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Research Article

Isolation and Characterization of Whole-Cell Calcium Channel Currents in Cultured, Identified Neurones of Lymnaea

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Summary and Conclusions

1) Whole-cell Ca^{2+}-channel currents were studied using the patch-clamp technique, applied to cultured pedal I cluster neurones of the snail Lymnaea stagnalis.

2) Calcium channel currents were separated from other ionic currents pharmacologically.

3) Only a single type of calcium channel current could be observed in the cultured spherical Lymnaea neurones studied.

4) This current first appeared at ca. -30 mV and peaked at or around +10 mV, when Ba^{2+} was used as the charge carrier and the cell was held at either -50 mV or -80 mV. It was a long lasting, slowly inactivating current similar to the high-voltage-activated calcium currents in other preparations.

5) The HVA current was weakly sensitive to the dihydropyridine agonist, Bay K 8644 and the antagonist, Nifedipine, and could be completely blocked by cadmium chloride. Thus, it resembled the classical L-type calcium current described in other preparations.

Keywords: *Calcium channel; Calcium currents; Cultured neurons; Snail*

Abbreviations: ABS: Antibiotic Saline; DM: Defined Medium; DHP: Dihydropyridine; ep: end-pulse current; GA: General Anaesthetic; hp: Holding Potential; HEPES: 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid; HVA: High Voltage Activated; I-V curve: Current Voltage Curve; LVA: Low Voltage Activated; pk: Peak Current; SD: Standard Deviation; SEM: Standard Error of The Mean; tp: Test Potential; Vc: Command Voltage; VDCC: Voltage Dependent Calcium Channel or Alternatively; VGCC: Voltage-Gated Calcium Channel

Introduction

From a molecular biological viewpoint calcium channel subtypes are very diverse largely to due to multiple genes encoding calcium channel α1 subunits. However, in electrophysiological terms "voltage-gated calcium channels (VGCC) are the primary mediators of depolarization-induced calcium entry into neurons" [1] and in the vertebrates, the T-, N-, and L-, P/Q- and R- type Ca^{2+} channels are commonly described, based on their biophysical and pharmacological properties [2,3,4,5]. These are both electro physiologically and pharmacologically distinct from one another reflecting their structural specificity. The T-channels are low voltage activated (LVA) channels whilst the N and L channels are high voltage activated (HVA). Additionally, P-type channels from cerebellar Purkinje cells and the presynaptic terminals of the squid giant synapse have been described and found to be very similar to one another [6,7,8]. Furthermore, minor species

variations in calcium receptors clearly exist and more are likely to be found. However, both agonist and voltage gated Ca^{2+} channels occur in all highly evolved groups of animals, including vertebrates, and can be modulated by intracellular signalling pathways [9,10] as originally suggested by [11]. A newer, more systematic, gene–based nomenclature of calcium channels [12] based on the diverse structural relationships between α1 subunits is also in common usage [1,5]. However, the older system of channel nomenclature tends to be retained for biophysical and pharmacological studies of the type described here.

Both LVA and HVA channels are known to exist in molluscan neurones [9,13,14,15]. Acutely isolated cells may not possess the full complement of ion channels following preparation with proteolytic enzymes and for this reason we have chosen to use cultured neurones, according to the methods outlined by Syed., *et al*. [16]. Previously [17,18], we have demonstrated that cultured neurones from Lymnaea stagnalis are capable of normal action potentials when grown in culture, in isolation, and this is clear evidence that they maintain their normal repertoire of ion channels under these conditions. We have also demonstrated that general anaesthetics such as halothane modify the calcium dependent components of action potentials [17,19]. Since VGCCs are known to be one of many targets for general anesthetics [20], we report here the characterization of whole-cell calcium channel currents in identified, cultured neurones of Lymnaea stagnalis, prior to a study of the actions of anaesthetics on these currents.

Materials and Methods

Specimens of Lymnaea stagnalis were obtained from Blades Biological, Kent, maintained in aerated holding tanks at a temperature of 16 to 18°C and fed on lettuce. Spherical cells from right pedal I cluster, right parietal A group and some H, I, J, K cells were cultured for 1 - 4 days in defined medium, rather than in culture medium, so as to minimize neurite extension. Care was taken to select the I-cell cluster for culturing neurones because these cells are relatively small, easily accessible, and have type II action potentials, with a distinct calcium plateau on the repolarization phase [21]. The RPeI-cluster is homogeneous and forms a distinct lobe of about 60 cells on the anterolateral surface of the right pedal ganglion [22]. The neurones project into the penis nerve [23]. Typically, cells used for whole-cell patch-clamp recording ranged in diameter from 20 - 40 microns.

Preparation of isolated neurones: Snails were deshelled and surface sterilized by placing them in 12.5 % Listerine solution in ABS for 7 to 10 minutes. They were then placed in ABS for another 10 to 15 minutes for recovery. They were then dissected in a laminar flow hood (Complete Clean Environments) using sterile dissecting techniques. Thick connective tissue was removed from the brain by microdissection and it was then incubated in 0.2 % trypsin type III (T-8253, Sigma) in defined medium for 25 to 30 minutes. The tissue was then rinsed in three or four changes of defined medium and left for 30 minutes to an hour in a final change of defined medium. Neurones were then isolated by methods similar to those of Kater and Mattson [24]. The nerves and connectives were then crushed to break all the axons and to free the cells from connections. Cells of choice were then identified and the soft connective tissue sheath overlying those cells was torn with the help of fine forceps and/or a tungsten micro-knife close to those cells. Cells were nudged towards this opening and then sucked into a glass micro-pipette which had been coated with Sigmacote (Sigma) or snails' blood to prevent the cells from adhering to its surface. The isolated neurones were then transferred to another dish containing DM and coated with Sigmacote or snails' blood for temporary storage. Healthy cells which appeared as robust regular surfaced yellowish cells with small stumps of axons or as small spheres were selected for culturing.

To prepare spherical cells with no neurites, neurones were finally transferred to poly-L-lysine (PLL) coated Falcon #3001 dishes (Beckton Dickenson and Company) containing DM only. These dishes were placed in an incubator (Gallenkemp) at 16 to 20oC and left undisturbed for at least 24 hours or until recording commenced. Normally four to six cells were cultured in one dish in previously marked areas to ease identification.

Salines and Culture media

Antibiotic saline (ABS) consisted of sterile normal saline [25] fortified with gentamicin sulphate to give a final gentamicin concentration of 150 µl/ml.

Cell culture media: The cell culture procedures were adapted from the techniques of Wong., *et al*. [26], Haydon, [27] and Syed., *et al.* [16]. Defined medium (DM) contained 50% Leibovitz, L-15 medium with glutamine (Sigma), 25% 4x saline (containing in mM: NaCl, 79.9; CaCl2, 11.02; KCl, 6.7; MgCl2, 6.0; HEPES (Sigma), 40; pH adjusted to 7.5) and 25% double distilled sterile water. Gentamicin Sulphate (Sigma) was added to give a final concentration of 50 µl/ml. A small amount of glucose (0.3 mM) was added at this stage.

This solution was shaken vigorously and then stored in 115 ml capacity Nalgene filter bottles (Nalge, U.K. Ltd) after filtration for later use. Fresh medium was prepared every three to four weeks.

Cultured neurones: The culture dishes were used as recording chambers and exchange of solutions was accomplished by flushing through at least 30 to 40 ml of solution which could be done in about 1 to 2 minutes.

Preparation of culture dishes: 1 ml of 0.5 mg/ml or 1.0 mg/ml of poly-L-lysine hydrobromide (P-2636, Sigma) in tris solution (prepared from Trizma base, Sigma) at 8.4 pH was pipetted into each of the Falcon #3001 dishes and taking care that it spread evenly over the whole bottom surface of the dish. It was left for about 16 hours after which poly-L-lysine was pipetted out completely and rinsed with sterile, double distilled water twice. Dishes were then incubated in the antibiotic saline for half an hour and then rinsed thoroughly with sterile, double distilled water again and then left to air dry in the laminar flow hood. Once dried these dishes were exposed to ultraviolet light for a period of 15 minutes and then stored in a sterile box at room temperature for later use. The dishes were used after at least a week of preparation as it was observed that cell growth was not good if dishes were used in the initial days after preparation. This could be due to some toxicity of poly-L-lysine in the initial few days.

Whole-cell patch-clamp recordings: Patch pipettes (2-5 MΩ) were fabricated from soda-lime glass microhaematocrit capillary tubing of outer diameter 1.6 mm, using a two-stage vertical pipette-puller (pp-83, Narishige, Japan) and were coated to within 100 *m of the tip with Sylgard resin to reduce the capacitance. Whole-cell currents were recorded according to the method of Hamill., *et al*. [28] using a List EPC-7 amplifier (List Medical, Germany). Culture dishes were mounted on the stage of an inverted microscope (Olympus, CK2) and cells viewed using phase contrast optics at a total magnification of 200. A Gould digital storage oscilloscope (OS 4040) was used to monitor the electrical signals. Seals were achieved by applying light suction to patch pipettes pressed gently against the neurons. Formation of a satisfactory seal was observed as a large increase in resistance as measured by the current response to small voltage pulses. Seals of 1 to 10 G* were routinely obtained. Current signals were low-pass filtered through an eight pole Bessel filter (Frequency devices, 902) before being viewed on the oscilloscope. The series capacitance was then adjusted to compensate for the additional whole-cell capacitive current. At least 65% of series resistance compensation was routinely performed. The bath electrode consisted of a Ag-AgCl wire connected to the bathing solution via an agar bridge filled with normal Hepes saline.

Current Separation: Most of the salts were obtained from "BDH" unless specified otherwise. The whole-cell calcium channel current was separated from other ionic currents by blocking all other currents as far as possible. Neither internal nor external solutions contained any added Na^+ or K^+. The external solution contained tetraethyl-ammonium (TEA) and 4-aminopyridine (4-AP), which are known to block various types of K^+ currents. Because Cs^+ is much less permeable than K^+ in most K^+ channels and can block K^+ channels when applied inside cells K^+ was replaced with Cs^+ inside the pipette. In some of the experiments Ba^{2+} was used as the charge carrier because Ba^{2+} fails to activate Ca^{2+}-activated K^+ channels and is also a potent blocker of K^+ channels when applied externally [29]. With these solutions, the currents elicited from a holding potential of -50 mV were inward at relatively positive voltages (from -30 mV to +50 mV), and readily blocked by 0.1 mM Cd^{++}. Thus, it could be concluded with reasonable surety that we were dealing with an isolated calcium current [13,30]. The following solutions were used to study the calcium currents in isolation (in mM):

Bath solution		Pipette solution	
TEA-Cl	40	CsCl	40
CaCl2	4	EGTA	5
MgCl2	1.5	MgCl2	5

4-AP	10	ATP	3
Glucose	10	GTP	1
HEPES	10	HEPES	10
	(Sigma)		

The pH of all the solutions was adjusted to 7.4 with either hydrochloric acid (HCl, 5 M) in case of bath solutions, or with cesium hydroxide (CsOH, Aldrich) in the case of the pipette solution. The osmolality of all the solutions was adjusted to ca. 150 milliosmoles with sucrose.

Seals were usually initiated in 4 mM Ca^{2+} saline in the bath. Some experiments were also performed where Ca^{2+} was replaced by equimolar concentrations of Ba^{2+} as the charge carrier. Cadmium chloride ($CdCl_2$, Sigma) was added in a concentration of 0.1 mM to the calcium saline containing either Ba^{2+} or Ca^{2+} as the charge carrier with no difference in results. As $CdCl_2$ caused a near-total inhibition of the calcium channel current, no attempt was made to subtract cadmium-sensitive current from the total current for further analysis. In most of the experiments with Bay K 8644 and nifedipine, 4 mM Ca^{2+} was used as the charge carrier.

Drugs: Nifedipine and Bay K 8644 were obtained from Calbiochem. Nifedipine and Bay K 8644 were prepared as 1 - 10 mM stock solutions in ethanol. Dilutions were made immediately prior to use in calcium saline. These drugs were prepared and used under low light conditions. Control experiments using the same concentrations of ethanol as would be obtained in experiments with Bay K 8644 and nifedipine yielded no appreciable effect of ethanol on calcium channel current. ATP (adenosine triphosphate) and GTP (guanosine triphosphate) were obtained from Sigma and were kept in solution below 0oC. They were added to the pipette solution just before the start of experiments. To enhance the probability of obtaining the gigaseal, all pipette solutions were filtered (0.22 μm) before use, as was the solution bathing the cell.

Data acquisition

A microcomputer (Opus V in the initial experiments and later on Elonex PC-320X) and CED Patch and Voltage Clamp software were used for pulse generation and data acquisition and analysis. Voltage-pulse protocols were applied to the stimulus input of the List EPC-7 amplifier following digital to analogue conversion by a CED 1401 laboratory interface using Patch and Voltage Clamp software by CED. Command pulses were delivered every 5 or 10 seconds and the signal amplified through the EPC-7 was recorded digitally on line to the hard disc of the computer without subtraction of the leak current. Currents generated by hyperpolarizing pulses of -10 mV were recorded with every set of experiments and stored with them for subsequent off-line digital subtraction of leakage and capacitive currents. In most of the experiments the leakage current was very small in comparison to the actual current records (typically less than 1% of the peak current) and leakage subtraction was not usually performed for constructing current traces because it introduced a lot of noise. However, the data used to construct the current-voltage plots was always leak-subtracted. Data was routinely acquired by the computer at 5 - 10 kHz and stored initially on hard disc. Analog data was filtered at 3 kHz and usually sampled at a minimum of 18 μsec/point.

The EPC-7 has capacity compensation for up to 100 picofarads (pF) which roughly corresponds to a spherical cell of about 30 - 35 microns. This necessitated the use of cells of relatively smaller size with a soma diameter of not greater than 38 - 40 microns. It was possible to get cells of such size if very small snails with a shell length of 1 - 1.5 cm were used.

Data analysis: Voltage-clamp data was analyzed with the help of CED Patch and Voltage clamp software. The % inactivation value as used in this study, gives a measure of the rate of inactivation of the current during the depolarizing pulse, and was calculated as follows:

$$\% \text{ inactivation value} = (I_{peak} - I_{end})/I_{peak},$$

where Ipeak is the peak current amplitude, Iend the current amplitude at the end of the depolarizing pulse. Statistical analysis of significance was done with Student's paired or unpaired t-tests using the `Oxstat' program. Data is either expressed as mean ± S.D. or mean ± S.E.M.

Results

1. Properties of whole-cell calcium currents

Time course of Ca^{2+} channel current: In the presence of appropriate internal and external solutions, a strong depolarizing voltage step from a negative holding potential (-50 mV) produced an inward current that activated rapidly and decayed to a non-zero level by the end of a 180 msec pulse. The threshold at which inward current could be evoked was ca. -30 mV. The current showed little inactivation over 180 msec near threshold, and inactivated incompletely with larger depolarizations. Figure 1a shows an example of a Ca^{2+} channel current produced in response to a 180 msec depolarizing voltage step to +20 mV from a holding potential of -50 mV. It produced a relatively slowly activating current which typically reached peak amplitude in 10 - 15 msec at this voltage, and then partially inactivated to a non-zero level gradually with time. When using the standard ATP-GTP containing patch electrode filling solution and 4 mM Ca2+ as the charge-carrier in the bath solution, the percentage decline in amplitude at the end of the depolarizing pulse of 180 msec (i.e. %inactivation value) averaged 39.56% ± 5.09% S.E.M. (n = 8). The current did not reach zero level even at the end of a 3.5 seconds long depolarizing pulse to +20 mV from a holding potential of -50 mV (Figure 1b).

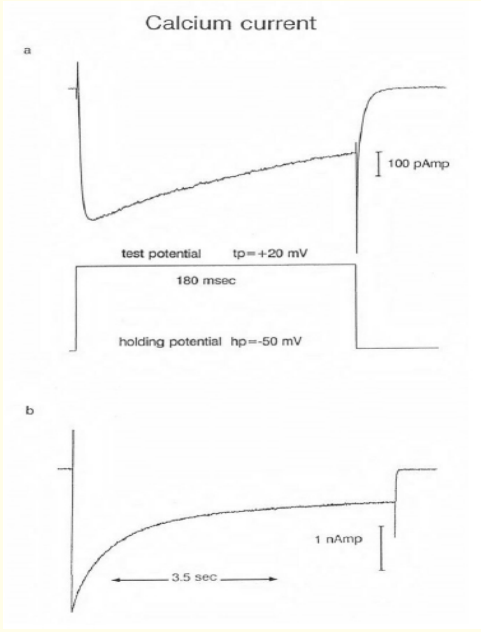


Figure 1: *Typical calcium channel current in a cultured Lymnaea neurone. (a) During whole-cell recording in calcium saline the cell was held at a holding potential (hp) = -50 mV and a 180 msec long depolarizing voltage step was applied to a command voltage (VC) of +20 mV. This elicited the maximal peak current response which showed a rapid activation and a slow and incomplete inactivation of calcium current. (b) In another cell held at -50 mV a prolonged depolarizing pulse of 3.5 seconds was applied to VC of +20 mV. The current did not inactivate fully even during this long pulse. Calcium saline contained (in mM): TEA-Cl 40, 4-AP 10 mM, CaCl2 4, MgCl2 1.5, Glucose 10, HEPES 10. The pipette solution contained (in mM): CsCl 40, EGTA 5, MgCl2 5, HEPES 10, Mg-ATP 3, Na2-GTP 1. Data acquired at 10 kHz in case of (a) and at 1 kHz in (b). The current traces were not leak subtracted.*

A plot of either the peak current or the current at the end of the pulse as a function of test potential had a single maximum near +20 mV when 4 mM Ca^{2+} was used as the charge carrier (Figure 2). The I-V curve shown in figure 2 shows that the current first appeared at -30 mV and peaked at around +20 mV under these conditions. It can be seen that the early peak current and the time-dependent current decay both disappeared at about the same null potential, i.e., +60 to +70 mV. At voltages, more positive to +60 to +70 mV, net outward currents

were visible. Since there is too little intracellular Ca^{2+} to carry appreciable outward current, true calcium I-V relations must approach the voltage axis at a very narrow angle at these positive potentials [29].

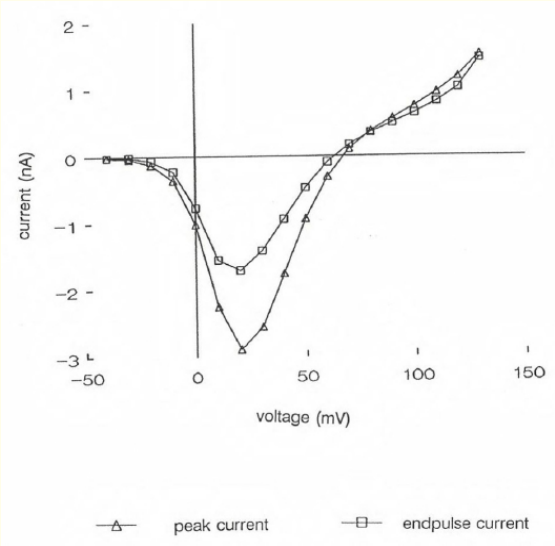


Figure 2: *Typical current voltage relationship of peak calcium channel current and endpulse current in a Lymnaea neurone. Hp = -50 mV, with 100 msec voltage commands in 10 mV increments from -40 mV to +130 mV. Measurement of peak calcium current (open triangles) and late calcium current at the end of the command pulse of 100 msec (open squares) were taken and plotted as a function of command potential. The peak of the I V curves in both cases were attained at around +20 mV when 4 mM Ca^{2+} was used as the charge carrier as in this case. The current reversed its sign at ca. +70 mV.*

Effect of permeant ions on the time course of Ca^{2+} channel current: Figures 3 and 4 show the results of experiments in which the effect of the species of inward charge carrier on the peak amplitude and the decay of current during the pulse was investigated. The bathing solution first contained 4 mM Ca^{2+} as the charge carrier and currents were elicited by depolarizing pulses of 100 msec duration from a constant holding potential of -50 mV. Depolarizing pulses were applied in incremental steps of 10 mV, delivered at intervals of 5 sec to allow for recovery of calcium channels from the effects of previous stimulation. For simplicity, current traces elicited in response to voltage steps to -40 to +50 mV are shown though the full voltage protocol included voltage steps to +130 mV - beyond the apparent reversal potential of calcium currents. Then the calcium saline was exchanged with a solution containing 4 mM Ba^{2+} and the same protocol repeated. The currents produced in the presence of 4 mM Ba^{2+} were larger than those elicited with 4 mM Ca^{2+} (Figure 3b). The average ratio of the peak Ba^{2+} (4 mM) current to peak Ca^{2+} (4 mM) current was 1.72 ± 0.22 S.E.M. (n = 7).

When the peak current as well as the end pulse current was plotted against the test potentials an increase of Ca^{2+} channel current could be observed over the whole voltage range (Figure 4). The calcium current depended upon the external charge carrier concentration. A Higher concentration of Ba^{2+} (10 mM) further enhanced the calcium channel current as is illustrated in figure 3c and 4. The same would happen if the concentration of Ca^{2+} in the bath solution was increased [30,31]. Thus, equimolar concentrations of Ba^{2+} could be used to replace Ca^{2+} as the charge carrier. The increase of amplitude of the current by using Ba^{2+} as the charge carrier has been demonstrated as a property of HVA Ca^{2+} channels, the LVA channels showing an equivalent conductance for both Ca^{2+} and Ba^{2+} [31,32,33]. Use of 10 mM Ba^{2+} enhanced the Ca^{2+} channel current (Figure 3c) and the ratio of 10 mM Ba^{2+} to 4 mM Ca^{2+} was 2.46 ± 0.33 (±S.E.M.) (n = 5).

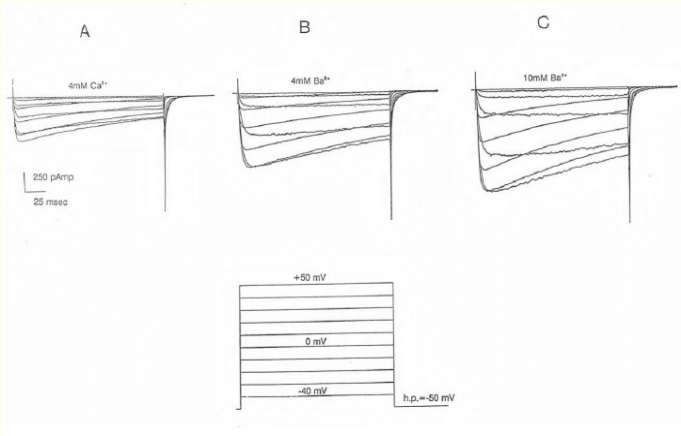


Figure 3: *Effect of replacing Ca^{2+} with Ba^{2+} as the charge carrier on calcium channel current. Holding potential was -50 mV and 180 msec voltage commands in 10 mV increments were applied from -40 mV to +50 mV, first in 4 mM Ca^{2+} (A), 4 mM Ba^{2+} (B) and then in 10 mM Ba^{2+} (C). The amplitude of the calcium current was almost doubled when equimolar concentration of Ba^{2+} was replaced for Ca^{2+} in the bath (B). Amplitude of the current increased to about 3 times that of (A) when 10 mM Ba^{2+} was present in the bathing solution. Pipette solution same as in figure 4.2. Data acquired at 10 kHz and was leak-subtracted.*

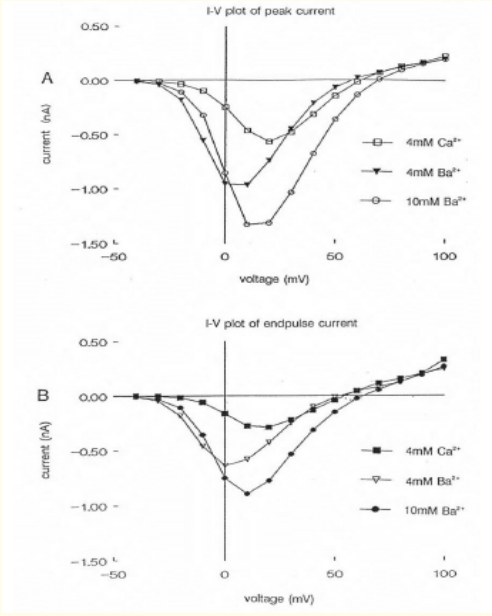


Figure 4: *The peak amplitude of calcium channel currents and the amplitude of the current at the end of 180 msec pulse recorded in figure 4.4 are plotted as a function of membrane potential. (A) Currents were activated at ca. -30 mV and maximal amplitudes were reached at about +20 mV when 4 mM Ca^{2+} (open squares) was used as charge carrier. When 4 mM Ba^{2+} was used to carry the charge the maximal amplitudes were reached at about +5 mV (closed triangles), a shift to the left of about 15 mV. Using 10 mM Ba^{2+} as the charge carrier (open circles) shifted the peak of the I-V curve slightly back to the right as the maximum currents were elicited between +10 and +15 mV. (B) shows the I-V plots of end pulse current for the corresponding situations as in (A), exhibiting exactly the same behaviour except that the peaks of I-V curves of endpulse currents in Ba^{2+} were attained at relatively negative voltages as compared to the I-V curves of peak currents.*

When the Ca^{2+} current (4 mM Ca^{2+}) elicited by a voltage step near the peak in the current-voltage relationship was scaled by a constant to match the peak Ba^{2+} current (4 mM Ba^{2+}) (Figure 5a), the time course of inactivation was different. The extent of decay during positive voltage steps varied to some extent from cell to cell, but there was a decrease in this decay when 4 mM Ba^{2+} was used instead of 4 mM Ca^{2+} and was significantly reduced when 10 mM Ba^{2+} was used as charge carrier (Figure 5 b). This suggests that the species of divalent cation does determine the time course of the decay of current. The most likely explanation for this is a decreased calcium-induced calcium channel inactivation as described by Kostyuk & Krishtal [34] and Byerly & Moody [35]. The shift of the peak of current-voltage relationship to hyperpolarizing direction by 10 to 15 mV when Ba^{2+} was used as the charge carrier instead of Ca^{2+}should also be noted. These findings are similar to that observed for high-threshold Ca^{2+} currents in other preparations [32] and in large, unidentified Lymnaea neurones [13,30]. This shift of the peak of I-V curve has been ascribed to a change in surface charge potential. Increasing the concentration of charge carrier shifted the peak of the I-V curve to the depolarizing voltages [34,30]. In high Ba^{2+} solution where currents were about twice or two and a half times larger, maximal peak amplitude and apparent reversal potential were shifted to depolarizing direction along the voltage axis (Figure 4). A reduction in the concentration of Ca^{2+} in the bath led to a decrease in the amplitude of current. Figure 6 shows the effect of replacing the 4 mM Ca^{2+} bath solution with a bath solution containing 0 mM Ca^{2+} (i.e., no added calcium). From a holding potential of -50 mV a voltage step to the test potential of +20 mV elicited a peak current of about 2 nanoamperes in 4 mM calcium saline (Figure 6a). In zero calcium, the current trace was almost flat. When the current was plotted as a function of test potential very little current was produced by voltage steps between -30 mV to +20 mV (Figure 6b). At potentials, more positive than +20 or +30 mV an outward current was visible, which is most likely to be the non-specific current as described elsewhere [13,29,30]. This current is probably carried by Cs^{+} ions through Ca^{2+} channels [29,36].

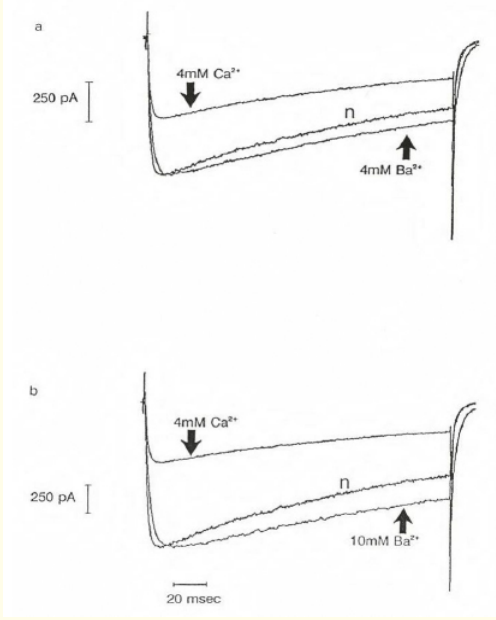


Figure 5: *Effect of changing the charge carrier on current amplitude and current inactivation. (a) Ca^{2+} current (4 mM Ca^{2+}) was elicited by a 180 msec long voltage step to +10 mV from a holding potential of -50 mV. A similar pulse was then applied when Ca^{2+} was replaced with an equimolar concentration of Ba^{2+} and the current trace superimposed. the trace obtained in Ca^{2+} was then scaled by a constant to match the peak Ba^{2+} current (marked by n). The time course of inactivation came out to be different in the two situations in that there was a decrease in the decay of calcium channel current when 4 mM Ba^{2+} was used instead of 4 mM Ca^{2+}. (b) Exactly the same procedure as in case of (a) except that the concentration of Ba^{2+} used was increased to 10 mM. The rate of inactivation was significantly reduced when 10 mM Ba^{2+} was used as charge carrier.*

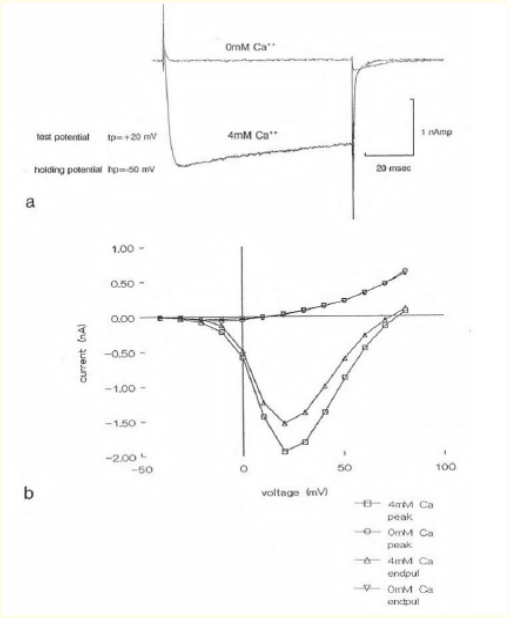


Figure 6: *Effect of decreasing the bath concentration of Ca^{2+} to zero. (a) From a holding potential of -50 mV, a 100 msec long VC was applied to test potential of +20 mV, first in normal calcium saline and then in saline with no added Ca^{2+}. Almost no current flowed when the external solution had zero calcium. (b) In the same cell as in (a) 100 msec voltage commands in 10 mV increments from -40 mV to +80 mV were applied from a holding potential= -50 mV. Measurement of peak calcium current (open squares) and late calcium current at the end of the command pulse of 100 msec (open triangles) were taken and plotted as a function of command potential. Same protocol repeated with `zero' Ca^{2+} in the bath. The I-V curves for the peak current (open circle) and the endpulse current (inverted open triangles) demonstrated very little inward current at voltages between -30 to +20 mV. There was evidence of outward currents in response to command voltages beyond +20 mV in this case.*

The rate of inactivation of the current during a depolarization to a given voltage was a function of the solution bathing the neurone. Currents in 10 mM Ba^{2+} solution inactivated very slowly at potentials that elicited maximal currents. Figure 7 shows a histogram of the mean percentage-inactivation ± S.D. of the peak calcium channel current measured in 4 mM Ca^{2+} 4 mM Ba^{2+} and 10 mM Ba^{2+}. A paired t-test (n = 6) showed a significant difference in % inactivation between 4 mM Ca^{2+} and 10 mM Ba^{2+} ($p =< 0.05$), the %inactivation value decreasing from ca. 43% in Ca^{2+} down to ca. 27% in Ba^{2+}. A significant decrease in the % inactivation value of calcium channel current was also observed by using 10 mM Ba^{2+} as compared to 4 mM Ba2+ ($p = < 0.05$) (Figure 7). A similar effect has been reported in ventricular myocytes [37] where they also observed a slowing down of activation, as measured by time-to-peak, in high Ba^{2+} concentration.

The results above make it clear that the amplitude of the current increases and the rate of decay of calcium channel current is reduced if Ba^{2+} is used as the charge carrier instead of Ca^{2+}, especially when a relatively higher concentration (10 mM) of Ba^{2+} is used. Ba^{2+} in small concentrations acts as a blocker of IK and also favours a better survival of Ca^{2+} channel function [29].

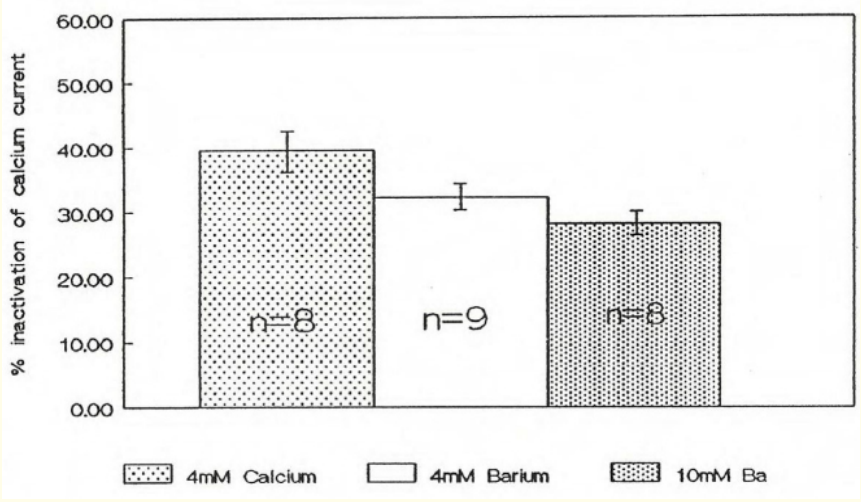
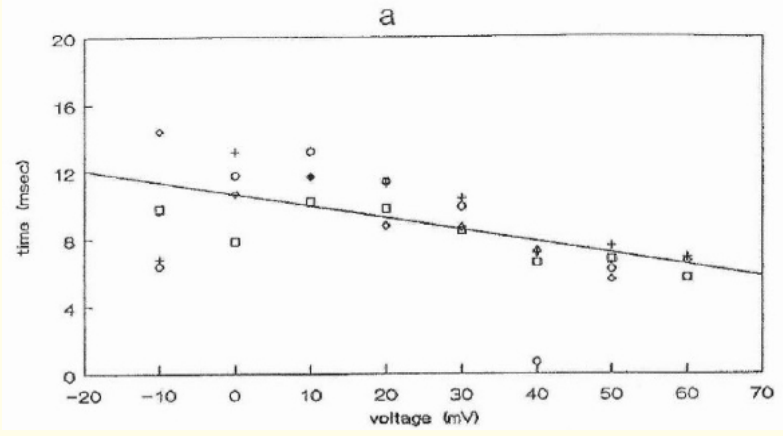


Figure 7: *Histogram showing the mean ± S.D. of percentage inactivation in 4 mM Ca^{2+} and 4 mM Ba^{2+}& 10 mM Ba^{2+}. `n' denotes the number of experiments performed. The percentage inactivation was calculated as*

*%Inactivation = [(Ipeak-Iend)/Ipeak]*100*

where I peak is the current at the peak of maximal calcium channel current, Iend is the current at the end of the 180 msec of maximal calcium channel current. The % inactivation was significantly less when 10 mM Ba^{2+} was used as the charge carrier as compared to 4 mM Ca^{2+}.

Effect of the test potential on the rate of activation and decay of Ca^{2+} channel current: The rate of activation of the calcium current and the rate of decay (or inactivation) was found to be dependent on the test potentials. This phenomenon can be observed in Figure 3 and is better illustrated in figures 8a where time-to-peak is plotted against membrane potential. At depolarizing voltages to -20, -10, 0 and +10 mV, the calcium channel current activated relatively slowly as compared to that seen at voltages above +30 mV. At low divalent ion concentrations the rate of inactivation was faster at low test potentials than at more depolarized potentials. For example, the rate of decay of calcium channel currents was minimal at voltages between -30 mV and -10 mV; once activated to the maximum the current remained more or less in a steady state throughout the voltage step. This is better illustrated in figure 8b where ratio of the current at the end of 180 msec pulse to the respective peak current is plotted as a function of membrane voltage. The effect of slower activation of calcium channel current was greatly magnified when Ba^{2+} was used as charge carrier.



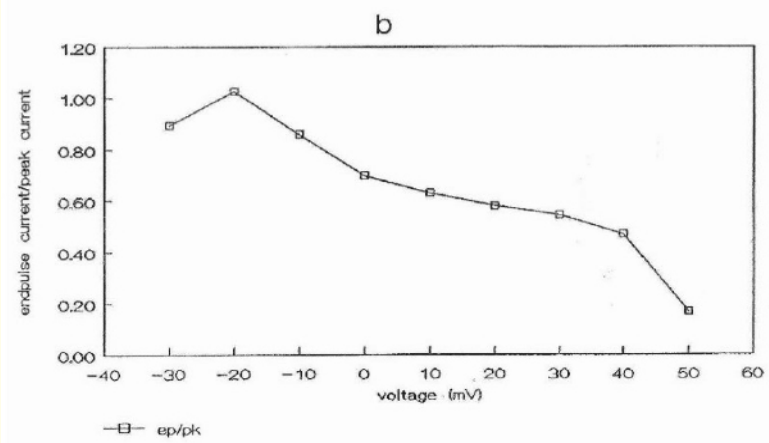


Figure 8: *Dependence of the rate of activation and the rate of inactivation of the calcium channel currents on test potential. Currents were activated in Lymnaea neurone by 180 msec depolarizing pulses from a holding potential of -50 mV to test potentials between -30 to +80 mV. (a) Time-to-peak was plotted vs. membrane potential. Time-to-peak was strongly voltage-dependent as illustrated, decreasing with stronger depolarizations (indicated by the slope of the line of best fit drawn through data points). Data from four cells using 4 mM Ca^{2+} as the charge carrier. (b) The ratio of the endpulse current to the respective peak currents was plotted as a function of membrane voltage to clarify that there was minimal inactivation of calcium channel currents at hyperpolarized voltages even at the end of 180 msec long pulse. Data from one representative experiment using 4 mM Ca^{2+} as the charge carrier. As the command voltage was stepped up the rate of activation became faster and there was greater inactivation of current.*

Reversible blockade by cadmium chloride: Cadmium blocks the Ca^{2+} channel current in neurones [3,13,38], as well as in cardiac myocytes [39]. In micromolar concentrations it is a specific inorganic blocker for calcium channels. Though Co^{2+} and Ni^{+} can also be used for blocking Ca^{2+} channels, the concentrations required for these ions are about 200 times greater than for Cd^{2+} [30]. Using a 100 μM concentration of cadmium chloride it was possible to abolish the calcium current almost completely. Figure 9 shows current traces where peak Ca^{2+} channel current was evoked from a holding potential of -50 mV to test potentials between -20 mV to +60 mV, first in the absence and then in the presence of 0.1 mM CdCl2. A near total abolition of calcium channel current was quite obvious. This effect was reversible when cadmium saline was replaced with normal calcium saline (Figure 9c). The current-voltage relationship of the peak and end pulse current has been plotted in (Figure 10 a, b). These show a typical recording from 20 cells in which a complete abolition of calcium channel current through out the voltage range studied could be observed. The blocking effect was also exerted on the outward currents at voltages positive to the point where the current reverses its sign. This is as expected from a true channel blocker. Byerly and Hagiwara [13] observed a similar block of calcium channels in relatively larger, unidentified neurones from Lymnaea, but at much higher concentration of CdCl2 (1 mM).

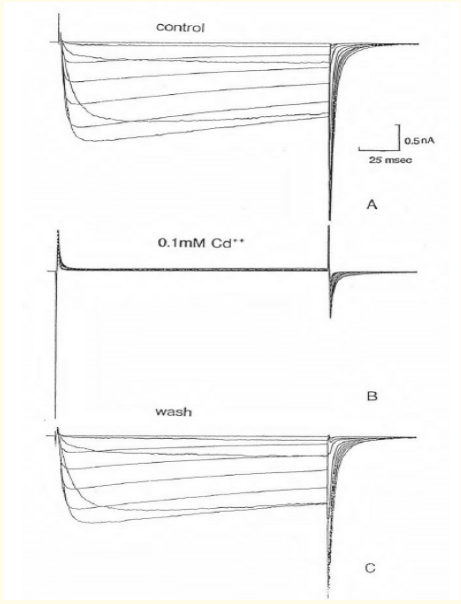


Figure 9: *Effect of 0.1 mM $CdCl_2$ on calcium channel current. The membrane potential was stepped to test potentials between -20 to +60 mV in 10 mV increments from a holding potential of -50 mV by 180 msec long pulses. (A) Current traces recorded under normal circumstances, (B) after application of $CdCl_2$ and (C) after washing away the $CdCl_2$ with normal calcium saline. There was almost a complete abolition of calcium current in the presence of CdCl2 and recovery was also rapid and complete.*

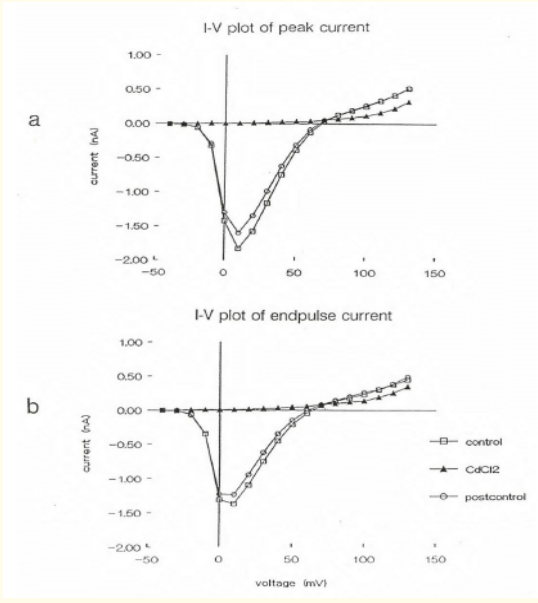


Figure 10: *Reversible blockade of calcium current by CdCl2. I-V plot constructed from current responses of the same cell as in figure 4.10 to voltage steps to -40 mV to +130 mV. Holding potential was -50 mV and the pulse duration was 180 msec. (a) shows the reversible blocking effect of CdCl2 on the peak currents (closed triangles) whereas (b) demonstrates the same effect on the current at the end of pulse (closed triangles).*

Reversal potential: As described earlier and observed in figures in the preceding sections, the calcium channel current started declining in amplitude in response to depolarizing voltage steps after attaining a peak at around +20 mV. At voltages more positive to +60 to +70 mV net outward currents were visible (Figures 2 and 4), though there were some variations. The I-V relation of the calcium current at large positive potentials is close to the zero line, as should be expected from the very low concentration of Ca2+ inside the membrane. This is in agreement with the observation of Byerly & Hagiwara [13].

Effect of the holding potential on Ca^{2+} channel current: To separate the components of calcium channel currents at the whole-cell level various methods can be used such as manipulations of the holding potential [40], block by dihydropyridines [3,40] and a preferential block by Cd^{2+} or Ni+ [32,41]. Ca^{2+} channels have been broadly divided into the high-voltage-activated (HVA) and the low-voltage-activated (LVA) calcium currents depending upon the voltage at which they can be elicited. The presence or absence of various types of Ca^{2+} channel currents was examined by studying the effects of different holding potentials on calcium channel current and measuring peak current and end pulse current elicited in response to pre-determined test potentials from different holding potentials. Figure 11a shows current traces evoked by a 100 msec depolarizing pulse to +20 mV from holding potentials of -80 mV and -50 mV. The current responses evoked from both these holding potentials clearly superimpose. Usually, if the T-type current is also present then an early rapidly decaying phase (LVA calcium current) can be seen followed by a sustained component (HVA calcium current). The current voltage relationship (Figure 11.b) shows an absence of any current in the voltage range of -70 to -40 mV, the usual range of activation of T-type calcium current. The absence of the typical shoulder in the I-V plots in the voltage range of -60 to -40 mV, is indicative of presence of only HVA calcium channel current. In Figure 11 c traces of peak current evoked by depolarizing the cell to +20 mV from a holding potential of either -50 mV or -100 mV are superimposed.

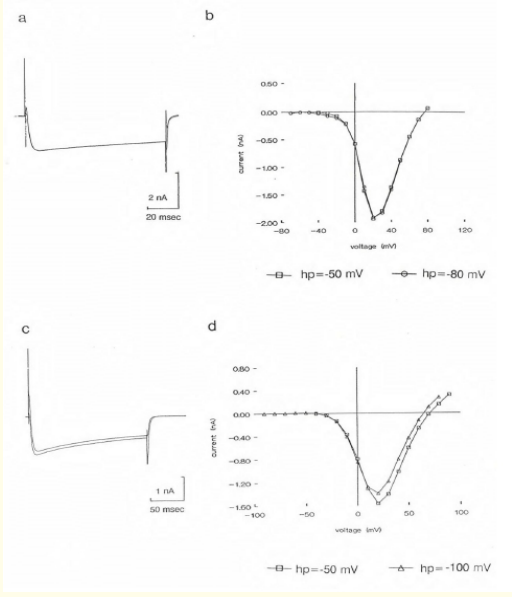


Figure 11: *Effect of two different holding potentials on calcium channel current. (a) Current traces evoked by 100 msec depolarizing pulse to +20 mV from holding potentials of -80 mV and -50 mV. The current responses evoked from both these holding potentials superimpose very nicely. (b)Current-voltage relationship constructed from current responses elicited by depolarizing the cell to voltages between -40 mV to +80 mV in 10 mV steps from a holding potential of -50 mV (open squares) and then by depolarizing to -70 through to +40 mV from a holding potential= -80 mV (open circles). There was no appreciable difference between the two curves which indicated an absence of typical low-voltage-activated calcium currents. (c) Current traces obtained by depolarizing the cell to +20 mV from a holding potential= -50 mV and then from a holding potential= -100 mV. Again the two current traces were very similar. (d) I-V relationship obtained in the same manner as in (b) except for the difference in the holding potential. The typical shoulder characteristic of LVA current was absent.*

2. Modifications of Ca^{2+} channel currents by dihydropyridines (DHPs)

Effect of Nifedipine: The high-voltage-activated calcium channel current observed in pedal I-cluster cells was weakly sensitive to dihydropyridine derivatives. Nifedipine, a commonly used DHP antagonist of calcium currents, was used in a concentration ranging from 0.1 to 4 μM. Higher concentrations had no additional effect. It suppressed the maximal peak amplitude current from a holding potential of -50 mV by about 18% (n = 7) when used in micromolar concentrations. In the case illustrated in figure 12(a) 2 μM of nifedipine was used. It shows the current response evoked by a depolarizing pulse to +20 mV from a holding potential of -50 mV, first in the absence and then in the presence of nifedipine. A reversible block of a portion of total calcium channel current is quite obvious. The current-voltage relationship was produced by depolarizing the cell to test potentials of -40 to +100 mV from a holding potential of -50 mV (Figure 12.b). It shows a partial blockade of calcium channel current, the block being more prominent in the voltage range of -30 to +30 mV. This block was reversible on wash out of nifedipine. This type of response of calcium channel currents to nifedipine was observed in other experiments to variable degree, some cells showing a complete refractoriness to the drug while in others there was a slight increase in the current on exposure to nifedipine. It comes as no surprise since the effects of DHPs on ICa have been reported to be complicated by mixed agonist/ antagonist actions [42].

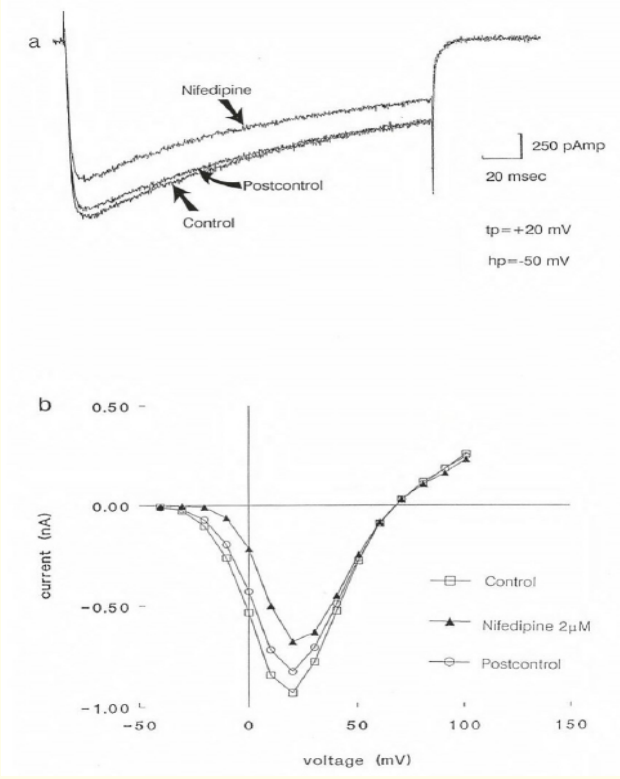


Figure 12: *Effect of nifedipine on calcium current in a cultured right pedal I-cluster cell. Antagonist dihydropyridine, nifedipine, blocked whole-cell calcium current in cultured RPeI-cluster cells. (a) Using 4 mM Ca2+ as the charge carrier, nifedipine (2 μM) inhibited 20 % of the current elicited by a 180 msec voltage step from a holding potential of -50 mV to command potential = +20 mV. (b) I-V plot for the same cell showing the antagonism of nifedipine on currents evoked from a holding potential of -50 mV to a variety of test potentials. The effect of nifedipine was reversible as shown both in (a) and (b).*

Effect of Bay K 8644: In a dose of 1-2 μM, Bay K was able to increase the maximal peak amplitude calcium channel current by 15.93% ± 1.44 (mean ± S.E.M., n = 7) at a holding potential of -50 mV (Figure 13), and also shifted the peak of the I-V curve (Figure 13 b) by 10 to 15 mV in the hyperpolarizing direction, a phenomenon typical of Bay K and other DHP agonists. This effect of increase of current in the hyperpolarizing direction is clearly illustrated in (figure 13 a) Calcium current was elicited from a holding potential of -50 mV by depolarizing to potentials between - 40 mV to +100 mV (current traces are only shown for voltage steps to -10 to +50 mV). Maximum current was observed at +30 mV under control conditions when using Ca2+ as the charge carrier. Bay K 8644 had a much more pronounced effect at test voltages of -10, 0, +10 and +20 mV as compared to +30, +40 or +50 mV. Increasing the dose of Bay K did not further enhance the Ca2+ channel current. The effects were induced rapidly but very difficult to wash out in some experiments, a phenomenon observed by other investigators [43]. In certain cases the enhancement of calcium current was very transitory, the effect disappearing within a minute or so. Figure 13.a illustrates that at test potentials of +10, +20 and +30 mV the time to peak was shorter and the peak inward current larger after application of 2 μM Bay K 8644. At almost all voltages tested the endpulse current was increased to a significantly lesser extent compared to the peak current ($p = < 0.001$). Rather in certain cases the end pulse current elicited at maximum amplitude of calcium current was even smaller than the control end pulse current. The inactivation of calcium channel current was, thus, enhanced by Bay K 8644 in most cases.

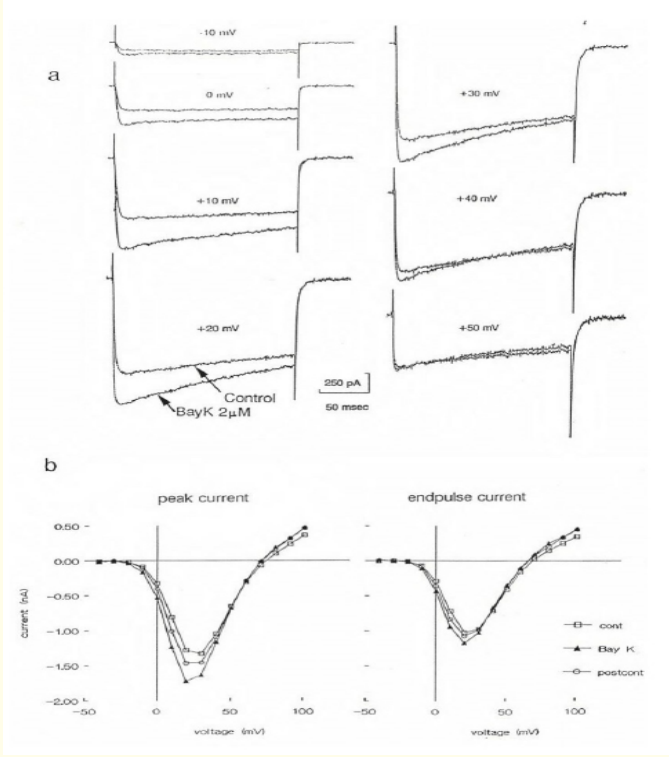


Figure 13: *Sensitivity of the HVA Ca^{2+} current to the dihydropyridine agonist Bay K 8644. (a) during whole-cell recording, test potentials of 180 msec durations were applied to -10 to +50 mV (test potentials shown at the top of each pair of traces) from a holding potential of -50 mV. With 4 mM Ca^{2+} as the charge carrier, control currents were evoked at each potential. Bay K 8644 (2 μM) was then applied and effects seen by applying similar voltage steps as in the control. An appreciable enhancement of the calcium current was manifest during test pulses to 0, +10, +20 and +30 mV in the presence of Bay K 8644 (lower traces in each set). (b) I-V characteristic for a family of calcium currents in the same cell obtained from holding potential of -50 mV for the peak current and the endpulse currents. Plots were obtained before and after addition of Bay K 8644 and then after washing Bay K. In this representative case, after drug treatment the peak of the I-V curve was shifted to the left and the maximal current was increased more in the region of test potentials of -10 to +30 mV.*

Stability of Recording: A substantial degree of Ca^{2+} current rundown has perviosly been reported [13] in internally perfused unidentified Lymnaea neurones of large diameter (80-120 μM). In their experiments the current reduced to one tenth of the original in 30 minutes and a number of other investigators have reported similar finding. By using ATP and GTP in the patch pipette it was possible to have a reasonably stable recording over long periods, sometimes for four to five hours (Figure 14).

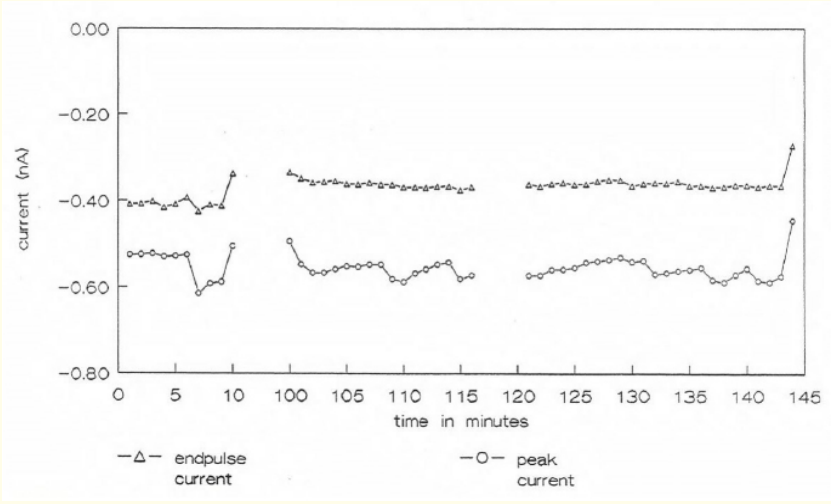


Figure 14: *Stability of recording of calcium channel activity. The maximal calcium current was elicited by depolarizing the cell from a holding potential of -50 mV to a test potential of +20 mV and peak current (open circles) and the current at the end of the pulse (open triangles) recorded. Test pulses were applied every minute for the first 10 minutes. There was a gap of 90 minutes before the same protocol was repeated for another 16 minutes. After another interval of 6 minutes similar depolarizing pulses were applied every minute for the next 25 minutes. There was only a small difference in the amplitude of the peak as well as the end pulse current between these sets of observations.*

Discussion

In this paper, we have presented strong evidence for a dihydropyridine-sensitive L-type Ca^{2+} current in cultured pedal I cluster neurones of Lymnaea, using the whole-cell patch-clamp technique. A combination of solutions in the bath and pipette was utilized to ensure complete isolation of Ca^{2+} currents from other currents that have been described in molluscan neurones [44,45]. Under these conditions, robust currents through Ca^{2+} channels were recorded using either Ca^{2+} or Ba^{2+} as the charge carrier. The calcium current isolated in this manner could be completely blocked by Cd^{2+} (100 μM). During the whole-cell recordings the calcium current activated in all cells at -35 to -25 mV from a holding potential of either -50 mV or -80 mV. This high-voltage-activated current was substantially larger when equimolar Ba^{2+} replaced Ca^{2+} as the charge carrier, and this substitution resulted in a 10 - 15 mV shift to the left of the I-V curves for both the peak and the end pulse currents. This current was sensitive to the dihydropyridine (DHP) agonist Bay K 8644 (0.1 to 2 μM) and antagonist nifedipine (0.5 to 4 μM). Thus, the dihydropyridine pharmacology of this prolonged, slowly inactivating current resembled that of the L-type calcium current found in dorsal root ganglion neurones [40] and in heart cells [41]. The inactivation of HVA currents in Lymnaea neurones showed a sensitivity to the level of free $[Ca^{2+}]i$. Inactivation was largely incomplete in the presence of internal Ca^{2+}-EGTA buffers and Ba2+ further reduced the inactivation [46].

Calcium channels in Lymnaea neurones: The classification and functional roles of VGCCs in molluscs were reviewed by Kits and Mansvelder in 1996 [9], who indicated the prescience of T-, N-, L- and P-type channels in molluscan neurones and muscles, particularly those of Aplysia, Helix and Lymnaea and the squid giant synapse. The initial description of calcium current in acutely isolated neurones from

Lymnaea, given by Kostyuk and Krishtal [34], showed it to be partially sensitive to organic calcium channel antagonists like verapamil, and selectively blocked with Cd^{2+} ions when applied in concentration of 1-2 mM. The concentration of verapamil that was found to be effective by Kostyuk & Krishtal in partially blocking the Ca^{2+} channels was 0.5 mM. Organic Ca^{2+} channel blockers have been reported to be relatively ineffective in blocking Ca^{2+} currents in Lymnaea neurones [13]. Even 100 μM nifedipine was found to exert only a weak, slow and largely irreversible effect on Ca^{2+} currents. The description of the calcium current observed by Byerly., *et al.* (1985) was more like a high-voltage-activated slowly inactivating long-lasting current. Acutely isolated, large, unidentified neurones from Lymnaea were also observed by other workers to have only one type of calcium current resembling the classical high-voltage-activated calcium current [13,30]. The Ca^{2+} channel currents in caudodorsal cells from the brains of Lymnaea stagnalis were also found to be of HVA type [47].

The calcium channel density may vary in large and small cells [48] and the expression of various channel types may be different in different cells [49] of the same structure. The majority of the cells in the ganglia of the brain of Lymnaea stagnalis are not very large. Therefore, for the current study, we chose the pedal I-cell cluster, which is a homogeneous group of cells with relatively small size, for characterization. Calcium currents in the right pedal I-cluster cells of Lymnaea stagnalis were similar to those in other large unidentified Lymnaea neurones [13,44,50]. They were activated to maximal level at +20 to +30 mV of membrane potential. Inward calcium current was typically and kinetically similar to L-type current and sensitive to dihydropyridine agonist, Bay K 8644, and antagonist, nifedipine [40]. Our results confirm the earlier finding of the existence of only an HVA Ca^{2+} current and extend it to small diameter cells in culture. Ca^{2+} currents in cultured Pedal-I cluster cells were found to activate relatively slowly at less depolarized voltages and the rate of activation increased at stronger depolarizations. Similarly Ca^{2+}currents inactivated very slowly at lower voltages, whereas inactivation was hastened when stronger depolarizations were used. This is a clear indication of voltage- and time-dependent inactivation of Ca^{2+} currents as the effect of Ca^{2+}-induced inactivation was reduced to a minimum by using EGTA in the pipette solution and Ba^{2+} as the charge carrier in external solutions in some of the experiments. The inward currents measured by Byerly & Hagiwara (1982) showed little apparent inactivation and at +20 mV test potential the typical decay was less than 50% during a 60 msec pulse. The calcium channel current switched on with a sigmoidal time course which is suggestive of more than one closed state in which calcium channels can exist [13]. Findings of the present study are also in line with those of Byerly & Hagiwara [13] and Byerly., *et al*. [30,50].

An acceleration of inactivation of calcium channel currents was observed with Bay K 8644 at strong test depolarizations of +20 mV or above, probably because of the partial antagonist action of Bay K 8644 [42]. Bay K 8644 (a DHP agonist) elevates calcium influx by promoting a pattern of calcium channel gating with very long opening and brief closing events in neurones and cardiac cells [40,41,42]. Bay K 8644 probably acts by inhibiting dephosphorylation of calcium channels as it has been shown to delay the loss of channel activity in minimal solution [51]. The dihydropyridine antagonists such as nifedipine, nitrendipine, nisoldipine, are potent blockers of cardiac HVA Ca^{2+} currents with little effect on LVA currents [32] and selectively block L-type currents in neurones [3]. These DHP antagonists increase the proportion of sweeps where the channel fails to open [42]. These blockers of L-type calcium channels are thought to have more potent actions on vertebrate cells than on invertebrate cells [52] and we have tested them. Nifedipine had both type of effects. This is not unusual because of the mixed agonist/antagonist action of dihydropridines.

It is possible that the data we present here, could either be interpreted to indicate the existence of two types of HVA Ca^{2+} currents in the right pedal I-cluster neurones: a dihydropyridine-sensitive and an insensitive component, or alternatively the single type of calcium channel present shows a limited sensitivity to dihydropyridines, and thus differs from the classically described L-type calcium channel [40]. Probably the neurones of Lymnaea stagnalis possess calcium channels which do not strictly follow the T, N and L classification in other cells. The other types have intermediate properties of the typical T, N and L type channels and some of their own unique ones, e.g. P-type channel. It is suggested that further characterization of Ca^{2+}channels should be done using pharmacological tools such as omega-conotoxin and funnel-web spider toxin.

Like Na^+ and K^+ channels various structurally and functionally distinct isoforms of Ca^{2+} channels are expressed in different tissues [53] that are suitable for some specific purpose in that particular cell. Some cells apparently express only one of VGCC type, e.g., Swandulla & Armstrong [54] found evidence for only one type of HVA current in chick dorsal root ganglion (DRG) neurones while Helix pomatia [55] and Helix aspersa neurones have been reported to contain only an HVA calcium channel [56,57,58].

Differential distribution of Ca^{2+} channels: The contention that some Lymnaea neurones may possess only one type of Ca^{2+} channel is open to question in the wake of reports of non-uniform distribution of Ca^{2+} channels over the soma and neurites [3,59]. Haydon and Man-Son-Hing [14], reported that neurones with extended neurites had both HVA and LVA calcium current in their soma but only HVA current in the distal secretory processes. When neurones were grown as spheres such that the somata attained the ability to release neurotransmitter then only the HVA current could be detected in the soma. All the neurones used in the present study were grown as spheres with no neuritic extension in order to achieve a better space clamp. Thus, it is probable that in this configuration only HVA Ca^{2+} channels were expressed. In hippocampal neurones both LVA and HVA Ca^{2+} channels were suggested to be located on the soma membrane and the neuritic outgrowths contained only HVA current [60]. On the other hand, excitable growth cones of dorsal root ganglion (DRG) neurones were reported to contain about as many LVA Ca^{2+} channels as the soma [61]. N-type channels were predominantly found in the growth cones of PC12 cell clones [62]. Muller., *et al*. [63] reported a lack of LVA current in the somata of redissociated neurones which had lost their dendrites as compared to those in network which possessed both types of Ca^{2+} channels. In hypothalamic neurones their distribution in the dendritic membranes might be related to the outgrowth of dendrites and the formation of synapses in the neural network. Those on nerve terminals probably have different electrophysiological characteristics and pharmacological susceptibility, and were most likely to be the N-type calcium channels. Those on the cell bodies and the dendrites were probably of L- and T-types indicating that HVA, but not LVA, Ca^{2+} currents were required for synaptic transmission in the network of cultured neurones. Westenbroek., *et al.* [64] have demonstrated a clustering of L-type channels on the basal areas of the dendrites and the cell bodies. It is most likely that the distribution of Ca^{2+} channels on various regions of a neurone is dictated by their functional requirements.

Variability in properties of calcium channels: Though the biophysical and pharmacological studies have provided ample evidence for the existence of neuronal LVA and HVA Ca^{2+} channels in neurones, further distinction into possible multiple HVA channels on the basis of Ca2+ current kinetics is a controversial point [65]. Furthermore, neuronal Ca^{2+} currents of different species and even neurones of the same species are not equally affected by specific pharmacological agents. Minor species variations in calcium receptors clearly exist [1] and expression of various combinations of L-type Ca^{2+} channel subunits [1,5] can give rise to a variety of calcium channels that differ in their calcium permeation properties and in the characteristics of their dihydropyridine binding sites [66]. Such diversity in the structure of channel subunits permit the execution of various functions of Ca^{2+} channels in different tissues. These variations would not extend to structures that determine basic biophysical properties of the channels, such as activation and inactivation gating and ion permeation [65]. Some cells apparently express only one of the three classical types of calcium channels, e.g., L-type in adrenal chromaffin cells [36], while in other cases a wide range of Ca^{2+} channels are found [67]. In the presynaptic terminals of the squid giant synapse show HVA currents that are insensitive to DHPs or omega-conotoxin [68]. Unidentified neurones of Helix pomatia [55] and Helix aspersa have been reported to contain only one class of voltage-dependent calcium channels, the HVA calcium channel [56,57,58]. Swandulla & Armstrong [54] found evidence for only one type of HVA current in chick dorsal root ganglion (DRG) neurones which was not affected by Bay K 8644 [46]. The complement of ion channels may vary from species to species and from neurone to neurone in the same species and, as mentioned above may be different in different parts of the same neurone.

Lymnaea as a model system for anaesthetics research: Lymnaea has proved to be a tractable preparation for research on the cellular basis for inhalational [19,69,70] and intravenous [70,71,72] general anaesthetics (GAs). As mentioned above, previous experiments have indicated that VGCCs are likely targets for the action of general anaesthetics [20] and are known to have actions at both electrical and chemical synapses [70,73,74,75,76,77,78], both in vitro and in culture. In a further paper (in preparation) we will explore the actions of the inhalational anaesthetic halothane on the dihydropyridine sensitive calcium currents described here.

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Bibliography

1. Simms BA and Zamponi GW. "Neuronal, voltage-gated calcium channels: structure, function and dysfunction". *Neuron* 82.1 (2014): 24-45.

2. Carbone E and Lux HD. "Single low-voltage-activated calcium channels in chick and rat sensory neurones". *Journal of Physiology* 386 (1987): 571-601.

3. Fox AP., *et al.* "Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones". *Journal of Physiology* 394(1987): 149-172.

4. Tsien RW. "Multiple types of neuronal calcium channels and their selective modulation". *Trends in Neurosciences* 11.10 (1988): 431-438.

5. Gurkoff G., *et al*. "Voltage-gated calcium channel antagonists and traumatic brain injury". *Pharmaceuticals* 6.7 (2013): 788-812.

6. Llinas RR., *et al*. "Voltage dependent calcium conductances in mammalian neurons. The P channel". *Annals of the New York Academy of Sciences* 560 (1989): 103-111.

7. Regan LJ., *et al*. "Ca2+ channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and omega conotoxin". *Neuron* 6.2 (1991): 269-280.

8. Sah DW. "Neurotransmitter modulation of calcium current in rat spinal cord neurons". *Journal of Neuroscience* 10.1 (1990): 136-141.

9. Kits KS and Mansvelder HD. "Voltage gated calcium channels in molluscs: classification, Ca2+ dependent inactivation, modulation and functional roles". *Invertebrate Neuroscience* 2.1 (1996): 9-34.

10. Striessnig J., *et al*. "L-type Ca2+ channels in heart and brain". *WIREs Membrane Transport and Signaling Signal* 3.2 (2014): 15-38.

11. Hille B. "The Sharpey Schafer Lecture. Ionic channels: evolutionary origins and modern roles". *Quarterly Journal of Experimental Physiology* 74.6 (1989): 785-804.

12. Ertel EA., *et al*. "Nomenclature of voltage-gated calcium channels". *Neuron* 25.3 (2000): 533-535.

13. Byerly L and Hagiwara S. "Calcium currents in internally perfused nerve cell bodies of Lymnaea stagnalis". *Journal of Physiology* 322 (1982): 503-528.

14. Haydon PG and Man Son Hing H. "Low and high-voltage-activated calcium currents: their relationship to the site of neurotransmitter release in an identified neuron of Helisoma". *Neuron* 1.10 (1988): 919-927.

15. Spafford JD., *et al*. "Expression and modulation of an invertebrate presynaptic calcium channel α1 subunit homolog". *Journal of Biological Chemistry* 278 (2003): 21178-21187.

16. Syed NI., *et al*. "In vitro reconstruction of neuronal circuits: a simple model approach". *In: Modern Techniques in Neuroscience Research* (1999): 361-377.

17. Yar T., *et al*. "Effects of general anaesthetics on cultured Lymnaea neurones". *Acta Biologica Hungarica* 44.1 (1993): 33-36

18. Yar T and Winlow W. "Halothane depresses a high-voltage-activated calcium current in cultured neurones Lymnaea stagnalis". *Journal of Physiology* 459(1993): 12.

19. Girdlestone D., *et al*. "The actions of halothane on spontaneous activity, action potential shape and synaptic connections of the giant serotonin-containing neurones of Lymnaea stagnalis (L)". *Comparative Biochemistry and Physiology C* 93.2 (1989): 333-339.

20. Orestes P and Todorovicz SM. "Are neuronal voltage-gated calcium channels valid cellular targets for general anesthetics?". *Channels (Austin)* 4.6 (2010): 518-522.

21. Winlow W., *et al*. "Characterization of Lymnaea neurones by determination of action potential trajectories". *Journal of Experimental Biology* 99 (1982): 207-221.

22. Slade CT., *et al*. "The neuronal organisation of the paired pedal ganglia of Lymnaea stagnalis (L.)". *Comparative Biochemistry and Physiology* 69A (1981): 789-803.

23. Kyriakides M., *et al*. "The morphology and electrophysiology of the neurones of the paired pedal ganglia of Lymnaea stagnalis (L.)". *Comparative Biochemistry and Physiology* 93.4 (1989): 861-876.

24. Kater SB., *et al*. "Calcium regulation of the neuronal growth cone". *Trends in Neurosciences* 11.7 (1988): 315-321.

25. Benjamin PR and Winlow W. "The distribution of three wide-acting synaptic inputs to identified neurons in the isolated brain of Lymnaea stagnalis (L)". *Comparative Biochemistry and Physiology* 70.3(1981): 293-307.

26. Wong RG., *et al* "Neurite outgrowth in molluscan organ and cell cultures: the role of conditioning factor(s)". Journal of Neuroscience1.9 (1981): 1008-1021.

27. Haydon PG. "The formation of chemical synapses between cell cultured neuronal somata". *Journal of Neuroscience* 8.3 (1988): 1032-1038.

28. Hamill O., *et al.* "Improved patch-clamp techniques for high resolution current recordings from cells and cell-free membrane patches". *Pflugers Archiv* 391.2(1981): 85-100.

29. Lee KS and Tsien RW. "High selectivity of calcium channels in single dialysed heart cells of the guinea-pig". *Journal of Physiology* 354(1984): 253-272.

30. Byerly L., *et al*. "Calcium current activation kinetics in the neurones of the snail Lymnaea stagnalis". *Journal of Physiology* 348 (1984): 187-207.

31. Tsien RW., *et al*. "Calcium channels: mechanisms of selectivity, permeation, and block". *Annual Review of Biophysics & Biophysical Chemistry* 16 (1987): 265-290.

32. Bean BP. "Two kinds of calcium channels in canine atrial cells". *Journal of General Physiology* 86.1 (1985): 1-30.

33. Fox AP., *et al*. "Single channel recordings of three types of calcium channels in chick sensory neurones". *Journal of Physiology* 394 (1987): 173-200.

34. Kostyuk PG and Krishtal OA. "Effects of calcium and calcium-chelating agents on the inward and outward currents in the membrane of mollusc neurones". *Journal of Physiology* 270.3 (1977): 569-580.

35. Byerly L and Moody WJ. "Intracellular calcium ions and calcium currents in perfused neurones of the snail Lymnaea stagnalis". *Journal of Physiology* 352 (1984):637-652.

36. Fenwick EM., *et al*. "Sodium and calcium channels in bovine chromaffin cells". *Journal of Physiology* 331(1982): 599-635.

37. Mcdonald Tf., *et al*. "Voltage-dependent properties of macroscopic and elementary calcium channel currents in guinea pig ventricular myocytes". *Pflugers Archiv* 406.5 (1986): 437-448.

38. Hirning LD., *et al*. "Dominant role of N type Ca^{2+} channels in evoked release of norepinephrine from sympathetic neurons". *Science* 239 (1988): 57-61.

39. Lansman JB., *et al*. "Blockade of current through single calcium channels by Cd2+, Mg2+ and Ca2+. Voltage and concentration dependence of Ca2+ entry into the pore". *Journal of General Physiology* 88.3 (1986): 321-347.

40. Nowycky MC., *et al*. "Three types of neuronal calcium channels with different calcium agonist sensitivity". *Nature* 316 (1985): 440-443.

41. Nilius B., *et al*. "A novel type of cardiac calcium channel in ventricular cells". *Nature* 316 (1985): 443-446.

42. Hess P., *et al*. "Different modes of Ca channel gating behaviour favoured by Ca agonists and antagonists". *Nature* 311 (1984): 538-544.

43. Taylor RW. "Two-suction-electrode voltage-clamp analysis of the sustained calcium current in cat sensory neurones". *Journal of Physiology* 407 (1988): 405 432.

44. Kostyuk PG and Krishtal OA. "Separation of sodium and calcium currents in the somatic membrane of mollusc neurones". *Journal of Physiology* 270.3 (1977): 545-568.

45. Adams DJ., *et al*. "Ionic currents in molluscan soma". *Annual Review of Neuroscience* 3 (1980): 141-167.

46. Hess P. "Calcium channels in vertebrate cells". *Annual Review of Neuroscience* 13 (1990): 337-356.

47. Brussaard AB., *et al*. "Inhibitory modulation of neuronal voltage-dependent sodium current by Phe-Met-Arg-Phe-amide". *Neuroscience Letters* 111.3 (1990): 325-332.

48. Scroggs RS and Fox AP. "Multiple Ca2+ currents elicited by action potential waveforms in acutely isolated adult rat dorsal root ganglion neurons". *Journal of Neuroscience* 12.5 (1992): 1789-1801.

49. Mogul DJ and Fox AP. "Evidence for multiple types of Ca2+ channels in acutely isolated hippocampal CA3 neurones of the guinea-pig". *Journal of Physiology* 433 (1991): 259-281.

50. Byerly L., *et al*. "Permeation and interaction of divalent cations in calcium channels of snail neurones". *Journal of General Physiology* 85.4 (1985): 491-518.

51. Armstrong DL. "Calcium channel regulation by calcineurin, a Ca2+ activated phosphatase in mammalian brain". *Trends in Neurosciences* 12.3 (1989): 117-122.

52. Hille B. "Ionic Channels of Excitable Membranes". *Sunderland, Massachusetts: Sinauer Associates, Inc.* (1992).

53. Chen CF and Hess P. "Mechanism of gating of T type calcium channels". *Journal of General Physiology* 96.3 (1990): 603-630.

54. Swandulla D and Armstrong CM. "Fast-deactivating calcium channels in chick sensory neurons". *Journal of General Physiology* 92.2 (1988): 197-218.

55. Doroshenko PA., *et al.* "Intracellular metabolism of adenosine 3',5'-cyclic monophosphate and calcium inward current in perfused neurones Helix pomatia". *Neuroscience* 7.9 (1982): 2125-2134.

56. Chad J and Eckert R. "An enzymatic mechanism for calcium current inactivation in dialysed Helix neurones". *Journal of Physiology* 378 (1986): 31-51.

57. Gutnick MJ., *et al*. "Voltage-dependent and calcium-dependent inactivation of calcium channel current in identified snail neurones". *Journal of Physiology* 412 (1989: 197-220.

58. Yakel JL. "Inactivation of the Ba2+ current in dissociated Helix neurons: voltage dependence and the role of phosphorylation". *Pflugers Archive* 420.5-6 (1992): 470-478.

59. Llinas R and Yarom Y. "Properties and distribution of ionic conductances generating electroresponsiveness of mammalian inferior olivary neurones in vitro". *Journal of Physiology* 315(1981): 569-584.

60. Yaari Y., *et al*. "Development of two types of calcium channels in cultured mammalian hippocampal neurons". *Science* 235 (1987): 680-682.

61. Gottmann K and Lux HD. "Low- and high-voltage-activated Ca2+ conductances in electrically excitable growth cones of chick dorsal root ganglion neurons". *Neuroscience Letters* 110.1-2 (1990): 34-39.

62. Reber BF and Reuter H. "Dependence of cytosolic calcium in differentiating rat pheochromocytoma cells on calcium channels and intracellular stores". *Journal of Physiology* 435 (1991): 145-162.

63. Muller TH., *et al*. "Ionic currents in cultured rat hypothalamic neurones". *Journal of Physiology* 450 (1992): 341-362.

64. Westenbroek RE., *et al*. "Clustering of L-type calcium channels at the base of major dendrites in hippocampal pyramidal neurones". *Nature* 347 (1990): 281-284.

65. Swandulla D., *et al*. "Do calcium channel classifications account for neuronal calcium channel diversity?". *Trends in Neuroscience* 14.2 (1991): 46-51.

66. Varadi G., *et al*. "Acceleration of activation and inactivation by the beta subunit of the skeletal muscle calcium channels". *Nature* 352 (1991): 159-162.

67. Neelands TR., *et al*. "Functional expression of L-, N-, P/Q- and R-type calcium channels in the human NT2-N cell line". *Journal of Neurophysiology* 84.6 (2000): 2933-2944.

68. Augustine GJ., *et al*. "Calcium action in synaptic transmitter release". *Annual Review of Neuroscience*10 (1987): 633-693.

69. Girdlestone D., *et al*. "The actions of three volatile general anaesthetics on the withdrawal reflexes of Lymnaea stagnalis (L)". *Comparative Biochemistry and Physiology C* 92.1 (1989): 39-43.

70. Qazzaz MM and Winlow W. "Differential Actions of Volatile Anaesthetics and a Systemic Barbiturate on Strongly Electrically Coupled Neurons". *EC Neurology* 2.4 (2015): 188-204

71. Woodall AJ And McCrohan CR. "Excitatory action of propofol and ketamine in the snail Lymnaea stagnalis". *Comparative Biochemistry and Physiology C* 127.3 (2000): 297-307.

72. Woodruff KT., *et al*. "Short Term Exposure to Ketamine Reduces Cholinergic Synaptic Transmission, But Not Post-Tetanic Potentiation in Central Neurons". *EC Neurology* 2.6 (2015): 248-259.

73. Spencer GE., *et al*. "Halothane affects both inhibitory and excitatory synaptic transmission at a single identified molluscan synapse, in vivo and in vitro". *Brain Research* 714.1-2 (1996): 38-48.

74. Spencer GE, *et al*. "Halothane-induced depression at both an in vivo and in vitro reconstructed synapse between neurons of Lymnaea". *Journal of Neurophysiology* 74.6 (1995): 2604-261.

75. Woodall AJ., *et al*. "Anesthetic treatment blocks synaptogenesis but not neuronal regeneration of cultured Lymnaea neurons". *Journal of Neurophysiology* 90.4 (2003): 2232-2239.

76. Winlow W., *et al*. "Novel and orthodox responses of in vitro reconstructed synapses to volatile anaesthetics". *Progress in Anaesthetic Mechanisms* 6 (2000): 299-305.

77. Winlow W., *et al*. "Modulation of reconstructed peptidergic synapses and electrical synapses by general anaesthetics". *Toxicology Letters* 100-101 (1998): 77-84.

78. Spafford JD., *et al*. "In Vitro Characterization of L-Type Calicum Channels and their contribution to firing behaviour in invertebrate respiratory neurons". *Journal of Neurophysiology* 95 (2006): 42-52.

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