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Voltage-sensitive potassium channels expressed after 20-Hydroxyecdysone treatment of a mosquito cell line

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ABSTRACT

The goal of this research was to express receptors and ion channels in hormone-treated insect cell lines. Treatment of *Anopheles gambiae* Sua1B cells with 20-hydroxyecdysone showed an inhibition of cell growth over a time course of three days, with no change in cellular morphology. The effect of 20-hydroxyecdysone was enhanced in the presence of the potassium channel blocker 4-aminopyridine, but not tetraethylammonium. Concentration-response curves of 4-aminopyridine in the presence of 42 μM (1 mg/ml) 20-hydroxyecdysone showed similar IC_{50} values (6–10 μM) across 3 day exposures. Whole cell patch clamp confirmed the expression of delayed-rectifier (K_{v2}) potassium channels in hormone-supplemented Sua1B cells, whereas untreated Sua1B cells showed no evidence of Kv2 expression. The hormone-induced expression of K_{v2} channels occurred in as little as 4 h after treatment, but were not observed after 24 h of exposure to 20-hydroxyecdysone, suggesting they played a role in cell death. The expressed channels had current-voltage relationships diagnostic for the K_{v2} subtype, and were inhibited with an $IC_{50} = 13$ mM of tetraethylammonium. Overall, these parameters were similar to *Anopheles gambiae* Kv2 potassium channels expressed in HEK-293 cells. The induced presence of ion channels (and possibly receptors) in these cells has potential utility for high throughput screening and basic neuroscience research.

1. Introduction

Mosquitoes and ticks, which account for 85% of known animal species, are important vectors of disease (Lonc et al., 2011). The worldwide resurgence of vector-borne diseases has been a growing problem for a quarter of a century since its initial rise in the 1970s (Gubler, 2008). Many factors, including insecticide and drug resistance, have played a role in the resurgence of zoonotic diseases leaving researchers looking for new prevention, surveillance, and control techniques (Gubler, 1998). Another major contributor of resurgence is the introduction and changes in arthropod disease vector distribution, especially in the Americas. For example, the yellow fever mosquito (*Aedes aegypti*), the Asian tiger mosquito (*Aedes albopictus*), which transmits dengue fever, and the black-legged tick, *Ixodes scapularis*, which transmits Lyme disease have all changed in geographical distribution in the past decades (Norberg et al., 2013). These factors challenge the control and prevention techniques currently employed, and demonstrate clearly the need for new insecticides.

The success rate of insecticide development has declined due to increased cost of chemical synthesis and the difficulty of finding novel compounds having both high toxicity and selectivity for target organisms (Hammock and Soderlund, 1986). Ridley et al. (1998) indicate that companies have typically screened over 100,000 compounds per year, per assay, including both *in vitro* and *in vivo* testing. High-throughput screening (HTS) techniques have been adapted for the drug discovery process to reduce the amounts of media and compound used in 96-, 384-, and 1536-well microplate formats (Woods et al., 2010). At present, the discovery and development of insecticides is limited by the available technologies and the procurement of large quantities of nervous tissue or target site proteins.

Primary cell cultures, which are laborious to produce and maintain, and have poor tissue longevity, are inadequate for insecticide screening. Cloning, along with transient and

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heterologous expression of ion channel and receptor proteins in cell culture has been an important avenue used in the drug discovery pipeline (Eglan, 2009). However, the use of such expression techniques for insecticide development comes with disadvantages. Many insect target site proteins have poorly defined subunit composition and screening homomultimeric or chimeric targets, while convenient, may express altered pharmacology compared to "native" targets, all of which raise possible issues of assay fidelity. The goal of this research was to evaluate the presence of insecticide target proteins in hormone-treated insect cells, which could lead to new HTS methods, as well as a source of insect proteins for basic research.

2. Materials and methods

2.1. Chemicals

Chemicals used were 20-hydroxyedcysone (20-HE), veratridine (VTD), tetraethylammonium (TEA), and 4-aminopyridine (4-AP). All chemicals had a purity of ≥90% and were obtained from Sigma-Aldrich (St. Louis, Missouri, USA.). They were dissolved in DMSO (Fisher Scientific, Pittsburgh, PA, USA) as stock solutions, except for TEA, which was dissolved in buffer. Other biochemicals and salts were obtained from various commercial suppliers.

2.2. 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 *An. gambiae* neonate larvae and maintained as described previously (Dimopoulos et al., 1997). They were grown in a log phase culture in tissue culture flasks (BD Falcon Tissue Culture Flasks, Fisher Scientific, Suwanee, Georgia, USA) maintained on Schneider's insect media (Sigma-Aldrich). Insect media was supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 U/ml penicillin and streptomycin (Sigma-Aldrich). Cells were maintained at 28 °C in a non-humidified environment without CO_2 amendment and passed every 3–5 days.

Human embryonic kidney (HEK)-293 cells (CRL-1573) were maintained on Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, as described previously (Larson et al., 2017). Briefly, cells were stably transformed to express the *Anopheles gambiae* Kv2 (*Ag*Kv2) channel gene (Accession # XM_315955.4) by combining it with a CMV promoter and tetracycline-regulated expression mechanism. The cell line was engineered by Dualsystems Biotech AG (Schlieren, Switzerland) under commercial contract. Tetracycline for induced expression was obtained from Sigma-Aldrich. Cells were maintained at 37 °C in a non-humidified environment with 5% CO_2 amendment. Cells were passed every 3 days according to the manufacturer's instructions.

2.3. Application of 20-hydroxyecdysone and cell counting

Once a confluent monolayer of Sua1B 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 containing either 0.1% DMSO (control), 20-HE in DMSO, or 20-HE and drug in DMSO or other vehicle, was then added to the culture. For electrophysiological studies, 20-HE was added immediately after maintenance (24 h experiments) or to fresh growth medium for cells grown in dishes 3 h before experiments were performed. Growth and differentiation studies were conducted with the same methods described in Jenson et al. (2012). For cell counts, each plate was scored with a razor blade prior to plating so that unbiased samples were taken. An ocular grid (0.221 mm^2) was lined up 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 within the grid area were recorded visually every day for 3 days, with at least three replicate flasks per treatment.

2.4. Patch clamp electrophysiology

Currents were amplified and filtered by patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale CA, USA) connected via A/D-converter (Digidata 1440A) 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 were sampled at 10 kHz and underwent series resistance compensation and low-pass filtering at 2 kHz. For recording and analysis, pClamp 10.0 software (Molecular Devices) 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 (ca. 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 desired concentrations, with no more than 0.1% 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 flow from the reservoir into the drug ejection pipette (ca. 0.8 mm) for microperfusion over the target cell.

2.5. Statistical analysis

Cellular growth studies were determined with single chemical treatment or multiple agents in combination, over time. The number of cells over three days were analyzed by calculating the mean and standard error of the mean (SEM) for each treatment alone or in combination, using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). If a significant treatment effect was observed among treatments, the means were compared using a one-way analysis of variance procedure (ANOVA) followed by a Student-Neuman Keul's (SNK) multiple comparison test. Similarly, measurements of potassium current amplitude were analyzed as mean ± SEM, with one-way ANOVA or paired *t*-test used to evaluate them for statistically significant differences. Concentration-response curves were fit by non-linear regression to a four parameter logistic equation using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). Data were analyzed for each curve to determine the IC_{50} or EC_{50}, 95% confidence intervals (CI), and goodness of fit parameter (R^2).

3. Results

3.1. Cell growth and morphology studies

As reported previously (Diykov et al., 2013), Sua1B cells in culture displayed a variety of morphological shapes, typically non-spherical and often with fine processes, in many cases superficially resembling monopolar, bipolar, and multipolar neurons (Fig. 1A). The cells showed no obvious morphological changes after 48 h in 42 μM 20-HE, but inhibition of cell growth was apparent through reduction in cell density (Fig. 1B).

In studies of hormone and ion channel probes on cell growth, Sua1B cells were exposed to 20-HE (42 μM), 4-AP (100 μM), or VTD