

Nitromethylene actions on in situ and expressed insect nicotinic acetylcholine receptors

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Single channel recordings from dissociated housefly (*Musca domestica*) neurons show that a novel type of nitromethylene insecticide, 2(nitromethylene)tetrahydro-1,3-thiazine (NMTHT) gates a channel, the conductance and open time histogram of which resemble those obtained when acetylcholine is the agonist. Injection into *Xenopus* oocytes of a locust (*Schistocerca gregaria*) α -subunit mRNA results in the expression of functional nicotinic receptors sensitive to NMTHT. Control oocytes injected with distilled water are insensitive to the same concentration of this compound. Thus NMTHT exhibits agonist actions at both in situ and expressed insect nicotinic receptors, and one site of action of this compound is on an insect nicotinic receptor α -subunit.

Nicotinic receptor; Receptor subunit; Nitromethylene; Insect

1. INTRODUCTION

Insect ion channels are targets for many insecticides [1] including: pyrethroids and DDT [2]; cyclodienes and cyclohexanes [3]; cartap [4]. Nitromethylenes [5] are a new class of molecules that modify cholinergic synaptic transmission in insects [6,7]. Recently, both the nicotinic receptor agonist [6,8] and antagonist actions [8,9] of nitromethylenes have been detected. Since this series of compounds includes insecticidally-active molecules, it is of considerable interest to understand their mechanism of action at the receptor, and to identify the particular receptor subunit(s) involved.

Several laboratories have described acetylcholine-gated cationic channels on insect neurones (e.g. [10,11]). Three distinct channel conductances (20 pS, 32 pS, 80 pS) have been detected in cell-attached patches from dissociated housefly (*Musca domestica*) neurones [12]. As these cell body membranes are the only insect cells to date to exhibit all three conductances, they have been chosen for single channel studies of the actions of NMTHT on in situ nicotinic receptor/channel molecules.

Recently poly(A)⁺ mRNA synthesized from cDNA encoding an α -subunit (α L1) of an insect neuronal nicotinic acetylcholine receptor/channel molecule has been expressed in oocytes of *Xenopus laevis* [13,14]. Functional receptors expressed in this way differ in their

pharmacology from other nicotinic acetylcholine receptors described to date, but exhibit several of the properties of a native insect receptor/channel molecule [15]. In the present study, we describe unitary conductance measurements on in situ nitromethylene-activated channels of insect neurones. Also, an expressed insect nicotinic receptor provides a test of whether or not the α L1 subunit carries a functional binding site for a nitromethylene insecticide.

2. MATERIALS AND METHODS

2.1. Patch-clamp recordings from dissociated neurones

Neurones from embryonic houseflies (*Musca domestica*) were prepared and maintained in short-term culture as previously described [16]. Briefly, eggs were dechorionated by immersion in bleach, washed with saline, transferred into sterile polypropylene tubes and dissociated by trituration through siliconized Pasteur pipettes. Cells were pelleted by centrifugation and resuspended in Liebovitz's L-15 medium (Gibco) supplemented with 10% heat-inactivated foetal calf serum (Gibco), 100 U·ml⁻¹ penicillin/streptomycin (Sigma) and 2.5 μ g·ml⁻¹ fungizone (Gibco). Cells were washed twice with this medium, and plated on coverslips coated with 1 mg·ml⁻¹ concanavalin A (Aldrich) in medium plus 0.6% Methocel EM4 (Dow).

Cells on coverslips were transferred into saline of the following composition before patch-clamp recording: NaCl, 128 mM; KCl, 2.0 mM; CaCl₂, 1.8 mM; MgCl₂, 4 mM; trehalose, 5 mM; sucrose, 15 mM; N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer, 5.0 mM adjusted to pH 7.2 with NaOH. Patch pipettes were pulled from borosilicate glass (Clark Electromedical) and filled with saline containing the nitromethylene insecticide 2(nitromethylene)tetrahydro-1,3-thiazine (NMTHT). Cell-attached records [17] from the cell bodies of dissociated neurones were obtained at room temperature (22–26°C). Records were filtered at 1 kHz (–3 db) using an 8-pole Bessel filter and stored on an FM tape recorder (Rascal Store 4). Data were subsequently digitized at 5 kHz for analysis using pClamp soft-

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ware (Axon Instruments) on an IBM AT computer. Samples of NMTHT were synthesized at the Sittingbourne Research Centre, Shell Research, UK.

2.2. *Xenopus* oocytes: mRNA injection and electrophysiological recording

Oocytes were obtained from mature *Xenopus laevis* females by either partial or complete removal of the ovary, and cells at stage 5 or 6 were selected for mRNA expression studies. Exposure to 1 mg·ml⁻¹ collagenase for 30 min was followed by manual defolliculation. Modified Barth's medium [18] with added pyruvate (92.5 mM) and gentamycin (20 mg·ml⁻¹) was used to maintain oocytes at 18–19°C.

Poly(A)⁺ mRNA for injection was prepared from cDNA encoding the locust (*Schistocerca gregaria*) nicotinic α -subunit (α L1), as described earlier [15]. Locust poly(A)⁺ mRNA was dissolved in distilled, sterile water and stored in 2–5 μ g aliquots at 1 μ g·ml⁻¹ concentration to a temperature of –70°C. Individual oocytes were injected with 50 μ l quantities of this material. Microinjection of the mRNA material was carried out using a microsyringe (Drummond series 500). Following injection, oocytes were stored in a 5.0-cm diameter Petri dish at 18°C with a complete change of medium every 24 h.

For electrophysiological studies, an oocyte was surrounded by 6–8 small pins to restrain it to the centre of an experimental chamber (0.5 ml volume). The chamber was perfused continuously at a rate of 3.0 ml·min⁻¹. Changes in membrane potential were monitored by a single intracellular microelectrode filled with 2.0 M KCl (resistance 5–10 M Ω) linked to a high-impedance DC amplifier, and the output was displayed on a Gould BS-272 chart recorder.

3. RESULTS

3.1. Action of 2(nitromethylene)tetrahydro-1,3-thiazine (NMTHT) on *in situ* insect nervous system nicotinic receptors

Three conductances activated by acetylcholine and nicotine have been observed in cell attached patches from *Musca* neurones in culture. The nitromethylene insecticide, NMTHT, which has been shown to act as a nitotinic agonist [6], also activates single channels in these cells. Fig. 1 shows single channel records from a

cell-attached patch at the cell resting potential with a pipette solution containing 200 μ M NMTHT. The current–voltage relation for this patch yielded a conductance of 30 pS. The mean conductance from four patches was 32 ± 2 pS, a value very close to that obtained with acetylcholine as the agonist (32 ± 2 pS, [16,19]). The amplitude histogram of sampled points for this patch showed three peaks, but the larger conductance channel exhibited only brief and infrequent openings which made it impossible to obtain accurate estimates for the single channel conductance. Kinetic analysis of channels activated by NMTHT was limited to the lower conductance channels. A window discriminator was used to trigger data acquisition to select currents from the 30 pS conductance and any remaining multiple openings or events from the larger conductance channel were excluded from the open time histogram. A minimum event duration of 0.35 ms was employed to limit interference from brief events which would not have been fully resolved due to the limited bandwidth of the recordings. Open time distributions were best fitted by two exponentials (Fig. 2a). Closed times (Fig. 2b) will be subject to distortion by the presence of the two channel types; these required three exponential components to fit the distribution histograms. Results obtained with NMTHT were similar to those reported for *Musca* channels activated by acetylcholine [12,19].

3.2. Actions of 2(nitromethylene)tetrahydro-1,3-thiazine (NMTHT) on insect nicotinic receptors expressed in *Xenopus* oocytes

As shown in Fig. 3, control oocytes injected with 50 nl distilled water gave no response to either nicotine or 2(nitromethylene)tetrahydro-1,3-thiazine (NMTHT) when tested at concentrations in the range 1.0×10^{-8} M to 1.0×10^{-4} M. However, when oocytes were injected

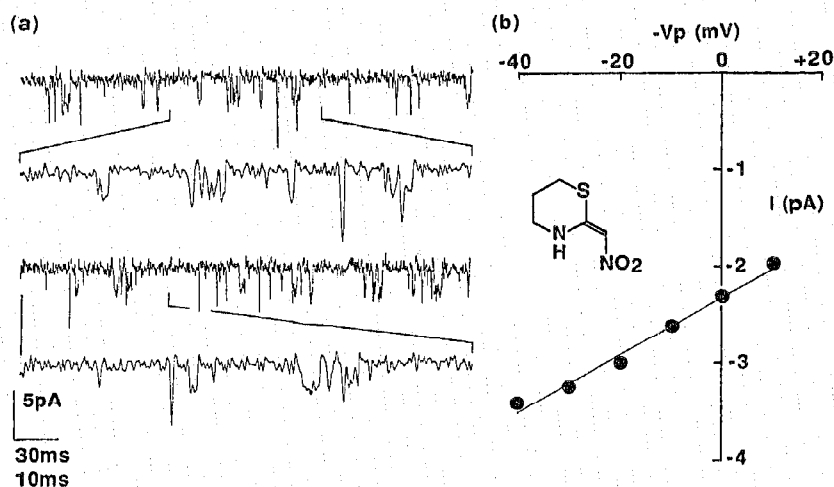


Fig. 1. (A) Records of currents from a cell-attached patch at the cell resting potential, the pipette solution contained 200 μ M NMTHT. Events with two different amplitudes, which were sometimes superimposed, can be seen. Records were filtered at 1 kHz. Scale bars, 5 pA (vertical) and 30 ms (horizontal, 10 ms for expanded traces). (B) Current–voltage relation from the same patch as in (A), the chord conductance (fitted by linear regression) for the smaller of the two channels in this patch was 30 pS. The larger events were too brief to allow accurate estimates of the current amplitude. Inset shows the chemical structure of 2(nitromethylene)tetrahydro-1,3-thiazine (NMTHT).

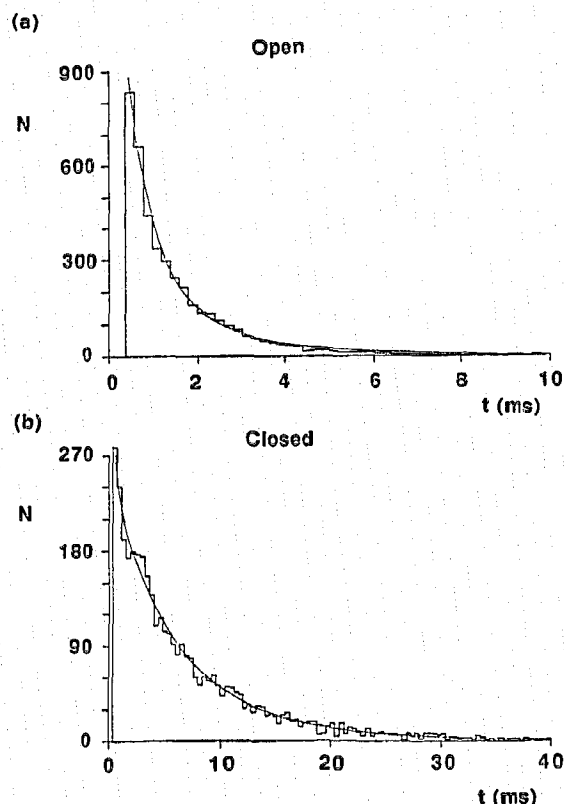


Fig. 2. (A) Open times for the 30 pS channel in a cell attached patch at the resting potential. Multiple openings or openings of the larger channel were excluded as were events of duration less than 0.35 ms. The histogram was fitted with two exponentials whose time constants were 0.53 ms and 1.82 ms (Mean values from 7 patches were 0.62 ± 0.09 ms and 2.46 ± 0.20 ms). (B) Closed times from the same patch as in (A). The histogram was fitted with three exponentials of 0.31 ms, 5.48 ms and 15.1 ms. These values will not be reliable because of the presence of more than one channel in the patch.

with mRNA encoding the α L1 nicotinic receptor subunit, depolarizing responses to both compounds were detected. As shown (Fig. 3), NMTHT was less effective than nicotine in its depolarizing actions on the expressed receptor.

In voltage-clamp experiments on oocytes injected with poly(A)⁺ mRNA encoding α L1, nicotine induced either monophasic inward currents, or biphasic (inward followed by outward) currents (Fig. 4a). When the nicotinic antagonist α -bungarotoxin was applied for 15 min at a concentration of 1.0×10^{-6} M both inward and outward currents were abolished. The basis for the outward current is not known but its variability indicates that it is likely to be an endogenous oocyte membrane current such as a Ca^{2+} -activated Cl^- current [18]. The outward current may be triggered by the entry of Ca^{2+} via the nicotinic receptor/channel, but further studies are needed to establish the nature of the biphasic currents seen in some expression experiments.

When NMTHT was applied at concentrations in the range 5.0×10^{-7} M– 2.0×10^{-5} M currents similar to those detected with nicotine were recorded. Thus injection

of mRNA encoding the locust α L1 nicotinic receptor subunit results in the expression of an NMTHT-sensitive nicotinic acetylcholine receptor.

4. DISCUSSION

2(Nitromethylene)tetrahydro-1,3-thiazine (NMTHT) activates channels in dissociated neurones from *Musca* which resemble channels gated by acetylcholine. One class of channels activated by both compounds exhibits similar conductances (32 pS) and open time histograms. Cultured neurones from the fly and the cockroach exhibit multiple channel types in response to acetylcholine [6,19] and also in response to nitromethylenes (this study). The fly and cockroach nicotinic receptor channels also appear to be similar in that open time histograms require at least two exponentials to produce an adequate fit. Single channel slope conductances in *Musca* neurones were identical with acetylcholine or NMTHT as the agonist, a similar lack of effect on conductance of the agonist has been reported in a more extensive survey on vertebrate muscle [20]. Cheung et al. [21] reported a slight increase in the single channel current with the nitromethylene WL136993 as the agonist, compared to acetylcholine, though no values for the slope conductances were given. These authors [21] also noted that the nitromethylene used in their study caused an increase in the mean open time compared to acetylcholine, whereas we noted a small reduction (Table I). Whether these changes are significant or simply due to the small sample size remains to be resolved. Such a discrepancy could be due to the different nitromethylenes used in each study, as there is a range of these compounds with activity which varies from ago-

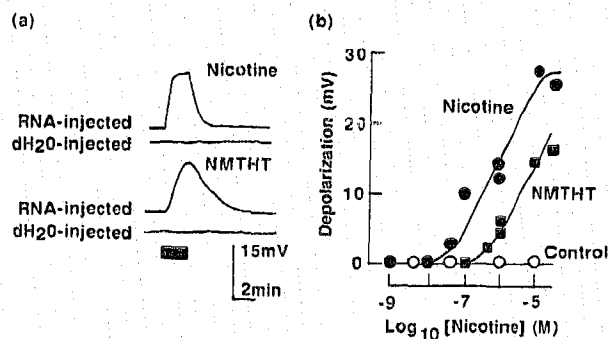


Fig. 3. Depolarizing actions of nicotine and 2(nitromethylene)-tetrahydro-1,3-thiazine (NMTHT) on *Xenopus* oocytes injected with mRNA derived from a cloned locust (*Schistocerca gregaria*) α subunit (α L1). (a) The rapid depolarization in response to 1.0×10^{-6} M nicotine detected in mRNA injected (50 nl $1 \mu\text{g}/\mu\text{l}$ mRNA) oocytes was not seen when oocytes injected with 50 nl distilled water (dH₂O) were exposed to the same concentration of nicotine. Depolarizing responses to 1.0×10^{-5} M NMTHT were also observed in mRNA injected oocytes, but not in oocytes injected with the same volume of distilled water. (b) Dose-response curves for the depolarizing actions of nicotine (data pooled from 3 separate oocytes) and NMTHT (data pooled from 2 separate oocytes) show that nicotine is more effective than NMTHT on the expressed receptor.

nist to antagonist [9]. Thus nitromethylenes may prove to be useful for characterization of agonist/antagonist interactions at nicotinic receptors, especially if coupled with studies on specific receptor subunits.

Injection of the locust α L1 receptor subunit into *Xenopus* oocytes leads to functional expression of a nicotinic receptor-channel complex [13–15], that is sensitive to NMTHT. The simplest interpretation of this observation is that α L1 can form a homo-oligomeric complex which carries transmitter binding sites and that nitromethylenes can bind to these sites. At present, we cannot exclude the possibility that one or more endogenous components from the oocyte are needed to allow functional expression, though expression of a functional homo-oligomer has also been reported following injection of the α 7 subunit from chick brain neuronal nicotinic receptors [22]. The responses of α L1 injected oocytes to bath-applied agonists and antagonists strongly resemble those of in situ receptors [15]. A further example is the finding that in both cases nicotine is a more effective agonist than NMTHT. Thus, α L1 either forms a homo-oligomer which possesses a nitromethylene binding site, or it forms part of a hetero-oligomer, the pharmacology of which is largely determined by the injected insect subunit.

Nitromethylene insecticides are capable of acting on both in situ nicotinic receptors of several insect species and also of gating a presumed locust α L1 homo-oligomer expressed in *Xenopus* oocytes. We suggest that a nitromethylene binding site resides on the α -subunits of insect nicotinic receptors. Further studies will be needed to locate binding sites for nitromethylenes on such polypeptides. These insecticidally-active compounds may

Table I

Mean open times calculated from single channel data recorded from dissociated *Musca* and *Periplaneta* neurones, using cell attached patches containing either acetylcholine or a nitromethylene compound

Agonist	Insect	Mean open times (ms)		n
		(1)	(2)	
Acetylcholine				
<i>Musca domestica</i>		0.70 ± 0.08	3.25 ± 0.83	7
<i>Periplaneta americana</i>		0.69 ± 0.09	2.55 ± 0.10	3
Nitromethylenes				
NMTHT				
<i>Musca domestica</i>		0.62 ± 0.04	2.46 ± 0.20	7
WL 136993				
<i>Periplaneta americana</i>		0.50 ± 0.03	3.50 ± 0.06	3

Abbreviations: n, number of patches studied; NMTHT, 2(nitromethylene)tetrahydro-1,3 thiazine.

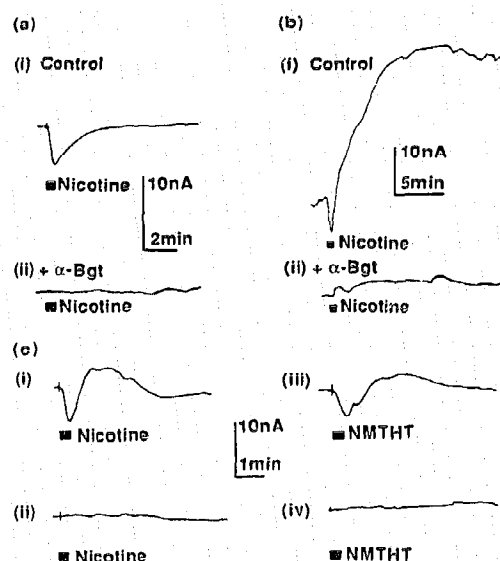


Fig. 4. Currents induced by nicotine and 2(nitromethylene)tetrahydro-1,3-thiazine (NMTHT) recorded under voltage-clamp from *Xenopus* oocytes injected with mRNA derived from a cloned locust (*Schistocerca gregaria*) α subunit (α L1). (a) Monophasic (inward) currents recorded at a holding potential (E_h) of -60 mV were blocked by a 30 min exposure to 1.0×10^{-7} M α -bungarotoxin. (b) Biphasic (inward and outward) currents were also blocked by a 15 min exposure to 1.0×10^{-6} M α -bungarotoxin. (c) Nicotine (1.0×10^{-6}) induced biphasic responses in an mRNA injected oocyte (i), but no currents were induced in a distilled water injected oocyte (ii). When tested with NMTHT (1.0×10^{-5} M), the same oocyte from which biphasic responses were detected in c (i) also yielded biphasic responses (iii), whereas controls injected with the same volume of distilled water showed no response (iv).

also enhance our understanding of agonist and antagonist interactions at neuronal nicotinic acetylcholine receptors.

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