



Short communication

Anopheles gambiae mosquito isolated neurons: A new biological model for optimizing insecticide/repellent efficacy

Céline Lavalie-Defaix^{a,1}, Véronique Apaire-Marchais^{a,1}, Christian Legros^a, Cédric Pennetier^b, Aly Mohamed^{a,c}, Patricia Licznar^a, Vincent Corbel^b, Bruno Lapied^{a,*}

^a Laboratoire Récepteurs et Canaux Ioniques Membranaires (RCIM) - UPRES EA 2647/USC INRA 2023, IFR 149 QUASAV, Université d'Angers, UFR Sciences, 2 boulevard Lavoisier, 49045 Angers Cedex, France

^b Institut de Recherche pour le Développement (IRD), MIVEGEC (UM1-CNRS 5290-IRD 224), Centre de Recherche Entomologique de Cotonou, 01 BP 4414 RP Cotonou, Benin

^c Plant Protection Department, Faculty of Agriculture, Assiut University, 71516 Assiut, Egypt

ARTICLE INFO

Article history:

Received 13 April 2011

Received in revised form 23 May 2011

Accepted 8 June 2011

Keywords:

Malaria
Mosquito
Isolated neurons
Insecticides
DEET
Vector-borne diseases

ABSTRACT

To understand better the mode of action of insecticides and repellents used in vector-borne diseases control, we developed a new biological model based on mosquito neurons isolated from adults *Anopheles gambiae* heads. This cellular model is well adapted to multidisciplinary approaches: electrophysiology, pharmacology, molecular biology and biochemical assays. Using RT-PCR, we demonstrated that isolated neurons express the nicotinic acetylcholine receptor subunit $\alpha 1$ (Ag $\alpha 1$ nAChR), two acetylcholinesterases (AChE-1 and AChE-2) and three voltage-gated ion channels required for membrane excitability (AgCav1, AgNav1 and AgKv1). In order to correlate the expression of the different transcripts, encoding functional AgNav channel, nAChR receptor and AChE enzymes detected by RT-PCR, with electrophysiological activity we used patch-clamp technique. We revealed that AgNav and AChE which are targeted by insecticide and/or repellent were sensitive to the pyrethroid permethrin and to the repellent DEET, respectively. In addition, using colorimetric method, we also showed that AChE was sensitive to the carbamate propoxur. These results indicated that this novel neuronal mosquito model will lead to molecular and functional characterization of insecticide/repellent targets and appears as a powerful tool to investigate the development of highly specific and effective strategies for disease vector control.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Despite considerable efforts in recent years dedicated throughout the world to control vector-borne diseases, malaria is still a major public health problem with nearly 250 million cases a year and 800,000 deaths among them, 85% are children under five years old (WHO, 2010). The only chemicals recommended by World Health Organization (WHO) to control adult mosquito vectors are neurotoxic insecticides, which target the sodium channels (pyrethroids) and the acetylcholinesterases (organophosphates and carbamates) (WHO, 2006). Unfortunately the main malaria vector species *Anopheles gambiae* developed multiple resistance mechanisms to these insecticides (Ranson et al., 2011). This is worrying because it may impede the WHO Global Action Plan for malaria control and its eradication. In this context, there is an urgent need to deeply investigate insecticide/repellent neurological effects and interactions, at both molecular and cellular levels,

for developing new research strategies to ensure better control of insecticide-resistant malaria vectors (Lapied et al., 2009).

Although, behavior observations, phenotypic and genotypic studies brought important knowledge allowing the development of alternative strategies against mosquitoes (Ranson et al., 2011), there is a real lack of data describing the physiology and pharmacology of insecticide/repellent targets-interactions at mosquito neuronal level. So it is crucial to find out new methods to overcome this limitation.

Until now, primary neuronal cultures have been developed from numerous insect species (Beadle, 2006). Because they express membrane receptors and ion channels targeted by insecticides, they constitute ideal biological models to investigate the mode of action of insecticides. More recently, the use of such biological models have highlighted that intracellular regulatory mechanisms have fundamental consequences for the sensitivity of insecticide targets (Lapied et al., 2009; Lavalie-Defaix et al., 2010). However, among insect neuronal preparations available, the fully differentiated Mosquito Isolated Neurons (MIN) model is still lacking. We set up a novel biological model of *A. gambiae* adult MIN which is offering all facilities to study the mode of action of insecticides/repellents, to investigate the impact of resistance mechanisms at neurologi-

* Corresponding author. Tel.: +33 241 73 54 38; fax: +33 241 73 52 15.

E-mail address: bruno.lapied@univ-angers.fr (B. Lapied).

¹ Equally contributed to this work.

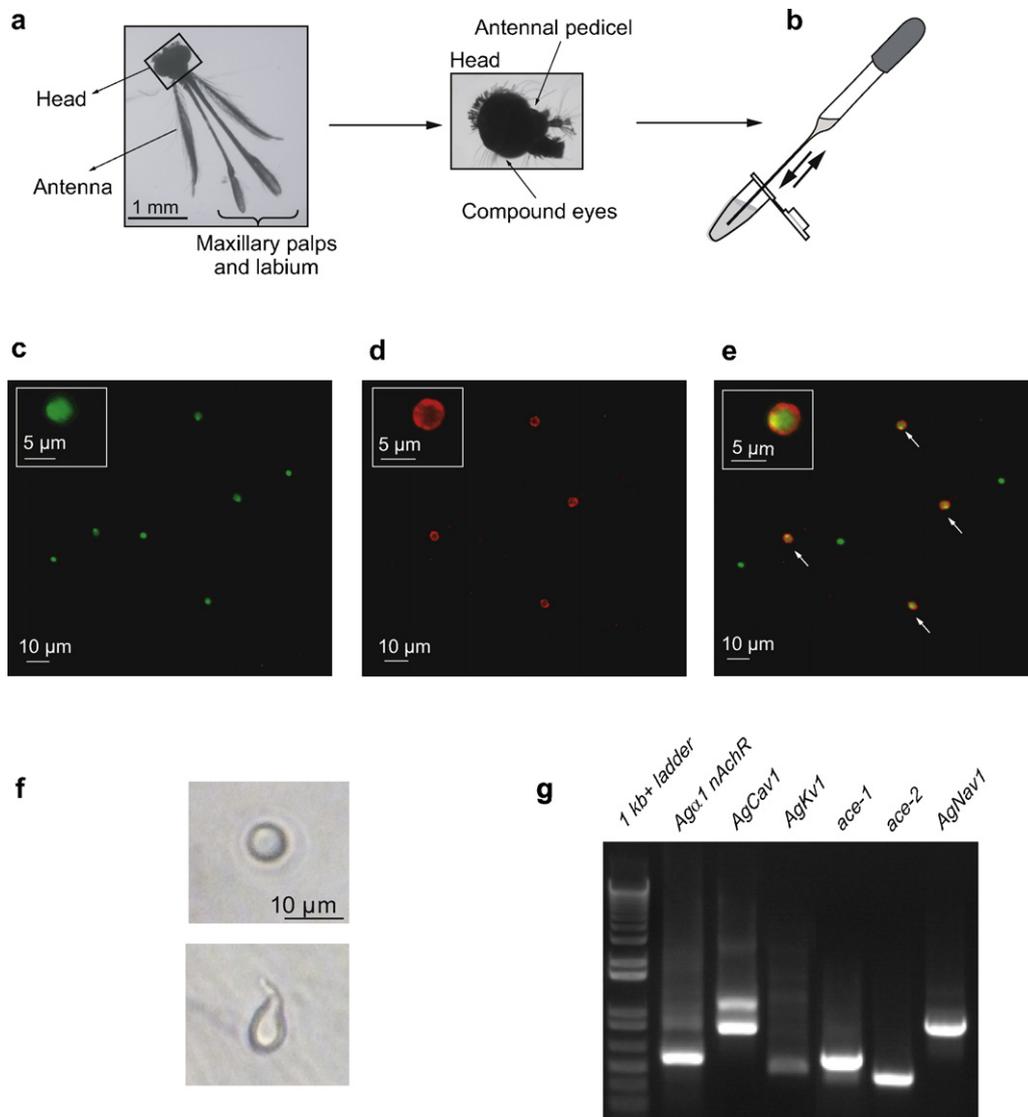


Fig. 1. Adult mosquito neuron isolation procedure and characterization. (a) *Anopheles gambiae* head. (b) Mechanical dissociation of neurons from mosquito head. (c–e) Confocal laser-scanning images: (c) counterstaining with acridine orange binds DNA in the nuclei of all cells. (d) HRP-staining in neurons among isolated cells. (e) Fluorescent double-labeling as a result of superimposing (c) and (d) identified cells as neurons (arrows). (f) Light photomicrograph of isolated adult mosquito neurons observed 24 h after dissociation. (g) Electrophoretic analysis of amplicons obtained by multiplex PCR using cDNA of isolated neurons (*Agα1 nAChR*: 548 bp; *Ag Cav*: 777 bp; *AgKv1*: 470 bp; *ace-1*: 558 bp; *ace-2*: 433 bp; *AgNav1*: 895 bp).

cal level and to characterize intracellular regulation mechanisms involved in the modulation of the sensitivity of insecticide targets.

2. Materials and methods

2.1. Mosquitoes

The reference susceptible strain of *A. gambiae* (Kisumu) provided by CCPV/IRD Montpellier, was used in this study and maintained under laboratory conditions: 26 ± 1 °C and $68 \pm 1\%$ relative humidity. This strain, originated from Kisumu in Kenya, was colonized many years ago and is free of any detectable insecticide resistance mechanism.

2.2. Cells isolation procedure

The adult mosquito heads were carefully cut under a binocular microscope. Antennae, maxillary palps, labium and antennal pedicels were removed. The heads were excised and placed in mosquito saline buffer containing (in mM) 130 NaCl, 2.5 KCl,

3 MgCl₂, 2 CaCl₂, 35 sucrose, 5 HEPES, 5% fetal bovine serum, 50 UI ml⁻¹ penicillin and 50 μg ml⁻¹ streptomycin. Dissociation of neuronal cells from head was performed mechanically by repetitive gentle suction through fire-polished Pasteur pipettes (Fig. 1).

2.3. Cell culture stainings

Isolated cells were placed in chamber slides pre-coated with poly-D-lysine hydrobromide (Sigma) and fixed for 15 min with 2% paraformaldehyde containing 2% (w/v) sucrose in phosphate-buffer saline (PBS, pH 7.2). After fixation, cells were washed 3 times for 5 min in PBS and 5 min in PBS containing 0.2% Triton X-100 (PBS-T). In order to block non-specific binding of the primary antibody, cells were pre-incubated with 4% bovine serum albumin (BSA, Sigma) in PBS-T for 1 h. Primary rabbit anti-horseradish peroxidase (HRP) antibodies (Sigma) used at a dilution of 1:5000 in PBS-T, was applied overnight at 4 °C. After washing in PBS-T, cells were incubated with secondary antibody (Cy3-conjugated goat anti-rabbit IgG, Chemicon International) used at a dilution of 1:1000 in PBS-T containing 1% BSA for 40 min in the dark at 20 °C. Then, the

Table 1
Biophysical characteristics of the inward sodium current of adult isolated mosquito neurons.

Current–voltage relationship		INa amplitude (pA) at –10 mV	Midpoint of steady-state activation	Slope factor of activation, <i>k</i>	Midpoint of steady-state inactivation (<i>V</i> _{0.5} , mV)	Slope factor of inactivation, <i>k</i>	Time constant of recovery from inactivation (ms)
Activation threshold (mV)	Reversal potential (mV)						
–40 ± 2 (<i>n</i> = 12)	+46.0 ± 5 (<i>n</i> = 12)	–196.7 ± 23.7 (<i>n</i> = 12)	–27.04 ± 0.10 (<i>n</i> = 11)	5.8 ± 0.1 (<i>n</i> = 11)	–47.4 ± 0.2 (<i>n</i> = 9)	8.6 ± 0.1 (<i>n</i> = 9)	2.7 ± 0.2 (<i>n</i> = 12)

secondary antibody solution was removed and the cells were incubated with acridine orange at 0.01% in PBS for 2 min. Subsequently, the cells were rinsed in PBS and mounted on glass slides using glycerol/PBS. Double-labeled preparations were analyzed using a confocal laser scanning microscope (Olympus BX50). We used the helium/neon laser (excitation wavelength 543 nm, detection range 555–700 nm) for imaging of Cy3 fluorescence and the argon laser (excitation wavelength 488 nm, detection range 500–650 nm) for imaging of acridine orange. Typical cells were chosen using the transmitted light scan mode of the confocal microscope (magnification 600×).

2.4. Gene expression profiling of mosquito neurons by multiplex PCR

Total RNA was extracted from cells isolated from 10 mosquito heads with the NucleoSpin RNA XS system (Macherey-Nagel) including a DNaseI treatment and transcribed into cDNA using protocols described previously (Moignot et al., 2009). Amplification was performed by multiplex semi-nested or nested PCR with specific primers sets (Supplementary Table 1) matching genes encoding the α 1 nicotinic acetylcholine receptor (Ag α 1 nAChR), the α subunit of the voltage-gated Ca²⁺ channel (AgCav1), the Shaker-related K⁺ channel (AgKv1), the acetylcholinesterase 1 (AChE-1), the acetylcholinesterase 2 (AChE-2) and the α subunit of the voltage-gated Na⁺ channel (AgNav1). To amplify simultaneously the different cDNAs, the first round of PCR was performed in a single tube, containing 2 μ l cDNA, 2 μ l 10× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.25 μ M of each gene-specific primer in a 20 μ l final volume and 0.3 U EuroTaq DNA polymerase (BioCat GmbH). The second round of PCR was done in separate tubes using 0.25 μ l of the first PCR as template and a second nested or semi-nested gene specific primer set in a 50 μ l final volume. For both PCR, an initial denaturation was carried out at 94 °C for 4 min, followed by 35 cycles (20 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C) of amplification and a final cycle of 10 min at 72 °C. The PCR products of expected size were separated on agarose gel and purified using NucleoSpin Extract II® (Macherey-Nagel) for direct sequencing (Millegen).

2.5. Electrophysiological recordings

The glass coverslips were mounted in a recording chamber (Warner Instruments) connected to a gravity perfusion system allowing drug application. The isolated neuron cell bodies, maintained at 27 °C, were used for recordings 4 h after dissociation. Patch-clamp technique in the whole-cell recording configuration (Hamill et al., 1981) was used to record both voltage-dependent and ACh-induced ionic currents (voltage-clamp mode) Ionic currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz using a 4-pole lowpass Bessel filter. Patch pipettes were pulled from borosilicate glass capillary tubes (GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) using a Sutter P-97 (Sutter Instruments). Pipettes had resistances ranging from 6 to 8 M Ω when filled with

internal solutions. The liquid junction potential between bath and internal solution was always corrected before the formation of a gigohm seal (>5 G Ω). In all experimental conditions, cells were bathed in external solution containing (in mM) 130 NaCl, 2.5 KCl, 3 MgCl₂, 2 CaCl₂, 5 HEPES, pH was adjusted to 7.2 with NaOH 1 M, except otherwise stated (see below). The internal solution contained (in mM): 140 K-gluconate, 10 NaCl, 3 MgCl₂, 0.1 CaCl₂, 1.5 EGTA, 1 ATP-Mg and 10 HEPES buffer; pH was adjusted to 7.2 with KOH 1 M.

The solutions used to record sodium current were designed to eliminate interference from outward potassium and inward calcium currents. The extracellular solution superfusing the cells contained (in mM) 130 NaCl, 50 TEA-Cl, 5 KCl, 2 MgCl₂, 0.5 CaCl₂, 0.5 CdCl₂, 5 4-AP, 10 HEPES, pH was adjusted to 7.2 with NaOH 1 M. Patch pipettes were filled with an internal solution containing (in mM) 140 CsCl, 7 NaCl, 2 MgCl₂, 2 EGTA, 10 HEPES, 1 ATP-Mg, pH was adjusted to 7.2 with CsOH 1 M. Although leak and capacitive currents were compensated electronically at the beginning of each experiment, subtraction of residual capacitive and leakage currents was performed with an on-line P/6 protocol provided by pClamp. Mosquito neurons were voltage-clamped at a steady-state holding potential of –70 mV and 15-ms test pulses were applied from the holding potential at a frequency of 0.3 Hz. Patch-clamp experiments were conducted at room temperature (20–22 °C). Data analyses were presented in Supplementary data.

2.6. Pneumatic pressure ejection application of the neurotransmitter acetylcholine

Acetylcholine (ACh, 100 μ M, Sigma Chemicals) was applied by pneumatic pressure ejection (15 psig, 200-ms pulses), with a pneumatic pressure system (Miniframe, Medical System Corporation, NY) to minimize receptor desensitization resulting from bath application of agonist (Lapied et al., 1990). The pressure ejection was made through a glass micropipette (resistance of 2 M Ω when filled with agonist) positioned in solution within 40 μ m from the isolated neuron cell body. In no experiment did the pressure ejection of normal saline realized with the same protocol affect the current baseline. Repeated applications of ACh were made with an interval of 2 min between the end of one application and the beginning of the next.

2.7. Biochemical assay of AChE activity

Seven heads from *A. gambiae* mosquito adults were placed in 700 μ L of the mosquito saline buffer and isolation of neuron cells was performed using mechanical dissociation as described (Fig. 1). Hundred μ L of the suspension were incubated for 15 min with 10 μ L of a carbamate insecticide (propoxur). Dilutions (10^{–4} M to 10^{–8} M) from the initial concentration (1 M) were used for propoxur. AChE residual activity was determined spectrophotometrically (Ellman et al., 1961) at 405 nm at 30 min using 100 μ L of 1 mM acetylthiocholine (ATC) (Sigma) and 100 μ L of 1 mM DTNB (Sigma) for each dilution, and was expressed as a percentage of ini-

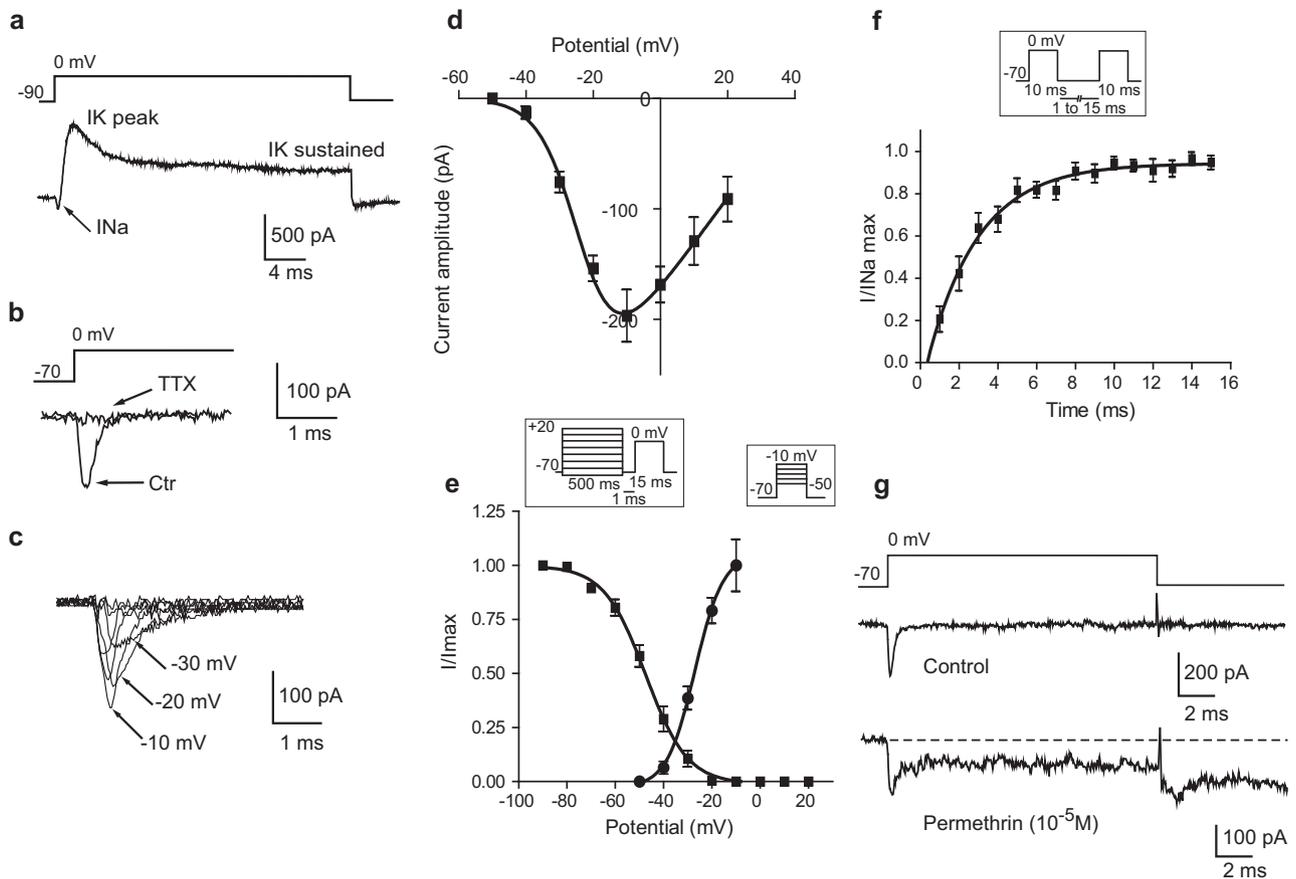


Fig. 2. Electrophysiological properties of the isolated mosquito neurons studied with the patch-clamp technique in whole-cell recording configuration. (a) Typical example of the whole cell currents activated by a depolarizing pulse to 0 mV from a holding potential of -90 mV. (b–e) Biophysical properties of the TTX-sensitive inward sodium current studied under voltage-clamp condition, in the presence of potassium and calcium channel blockers. (b) Blocking effect of TTX (10^{-7} M) on the control inward current elicited by a depolarizing pulse to 0 mV applied from a holding potential of -70 mV ($n=4$). Currents are capacity- and leak-corrected. (c) Currents are capacity- and leak-corrected. (d) Current–voltage relationship constructed from values of maximum current amplitude shown in (c) and plotted as a function of test pulses. The smooth curve represents the best fit according to Eq. (1) (Supplementary data). (e) Voltage-dependence of the steady-state activation (right curve) and inactivation (left curve) of the TTX-sensitive sodium current according to the protocols shown in inset. Smooth curves were fitted through the data points using single Boltzmann distributions (Eqs. (2) and (3) for activation and inactivation, respectively; see Supplementary data). (f) Time course of recovery from inactivation of the TTX-sensitive sodium current at a holding potential of -70 mV. The inset shows the experimental protocol used. (g) Typical example of the inward sodium currents recorded in control (upper trace) and after application of the pyrethroid insecticide, permethrin (10^{-5} M, lower trace). Currents were elicited by a depolarizing pulse according to the protocol shown above current traces. In all cases, data are means \pm S.E.M.

tial activity (without inhibitor). Values obtained are means of three independent experiments, each performed in triplicate.

3. Results and discussion

3.1. Characterization of the *A. gambiae* neurons

MIN were obtained from adult *A. gambiae* heads using mechanical dissociation (Fig. 1a and b). The proportion of neurons among dissociated cells was evaluated by a double immunocytochemical labeling method, using a rabbit anti-horseradish peroxidase (HRP) primary antibody as neuronal cell surface marker (Loesel et al., 2006). Counterstaining with acridine orange, that binds to DNA in the nuclei of all cells, yielded the total number of cells. 53.4% of the cell population ($n=196$) were double-labeled and were therefore identified as neurons (Fig. 1c–e). In this case, neurons appeared as small spherical cells with a mean diameter of $5.1 \pm 0.1 \mu\text{m}$ (Fig. 1f).

To demonstrate that our new mosquito neuronal model (i) expresses the main targets for insecticides and repellents, including voltage-gated Na^+ channels (Nav channels), acetylcholinesterases (AChE) and cholinergic receptor and (ii) is suitable for the development of molecular, electrophysiological and biochemical approaches, essential to study the mode of action of insecticides and repellents, we first adapted RT-PCR on such preparation. Using

cDNA from isolated cell, we performed a multiplex nested or semi-nested PCR with specific primers for genes encoding the $\alpha 1$ nicotinic acetylcholine receptor subunit (Ag $\alpha 1$ nAChR), the alpha subunit of voltage-gated Ca^{2+} channel (AgCav1), the Shaker-related K^+ channel (AgKv1), the acetylcholinesterase-1 (AChE-1), the acetylcholinesterase-2 (AChE-2) and the alpha subunit of Nav channel (AgNav1). The second round of PCR amplification revealed the simultaneous expression of all of these transcripts in the isolated cells from mosquito heads (Fig. 1g). Each PCR product was detected with the expected size and direct sequencing confirmed their molecular identity.

3.2. Electrophysiological properties of *A. gambiae* isolated neurons

In addition, in order to correlate the expression of the transcripts detected by RT-PCR with electrophysiological activity, we adapted whole-cell patch-clamp technique to MIN. The isolated neurons were used for electrophysiological recordings 4 h after dissociation. In our experimental conditions, isolated neurons can survive with stable electrophysiological properties for 24 h. According to the double immunocytochemical labeling data, all neurons investigated had spherical diameter cell body geometry of about $5 \mu\text{m}$ in which an adequate space-clamp is easily attained. In 100% of isolated neurons tested, depolarizing pulse from a holding potential

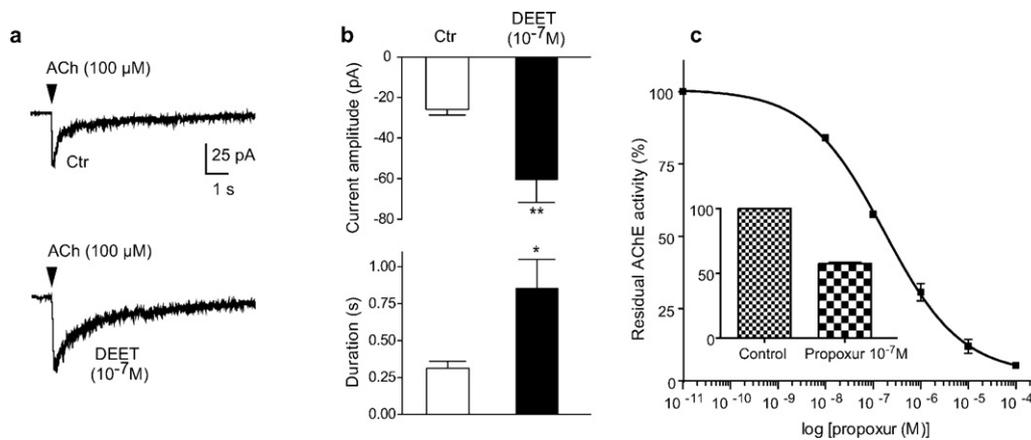


Fig. 3. Anticholinesterasic effects of the repellent DEET and the carbamate insecticide, propoxur on adult isolated mosquito neurons. (a) Typical examples of steady-state recordings of the acetylcholine (ACh) induced inward current obtained in whole cell voltage-clamp mode at a holding potential of -50 mV in control and after bath application of the repellent DEET (10^{-7} M). The cholinergic agonist was applied by pressure-ejection onto isolated neuron from a patch-pipette filled with saline containing $100 \mu\text{M}$ ACh. (b) Histogram illustrating the effects of DEET (10^{-7} M) on both amplitude and duration of the ACh-induced inward current. Data are means \pm S.E.M. ($n = 7$). (c) Dose-dependent anticholinesterasic effect of the carbamate propoxur on the neuronal residual AChE activity. The inset illustrates the comparative histogram giving the IC_{50} value estimated at 2×10^{-7} M.

of -90 mV to 0 mV elicited global currents consisting of a fast small inward sodium current followed by a biphasic outward potassium current (Fig. 2a). The mean amplitudes of the peak outward current was 620.1 ± 127.6 pA ($n = 10$) and 311.3 ± 68.8 pA ($n = 10$) for the sustained component. To isolate the inward sodium current, calcium and potassium current inhibitors were used. As illustrated in Fig. 2b, application of 10^{-7} M TTX, the well known Nav channel blocker, completely inhibited the residual inward current, indicating that this latter resulted of Na^+ influx through voltage-gated channel. The TTX-sensitive Na^+ current had an amplitude of -193.1 ± 23.2 pA ($n = 21$) in response to a depolarizing pulse from a holding potential of -70 mV to 0 mV. Fig. 2c shows a typical example of a transient inward current from an isolated neuron in response to a series of voltage-clamp steps applied from a holding potential of -70 mV. The corresponding peak current–voltage (I – V) relationship was illustrated in Fig. 2d. The voltage-dependence of the steady-state inactivation and activation of the sodium current together with the recovery time from inactivation were studied according to the protocols shown in Fig. 2e and f. All the biophysical characteristics of the voltage dependence of the Na^+ current were summarized in Table 1. These results indicate that the Nav channels expressed in *A. gambiae* MIN were functional with an inward current which displayed general electrophysiological properties very similar to those of other invertebrate neurons (Wicher et al., 2001).

3.3. Neurotoxic effects of insecticide and repellent on *A. gambiae* isolated neurons

Nav channels represent the key target sites for the insecticide pyrethroids (Soderlund, 2008). Permethrin is one of the most used insecticides among pyrethroids used in vector control (WHO, 2006). Application of permethrin on MIN evidenced that the Na^+ current is sensitive to this pyrethroid. As shown in Fig. 2g, compared to the control current, the decrease of the Na^+ current recorded in the presence of permethrin (10^{-5} M) presented a biphasic aspect composed of a fast component followed by the development of a slow maintained one. The deactivation of the slow component was represented by a large inward tail current when the membrane was stepped back to the holding potential. These typical effects of permethrin observed on the Na^+ current fit well with the effect of pyrethroids described in numerous insect neuronal preparations (Soderlund, 2008). Altogether, our results confirm that MIN are a suitable model to investigate the effects of insecticides acting on the

Nav channels. This is of great interest in order to understand further the neurological and molecular processes involved in target-site resistance to pyrethroids (*Kdr* and *super-Kdr*) and contribute to the design of novel insecticidal compounds on the same target.

We next focused on the characterization of AChE, another crucial target involved in the cholinergic neurotransmission in insect by specifically hydrolyzing ACh in the cholinergic synapses. AChE is the molecular target for insecticides including organophosphates, carbamates and more recently the most commonly used insect repellent, DEET (N,N-diethyl-3-methylbenzamide) (Corbel et al., 2009). To show that the different transcripts encoded functional nAChR and AChEs, we investigated the anticholinesterasic effects of DEET on MIN. We used the whole-cell patch-clamp technique combined with the pneumatic pressure ejection of the neurotransmitter ACh, as previously described elsewhere (Lapied et al., 1990). As expected, the results illustrated in Fig. 3a and b demonstrated a clear and significant inhibition of AChE after bath application of DEET (10^{-7} M). The ACh-induced inward current recorded under voltage-clamp condition following pressure ejection of ACh onto the isolated cell was significantly increased in both amplitude and duration (Fig. 3a and b), in accordance with a typical anti-cholinesterasic effect. At the same time, we implemented a biochemical assay to demonstrate that it is possible to measure the AChE enzymatic activity on MIN. The enzymatic activity was measured by using colorimetric method in the presence of the carbamate propoxur. AChE residual activity of mosquito neurons was determined for increasing concentrations of propoxur. The inhibitory concentration 50 (IC_{50}) value of propoxur was estimated at about 2×10^{-7} M and a decrease of 42.4% of AChE activity was observed in the presence of 10^{-7} M of propoxur (Fig. 3c). Altogether, these data indicate that MIN express functional nAChR and AChE.

4. Conclusion

We proposed the first mosquito neuronal model for the malaria vector *A. gambiae*. By using electrophysiology, pharmacology, molecular biology and biochemistry, we demonstrated that *A. gambiae* MIN express some of the most important functional receptors, ion channels and enzymes targeted by insecticides and repellents. Such approach can be used to build neuronal models of the other major mosquito vectors such as *Aedes aegypti*, *Aedes albopictus* (Dengue and Chikungunya viruses) or *Culex quinquefasciatus* (Filar-

iasis). The development of this neuronal mosquito model will have direct practical applications on insecticidal treatment. It will be a powerful neurobiological tool to bring new highlights on (i) the mode of action of both insecticides and repellents, (ii) the effect of mixtures of two unrelated insecticides and/or insecticide/repellent, which are proposed as promising novel strategies for the control of vector-borne diseases (Pennetier et al., 2009) and (iii) the management of insecticide-resistant malaria vectors.

Acknowledgements

Céline Laviaille-Defaix was supported by a post-doctoral grant of the Agence Nationale pour la Recherche (ANR, programme REAC SEST06 030 01). We thank Sophie Quinchard from RCIM laboratory, Angers University and Robert Filmon from the microscopy department, SCIAM, Angers University, for technical assistance. *Anopheles gambiae* eggs were provided by IRD, UR016, Montpellier, France. Aly Mohamed was supported by a doctoral fellowship of the Egyptian Government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jneumeth.2011.06.003](https://doi.org/10.1016/j.jneumeth.2011.06.003).

References

- Beadle DJ. Insect neuronal cultures: an experimental vehicle for studies of physiology, pharmacology and cell interactions. *Invert Neurosci* 2006;6:95–103.
- Corbel V, Stankiewicz M, Pennetier C, Fournier D, Stojan J, Girard E, Dimitrov M, Molgo J, Hougard JM, Lapied B. Evidence for inhibition of cholinesterases in insect and mammalian nervous systems by the insect repellent DEET. *BMC Biol* 2009;7:47.
- Ellman GL, Courtney KD, Andres JV, Featherstone BB. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961;7:88–95.
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflüger Arch* 1981;391:85–100.
- Lapied B, Le Corrion H, Hue B. Sensitive nicotinic and mixed nicotinic–muscarinic receptors in insect neurosecretory cells. *Brain Res* 1990;533:132–6.
- Lapied B, Pennetier C, Apaire-Marchais V, Licznar P, Corbel V. Innovative applications for insect viruses: towards insecticide sensitization. *Trends Biotechnol* 2009;27:190–8.
- Laviaille-Defaix C, Moignot B, Legros C, Lapied B. How does calcium-dependant intracellular regulation of voltage-dependant sodium current increase the sensitivity to the oxadiazine insecticide indoxacarb metabolite decarbomethoxylated JW062 (DCJW) in insect pacemaker neurons? *J Pharmacol Exp Ther* 2010;333:264–72.
- Loesel R, Weigel S, Brautig P. A simple fluorescent double staining method for distinguishing neuronal from non-neuronal cells in the insect central nervous system. *J Neurosci Methods* 2006;155:202–6.
- Moignot B, Lemaire C, Quinchard S, Lapied B, Legros C. The discovery of a novel sodium channel in the cockroach *Periplaneta americana*: evidence for an early duplication of the para-like gene. *Insect Biochem Mol Biol* 2009;39:814–23.
- Pennetier C, Constantini C, Corbel V, Licciardi S, Dabiré RK, Lapied B, Chandre F, Hougard JM. Synergy between repellents and organophosphates on bed nets: efficacy and behavioural response of natural free-flying *Anopheles gambiae* mosquitoes. *PLoS One* 2009;4:e7896.
- Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African *Anopheline* mosquitoes: what are the implications for malaria control? *Trends Parasitol* 2011;27:91–8.
- Soderlund DM. Pyrethroids, knockdown resistance and sodium channels. *Pest Manag Sci* 2008;64:610–6.
- Wicher D, Walther C, Wicher C. Non-synaptic ion channels in insects—basic properties of currents and their modulation in neurons and skeletal muscles. *Prog Neurobiol* 2001;64:431–525.
- WHO. Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets. In: WHO/CDS/NTD/WHOPES/GCDDP/2006.3. Geneva: WHO Press; 2006. p. 60.
- World Health Organization (WHO). World Malaria Report 2010. Geneva: WHO Press; 2010. p. 204.