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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 μ M 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.

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

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.

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 *An. 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 CO₂ 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/cm²), as in a normal passage. Fresh growth medium was added and the cells were allowed to attach for 30 minutes 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 mm²) 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 MgCl₂, 2 mM CaCl₂, 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 MgCl₂, 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 were between 50%-80%. Cells were washed with 1x 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 $1 \times 10^6 - 5 \times 10^7$ 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 M Ω) 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 PrismTM (GraphPad Software, San Diego, CA, USA). Data were analyzed for each curve to determine the IC₅₀ or EC₅₀, 95% CI, and R² (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 μ M). 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 μ M showing the highest amount of inhibition on days 2 (73%) and 3 (82%), with less inhibition of cell growth as concentrations decreased to 50 μ M. No statistical significance was found in the response to lindane for lower concentrations (0.5-10 μ M) 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 IC₅₀ value could

not be reliably determined. The IC_{50} (CI) value for day 2 was 87 (44-172) μ M, $R^2 = 0.84$, and Hill slope of -0.67; and for day 3 was 68 (34-135) μ M, $R^2 = 0.85$, and a Hill slope = -0.72 (Fig. 1B).

Sua1B cells displayed an increased Cl^- current amplitude after the application of lindane (Fig. 2A,B), whereas DMSO (0.1%) showed no change. The effect of lindane varied with concentration, and was elevated significantly above basal levels at 100 μ M (t-test, $P < 0.05$). Lindane stimulated chloride currents to a calculated maximum of 144% of control, with an EC_{50} of 116 (21-658) nM, $R^2 = 0.23$, and a Hill slope of 1.6 (Fig. 2C). Lindane enhancement of chloride currents was blocked in extracellular solution without calcium ions (Fig. 2C). Current amplitudes in 1 mM lindane were lower than expected, and the current amplitude declined both with and without calcium. This effect may result from chloride current block by lindane at this concentration, and these data distorted the plot and curve fit, as reflected in the low R^2 value (0.23).

DIDS, a VSCC blocker [10], showed significant inhibition of cell growth at high concentrations, with 100 μ M active on day 1 and declining in effect thereafter, while 200 μ M was effective on all 3 days of incubation, when compared to control (Fig. 3A). No significant differences were observed on cell growth in the presence of 50 μ M DIDS. A concentration-response plot for DIDS was run on Sua1B cell growth at day 2 of exposure, which showed an $IC_{50} = 53$ (48-80) μ M, $R^2 = 0.93$, and a Hill slope = 0.75 (Fig. 3B). The calculated minimum for the curve was 439 cells, for a maximal 46% inhibition of cell growth by DIDS.

Because DIDS is a known blocker of Cl^- currents and reduced the number of Sua1B cells, its effects on Cl^- currents were studied using whole cell planar patch electrophysiological techniques. Cells displayed reduced current amplitude after the application of DIDS (100 nM-1

mM), especially at positive potentials, and with increased inhibition at higher concentrations (10 μ M-1 mM) (Fig. 4A). DMSO (0.1%) controls showed no effect on the amplitude of the chloride current. A concentration-response plot for DIDS was run on Sua1B cells on day 2 of exposure, which showed an $IC_{50} = 5.1 \mu$ M (0.15-174.5 μ M), a Hill slope = -0.5, and an $R^2 = 0.63$ (Fig. 4B). Sua1B cells only showed partial inhibition of Cl^- current (69%) with DIDS even at high concentrations (1 mM).

Some chloride currents (7 out of 19 patches) did not respond to DIDS inhibition, so recordings were performed in a low chloride buffer to confirm that they were chloride currents (Fig. 5A,B). In the presence of low chloride solution (35 mOsmol Cl^- in low chloride saline solution compared to 297 mOsmol Cl^- in regular external saline solution), DIDS-insensitive currents showed significantly reduced current amplitude, confirming that they were indeed chloride currents (Fig. 5A,B). No early inward currents consistent with fast sodium channels were observed in patch clamp studies.

Chloride currents were also exposed to a pyrethroid of both the type 1 (allethrin) and type 2 (FNV) categories, respectively [11, 12], and compared to control currents in DMSO (0.1%) to determine any significant effects on overall current amplitudes (Fig. 6). Control Cl^- currents (Fig. 6A) were reduced about 27% by 10 μ M allethrin (Fig. 6B), but substantially reduced by this same concentration of FNV (Fig. 6C). Replicated current-voltage relationships of different cells ($n = 6$) showed no significant reduction of current amplitudes in Sua1B cell recordings after application of 10 μ M allethrin (Fig. 6D) when compared to control Cl^- current amplitudes. In contrast, FNV (10 μ M) displayed 60-70% reduction in current amplitude at this concentration when compared to control currents at -100 and +100 mV (Fig. 6E).

In a previous study [13], we found that when applied to Sua1B cells alone, 1 μ M VTD (a sodium channel activator) and TTX (a sodium channel blocker) showed no effect on cell growth. In the present investigation, when VTD was applied in combination with varying amounts of FNV, there was inhibition in cell number that was fit by a two-site binding model (Fig. 7). A potent effect of $IC_{50} = 15$ nM (4.5-49 nM), was found for the high affinity site and a lower potency effect with $IC_{50} = 56$ μ M (25-124 μ M) was found for the second site. The biphasic curve fit had an $R^2 = 0.951$. Sua1B cells were then exposed to TTX over a 3 day period with combinations of VTD (1 μ M) + FNV (1 μ M or 100 μ M). In these experiments, 1 μ M TTX reversed the inhibitory effect of 0.1 μ M FNV + 1 μ M VTD on cell number, whereas high concentrations of FNV (100 μ M + 1 μ M VTD) showed inhibition of cell numbers that could not be reversed by TTX (Fig. 7, inset).

4. Discussion

Two types of chloride channels previously identified in Sua1B cells [7] were characterized within this study. Outwardly rectifying voltage sensitive currents observed in native Sua1B cells seem to be mediated either primarily by CACCs or by a combination of CACCs and volume-sensitive, outwardly-rectifying anion channels [7]. High concentrations of lindane, an organochlorine insecticide, inhibited growth of Sua1B cells, but the response did not occur in a typical concentration-dependent manner. Current amplitudes were increased at concentrations below 1 mM, which then reduced current amplitude (Fig. 2). The primary neurotoxic effects of lindane are usually attributed to its interference with γ -aminobutyric acid (GABA) receptor-ionophore complex; however, Abalis *et al.* [14] showed that lindane also binds with high affinity ($K_i = 0.04 \pm 0.01$ μ M) to VSCC in *Torpedo* electroplaque. Lindane, at the concentrations run in this study (50-200 μ M), also affects intracellular calcium mobilization in

rat myometrial smooth muscle cells (100-200 μM) [15], guinea pig macrophages (125 μM) [16] and human HL-60 cells [17]. Such calcium mobilization could explain the effects of lindane at high concentrations on Sua1B cell growth, as well as activation of CACC (Fig. 3). However, any lindane-specific alterations in calcium ion homeostasis or signaling remain to be determined. Future studies utilizing calcium fluorescence assays in the presence and absence of lindane should be run to verify any possible effects on calcium ion flux/homeostasis.

Outward Cl^- currents expressed in Sua1B cells were mostly blocked by DIDS. The insecticidal/nematicidal properties of DIDS have been documented by Boina *et al.* [18, 19], who observed paralysis in free living and plant parasitic nematodes, and was a stomach poison in European corn borer (*Ostrinia nubilalis*) larvae. Additional experiments on European corn borer disclosed that DIDS had a dose dependent effect on larval midgut $^{36}\text{Cl}^-$ ion transport ($\text{IC}_{50} = 7.4 \mu\text{M}$) and midgut alkalinity (ca. $\text{IC}_{50} = 29 \mu\text{M}$) [19]. This latter action was similar to the potency found for DIDS on cell growth in this study ($\text{IC}_{50} = 53 \mu\text{M}$). Using the planar patch clamp technique, the inhibitory effect of DIDS on Cl^- currents at +60 mV had $\text{IC}_{50} = 5.1 \mu\text{M}$, which is 10-fold greater potency than that observed in the cell growth studies, but similar to the IC_{50} for blocking $^{36}\text{Cl}^-$ uptake in European corn borer larval midgut. A similar potency of DIDS on Cl^- currents was shown in rat peritoneal mast cells with $\text{IC}_{50} = 2.3 \mu\text{M}$ and a Hill coefficient $n = 0.7$ [20]. Not all cells showed inhibition of current amplitude by DIDS, and this quality is shared by the cystic fibrosis transmembrane Cl^- conductance regulator [21] and a certain ClC-3 type of chloride channel expressed in CHO cells [22]. The identity of the DIDS insensitive Cl^- channel of Sua1B cells and the amino acids responsible for DIDS sensitivity in these channels remain to be determined.

When characterizing the chloride channels present in Sua1B cells, this study found the majority of channels to be VSCCs, while those Diakov *et al.* [7] found were predominantly the CACC channel subtype. Both chloride channel subtypes were found in each study, but are thought to activate in different physiological circumstances and occur independently of one another. We do not know why the variation in chloride channel expression occurs, but it could be due to different aliquots/passages of Sua1B cells. Both studies showed the same reduction in current amplitude in the presence of DIDS (69%), confirming that a similar population of DIDS-sensitive chloride channels was present.

Patch clamped Sua1B cells lack inward currents indicative of typical electrogenic sodium or calcium channels. However, in the presence of 1 μ M VTD and increasing concentrations of FNV, cell death was increased in a dose-dependent, biphasic manner (Fig. 7). The high potency effect ($EC_{50} = 15$ nM) was completely inhibited in the presence of TTX, suggesting the possible presence of an “electrically-silent” sodium channel, similar to that previously described in rat C6 [23] and C9 cells [24]. In these cells, sodium currents cannot be activated by voltage clamp depolarization, but radiotracers can be used to measure chemical activation of the channels by alkaloid or protein neurotoxins. Similar experiments could be used to characterize these putative sodium channels in Sua1B cells.

The signs of intoxication by pyrethroids differ between the two categories (type 1 and 2) in mammals, where compounds described as type 1 (e.g., allethrin) display a tremor syndrome, whereas type 2 (e.g., FNV) present choreoathetosis (sinuous writhing) and salivation [25]. The different intoxication syndromes of the two classes of pyrethroid led to the hypothesis that there are other mechanisms involved in the toxicity of pyrethroids than just an action on sodium channels. Relevant to the present study is a proposed effect on voltage-gated chloride channels.

Forshaw and Ray [26] showed a reduction of chloride conductance and an increase in membrane resistance in mammalian skeletal muscle, non-myelinated nerve fibers, and mouse neuroblastoma cells in the presence of a type 2 pyrethroid (deltamethrin), but not to a type 1 (cismethrin). In Sua1B cells, we observed an inhibition of chloride current amplitude after application of 10 μ M FNV (type 2), while no significant inhibition of the chloride current was shown in the presence of 10 μ M allethrin (type 1). Such an effect of FNV on VSCC could contribute to the low potency inhibitory effect of FNV on Sua1B cell growth, but could also be due to mitochondrial effects, since Gassner *et al.* [27] showed pyrethroids are micromolar inhibitors of mitochondrial complex 1 in rat hepatocytes. To our knowledge, this finding is the first report of type 2 pyrethroid inhibition of insect VSCCs, and further research is required to explore any role it plays in pyrethroid intoxication and lethality.

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References

- [1] B.M. Greenwood, D.A. Fidock, D.E. Kyle, S.H. Kappe, P.L. Alonso, F.H. Collins, P.E. Duff, Malaria: progress, perils, and prospects for eradication. *J Clin Invest* 118 (2008) 1266–1276.
- [2] J. Casida, G. Quistad, Golden age of insecticide research: past, present, or future? *Annu Rev Entomol* 43 (1998) 1-16.

306 [3] G. Smagghe, C.L. Goodman, D. Stanley, Insect cell culture and applications to research
307 and pest management. *In vitro Cell. Dev. Biol. Animal* 45 (2009) 93-105.

308 [4] L. Swevers, L. Kravariti, S. Ciolfi, M. Xenou-Kokoletsi, N. Ragoussis, G. Smagghe, Y.
309 Nakagawa, B. Mazomenos, K. Iatrou, A cell-based high-throughput screening system for
310 detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic
311 compounds. *FASEB J* 18 (2003) 134-136.

312 [5] L. Decombel, G. Smagghe, L. Tirry, Action of major insecticide groups on insect cell
313 lines of the beet armyworm, *Spodoptera exigua*, compared with larvacidal toxicity. *In*
314 *vitro Cell. Dev. Biol. Animal.* 40 (2004) 43-51.

315 [6] G. Dimopoulos, A. Richman, H-M Mueller, F.C. Kafatos, Molecular immune responses
316 of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc Natl Acad Sci*
317 *USA* 94 (1997) 11508-11513.

318 [7] D. Diakov, L.J. Jenson, J.R. Bloomquist, Voltage-sensitive chloride ion channels in
319 *Anopheles gambiae* Sua-1B cells. *Invert Neurosci* 13 (2013) 57-62.

320 [8] D.R. Boina, J.R. Bloomquist, Blockage of chloride channels and anion transporters with
321 pesticidal natural products and their synthetic analogs. *Phytochem Rev* 10 (2011) 217–
322 226.

323 [9] L.J. Jenson, S.L. Paulson, J.R. Bloomquist, Induction and inhibition of an apparent
324 neuronal phenotype in *Spodoptera frugiperda* insect cells (Sf21) by chemical agents.
325 *Invert Neurosci* 12 (2012) 119–127.

326 [10] Z. Qu, H.C. Hartzell, Functional geometry of the permeation pathway of Ca^{2+} -activated
327 Cl^- channels inferred from analysis of voltage-dependent block. *J Biol Chem* 276 (2001)
328 18423–18429.

329 [11] M.S. Schechter, N. Green, F.B. LaForge, Constituents of pyrethrum flowers. XXIII.
330 Cinerolone and the synthesis of related cyclopentenolones. J Amer Chem Soc 71 (1949)
331 3165–3173.

332 [12] N. Ohno, K. Fujimoto, Y. Okuno, T. Mizutani, M. Hirano, N. Itaga, T. Honda, H.
333 Yoshioka, A new class of pyrethroidal insecticides; α -substituted phenyl-acetic acid
334 esters. Agr Biol Chem 38 (1974) 881–883.

335 [13] L.J. Jenson, J.R. Bloomquist, Role of serum and ion channel block on growth and
336 hormonally-induced differentiation of *Spodoptera frugiperda* (Sf21) Insect Cells. Arch
337 Insect Physiol Biochem 90 (2015) 131-139.

338 [14] I.M. Abalis, M.E. Eldefrawi, A.T. Eldefrawi, Binding of GABA receptor channel drugs
339 to putative voltage-dependent chloride channel in *Torpedo* electric organ.
340 Biochem Pharmac 34 (1985) 2579-2582.

341 [15] K.A. Criswell, E.L. Stuenkel, R. Loch-Caruso, Lindane increases intracellular calcium in
342 rat myometrial smooth muscle cells through modulation of inositol 1,4,5-trisphosphate-
343 sensitive stores. J Pharmacol Exp Ther 270 (1994) 1015-1024.

344 [16] A. Holian, M.A. Marchiarullo, D. Stickle, Gamma hexachlorocyclohexane activation of
345 alveolar macrophage phosphatidylinositol cycle, calcium mobilization and O₂-
346 production. FEBS Lett 176 (1984) 151-154.

347 [17] J.J. Kang, I.L. Chen, H.F. Yen-Yang, Mediation of γ -hexachlorocyclohexane-induced
348 DNA Fragmentation in HL-60 Cells through Intracellular Ca₂₊ Release Pathway. Food
349 Chem Toxicol 36 (1998) 513-520.

350 [18] D.R. Boina, E.E. Lewis, J.R. Bloomquist, Nematicidal activity of anion transport
351 blockers against *Meloidogyne incognita*, *Ceanorhabditis elegans*, and *Heterorhabditis*
352 *bacteriophora*. Pest Manag Sci 64 (2008) 646-653.

353 [19] D.R. Boina, J.R. Bloomquist, Toxicity and disruption of midgut physiology in larvae of
354 the European corn borer, *Ostrinia nubilalis*, by anion transporter blockers. Arch Insect
355 Biochem Physiol 70 (2009) 151–161.

356 [20] J. Dietrich, M. Lindau, Chloride channels in mast cells: Block by DIDS and role in
357 exocytosis. J Gen Physiol 104 (1994) 1099-1111.

358 [21] E.A. Pasyk, J.K. Foskett, Mutant ($\Delta F508$) cystic fibrosis transmembrane conductance
359 regulator Cl⁻ channel is functional when retained in endoplasmic reticulum of
360 mammalian cells. J Biol Chem 270 (1995) 12347-12350.

361 [22] Li X, K. Shimada, L. Showalter, S. Weinman, Biophysical properties of ClC-3
362 differentiate it from swelling-activated chloride channels in chinese hamster ovary-k1
363 cells. J Biol Chem 275 (2000) 35994-35998.

364 [23] P.W.L. Tas, H-G Kress, K. Koschel, Halothane inhibits the neurotoxin stimulated [¹⁴C]-
365 guanidinium influx through ‘silent’ sodium channels in rat glioma C6 cells. FEBS Lett
366 182 (1985) 269-272.

367 [24] Y. Jacques, G. Romey, M.T. Cavey, B. Kartalovski, M. Lazdunski, Interaction of
368 pyrethroids with the Na⁺ channel in mammalian neuronal cells in culture. Biochimica et
369 Biophysica Acta 600 (1980) 882-897.

370 [25] D.M. Soderlund, J.M. Clark , L.P. Sheets, L.S. Mullin, V.J. Piccirillo, D. Sargent, J.T.
371 Stevens, M.L. Weiner, Mechanisms of pyrethroid neurotoxicity: implications for
372 cumulative risk assessment. Toxicology 171 (2002) 3–59.

- 373 [26] P.J. Forshaw, D.E. Ray, A novel action of deltamethrin on membrane resistance in
374 mammalian skeletal muscle and non-myelinated nerve fibres. *Neuropharmacol* 29 (1990)
375 75-81.
- 376 [27] B. Gassner, A. Wüthrich, G. Scholtysik, M. Solioz, The pyrethroids permethrin and
377 cyhalothrin are potent inhibitors of the mitochondrial complex I. *J Pharmacol Exp Ther*
378 281 (1997) 855-860
379
380

Figures

Figure 1. Effects of the organochlorine insecticide lindane on Sua1B insect cell number. (A) Time course curves for varying concentrations of lindane on Sua1B cells. (B) Concentration-response curves of lindane effects on Sua1B cell number over culture days 1-3. In B, the numbers of cells in solvent-treated controls are identical to those observed at the lowest concentration of lindane. In both plots, symbols are means $\pm$ SEM, and when error bars are absent, they are within the size of the symbol.

Figure 2. Illustration of chloride channel currents under control (A) conditions and in the presence of 1 mM lindane (B). C. Concentration-dependence of increased chloride current in Sua1B cells when exposed to lindane (n=9); extracellular solution containing calcium compared to the effect of lindane applied in extracellular solution without calcium (n = 6). Symbols are means $\pm$ SEM bars. Asterisks indicate a significant difference in lindane effects at a given concentration, in the presence and absence of calcium (unpaired T-test, $P < 0.05$).

Figure 3. Effects of DIDS on the number of Sua1B cells (A) at various incubation times (n=3). Bars are means $\pm$ SEM. Statistical analysis was performed for each post-treatment day using one-way ANOVA ($P < 0.05$) and a Dunnett's multiple comparison post test. Letters represent statistical difference for a given day of exposure, and bars labeled by different letters are significantly different. Concentration-response curve of DIDS (B) on the number of Sua1B cells, corresponding to data for day 2 of the incubation (n=3). Symbols are means $\pm$ SEM bars. When error bars are absent, they are within the size of the symbol.

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405 Figure 4. Electrophysiological effects of DIDS on chloride currents in Sua1B cells. (A) Current-
406 voltage relationship of chloride current in Sua1B cells and inhibition with DIDS ($n = 6$).
407 Symbols are means with error bars omitted for clarity. (B) Concentration-response curve
408 representing percent inhibition of DIDS at +60 mV ($n = 6$). Symbols are means $\pm$ SEM bars.

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410 Figure 5. DIDS insensitive chloride currents under planar patch conditions. Traces in (A) and
411 (B) illustrate currents in normal (297 mOsmol) and low (35 mOsmol) chloride extracellular
412 solutions, respectively. Plot (C) shows current-voltage relationship of DIDS-insensitive chloride
413 channels in control and low chloride solutions ($n=3$). Symbols are means $\pm$ SEM bars. Asterisks
414 indicate a significant difference in current amplitude at a given voltage step in control or low
415 chloride conditions (unpaired T-test, $P < 0.05$).

416

417 Figure 6. Effects of pyrethroids on Sua1B cells using manual whole cell patch clamping
418 technique. (A) shows control chloride current of a Sua1B cell before treatment. (B) and (C) are
419 examples of chloride current inhibition by type 1 (allethrin) and type 2 (FNV) pyrethroids,
420 respectively (10 μ M each). Panels (D) and (E) show current-voltage relationships of control
421 chloride currents and those currents in the presence of type 1 allethrin ($n=6$, 10 μ M) and type 2
422 FNV ($n=4$, 10 μ M) class of pyrethroids. Symbols are means $\pm$ SEM and asterisks indicate a
423 significant difference in current amplitude at a given voltage step (unpaired T-test, $P < 0.05$).

424

425

426 Figure 7. Inhibition of cell growth by FNV in the presence of a constant 1 μ M concentration of
427 VTD on cell number in Sua1B cells, and block of this effect by 1 μ M TTX. Concentration-
428 response curve of type 2 pyrethroid FNV taken on day 3 of incubation (n = 3 replicate
429 experiments run on different days with different flasks of cells) is fit by a two site model.
430 Symbols are means $\pm$ SEM bars. When error bars are absent, they are within the size of the
431 symbol. Inset: TTX sensitivity of high potency (10^{-7} M FNV) and low potency (10^{-4} M FNV)
432 effects, in separate experiments with matched controls (n = 3). Statistical analysis was
433 performed using one-way ANOVA (P<0.05) and a Student-Newman-Keul's multiple
434 comparison post test. Bars labeled by different letters represents statistical significance (P <
435 0.05).

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Figure(s)

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Photomicrograph of a typical Sua1B culture used in this study.