Calcium Channels in Insects

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1 INTRODUCTION

Calcium ions are known to be centrally involved in a wide range of cellular functions. In excitable cells they are intimately involved in functions such as: secretion, intracellular movement, neurotransmitter release, muscular contraction, enzyme activity, membrane potential, control of other ion channels and ultimately cell toxicity and death. It is thus apparent that careful regulation of intracellular calcium concentration is vital, and that manipulation of these processes might provide potent methodology for perturbing such functions for therapeutic or disruptive purposes.

2 [$^{45}Ca^{++}$] ENTRY INTO LOCUST SKELETAL MUSCLES VIA GLUTAMATE-GATED AND VOLTAGE GATED ION CHANNELS

The excitatory amino acid L-glutamate acts on depolarising junctional and extrajunctional receptors in locust skeletal muscle to gate non-specific cationic channels which are permeable to Na^+, K^+, and Ca^{++}[1,2]. Normally these quisqualate sensitive L-glutamate receptors desensitise rapidly in the presence of agonists, however this desensitisation can be blocked by preincubation in the lectin Concanavalin A (ConA). Incubation of locust muscle in L-glutamate has been shown to cause muscle cytotoxicity following ConA treatment, and this toxic response to glutamate has been shown to be calcium dependent. It has been proposed that the damage to muscle cells is caused by the entry of Ca^{++} through glutamate-receptor gated channels when they are prevented from desensitising and this system has been proposed as a model for excitotoxicity[3]. δ-Philanthotoxin (PTX-433) is a toxin from the venom of the digger wasp *Philanthus triangulum* which has been shown to block L-glutamate gated ion channels in a number of systems including locust skeletal muscle[4,5].

It has also been shown that insect skeletal muscles possess voltage-gated Ca^{++}

channels (VGCC) [6-10] whose pharmacology is broadly similar to vertebrate L-type Ca^{++} channels[11] and can be blocked by phenylalkylamines such as verapamil and dihydropyridines such as nifedipine. We have therefore carried out experiments to examine the influx of [$^{45}Ca^{++}$] through glutamate-gated and voltage-gated channels in locust skeletal muscle and to investigate the pharmacological differences between them.

2.1 Method

Metathoracic legs of female locusts (*Schistocerca gregaria)* were dissected to expose the *extensor tibiae* muscle. Muscles were incubated in standard locust saline containing (mM): NaCl (180); KCl (10); $CaCl_2$ (1); HEPES (10); pH 6.8; for 10 minutes followed by a further 30 mins in saline with or without ConA (1μM). Channel blockers, PhTX or nifedipine, were included in the preincubation for 20 minutes.

Muscles were incubated for 3 minutes in saline containing 0.037MBq/ml [$^{45}Ca^{++}$] and depolarised by addition of L-glutamate (100pM-10mM) or by elevating $[K^+]_o$ to 50mM, contralateral legs served as unstimulated controls.

After various times (optimum 15 minutes) muscles were washed (x3) with ice cold saline and finally with ethanol. Ethanol was discarded and the muscle was detached and allowed to dry overnight. The dry weight of the muscle was determined. Muscles were solubilised in 350ml Triton X100 1%v/v for 3-4 hrs, before addition of 4ml of aqueous scintillation fluid. Levels of [$^{45}Ca^{++}$] were determined by liquid scintillation counting and results were expressed as cpm/mg dry muscle weight.

2.2 Pharmacology of [$^{45}Ca^{++}$] Entry into Locust Skeletal Muscle

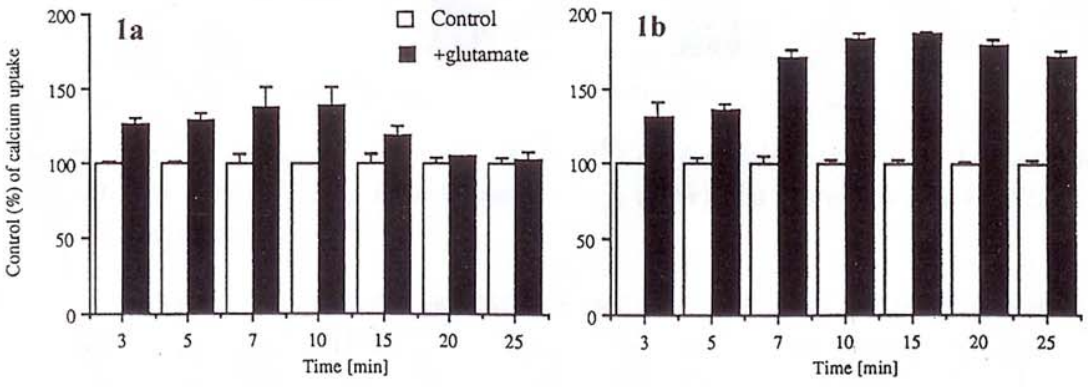

Figure 1 *Time course of [$^{45}Ca^{++}$] uptake following addition of L-glutamate 100μM. In the absence of ConA* **(Figure 1a)** *uptake peaks at 40% above control levels. Preincubation in 1μM ConA* **(Figure 1b)** *results in a higher level of uptake (87% above control) which persists for up to 25 minutes. Each bar represents mean ± SEM of at least six experiments.*

L-glutamate produced a dose dependent and time dependent increase in [$^{45}Ca^{++}$] entry into the muscle. [$^{45}Ca^{++}$] entry was maximally stimulated by 100μM glutamate and peaked after 10-15 minutes (Figure 1). Preincubation in ConA increased both the time course and amount of [$^{45}Ca^{++}$] entry.

Preincubation in PhTX-343 did not affect basal uptake but reduced L-glutamate (100μM) induced uptake dose dependently with an IC_{50} of approximately 10 nM. (Figure 2)

Elevated $[K^+]_o$ (50mM) induced an influx of [$^{45}Ca^{++}$] of similar magnitude to that produced by L-glutamate however this process was unaffected by preincubation in ConA. The dihydropyridine calcium channel blocker nifedipine reduced [$^{45}Ca^{++}$] entry in a dose dependent fashion with an IC_{50} of approximately 50 nM (Figure 3). In contrast to the results described above PhTX-343 has little effect on elevated $[K^+]_o$ induced [$^{45}Ca^{++}$] entry and nifedipine does not block [$^{45}Ca^{++}$] movement through L-glutamate gated channels (Fig 4).

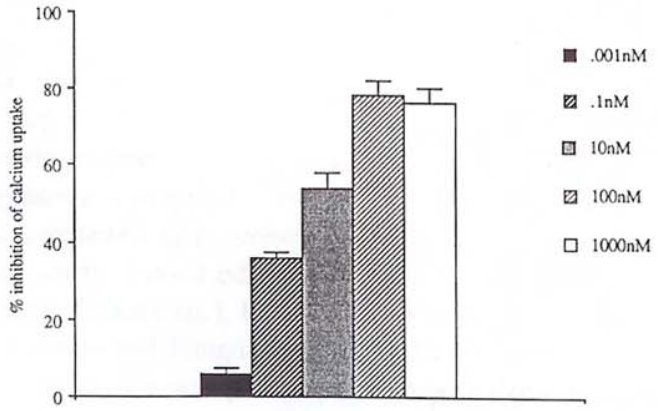

Figure 2 *PhTX-343 inhibited L-glutamate (100μM) induced [$^{45}Ca^{++}$] uptake dose dependently. Each bar represents mean ±SEM of at least six experiments for a particular dose of PhTX-343.*

It is thus apparent that [$^{45}Ca^{++}$] can enter locust muscles through both voltage and ligand gated ion channels, and the use of pharmacological tools can enable these two ionic fluxes to be isolated. Presumably during normal function excitability is regulated by both of these mechanisms, but their pharmacological distinctiveness may provide opportunities to target them individually as a potential control strategy.

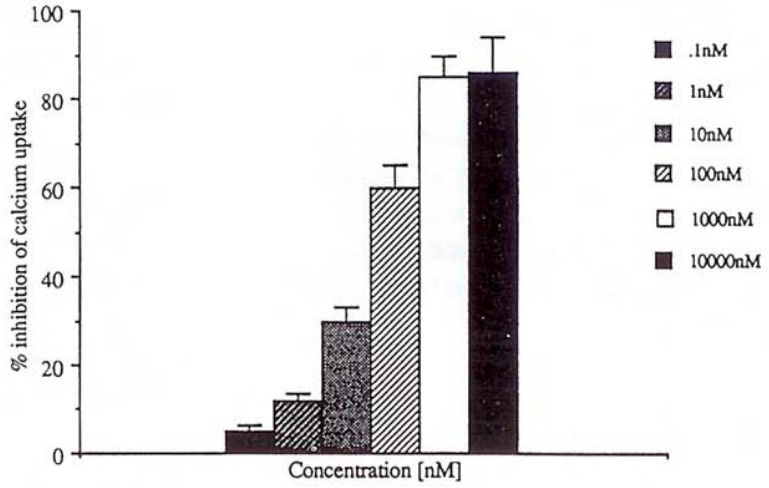

Figure 3 *Dose inhibition relationship for the action of nifedipine on elevated $[K^+]_o$ induced $[^{45}Ca^{++}]$ uptake by locust muscle. Each point represents mean ± SEM of at least six experiments.*

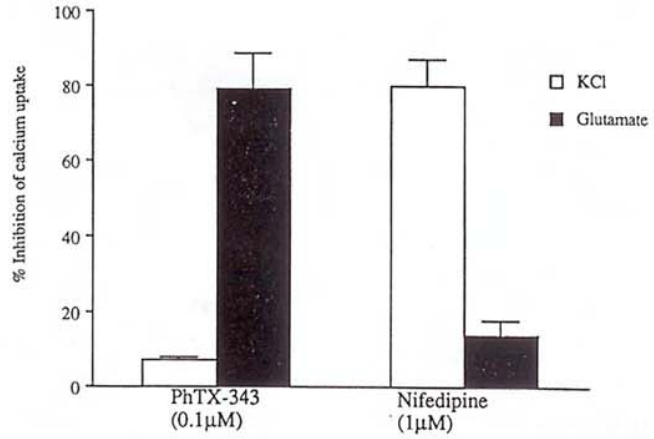

Figure 4 *PhTX-343 and Nifedipine inhibit $[^{45}Ca^{++}]$ uptake induced by L-glutamate (100μM) and elevated $[K^+]_o$ (50mM) respectively. Each point represents mean ± SEM of at least six experiments.*

3. INTERACTIONS OF HOUSEFLY NEURONAL CALCIUM CHANNELS WITH DELTAMETHRIN

VGCC are known to be widespread in the nervous systems of invertebrates[10] and whilst their properties resemble those of the well-characterised calcium channel sub-types identified in vertebrate preparations[12] functional differences have also been described[10]. Cloning studies have now enabled the molecular structure of vertebrate VGCC to be extensively characterised. They consist of a number of subunits, with the α_1–subunit being identified as the component responsible for forming the voltage gated pore. The α_1–subunit

consists of a peptide with 24 membrane spanning helices arranged in four repeating domains reminiscent of the structure of the α_1–subunit of the voltage-gated sodium channel. The α_1–subunits have been cloned and sequenced from a number of vertebrate VGCC[12], and more recently from the insects *Musca domestica* (Md1α)[13] and *Drosophila melanogaster* (Dmca1D)[14]. Comparisons of sequence homologies between insect VGCC α_1–subunits and those of mammals have suggested that the insect channels are most similar to L-type calcium channels.

It is firmly established that the pyrethroid insecticides act on insect voltage-gated sodium channels[15] to modify their gating properties. In view of the molecular similarities between voltage-gated sodium and calcium channels, a series of experiments was carried out to see if there was any interaction between the type II pyrethroid deltamethrin and housefly neuron calcium currents.

3.1 Method

Adult houseflies (Musca. domestica) were anaesthetised with CO_2, decapitated and the thoracic ganglia were transferred to ice cold sterile Ca^{++}/Mg^{++}-free dipteran saline containing (mM): NaCl (135), KCl (2.5), $NaH_2PO_3.H_2O$ (0.4), $NaHCO_3$ (1.25), Glucose (0.5), HEPES (5.0) pH 7.2 . 10-15 ganglia, were cut to disrupt the neural sheath, placed in 1ml centrifuge-tube and washed three times in saline. Following treatment with a mixture of 0.5mg/ml collagenase and 2mg/ml dispase for 10 minutes at 37°C, the ganglia were washed three times and transferred to a laminar air-flow cabinet. Ganglia were washed a further three times and slowly introduced into modified Schneider's medium (85% Schneider's Drosophila medium (Gibco GBL), 15% foetal bovine serum (FBS), 100 units/ml penicillin, 100mg/ml streptomycin and 50mg/ml insulin pH 7.2) by gentle repeated washes into a final volume of 1ml. Ganglia were gently triturated through a flame polished Pasteur pipette and allowed to settle to the bottom of the centrifuge-tube. The overlying supernatant was transferred to sterile poly-D-lysine coated 55mm Falcon dishes. Ganglia were resuspended and the procedure repeated twice. Cultures were incubated in a polystyrene container at room temperature for up to 36 hours.

Isolated neurons were bathed in saline containing (mM); NaCl (140), KCl (3), $MgCl_2$ (4), $CaCl_2$ (1), HEPES (5), potassium channel blockers tetraethyl ammonium chloride (TEA-Cl, 30) and 4-aminopyridine (1), pH 7.2. Cationic currents were measured using the whole-cell configuration of the patch clamp. Unpolished patch-electrodes (2-3MΩ) were filled with solution containing (mM); CsCl (70), CsF (70), EGTA, (1.1), $MgCl_2$ (2), $CaCl_2$ (0.1), adjusted to pH 7.2 with CsOH.

Neurons were maintained at a holding potential of -100mV and currents were evoked by depolarising steps to a test potential between -70mV and +60mV. Experiments were performed at room temperature and analysed on-line. Potentials were corrected for liquid junction potentials, leak currents and capacitive currents. For each cell voltage-protocols were performed in triplicate and the average used for further analysis. Voltage-dependence of activation was measured using the same method but results were normalised relative to the maximum current for each cell.

3.2 Properties of Voltage-Activated Currents

Three classes of voltage-gated current were evoked when cell membranes were step depolarised from a holding potential of -100mV to potentials between -70mV and +60mV. These currents exhibited properties characteristic of calcium currents and sodium currents and were studied following pharmacological isolation (figure 5; Table 1) with either 10mM $CoCl_2$ (sodium current, figure 5a) or 1mM tetrodotoxin (calcium current, figure 5b).

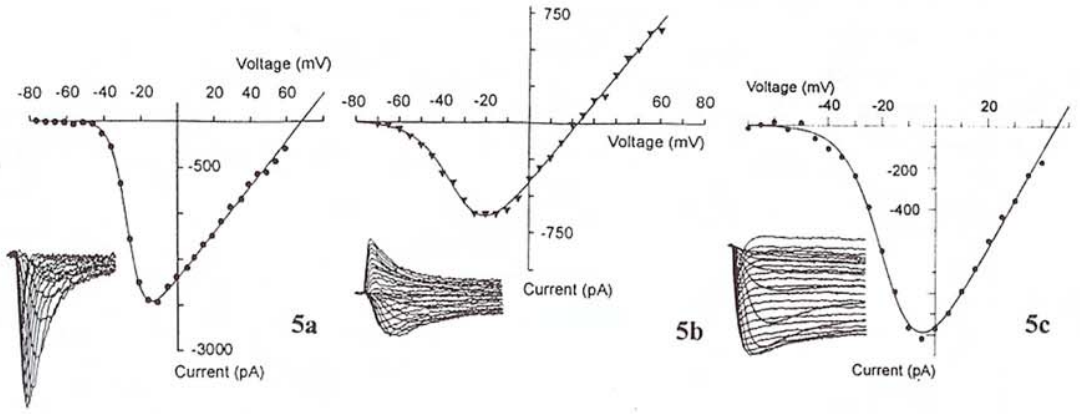

Figure 5 *Current-voltage (I/V) relationships for voltage-activated currents recorded in neurons cultured from the thoracic ganglia of M. domestica (Cooper). Data points represent the mean of triplicate experiments performed in the same cell. Curves were fitted with a Boltzmann function. Inset shows the family of currents used to construct the I/V curve.* **Figure 5a** *In the presence of 10mM $CoCl_2$, this current has properties consistent with a voltage-activated sodium current (Table 1).* **Figure 5b** *In the presence of 1μM TTX, this smaller current has properties consistent with a low-voltage activated calcium current (Table 1).* **Figure 5c** *shows a high-voltage activated calcium current which is blocked by $CoCl_2$, unaffected by TTX and is particularly sensitive to Cd^{++} ions, (EC_{50} 2.4μM)*

In some cells a further current (figure 5c) could be isolated which had somewhat different biophysical properties from the calcium current described above (figure 5b). It activated at -50 to -40 mV and reached a peak of activation at around -10 mV. The current was sensitive to divalent cations with EC_{50} values of 2.44μM for Cd^{++}, 762μM for Ni^{++} and 321μM for Co^{++}. At present a full pharmacological characterisation of these calcium currents has not been carried out; however the clear difference in activation voltage and the sensitivity of the second current to Cd^{++} suggest that the designation of low-voltage activated (LVA) (figure 1b) and high-voltage activated (HVA) (figure 1c) are appropriate.

Table 1 *Properties of Voltage-gated Channels from Fly Neurons*

Property	LVA Calcium current Figure 1b	Sodium Current Figure 1a
Onset of activation	-70 to -60mV (3)	-40 to -35mV (17)
Peak of activation	-35 to -30mV (3)	-10 to -5mV (17)
Time to max. peak I	5.03 ± 0.59ms (3)	2.61 ± 0.17ms (17)
Onset of inactivation	-85 to -80mV (3)	-70 to -65mV (15)
t for max. peak I	5.32 ± 0.18ms (3)	0.80 ± 0.07ms (15)
Recovery from inactivation	Up to 250ms (3)	Within 3ms (7)
Action of 10mM $CoCl_2$	Block	None
Action of 10nM TTX	None	Block

3.2 Modification of the whole-cell current by the pyrethroid insecticide Deltamethrin.

3.2.1 Sodium Currents.

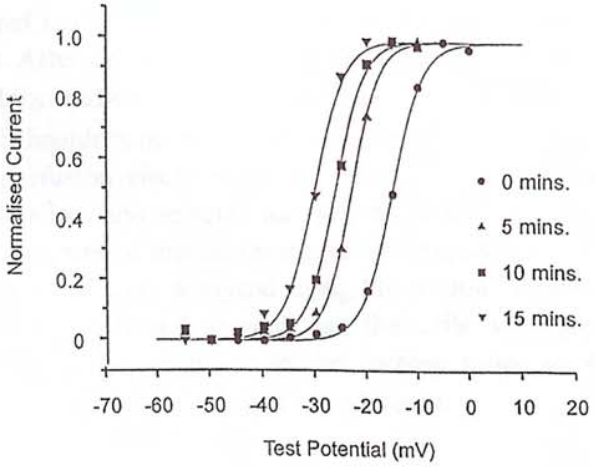

Figure 6 *1μM deltamethrin caused a time dependent hyperpolarising shift in activation voltage of the sodium current recorded in M. domestica neurons. Currents were elicited using step depolarisations from a holding potential of -100mV to potentials ranging between -70 and +60mV at 0.5Hz. After application of deltamethrin, the experiment was repeated in triplicate at 5 minute intervals for 15 minutes. Data were normalised relative to the maximum current and the curves were fitted with a modified Boltzmann function.*

The effects of deltamethrin on voltage-activated sodium currents were examined in isolated housefly neurons. Neurons were allowed to equilibrate for 10 minutes after entering the whole-cell configuration and current-voltage relationships were recorded in triplicate immediately prior to applying deltamethrin. Following the bath application of deltamethrin

at a concentration of 1μM (n=5) current properties were measured at 5 minute intervals. Voltage protocols were performed in triplicate, averaged and plotted against the corresponding test potential at each time interval

Deltamethrin resulted in a rapid hyperpolarising shift in the voltage-dependence of activation (figure 6). After 15 minutes half-maximal currents (mean ± SEM) were found to be significantly shifted ($P<0.01$, 1-way ANOVA) from a potential of -15.12 ± 3.01mV to -31.08 ± 3.69mV.

3.2.2 Calcium currents Application of 1μM deltamethrin to isolated cells from *M. domestica* caused a significant ($P<0.01$, 1-way ANOVA) hyperpolarising shift in the mid-point of the LVA-calcium current activation curve (figure 7a). This change was observed in all (n = 3) of the cells tested. Rundown prevented longer experimental time-courses. However even after 30 mins of application to cells exhibiting HVA-calcium currents no effect of 1μM deltamethrin was evident on the activation properties of the current (figure 7b).

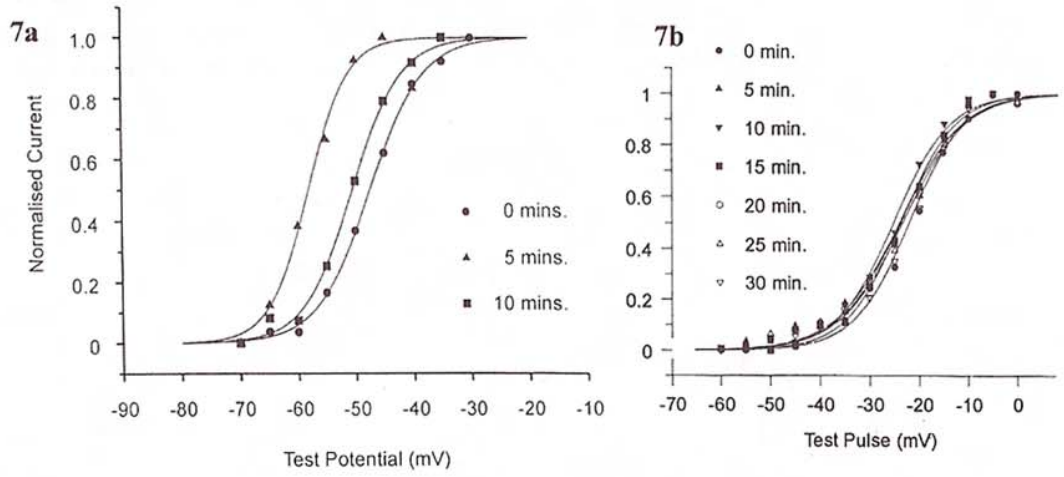

Figure 7. *Calcium currents were evoked using 30ms depolarising test pulses from a holding potential of -100mV to potentials from -70mV to +60mV in 5mV increments at a frequency of 0.5Hz. Voltage-protocols were performed in triplicate at each time interval and the mean current was normalised relative to the maximum current.* **Figure 7a** *1μM deltamethrin caused a time dependent hyperpolarising shift in activation voltage of the LVA-calcium current.* **Figure 7b** *1μM deltamethrin caused no change in the activation properties of HVA current.*

Two main conclusions may be reached from this study: i. that housefly neurons have at least two types of voltage activated calcium currents, in accordance with the two types of calcium current previously reported in cockroach DUM cells[16] ii. that the LVA channel type appears to be sensitive to pyrethroids and may be worthy of further study as a potential insecticide target.

4 ACTIONS OF DELTAMETHRIN ON INTRACELLULAR CALCIUM LEVELS IN ISOLATED HOUSEFLY NEURONS

The implication of the experiments described above is that pyrethroid insecticides might mediate some of their toxicity via a calcium-mediated mechanism. It could be argued that modification of Ca^{++} entry into cells would provide a particularly potent toxic pathway due to the potential for Ca^{++} dependent release of Ca^{++} from intracellular stores and perturbation of the numerous cellular processes involving Ca^{++}[17]. To establish whether pyrethroids are capable of modulating $[Ca^{++}]_i$ we have used ratiometric fluorescence imaging with the calcium sensitive dye FURA-2. This dye fluoresces under UV illumination, however the intensity of fluorescence varies as the dye binds Ca^{++}, the intensity at 340nm increasing whilst that at 380nm diminishes. The ratio of the fluorescence at these two wavelengths therefore gives a measure of $[Ca^{++}]_i$ which is independent of the cell volume or loading efficiency. Fluorescence imaging has been used to monitor changes in $[Ca^{++}]_i$ in response to activation of insect ligand gated ion channels[18,19].

4.1 Method

Insect neurons were dissociated as described above except that the final resuspension and plating was carried out in housefly normal saline onto the surface of clean glass 22mm square coverslips. After cells had adhered to the coverslip they were incubated for up to 36 hours in Schneider's medium. The medium was replaced with medium containing 2μM FURA-2AM in Schneider's medium and incubated for 30 mins. The coverslip was then transferred to a perfusion chamber on the stage of a Zeiss Epifluorescence microscope. Neurons were visualised and selected for ratiometric imaging. Fluorescence was imaged at 340 and 380 nm and pairs of images (averages of 8 exposures) were collected at 2 second intervals and their ratios were analysed using "Ionvision" software (Improvision). Drugs were applied via the perfusion system and the cells were depolarised by raising the extracellular potassium concentration in the bathing saline to 80mM. Where indicated sodium channels were blocked with 1μM tetrodotoxin.

4.2 Fluctuations in $[Ca^{++}]_i$ induced by Deltamethrin

Deltamethrin (100nM) affected neuronal $[Ca^{++}]_i$ in two ways: i. Initiation of spontaneous oscillations in $[Ca^{++}]_i$ (figure 8, figure 9) which can be blocked by the calcium channel blocker verapamil. ii. Potentiation of the amplitude of depolarisation induced increases in $[Ca^{++}]_i$ (figure 8). These actions of deltamethrin are unaltered in the presence of tetrodotoxin, implying that the mechanism does not involve the action of voltage gated sodium channels. Furthermore verapamil appears to inhibit the deltamethrin induced spontaneous oscillations pointing to the likely involvement of calcium channels in the actions of deltamethrin.

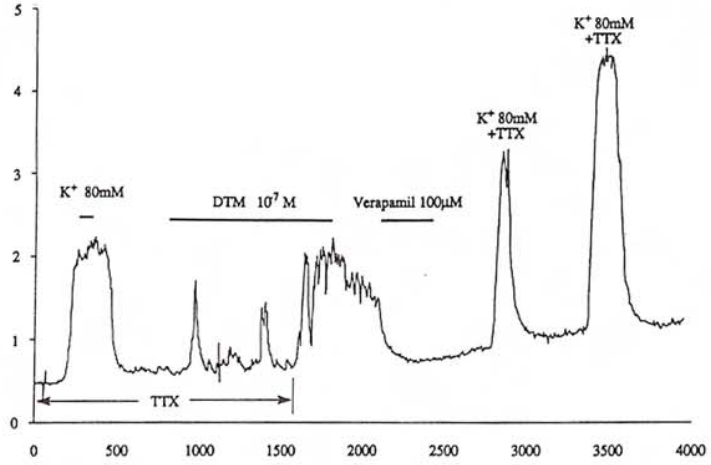

Figure 8 *Ratiometric analysis of FURA-2 fluorescence in isolated housefly neurons. Changes in intracellular calcium concentration $[Ca^{++}]_i$ are expressed as 340/380 ratiometric units (vertical axis) against time (horizontal axis). Depolarisation of the cells by elevating $[K^+]_o$ resulted in an increase in $[Ca^{++}]_i$. Addition of deltamethrin resulted in an increase in spontaneous oscillations of $[Ca^{++}]_i$ which can be blocked by verapamil. Subsequent K^+ depolarisations resulted in a potentiation of the induced elevation of $[Ca^{++}]_i$*

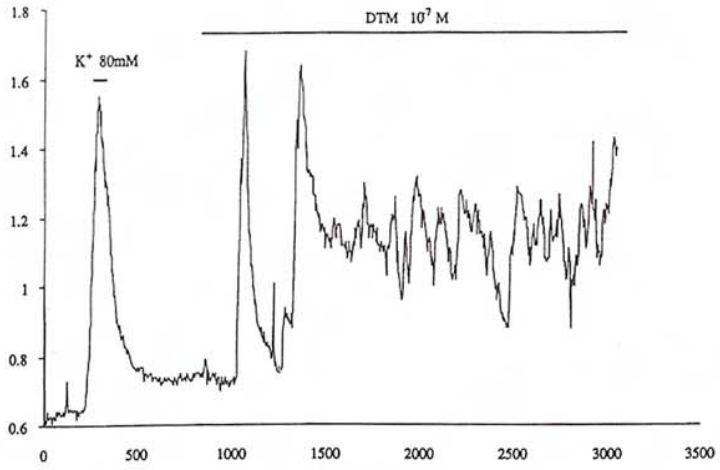

Figure 9 *Ratiometric analysis of FURA-2 fluorescence in isolated housefly neurons. Changes in intracellular calcium concentration $[Ca^{++}]_i$ are shown in 340/380 ratiometric units (vertical axis) against time (horizontal axis). Cells with spontaneous oscillations of $[Ca^{++}]_i$ often respond to deltamethrin by increrasing the frequency and amplitude of these oscillations.*

It is evident from these results that pyrethroids can modulate $[Ca^{++}]_i$ independently of their actions on sodium channels. These data could be interpreted in terms of a modification of VGCC seen above, however given the lipophilicity of pyrethroids it is also possible that they directly modulate intracellular calcium handling, as recently reported for

a number of other pharmacological agents in insect neurons[20]. Further work using both electrophysiology and fluorescence imaging in combination with selective pharmacological agents would enable these processes to be dissected. The use of housefly neurons for these studies also provides the opportunity to exmine the actions of pyrethroids in resistant strains.

In conclusion, excitable cells in insects express several types of channels with different properties which are capable of gating Ca^{++}. The variability of calcium channels, their pharmacological diversity and the crucial role of these ions in cell signalling should make them an attractive target for the discovery and development of insecticides.

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