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Insecticide sensitivity of native chloride and sodium channels in a mosquito cell line

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ABSTRACT

The aim of this study was to investigate the utility of cultured *Anopheles gambiae* Sua1B cells for insecticide screening applications without genetic engineering or other treatments. Sua1B cells were exposed to the known insecticidal compounds lindane and DIDS, which inhibited cell growth at micromolar concentrations. In patch clamp studies, DIDS produced partial inhibition (69%) of chloride current amplitudes, and an IC_{50} of 5.1 μM was determined for Sua1B cells. A sub-set of chloride currents showed no response to DIDS; however, inhibition (64%) of these currents was achieved using a low chloride saline solution, confirming their identity as chloride channels. In contrast, lindane increased chloride current amplitude (EC_{50} = 116 nM), which was reversed when cells were bathed in calcium-free extracellular solution. Voltage-sensitive chloride channels were also inhibited by the presence of fenvalerate, a type 2 pyrethroid, but not significantly blocked by type 1 allethrin, an effect not previously shown in insects. Although no evidence of fast inward currents typical of sodium channels was observed, studies with fenvalerate in combination with veratridine, a sodium channel activator, revealed complete inhibition of cell growth that was best fit by a two-site binding model. The high potency effect was completely inhibited in the presence of tetrodotoxin, a specific sodium channel blocker, suggesting the presence of some type of sodium channel. Thus, Sua1B cells express native insect ion channels with potential utility for insecticide screening.

1. Introduction

Due to the increasing resistance demonstrated by insects to pyrethroids, especially disease vectors [1], the need for insecticides with novel modes of action is becoming increasingly important. Development of new insecticides is also vital because regulations and use restrictions on conventional insecticides are becoming more stringent [2]. Invention of new insecticide chemical classes is limited by available chemical synthesis technologies, as well as the necessary procurement of large amounts of target site protein needed for high throughput testing. Smagghe et al. [3] reviewed the use of insect cell lines as tools in virus-related research, as models in the study of signal mechanisms and gene expression, and as platforms for screening novel insecticide activities, receptors, and ligands for pest control. When used for insecticide screening, cell lines typically contain the cloned gene of interest, coupled to a fluorescent reporter system [4]. However, there is a relative paucity of information available on the subunit composition of insect ion channels and receptors compared to their mammalian homologs, and they can sometimes be difficult to express in cell lines.

The main goal of this research was to assess any expression of insecticide target sites, especially Cl^- and Na^+ channels from an undifferentiated (native) mosquito cell line, in order to avoid cloning/expression issues. Previous screens of established insecticides on cell growth of an undifferentiated *Spodoptera exigua* cell line found it was sensitive to mitochondrial-directed compounds, but not insect growth regulators or agents acting specifically upon neuronal targets [5]. The present study used established pharmacological probes, cell growth, and patch clamp techniques, to detect expression of targets in Sua1B cells, a cell line first isolated from neonate *Anopheles gambiae* larvae [6]. Screens focused mostly on compounds affecting VSCCs, since these are known to exist in Sua1B cells [7], and blockers of these channels are insecticidal [8]. Special attention was made to correlate ion channel effects with changes in cell growth. The results could lead to new insecticides and/or high throughput screening methods.

Abbreviations: CACC, calcium-activated chloride channel; CI, 95% confidence intervals; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; EC_{50}, effective concentration at half maximal effect; IC_{50}, inhibitory concentration at 50% of initial effect; FNV, fenvalerate; GABA, gamma-aminobutyric acid; TTX, tetrodotoxin; VSCC, voltage-sensitive chloride channel; VTD, veratridine.

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2. Materials and methods

2.1. Chemicals

Lindane, DIDS, VTD, and FNV were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and were dissolved in DMSO (Fisher Scientific, Pittsburgh, PA, USA). TTX was obtained from Abcam (Cambridge, MA, USA), was dissolved in deionized water and stored as frozen 1 mM aliquots at −80 °C before use.

2.2. General cell culture techniques

The Sua1B insect cell line was obtained from Dr. Michael Povelones of Professor Fotis Kafatos' laboratory (Imperial College, London, UK). The cells were derived from triturated *A. gambiae* neonate larvae and maintained as described previously [6]. Cells were maintained in a log phase culture in tissue culture flasks (BD Falcon, Fisher Scientific, Suwanee, Georgia, USA) treated with Schneider's insect media from Sigma-Aldrich (St. Louis, Missouri, USA). Insect media was supplemented with 10% fetal bovine serum (Sigma) and 100 U/ml penicillin and streptomycin (Sigma). Cells were maintained at 28 °C in a non-humidified environment without CO_2 amendment (Amerex Instruments Inc., Lafayette, CA). Cells were passaged every 3–5 days.

2.3. Treatment of cells with insecticides

Once a confluent monolayer of cells formed in the culture flask, the cells were sloughed off and transferred to another sterile flask. The quantity of cells transferred was determined by the area of the growth surface to give a 1:5 dilution (cells/cm^2), as in a normal passage. Fresh growth medium was added and the cells were allowed to attach for 30 min in the incubator. The media was then removed and fresh media containing either 0.1% DMSO (control) or test compound in vehicle was added to the culture.

2.4. Cell counting

For cell number experiments, counts were performed using the methods of Jenson et al. [9]. Briefly, an ocular grid (0.221 mm^2) was aligned with the corners of the scored lines on the flask so that the same places and area were counted each day. The number of cells and the number differentiated within the grid were recorded visually every day for 3 days. Two counts were taken in areas at each of four intersections so that 8 samples were taken per flask. Each experiment used the same experimental design with at least three replicate flasks.

2.5. Manual whole cell patch clamp and chemical application

Pyrethroid effects on chloride currents were made by manual patch clamp. Currents were measured with an Axopatch 200B patch clamp amplifier (Molecular Devices, Sunnyvale, VA, USA), connected via A/D-converter (Digidata 1440A, Molecular Devices) to a personal computer. Cells were maintained at a holding potential of −100 mV, and stepped up to +100 mV in 10 mV increments and 500 msec duration. Recordings underwent low-pass filtering at 2 kHz, corrected for series resistance, and were sampled at 10 kHz. For recording and analysis, pClamp 10.0 software was used. Cells were bathed in an external buffer of the following composition: 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM *d*-glucose monohydrate, 10 mM HEPES, pH 7.4, osmolarity: 298 mOsmol. Patch pipettes (0.3 μm tip) were filled with solutions containing 10 mM NaCl, 140 mM KF, 2 mM $MgCl_2$, 20 mM EGTA, 10 mM HEPES, pH 7.2, osmolarity: 288 mOsmol.

Solutions of insecticide or drug were prepared in DMSO and diluted for patch clamping experiments into extracellular buffer to the corresponding concentrations with no more than 1% DMSO. After establishing a whole-cell clamp, the cell was perfused with drug via a gravity driven perfusion system. Fluid was controlled with pinch valves releasing fluid from the reservoir into the drug ejection pipette (0.83 mm) for microperfusion over the target cell.

2.6. Planar patch clamp

Planar patch clamp was used for all other electrophysiological experiments. Confluency of cells when harvested for planar patching was between 50%-80%. Cells were washed with 1× PBS (Ca^{2+} and Mg^{2+}-free). Schneider's insect media (9 mL) containing 10% fetal bovine serum (Sigma) and 100 U/ml penicillin and streptomycin (Sigma) was added to the cells and the flask was scraped. Dislodged cells were then centrifuged for 2 min at 100 g and supernatant was discarded. Cells were then re-suspended in external recording solution at a final density of 1×10^6–5×10^7 cells/ml (200–500 μL). Cells were pipetted onto a glass chip whose base was under negative pressure via Port-a-Patch® system (Nanion Technologies Inc., North Brunswick, NJ, USA). After sealing and membrane rupture to establish whole cell clamp, currents were amplified and filtered by a patch-clamp amplifier (HEKA EPC 10), connected to a personal computer. Cells were maintained at a holding potential of −80 mV, and stepped up to +100 mV in 10 mV increments. Steps were for 500 msec duration. Recordings underwent low-pass filtering at 2 kHz and were sampled at 10 kHz. For recording and data analysis, PatchMaster v2x60 software was used (HEKA Instruments, Bellmore, NY, USA). Chips (2–3.5 MΩ) were prepared with internal buffer of the following composition: 50 mM KCl, 10 mM NaCl, 60 mM KF, 2 mM $MgCl_2$, 20 mM EGTA, 10 mM HEPES, pH 7.2., osmolarity: 288 mOsmol. Cells were prepared and patch clamped in an external buffer of the following composition: 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose monohydrate, 10 mM HEPES, pH 7.4, osmolarity: 298 mOsmol.

2.7. Statistical analyses

The growth of cells was determined with single chemical treatment or multiple agents in combination, over time. Cell number over five days was analyzed for each treatment alone or in combination. The mean and SEM of treatment data points were then calculated. Treatment means were compared by using a one-way analysis of variance procedure (ANOVA), and a Student–Newman–Keul's multiple comparison test would follow if a significant treatment effect was observed. Concentration-response curves were constructed from growth measurements or voltage clamp currents, the latter taken at maximal current amplitude during a 500 msec activating pulse. Curves were generated by non-linear regression of log[agonist] vs. response or to a two-site binding model with GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). Data were analyzed for each curve to determine the IC_{50} or EC_{50}, 95% CI, and R^2 (goodness of fit), as well as maximal effect of the treatment.

3. Results

The known chloride channel blockers lindane and DIDS were tested against Sua1B cells to characterize any effects on cell growth (Fig. 1). A time-dependent response was demonstrated for Sua1B cells in the presence of varying concentrations of lindane (0.5–200 μM). No significant inhibition of cell number was observed on day 1 (Fig. 1A). Sua1B cell numbers were significantly inhibited at high concentrations of lindane, with 200 μM showing the highest amount of inhibition on days 2 (73%) and 3 (82%), with less inhibition of cell growth as concentrations decreased to 50 μM. No statistical significance was found in the response to lindane for lower concentrations (0.5–10 μM) on days 1–3 when compared to control (Fig. 1A). A concentration-response plot for lindane was run on Sua1B cell growth over a period of 3 days (Fig. 1B). Inhibition of cell growth after 1 day of exposure was modest and an IC_{50} value could not be reliably determined. The IC_{50} (CI) value for day 2