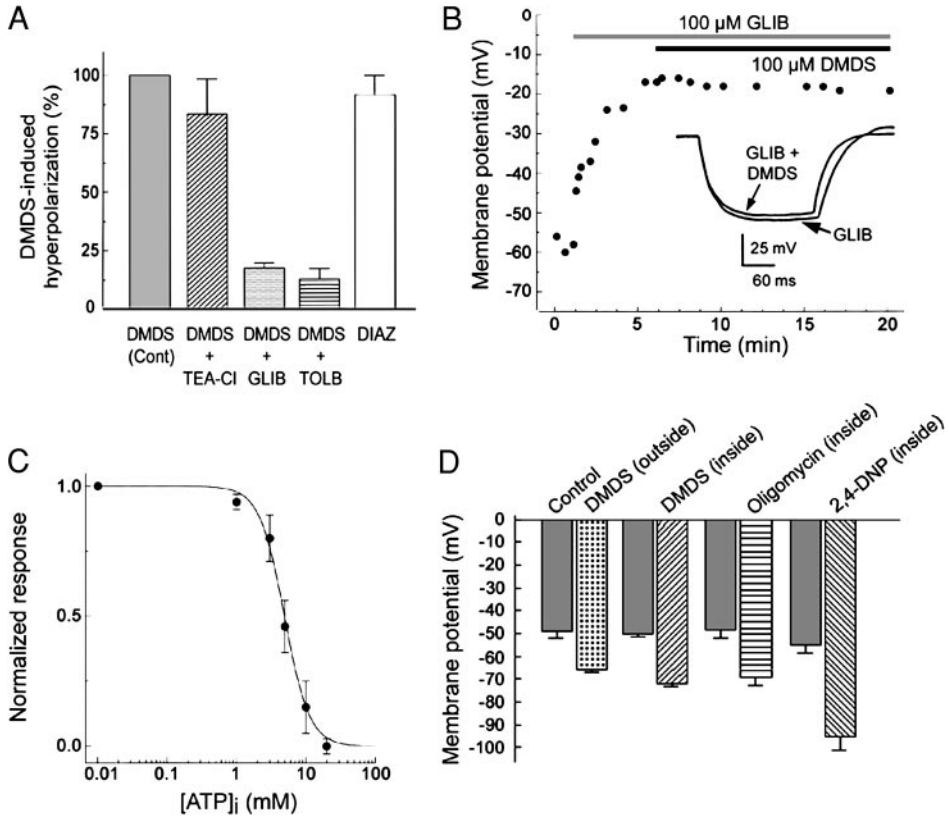

FIG. 4. DMDS activates K_{ATP} channels in DUM neuron cell bodies. *A*: histogram summarizing the mean percentage of DMDS-induced hyperpolarization recorded before (cont) and in the presence of 5 mM TEA-Cl (a nonselective potassium channel blocker), 100 μM glibenclamide (GLIB), and 100 μM tolbutamide (TOLB) known to block specifically the K_{ATP} channels. For comparison, the well known K_{ATP} channel opener diazoxide (DIAZ, 100 μM) was also tested. Note that diazoxide induced a similar hyperpolarization compared with DMDS. *B*: time course of depolarization of the membrane potential induced by 100 μM glibenclamide (GLIB) alone. Note that bath application of 100 μM DMDS in the presence of 100 μM glibenclamide only produced a slight effect on the membrane potential. *Inset*: membrane potential recorded in response to a hyperpolarizing current pulse (150 ms in duration) in glibenclamide (GLIB, 100 μM) and in the presence of 100 μM DMDS added to GLIB. *C*: study of the DMDS sensitivity of the DUM neuron K_{ATP} channels by combining incubation of isolated cell bodies with DMDS (100 μM) with dialysis of different ATP concentrations added in the patch pipette solution. The Hill plot of intracellular ATP sensitivity represents the concentration-response curve for ATP inhibition of DMDS effect. Note that the DMDS-induced hyperpolarization was almost fully inhibited for high ATP concentration. *D*: histogram illustrating the effects of DMDS applied externally or intracellularly through the patch pipette solution. These effects observed on the membrane potential were compared with those induced by intracellular application of 100 μM oligomycin (a mitochondrial ATPase inhibitor) and 100 μM 2,4-DNP (a nonspecific mitochondrial uncoupler). Data are means ± SE.

stable DMDS-induced hyperpolarizations were obtained depending on the ATP concentration pipette solutions. Figure 4*C* shows the corresponding construction of the ATP-dose inhibition curve. The DMDS-induced hyperpolarization almost was fully inhibited by internal ATP concentrations above 10 mM and was approximately half-maximal at 5 mM. At low ATP concentration (10 μM), the effect of DMDS on the membrane potential was maximum. The apparent IC_{50} for DMDS-induced hyperpolarization inhibition by internal ATP was 5.6 mM, according to the Hill equation ($r = 0.999$).

It is well known in the literature that K_{ATP} channels are an important class of ionic channels, linking bioenergetic metabolism to membrane excitability. Furthermore, K_{ATP} channels were sometimes associated with metabolic dysfunction since they were opened or closed in response to decrease and increase internal ATP concentrations, respectively. Consequently, intracellular factors such as mitochondrial dysfunction are believed to play an important role in the alteration of the K_{ATP} channels activity. In our context, it was tempting to suggest that the DUM neuron K_{ATP} channels might be one the targets acting as direct functional response element to DMDS-induced mitochondrial dysfunction, which thereby produced changes in intracellular ATP concentration. To substantiate this hypothesis, we first applied DMDS intracellularly (i.e., trough the intrapipette solution) or extracellularly (i.e., through the bathing solution superfusing the DUM neuron cell body). In both cases (Fig. 4*D*), DMDS (100 μM) induced an important hyperpolarization of the membrane potential [from –50.2 ± 1.2 ($n = 5$) to –71.9 ± 1.1 ($n = 7$) and from –49.3 ± 2.8 ($n = 5$) to –65.8 ± 1.5 ($n = 6$), respectively]. These results indicated that DMDS could cross the membrane to exert its neurotoxic effect on the K_{ATP} channels. Then we compared the effect of DMDS to oligomycin (100 μM; a mitochondrial ATPase inhibitor) and 2,4-DNP (100 μM), which were introduced into the cell body by diffusion through the patch pipette. Figure 4*D* illustrates that both compounds were capable of producing a hyperpolarization of the membrane potential [from –48.3 ± 3.8 ($n = 5$) to –68.6 ± 3.5 ($n = 5$) and from –55.2 ± 3.5 ($n = 5$) to –95.4 ± 5.6 ($n = 6$), respectively]. They also indicated that a decrease in intracellular ATP concentration,

following mitochondrial dysfunction, could activate DUM neuron K_{ATP} channels. It should be noted that we never observed any effect of bath applied DMDS using oligomycin in the patch pipette (data not shown). Together these results seemed to indicate that DMDS could indirectly activate K_{ATP} channels, leading to a hyperpolarization, via an inhibition of mitochondrial respiration that thereby decreased intracellular ATP concentration. To further understand this unusual neurotoxic mechanisms, we decided to examine further the effect of DMDS on the different mitochondrial respiratory chain complexes.

Effects of DMDS on cellular respiration

The effect of DMDS on animal cellular respiration was first investigated with cultured *D. melanogaster* S2 cells (Towers and Sattelle 2002), which gave us higher cell density than isolated DUM neurons for such investigation. The oxygen consumption of S2 cells in the exponential phase of growth was measured with an oxygen electrode. The respiration rate was constant and totally inhibited by 1 mM cyanide (results not shown). Addition of increasing concentrations of DMDS inhibited progressively the oxygen consumption (Fig. 5), which was reduced to 25% of the initial rate in the presence of 30 μM DMDS [i.e., from 11.2 nmolO_2/ml (control) to 2.7 nmolO_2/ml; see Fig. 5]. The residual oxygen consumption was abolished by 1 mM cyanide. When the cells were preincubated for 10 min with DMDS, the inhibition appeared stronger (i.e., 56% inhibition obtained with only 10 μM DMDS, not shown). This strong inhibition suggests that the existence of a diffusion barriers in intact cell prevents the rapid diffusion of DMDS toward its site of action. The inhibition of S2 cells respiration led us to further directly investigate the DMDS effect on the respiratory metabolism of isolated plant mitochondria.

EFFECTS OF DMDS ON PLANT MITOCHONDRIA. The effect of DMDS on the respiratory metabolism was tested using plant mitochondria isolated from imbibed pea (*P. sativum* L.) seeds. The great advantage of plant mitochondria with respect to their animal counterparts is that the respiratory electron-transport pathways comprise the cyanide-sensitive cytochrome pathway (like in animals) and the cyanide insensitive alternative pathway that consists of only one protein the alternative oxidase (AOX); beyond the branch point (ubiquinone, see for details Fig. 8*A*), the alternative pathway does not contribute to the generation of a proton-motive force, in contrast to the cytochrome oxidase pathway (Moller and Rasmusson 1998; Vanlerberghe and McIntosh 1997). Furthermore, in plant mitochondria, the NAD(P)H dehydrogenases is distinct from complex I (Moller and Rasmusson 1998). These additional features make plant mitochondria a suitable model to better identified which complex could be specifically affected by DMDS using one of the two pathways available in plant and not in animal mitochondria.

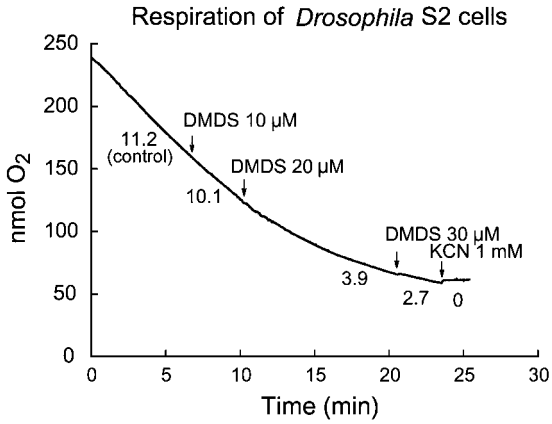

FIG. 5. Effect of DMDS on the cellular respiration of the cultured *D.melanogaster* S2 cell line. Graph represents the oxygen consumption of S2 cells recorded at 30°C using an oxygen electrode. Arrows indicate the addition of the different inhibitors tested (final concentration). The numbers below the trace indicate the rate of respiration in nmol O_2/ml.

The function of isolated mitochondria was assessed by measurement of oxygen consumption using different energy substrates in control and in the presence of DMDS (see Fig. 6). Isolated mitochondria will exhibit an initial slow rate of oxygen consumption in the presence of energy substrate. The addition of ADP will stimulate electron transport chain activity and will initiate a rapid consumption of oxygen. Consequently, using pea seed–purified mitochondria, we analyzed the effects of DMDS on the oxidation of various substrates that were monitored with the oxygen electrode. DMDS (10 μM) was diluted in the electrode buffer just before starting the experiment and placed in the reaction chamber containing mitochondria. The oxidation of succinate, measured as the state III rate (in the presence of ADP), was strongly reduced by 10 μM DMDS (44% inhibition; Fig. 6, *A* and *B*), suggesting an inhibition of electron transfer. The decreased rate was likely not due to an inhibition on the ATP synthetase since a strong uncoupler like P-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) did not reverse the DMDS inhibition (data not shown). A similar effect of DMDS was observed on the oxidation of exogenous NADH (51% of inhibition, Fig. 6, *C* and *D*) and pyruvate (40% of inhibition, Fig. 6, *E* and *F*). The oxidation of these three substrates (i.e., succinate, pyruvate, and NADH) involves different dehydrogenases that feed a common electron pathway from ubiquinones to complex III, cytochrome c, and complex IV. Therefore we thought that DMDS could either exert a general effect on all components or inhibit a specific component in the common pathway (i.e., cytochrome oxidase pathway). Careful observation of the pyruvate oxidation graph (Fig. 6, *E* and *F*) shows that the DMDS effect was less pronounced (21% of inhibition) on the respiration rate after cyanide addition. In fact, in the presence of pyruvate and when complex IV was blocked by cyanide, the electrons from reduced ubiquinones were directly transferred to oxygen through the cyanide-insensitive AOX pathway (Vanlerberghe and McIntosh 1998; Fig. 8*A*). This cyanide-insensitive respiration shown in Fig. 6, *E* and *F*, was inhibited by propylgallate, which is an inhibitor of the AOX pathway. The weaker inhibitory effect of DMDS on this cyanide-resistant pathway suggested that its site of action was more likely localized in the complexes III-IV segment of the electron transfer chain than in the set of initial dehydrogenases.

To evaluate a possible effect at the level of complex III, we analyzed the effect of DMDS on the electron transfer from exogenous NADH to cytochrome c, thus bypassing complex IV. This was made by following spectrophotometrically the NADH-dependent reduction of exogenous cytochrome c after blocking complex IV with cyanide and rupturing the outer membrane with a calibrated amount of Triton X 100. Even at

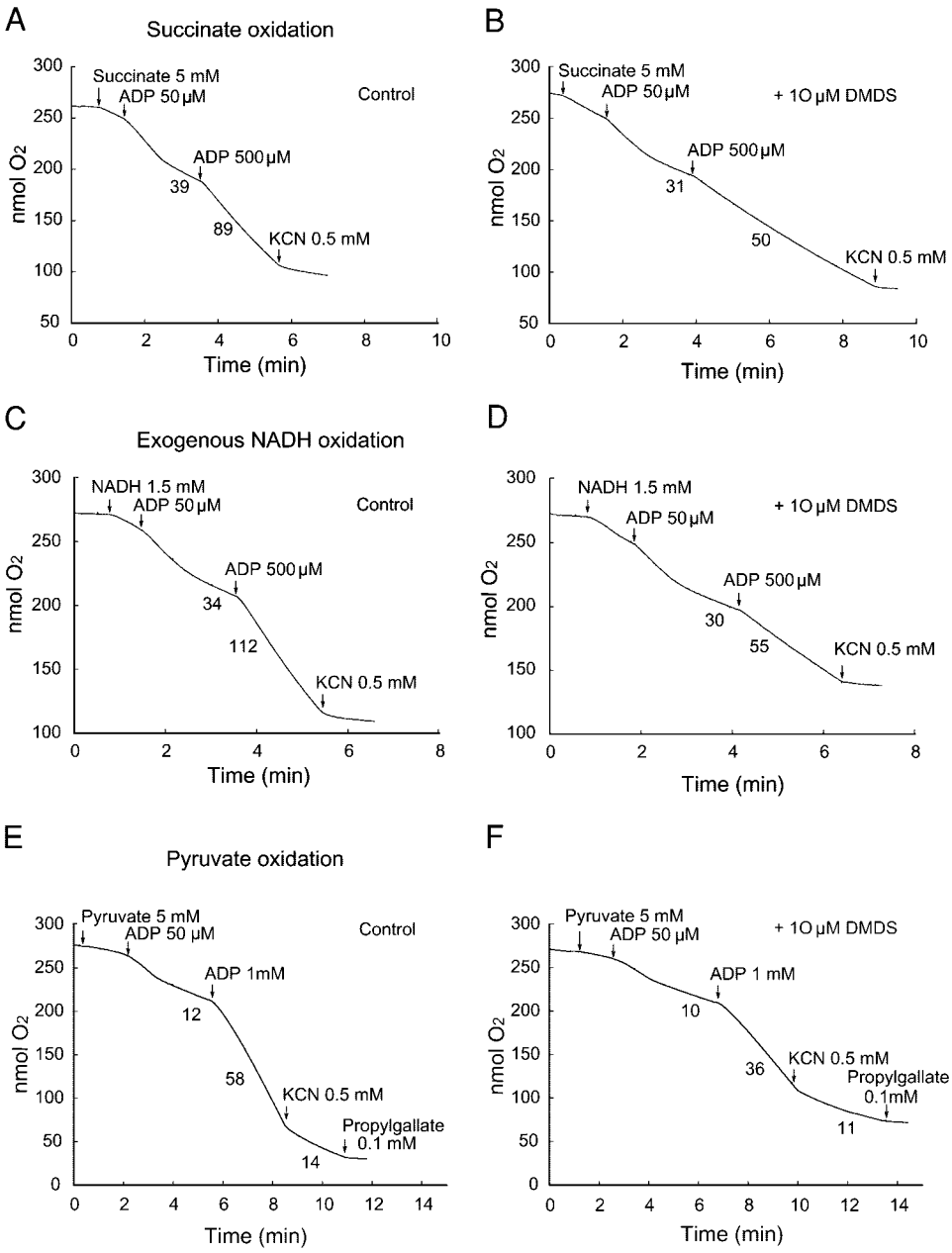

FIG. 6. Effect of DMDS on substrate oxidation by plant mitochondria. The oxygen consumption of purified pea seed mitochondria was monitored at 25°C using an oxygen electrode as described in METHODS. For each substrate, succinate (*A*), NADH (*C*), and pyruvate (*E*), the *left graph* represents control experiments and the *right graph* illustrates the same experiments performed in the presence of 10 μM DMDS (*B*, *D*, and *F*). Arrows indicate the addition of the different substrates, cofactors, and inhibitors tested. For pyruvate oxydation (*E* and *F*), the electrode buffer medium was supplemented with 1 mM NAD, 0.3 mM thiamine pyrrophosphate, and 0.5 mM malate. Numbers below traces refer to the rates of the oxygen consumption (nmol O_2/min/mg protein).

40 μM, DMDS was not able to inhibit the electron transfer from NADH to cytochrome c since the initial rate of reduction was almost identical to the control (Fig. 7*A*). The highest value obtained for the plateau is due to small variations in the cytochrome c concentration in the experiment. Since all these results focused on complex IV as the site of action of DMDS, we analyzed its impact on cytochrome oxidase activity. Mitochondrial membranes were solubilized by Triton X 100, and oxidation of reduced cytochrome c by cytochrome oxidase was followed spectrophotometrically. The spectra shows that 40 μM DMDS totally blocked the cytochrome oxidase, yielding the same effect with 0.8 mM cyanide (Fig. 7*B*). Lower concentrations of 4 and 0.4 μM were still effective in inhibiting the electron transfer through complex IV.

Taken together, these results obtained on plant mitochondria that display two respiratory electron-transport pathways including the cyanide-sensitive cytochrome pathway (common to animal) and the cyanide-insensitive alternative oxidase pathway allowed us to precisely identified within the electron transport chain the target site of DMDS. This sulfur compound, in the micromolar range, is a powerful inhibitor of complex IV (cytochrome oxidase).

EFFECTS OF DMDS ON DUM NEURON RESPIRATION. Because it was necessary to determine if DMDS neurotoxicity observed in DUM neurons occurred through mitochondrial respiratory chain complex IV dysfunction, we tested on isolated cell body KCN, which is commonly used in vitro as a rapid specific inhibitor cytochrome oxidase, the terminal enzyme of the electron-transport chain that catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen. As illustrated in Fig. 7*C*, bath application of 10 μM KCN produced an important hyperpolarization of the membrane potential [from –55.1 ± 0.6 mV (n = 4) to –82.1 ± 1.3 mV, n = 4]. It is

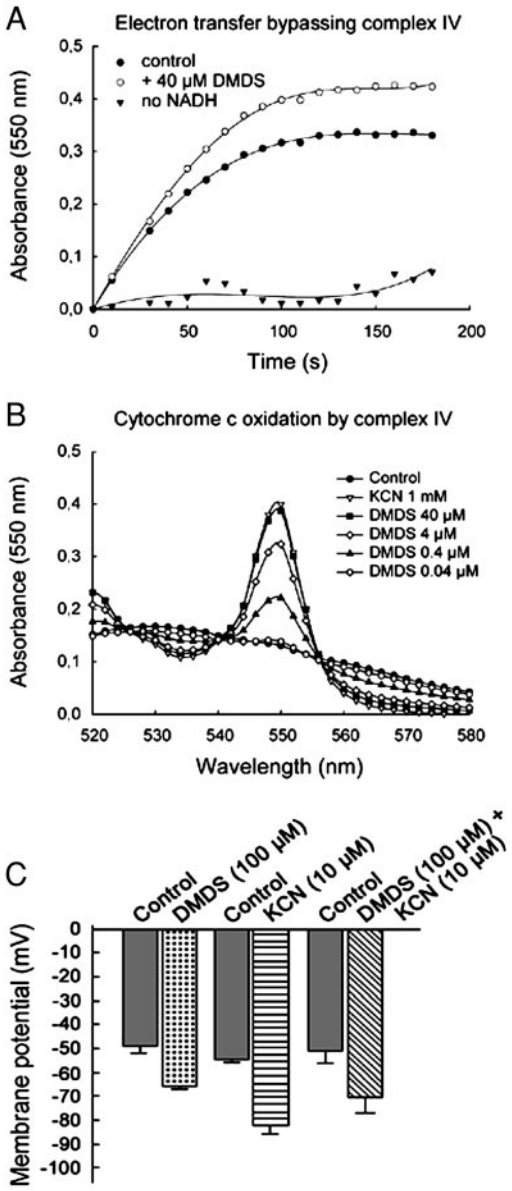

FIG. 7. Effect of DMDS on complex IV (cytochrome oxydase). *A*: time course of the mitochondrial electron transfer from exogenous NADH to cytochrome c was followed spectrophotometrically by measuring the decrease of absorbance of cytochrome c at 550 nm and hence the reduction of cytochrome c by the truncated electron chain transfer. *B*: to measure complex IV activity, the mitochondrial membranes were incubated for 3 min at 25°C in a reaction mixture containing 40 μM reduced cytochrome c and different concentrations of DMDS. Addition of 1 mM cyanide (KCN), known to block the electron transfer in complex IV, was also tested. The cytochrome c spectra were recorded at the end of the reaction to visualize the oxido-reduction state of cytochrome c (the reduced form absorbs at 550 nm). *C*: histogram illustrating the effects of external application of DMDS on the membrane potential compared with that induced by extracellular application of 10 μM KCN (a specific cytochrome oxidase inhibitor). Pretreatment of isolated DUM neuron cell body by 100 μM DMDS did not allow 10 μM KCN in producing additional effect on the membrane potential ($n = 4$). Data are means ± SE.

interesting to note that KCN did not produce any significant effect when DUM neurons were pretreated with 100 μM DMDS (Fig. 7*C*). These results strongly suggest that DMDS might affect the complex IV of the DUM neuron electron-transport chain.

DISCUSSION

The present study reports for the first time that a volatile natural compound, the plant-derived pesticide DMDS exerts a novel insecticidal activity via an inhibition of complex IV (cytochrome oxidase) in the mitochondrial electron transport system, which thereby affects neuronal K_{ATP} channel activity, never characterized, until now, in insect neurons. The precise mode of action of DMDS could be summarized in Fig. 8*A*, which illustrates the dissection of the electron transfer chain of plant mitochondria that was used in this study. Plant mitochondria are known to possess a flexible electron transfer chain with additional NAD(P)H dehydrogenases and a cyanide insensitive oxidase (AOX) pathway at the level of the ubiquinone pool. The functional dissection of the electron transfer chain led us to conclude that the site of action of DMDS is similar to cyanide and corresponds to the terminal oxidase of respiratory

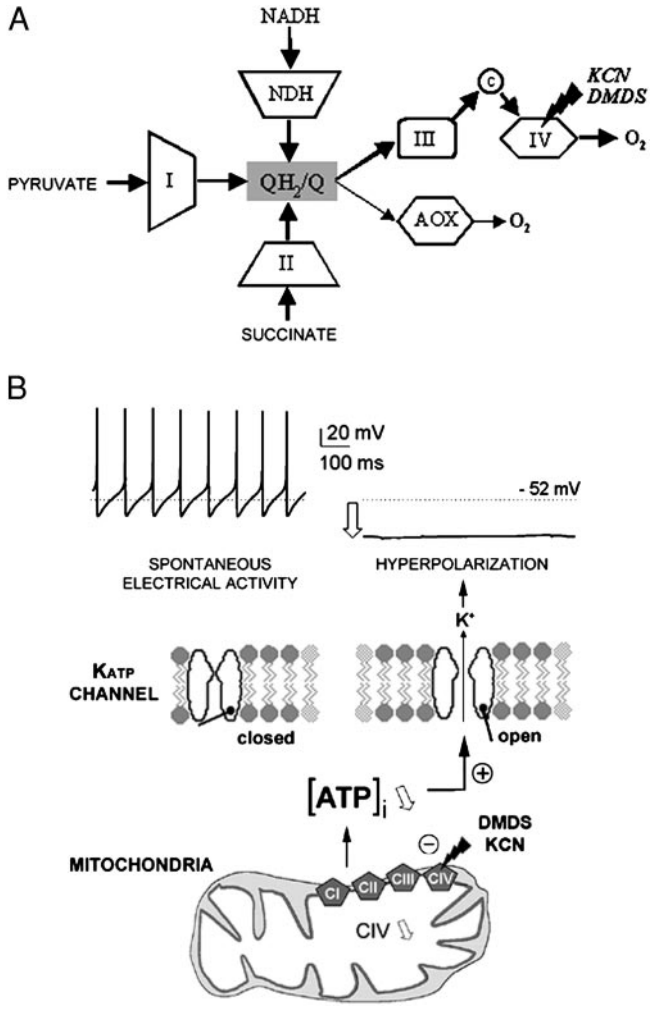

FIG. 8. *A*: simplified pattern of the electron transfer in plant mitochondria. Scheme illustrates the electron transfer pathway that was analyzed in this study. I, II, III, and IV represent the different mitochondrial respiratory chain complexes. NDH, NADH external dehydrogenase; QH_2/Q, ubiquinone pool; AOX, alternative oxidase (cyanide insensitive); c, cytochrome c; KCN, potassium cyanide. *B*: schematic illustration of the effect of complex IV (cytochrome oxidase) inhibition in single DUM neuron. *Left*: DUM neuron show pacemaker activity recorded under physiological conditions. Most K_{ATP} channels are closed. *Right*: in the presence of the complex IV inhibitor, DMDS (100 μM), K_{ATP} activate that leads to a membrane hyperpolarization of the DUM neuron associated with a complete loss of pacemaker activity.

electron transfer chains. At the cockroach CNS level, DMDS affects presynaptic processes that inhibit the neurotransmitter release and finally block the cholinergic synaptic transmission. In this case, the DMDS effect mimicked by the 2,4-DNP indicates that the disruption of energy metabolism is involved. However, in this case the toxic effect could not be attributed to the activation of pre- or postsynaptic K_{ATP} channels since diazoxide or glibenclamide did not mimic or block the effect of DMDS, respectively (data not shown). By contrast, in neurosecretory cells in which 2,4-DNP, oligomycin but also KCN (known to block the electron transport chain complex IV) produce similar effect to that of DMDS, the neurotoxic mode of action could be proposed according to the hypothetical scheme illustrated in Fig. 8*B*. Under physiological control conditions, octopaminergic DUM neurons show pacemaker activity and most of K_{ATP} channels are closed. The disruption of energy metabolism produced by the DMDS-induced inhibition of complex IV and the subsequent decrease of intracellular ATP concentration result in the activation of K_{ATP} channels. That leads to a membrane hyperpolarization of the DUM neurons, which is accompanied by a complete loss of spontaneous action potentials. Because cockroach DUM neurons are known to be involved in the control of vital physiological functions (Grolleau and Lapied 2000; Sinakevitch et al. 1996), DMDS, undoubtedly will produce crucial downstream consequences in insect. Moreover, because insect neuronal K_{ATP} channels have never been characterized before this study, our results suggest that these channels might correspond to a novel target site for such insecticides acting through mitochondrial dysfunction. This is reinforced by the fact that DMDS effects occur similarly following an external as well as an internal application mode. This might suggest that DMDS is highly membrane permeable, easy to diffuse over the cells and to penetrate into mitochondria. Such properties give to DMDS advantage for proposal as a good candidate as insecticidal compound.

Mitochondria enzymes as insecticide target

Many insecticidal activities occur due to the opening and closing of specific ion channel proteins embedded within the nerve membrane. The insect voltage-gated sodium channel is the well-established target of a variety of insecticides including DDT, pyrethroids, N-alkylamides, and the recently introduced oxadiazine Indoxacarb (Lapied et al. 2001; Zlotkin 1999). A large subtype diversity of cholinergic or GABA cell membrane receptors are also altered by other classes of insecticidally active molecules such as neonicotinoids and phenylpyrazoles (Bloomquist 2001; Nauen et al. 2001). Beside these most extensively known insecticidal targets, the mitochondria, which is responsible for most ATP production, is also targeted by pesticides (Schuler and Casida 2001). The disruption of energy metabolism usually results of either an inhibition of the electron transport system or an uncoupling of the transport system from ATP production. Some compounds block the production of ATP through an inhibition of the electron transport system and causes a decrease in oxygen consumption by the mitochondria. The common binding site in the electron transport chain is the complex I (NADH/ubiquinone oxidoreductase), which catalyzes the transfer of electrons from NADH to coenzyme Q through flavin mononucleotide. For instance, a number of insecticide or miticide synthetic compounds such as rotenone, pyridaben, fenazzaquin, and fenpyroximate act as complex I inhibitors. Furthermore, a small group of molecules called uncoupling agents (e.g., the original uncoupler dinitrophenol) are able to dissociate the oxidation of substrates from the manufacture of ATP. In this case, the transport system is not affected, the oxygen consumption increases but a dissipation of the proton gradient across the inner mitochondrial membrane does not allow a normal production of ATP. The newly developed compound chlofenapyr acts similarly, offering promise for further development of uncoupler for pest control. Finally, except for methyl bromide (CH_3Br) for which an effect on cellular respiration is only suspected, a number of fumigants including hydrogen phosphide (PH_3 or phosphine), are chemicals also known to induce mitochondrial dysfunction (Price 1985). However, as indicated below, such compounds display strong secondary effects and/or numerous disadvantages.

Can DMDS be used as fumigant?

Although fumigation has become an endangered technology due to pressures regarding environmental contamination and health concerns, it still remains one of the most effective methods for the protection of stored food against insect infestation. At present only two fumigants are still in use: CH_3Br and phosphine. However, as indicated in the Introduction, CH_3Br presents many disadvantages. As a consequence, the use of another fumigant such as phosphine was proposed. However, although phosphine does not interfere with germination (Sittisuang and Nakakita 1985), many investigations pointed out major disadvantages including the time required to eliminate the target pest (ranging from 3 to 7 days), the development of resistance in several species of stored product insects (Price 1991; Zettler 1991; Zettler et al. 1989) and the important oxidative damage observed in mammals. In this context, volatil *Allium* sulfurs such as DMDS is expected to increase in use due to the limited duration of legal use of both CH_3Br and phosphine. DMDS is highly volatile, mixes readily with air, and acts in gaseous state. Because of its small molecule, DMDS diffuses quickly and penetrates commodities more quickly than other fumigants. Furthermore, this study shows, for the first time, that DMDS, decreases ATP production via an inhibition of the mitochondrial respiratory chain complex IV (cytochrome oxidase). The fact that DMDS alters the electron transfer chain in a manner distinct from that of most of the compounds used (see Schuler and Casida 2001), it might be anticipated that resistant insects would show low cross-resistance to DMDS.

However, although one of the most attractive aspects of using DMDS as crop protectant is its high toxicity against a range of insect pests including the eggs, larvae, and adults in comparison with the well-known fumigants CH_3Br or thiosulfinates (Auger et al. 1994; Dugravot et al. 2002), it should be pointed out that DMDS also showed a mammalian toxicity. Previous findings established that DMDS was the causative agent in kale poisoning of cattle. Typical signs of kale poisoning were circulating of DMDS in the blood, Heinz body formation in the erythrocytes, and loss of body weight (Steven et al. 1981). Our study also reveals that the CL_{50} for the most susceptible insect (*D. basalis*) is only five times lower than that

measured for mice. Consequently, for the strategy of insecticide research, all together these results may implicate to look for DMDS derivatives with higher insecticidal activity. Finally, although the specific effect of DMDS as complex IV inhibitor make it ideal probe in the dissection of the function of the mitochondrial electron transport chain, another interesting facet of our study is that this toxic effect observed in pacemaker DUM neurons is associated with the K_{ATP} channel activation inducing hyperpolarization. It is known that chronic K_{ATP} channel activity could have fatal consequences particularly at neuronal level. Several compounds, which inhibit the mitochondrial electron transport chain, were considered probable suspect of nigral pathology in humans such as Parkinson's disease. For example, chronic brain infusion of low doses of the complex I inhibitor rotenone gives rats a Parkinsonian syndrome (Jenner 2001; Liss and Roeper 2001). Among the pathophysiological response to mitochondrial dysfunction previously observed, it seems now evident that the complex I inhibition is not the only reason for the vulnerability of neuronal cells to neurodegenerative process. Complex IV inhibition and its potential downstream consequences like K_{ATP} activation could also represent an epidemiological linkage between insecticide and the incidence of Parkinson's disease. Because previous studies indicated that aminergic pacemaker DUM neurons (Grolleau and Lapied 2000) were considered as interesting neuronal biomedical model for investigating the neurotoxic effect of anticancer agent (Grolleau et al. 2001), they could represent an exciting alternative way for studying, in our context, the place of complex IV deficiency in the neurodegenerative mechanism.

In conclusion, our results show that DMDS is a potent specific complex IV inhibitor in DUM neurons. Since alteration of the mitochondrial electron transport is a very generalized action, it seems conceivable to assume that other cells and/or physiological mechanisms are also affected (i.e., cockroach synaptic transmission between sensory fibers and GIs). It is also probable that DMDS has only little phytotoxicity since we showed that plant possess a DMDS-insensitive oxidative pathway. However, the mammal toxicity observed requires serious attention regarding a possible use of DMDS for pest control. More generally, research on new natural molecules for crops protection or pest management is becoming of growing interest in view of health hazard to humans, environmental insecurity as well as prevalence of insect resistance accounting for existing insecticides. Based on its great potential as fumigant, pest control using DMDS might be feasible and future work is highly recommended in this respect.

REFERENCES

Arthur FH. Grain protectants: current status and prospects for the future. *J Stored Prod Res* 32: 293–302, 1996.

Ashcroft FM and Gribble F. Correlating structure and function in ATP-sensitive K^+ channels. *Trends Neurosci* 21: 288–294, 1998.

Ashcroft FM and Gribble F. New windows on the mechanism of action of KATP channel openers. *TIPS* 21: 439–445, 2000

Auger J, Ferary S, and Huignard J. A possible new class of natural sulfur pesticide for fumigation. *Ecology* 25: 27–35, 1994.

Auger J, Lecomte C, and Thibout E. Leek odour analysis by gaz chromatography and identification of the most active substance for the leek moth *Acrolepiopsis assectella. J Chem Ecol* 15:1847–1854, 1989.

Bell CH. Fumigation in the 21st century. *Crop Protect* 19: 563–569, 2000.

Bloomquist JR. GABA and glutamate receptors as biochemical sites for insecticide action. In: *Biochemical Sites of Insecticide Action and Resistance*, edited by Ishaaya I. Berlin: Springer-Verlag, 2001, p. 17–41.

Brattsen LB. Metabolic defenses against plant allelochemicals. In: *Herbivores: Their Interaction with Secondary Plant Metabolites*, edited by Rosenthal GA and Janzen DH. New York: Academic, 1992, p. 175–241.

Buckingham SD, Lapied B, LeCorronc H, Grolleau F, and Sattelle DB. Imidacloprid actions on insect neuronal acetylcholine receptors. *J Exp Biol* 200: 2685–2692, 1997.

Carlini CR and Grossi-de-sa MF. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon* 40: 1515–1539, 2002.

Douce R, Bourguignon J, Brouquisse R, and Neuburger M. Isolation of plant mitochondria: general principles and criteria of integrity. *Methods Enzymol* 148: 403–415, 1987.

Dugravot S, Sanon A, Thibout E, and Huignard J. Susceptibility of *Callosobruchus maculatus* (Coleoptera: Bruchidae) and its parasitoid *Dinarmus basalis* (Hymenoptera: Pteromalidae) to sulphur-containing compound: consequences on biological control. *Environ Entomol* 31: 550–557, 2002.

Feyereisen R. Insect P450 enzymes. *Annu Rev Entomol* 44: 507–33, 1999.

Finney DL. *Probit Analysis*, 3rd ed. Cambridge, UK, 1971.

Grolleau F, Gamelin L, Boisdron-Celle M, Lapied B, Pelhate M, and Gamelin E. A possible explanation for a neurotoxic effect of the anticancer agent oxaliplatin on neuronal voltage-gated sodium channels. *J Neurophysiol* 85: 2293–2297, 2001.

Grolleau F and Lapied B. Dorsal unpaired median neurones in the insect central nervous system: towards a better understanding of the ionic mechanisms underlying spontaneous electrical activity. *J Exp Biol* 203: 1633–1648, 2000.

Hue B and Callec JJ. Electrophysiology and pharmacology of synaptic transmission in the central nervous system of the cockroach. In: *Cockroaches as Models for Neurobiology: Application in Biomedical Research*, edited by Huber I, Masler EP, and Rao BR. Boca Raton, FL: CRC Press, 1990, p. 149–168.

Hue B and Piek T. Irreversible presynaptic activation-induced block of transmission in the insect CNS by hemicholinium-3 and threonine-6-bradykinin. *Comp Biochem Physiol* 93C: 87–89, 1989.

Inagaki N, Gonoi T, Clement JP, Wang CZ, Aguilar-Bryan L, Bryan J, and Seino S. A family of sulfoxylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron* 16: 1011–1017, 1996.

Jenner P. Parkinson's disease, pesticides and mitochondrial dysfunction. *Trends Neurosci* 24: 245–246, 2001.

Lapied B, Grolleau F, and Sattelle DB. Indoxacarb, an oxadiazine insecticide, blocks insect neuronal sodium channels. *Br J Pharmacol* 132: 587–595, 2001.

Lapied B, Malécot CO, and Pelhate M. Ionic species involved in the electrical activity of single aminergic neurones isolated from the sixth abdominal ganglion of the cockroach *Periplaneta americana. J Exp Biol* 144: 535–549, 1989. *0*

Lapied B, Malécot CO, and Pelhate M. Patch clamp study of the properties of the sodium current in cockroach single isolated adult aminergic neurones. *J Exp Biol* 151: 387–403, 1990.

Lindroth RL. Hydrolysis of phenolic glucosides by midgut β-glucosidases in *Papilio glaucus. Insect Biochem* 18: 789–792, 1988.

Liss B and Roeper J. ATP-sensitive potassium channels in dopaminergic neurons: transducers of mitochondrial dysfunction. *News Physiol Sci* 16: 214–217, 2001.

Moller IM and Rasmusson AG. The role of NADP in the mitochondrial matrix. *Trends Plant Sci* 3: 21–27, 1998.

Nauen R, Ebbinghaus-Kintscher U, Elbert A, Jeschke P, and Tietjen K. Acetylcholine receptors as site for developing neonicotinoid insecticides. In: *Biochemical Sites of Insecticide Action and Resistance*, edited by Ishaaya I. Berlin: Springer-Verlag, 2001, p. 77–105.

Ouedraogo AP, Sou S, Sanon A, Monge JP, Huignard J, Tran MD, and Credland PF. Influence of temperature and humidity on population of *C. Maculatus* (Coleoptera:Bruchidae) and its parasitoid *D. Basalis* (Pteromalidae) in two zones of Burkina Faso. *Bull Entomol Res* 86: 695–702, 1996.

Pelhate M, Pichon Y, and Beadle DJ. Cockroach axons and cell bodies: electrical activity. In: *Cockroaches as Models for Neurobiology: Application in Biomedical Research*, edited by Huber I, Masler EP, and Rao BR. Boca Raton, FL: CRC Press, 1990, p. 131–148.

Price NR. The mode of action of fumigants. *J Stored Prod Res* 21: 157–164, 1985.

Price NR. Enzymatic factors in the resistance of stored-product pests to insecticides. *Biochem Soc Trans* 19: 759–762, 1991.

Rauscher MD. Natural selection and the evolution of plant-insect interactions. In: *Insect Chemical Ecology*, edited by Roitberg BD and Isman MB. New York: Chapman and Hall, 1992, p. 20–88.

Schuler F and Casida JE. The insecticide target in the PSST subunit of complex I. *Pest Manag Sci* 57: 932–940, 2001.

Sinakevitch IG, Geffard M, Pelhate M, and Lapied B. Anatomy and targets of dorsal unpaired median neurones in the terminal abdominal ganglion of the male cockroach *Periplaneta americana* L. *J Comp Neurol* 367: 147–163, 1996.

Sittisuang P and Nakakita H. The effect of phosphine and methyl bromide on germination of rice and corn. *J Pestic Sci* 10: 461–468, 1985.

Sogorb MA and Vilanova E. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicol Lett* 128: 215–228, 2002.

Steven FS, Griffin MM, and Smith RH. Disulphide exchange reactions in the control of enzymic activity. Evidence for the participation of dimethyl disulphide in exchanges. *Eur J Biochem* 119: 75–78, 1981.

Taylor RW. Commodity fumigation beyond the year 2000. *Pestic Outlook* 7: 31–34, 1996.

Towers PR and Sattelle DB. A *Drosophila melanogaster* cell line (S2) facilitates post-genome functional analysis of receptors and ion channels. *BioEssays* 24: 1066–1073, 2002.

Trounce A, Kim YL, Jun AS, and Wallace DC. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. *Methods Enzymol* 264: 484–509, 1996.

Vanlerberghe GC and McIntosh L. Alternative oxidase: from gene to function. *Annu Rev Plant Physiol Plant Mol Biol* 48: 703–734, 1997.

Watson RT. Methyl bromide. Science and technology and economic synthesis report. *Fumigant Pheromone* 29: 11–24, 1992.

Wicher D, Walther CH, and Wicher C. Non-synaptic ion channels in insects—basic properties of currents and their modulation in neurons and skeletal muscles. *Prog Neurobiol* 64: 431–525, 2001.

Zettler JL. Pesticide resistance in *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae) from flour mills in the United States. *J Econ Entomol* 83: 1677–1681, 1991.

Zettler JL, Halliday WR, and Arthur FH. Phosphine resistance in insects infecting stored peanuts in the southeastern United States. *J Econ Entomol* 82: 1508–1511, 1989.

Zlotkin E. The insect voltage-gated sodium channel as target of insecticides. *Annu Rev Entomol* 44: 429–455, 1999.