Effects of Sodium Pentobarbital on Potassium Currents and Intracellular Calcium Concentration of Isolated, Cultured Pedal I Cluster Neurons of *Lymnaea stagnalis*

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Abstract

   Sodium pentobarbital is currently used as a systemic veterinary anesthetic. Here we consider its actions on the potassium currents of isolated and on intracellular calcium concentration in cultured right pedal I (RPeI) cluster neurons of *Lymnaea stagnalis*. We demonstrate its dose-dependent effects on potassium currents, in the clinical range and compare them with the actions of the volatile anesthetics halothane and isoflurane that we have described previously on the same cell types. We also observe that pentobarbital can raise free intracellular calcium concentration in RPeI neurons, but this action appears to be cell specific.

Keywords: Potassium Currents; Molluscan Neurons; Whole Cell Patch Clamp; Pentobarbital; Intracellular Calcium Concentration; Fura II; Lymnaea stagnalis

Introduction

   In previous reports we have studied the actions of volatile anesthetics on the potassium [1] and calcium currents [2] of isolated, cultured pedal I cluster (RPeI) neurons of *Lymnaea stagnalis* using the whole cell patch-clamp technique. Here we consider the actions of the systemic anesthetic agent sodium pentobarbital on these neurons, again in single cell culture. These cells are known to exhibit up to four separate potassium currents [3] and a single high voltage activated L-type calcium current [4] in culture, all of which are diminished in 2% (v/v) halothane or 2% (v/v) isoflurane [1,2] in a dose-dependent manner, except that low clinical concentrations of isoflurane (0.5% v/v) enhance the L-type calcium current [1].

   Barbiturates have sleep-inducing properties causing depressant activity on the CNS similar to inhalational anesthetics [5]. They activate $GABA_A$ receptors but are non-selective and cause marked cardiovascular and respiratory depression, although they are poor analgesics. According to PubChem [6], sodium pentobarbital is a controlled substance used as a sedative, an anti-convulsive and a hypnotic. It is currently in use as veterinary anesthetic [7] and in animal euthanasia [8]. Volatile anesthetic agents are known to raise intracellular calcium concentration $[Ca^{2+}]_i$ in many cell types including B lymphocytes [9] and identified *Lymnaea* neurons [10,11] and Harris [12] found that sodium pentobarbital inhibited ATP-dependent uptake of Ca^{2+} by synaptic membranes, suggesting that this should raise the free $[Ca^{2+}]_i$.

   We recently demonstrated that sodium pentobarbital (0.5 - 2.0 mM) had divergent effects on identified neurons and was capable of generating paroxysmal depolarizing shifts (PDS) in motor neurons prior to quiescence [13] in the intact brain of *Lymnaea stagnalis.* PDS

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may also be generated by halothane in isolated, cultured neurons of the *Lymnaea* feeding system [14] and a preliminary study suggested that pentobarbital raises free intracellular calcium concentration $[Ca^{2+}]_i$ in isolated, cultured pedal I (RPeI) cluster neurons [15]. Here we consider the actions of clinical concentrations of sodium pentobarbital on identified potassium currents in RPeI cells of *Lymnaea* and compare them with our previous studies on the actions of halothane and isoflurane. In a separate experimental series we also investigate the effects of pentobarbital on $[Ca^{2+}]_i$ on the same neuron type. A preliminary report on the patch clamp studies has appeared elsewhere [16].

Materials and Methods

Pedal I cluster neurons which discharge irregular type II action potentials *in situ* [17] and have a small diameter (about 20 μm) have been chosen for this study. Pedal I cluster neurons were isolated and cultured for 1 - 5 days according to the methods of Walcourt-Ambakederemo and Winlow [18,19]. The L-15 medium used in cell culture was completely exchanged for bath solution which was continuously delivered to the dish and then removed using suction. The patch electrode was then pressed against the cell surface using a three-dimensional micromanipulator (Model MX-1 Narishige Scientific Instrument Laboratories, Japan). The seal was usually achieved spontaneously or was potentiated with slight continuous suction which was applied to the pipette interior. Tight seals with a resistance greater than 10 GΩ were normally achieved. In the whole-cell clamp configuration K^+ currents were measured and data were acquired with a CED 1401 interface and leakage currents were subtracted. Individual pedal I cluster cells were then clamped at -50 mV and, using a 500 ms command depolarization, were stepped from -90 mV to +90 mV in 10 mV intervals (See figure 1B).

The standard bath solution for K^+ currents contained NMDG 51.6, $CaCl_2$ 4, $MgCl_2$ 1.5, HEPES 10 and Glucose 10 mM. *N*- methyl- D- glucamine (NMDG) was used as a replacement for sodium ions as it has osmotic activity similar to sodium ions and cell are impermeable to it. In one series of experiments, 40 mM tetraethylammonium chloride (TEA) was also added to the bathing solution.

Whole cell patch pipettes had a resistance of 2 - 5 MΩ and standardly contained KCl 50, NaCl 1.6, $MgCl_2$ 1.5 HEPES 10, Mannitol 10 (to adjust osmolarity), but different amounts of EGTA, $CaCl_2$ or ATP as follows:

- Pipette solution A (zero ATP) contained KCl, 50; NaCl, 1.6; $MgCl_2$ 1.5; HEPES, 10; Mannitol, 10; EGTA, 0.5; and $CaCl_2$, 4.7 (in mM)
- Pipette solution B (Ca^{2+} and ATP) contained KCl, 50; NaCl, 1.6; $MgCl_2$ 1.5; HEPES, 10; Mannitol, 10; EGTA, 0.5; $CaCl_2$, 4.7 and ATP, 3 (in mM)
- Pipette solution C (ATP, but zero Ca^{2+}) contained (in mM) KCl, 50; NaCl, 1.6; $MgCl_2$, 1.5; HEPES, 10; Mannitol, 10; EGTA, 5; ATP, 3 (in mM).

Based on previous work by Schwarz and Passow [20], we determined that 4.7 mM Ca^{2+} is enough to activate I_{KCa} in snail neurons.

Pentobarbital was dissolved in the bath solution at concentrations between 10^{-10} - 10^{-3} M and used on the same day. The normal clinical concentration of pentobarbital is 50 mg/Kg to produce a range of 20 - 50 μM in mammalian CSF [21]. Using the CED Patch and Voltage Clamp Software (V clamp, version 5.0) potassium currents were measured at the sustained current on each trace.

Statistical analysis of electrophysiological data

The recorded data (n = 6 in each case) were averaged and were then leakage subtracted. Experimental values presented here were analysed by two way analysis of variance and a P value of ≤0.005 was considered to be a significant alteration of normal currents by the anesthetic.

Measurement and analysis of intracellular calcium concentration $[Ca^{2+}]_i$

Isolated, identified neurons were cultured for two days and then incubated in Fura-2 for 2 - 3h, according to the methods described by Ahmed., *et al*. [22]. Digital imaging of Fura-2 fluorescence from individual cells was carried out as described previously [22] using a Magical video imaging system. Tardis (version 7.35) software allowed us to record the subcellular alterations in $[Ca^{2+}]_i$ occurring on exposure of the cells to three different concentrations of pentobarbital (0.2, 0.5 and 1.0 mM) in continuously superfused HEPES buffered snail saline [23]. The protocol for our study was to first record the $[Ca^{2+}]_i$ in snail saline and then to superfuse the preparation with increasing concentrations of pentobarbital, each for at least 30min (maximum 1h). Throughout this procedure measurements of

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$[Ca^{2+}]_i$ were made at 6 min intervals. The neurons were then washed in snail saline for 30 min and $[Ca^{2+}]_i$ was determined every 10min. Data were expressed as mean standard deviation using a two-tailed significance test (MedCalc).

Results

Whole Cell Patch-clamp data

Delayed rectifier current (I_K)

Previous research had indicated that calcium activated currents are superimposed on the delayed outward current [24]. We therefore selectively blocked both I_{KCa} and I_{KATP} in order to study I_K. Using pipette solution C (which is rich in ATP) and K^+ free bath solution, potassium currents were recorded and I/V curves were constructed (Figure 1). Since there was ATP in pipette solution C, K_{ATP} was suppressed and there was Ca^{2+} in this pipette solution to activate the K_{Ca} current. Thus, the recorded current includes the delayed rectifier K^+ current, with a maximum current amplitude of ca. 8.5 nA, averaged over 6 preparations (Figure 1). We have applied a range of different concentrations ($10^{-10} - 10^{-3}$) of a racemic mixture of pentobarbital which is used clinically as a hypnotic and general anesthetic [25] and immediately recorded changes in I_k. Pentobarbital at 10^{-10} M had no significant effects (p value ≤ 0.005) on K^+ currents but they were significantly decreased by higher concentrations (P value ≤ 0.005) and 10^{-9}M pentobarbital was found to be the minimum effective dosage which was able to reduce potassium currents (P value ≤ 0.005). Using 10^{-9} and 10^{-3}M pentobarbital the K^+ current was reduced by 16% and 53% respectively at the highest step of depolarization, demonstrating dose-dependency of the current. With continuous wash out of the preparations no significant differences were found between pre-control and the post-control traces as shown in figure 1.
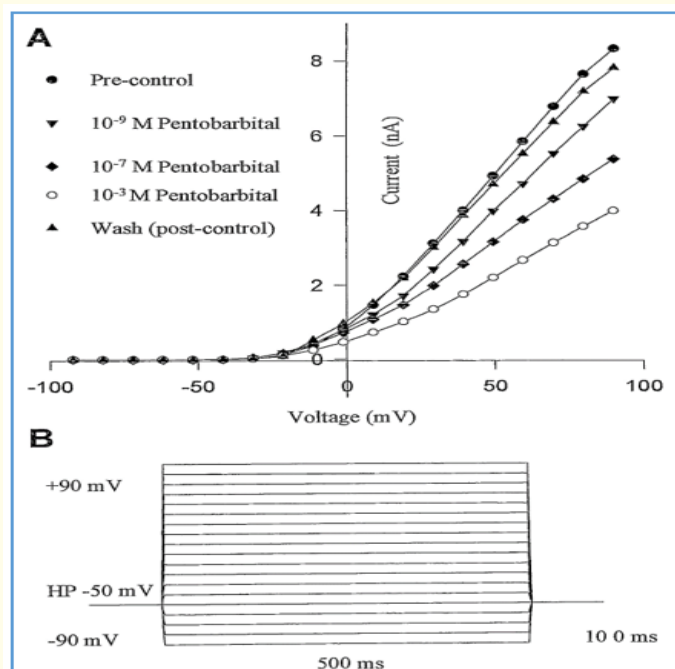

Figure 1: Concentration-dependent effect of pentobarbital on delayed rectifier K+ current of pedal I cluster neurons using pipette solution C, containing ATP, but zero Ca²⁺. A) The cells were clamped at -50 mV and stimuli were delivered every 10s. Depolarization steps were from -90 to +90 mV in 10 mV steps using a 500 ms command potential. The sequence of application of solutions: 1) Pre -control in normal bath solution; 2) 10^{-9} M pentobarbital; 3) 10^{-7} M pentobarbital; 4) 10^{-3} M pentobarbital; 5) Post-control (wash). B) The stimulus protocol showing the amplitude and duration of the command pulse. HP = holding potential. (A is reproduced from Winlow et al [11], under Creative Commons Attribution License (CC BY).

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Unidentified K⁺ current suppressed by pentobarbital

The current in figure 1 may contain other possible constituents other than I_{KCa} and I_{KATP} which were blocked. Since the delayed rectifier is known to be blocked by tetraethylammonium (TEA) in vertebrates and in *Lymnaea* [3,16,27,28], we also added 40 mM TEA to the bathing solution in a separate series of experiments (Figure 2) also using pipette solution C. We found that 40 mM TEA, which should ensure that I_K is completely blocked, suppressed the remaining current by 11% at +90 mV command potential. Then a mixture of 40 mM TEA and 10^{-5} M pentobarbital was applied and induced a reduction by 32%. Thus, pentobarbital suppresses the unidentified K⁺ current in a concentration-dependent manner, but it remains to be classified.
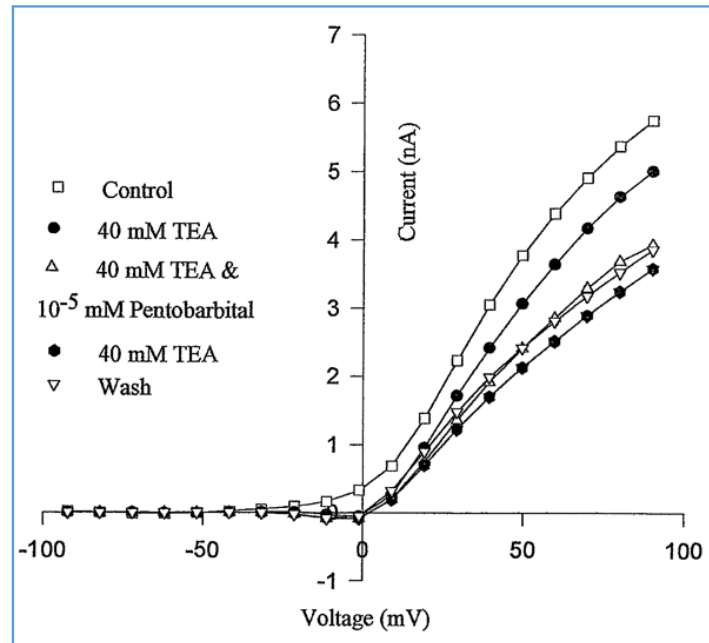

Figure 2: *Effect of TEA and pentobarbital on unidentified K⁺ current. A) Effect of TEA and pentobarbital on I_K, which was recorded with pipettes containing pipette solution C (5 mM EGTA to block $I_{(Kca)}$ and 3 mM ATP to block I_{ATP}). The sequence of application of solutions: 1) Pre-control in normal bath solution; 2) 40 mM TEA; 3) 40 mM TEA and 10^{-5} M pentobarbital; 4) 40 mM TEA; 5) Post-control (wash).*

K_{Ca} and K_{ATP}

A series of experiments was performed using pipette solution A, which contains calcium, but no ATP (Figure 3), thus allowing the expression of both K_{Ca} and K_{ATP}, as well as I_K. The control current reached a maximum of 20 nA at the highest depolarization step (Figure 3). Different concentrations of pentobarbital (10^{-9} - 10^{-3} M) were applied. The minimal effective concentration in this experiment was 10^{-8}, rather than 10^{-9} as shown in figure 1 and concentrations with significantly different effects from one another are shown. Pentobarbital at higher concentrations is not significantly more effective than 10^{-6}M. Pentobarbital at concentrations of 10^{-8}, 10^{-7} and 10^{-6} reduced I_k by 39, 65 and 74% respectively at +90 mV which is the highest depolarization step, suggesting that both I_{KCa} and I_{KATP} were suppressed by it.

To subtract the effect of K_{ATP} we used pipette solution B, which contains both calcium and ATP, thus leaving I_{KCa}. This reduced the maximum control current to ca. 16nA (Figure 4), indicating that K_{ATP} was suppressed by pentobarbital in a dose-dependent manner when compared with figure 3. In the absence of K_{ATP}, we again found that pentobarbital 10^{-8} M was the minimally effective concentration that decreases the remaining I_K (Figure 4). No significant differences in the current were found at concentrations between 10^{-8} and 10^{-4} pentobarbital, but 10^{-3} mM pentobarbital had a significantly stronger effect. The implication of these experimental manipulations is that both I_{KCa} and I_{KATP} are diminished by pentobarbital and I_{KCa} is clearly decreased in a concentration dependent manner.

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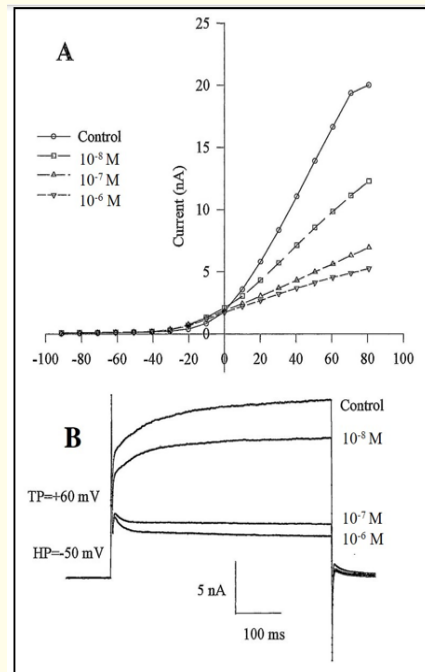
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Figure 3: Pentobarbital reduces gross K⁺ current in a dose dependent manner. Effect of pentobarbital on IK using pipette solution A which contains Ca^{2+} and zero ATP. A) IV curves generated under control conditions and in the presence of various concentrations of pentobarbital in a single cell (one of 6 repetitions). B) A whole cell patch recording from the same pedal I cluster neuron. The sequence of application of solutions: 1) Pre-control in normal bath solution; 2) 10^{-8}M pentobarbital (10 nM); 3) 10^{-7}M pentobarbital (100 nM); 4) 10^{-6}M pentobarbital. (1 μM). Original K⁺ current recording of the pedal I cluster neuron. HP = holding potential; TP = test potential + 60 mV from a holding potential of -50 mV.
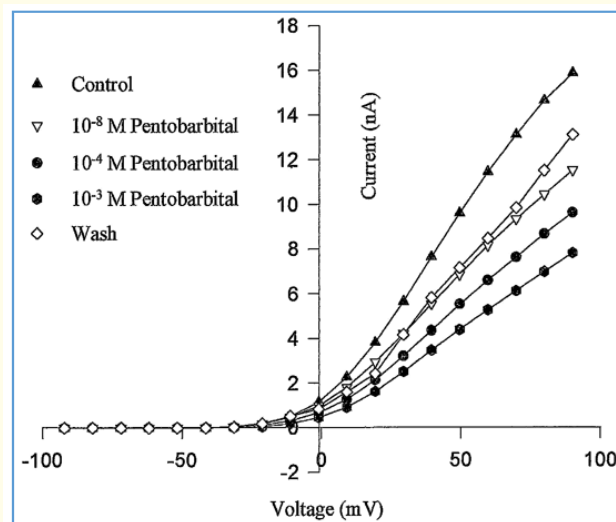

Figure 4: Effect of pentobarbital on I_{KCa} using a pipette solution containing Ca^{2+} and ATP (solution B). A) Effect of pentobarbital on I_K Sequence of application of solutions: 1) Pre-control in normal bath solution; 2) 10^{-8}M pentobarbital; 3) 10^{-7}M pentobarbital; 4) 10^{-6}M pentobarbital. A similar stimulation protocol to that shown in figure 1B was employed here.

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Is GABA associated with K$^+$ currents?

GABA is an important inhibitory transmitter in the vertebrate CNS. The GABA complex is usually associated with chloride channels and potassium channels may also be involved [29] and is known to modulate some *Lymnaea* neurons [30]. The effects of TEA and GABA on I_k, have been tested to find out which types of current are found in these cells. Figure 5 shows TEA at 40 mM can depress 40% of the I_k at +90 mV command potential but GABA at different concentrations (10^{-5} - 10^{-3} mM) has no significant effects.

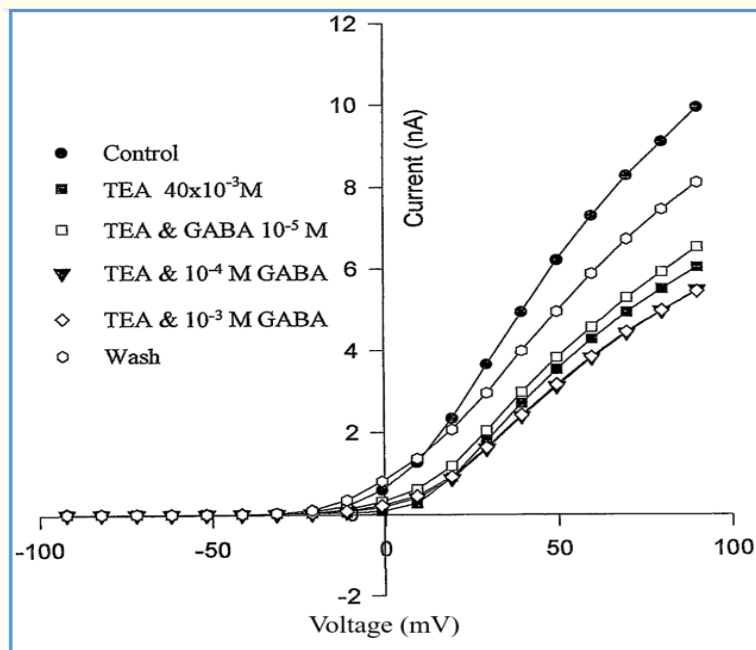*Figure 5:* *Effects of TEA and GABA on I_k, which was recorded with standard bath solution. Pipettes contained solution C (ATP, but zero Ca^{2+}). Sequence of application of solutions: 1) Pre-control in normal bath solution; 2) 40 mM TEA; 3) 40 mM TEA and 10^{-5} M GABA; 4) 40 mM TEA and 10^{-4} M GABA; 5) 40 mM TEA and 10^{-3} M GABA; 6) post control (wash). TEA at 40 mM concentration can partially depress I_k, but GABA at 10-5-10^{-3} mM concentration has no significant effects on it.*

Actions of pentobarbital on intracellular free calcium concentration [Ca^{2+}]$_i$ in RPeI cluster neurons

In a separate series of experiments the actions of pentobarbital on free [Ca^{2+}]$_i$ was measured using Fura-2 fluorescence both in normal saline and zero external calcium/EGTA saline. In normal saline application of 0.2 mM pentobarbital to pedal I cluster neurons (n = 5) (Figure 6) dramatically increased [Ca^{2+}]$_i$ from less than 50 nM to values of 1200 nM which declined over the next 6min to a stable level of about 600nM. Increasing the pentobarbital concentration had little further effect on [Ca^{2+}]$_i$, which remained elevated above baseline in all the experiments. [Ca^{2+}]$_i$ did not return to baseline values after washout, suggesting long term damage to the neurons.

At this stage we questioned whether these effects were specific to RPeI cluster cells and compared them with the with visceral M group motor neurons [13]. We demonstrated further clear differences (Figure 7a) in which an increase in [Ca^{2+}]$_i$ was only triggered when the pentobarbital concentration reached 5 x 10^{-4}M. A further comparison was made with the nearby pedal A cluster ciliomotor neurones [31], whose [Ca^{2+}]$_i$ was unaffected by pentobarbital at the same concentrations as those used on the RPeI neurons (Figure 7b). Thus, the actions of pentobarbital on [Ca^{2+}]$_i$ seem to be cell specific. Furthermore, the resting [Ca^{2+}]$_i$ levels were much less in the pedal A group cells than in either the RPeI or M group neurons.

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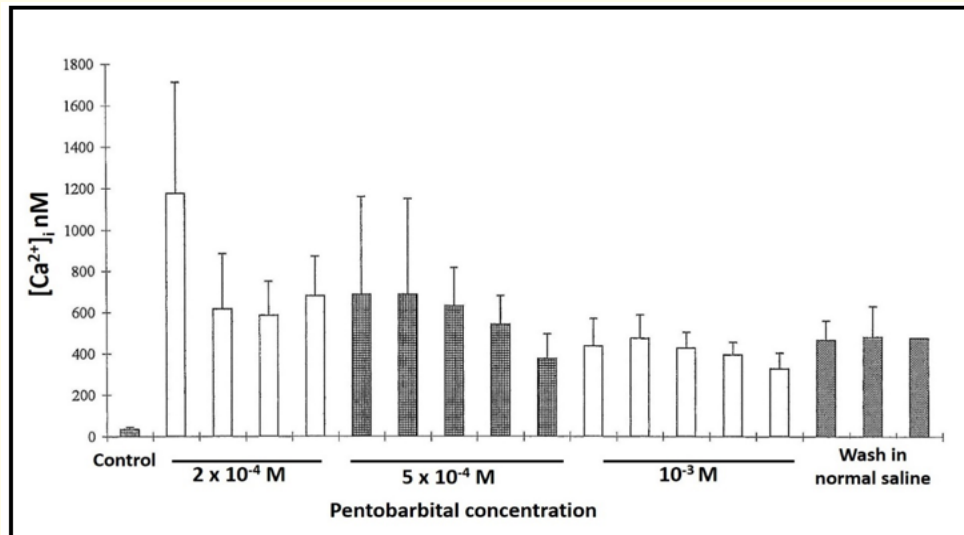
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Figure 6: *Pentobarbital increases $[Ca^{2+}]_i$ of isolated, cultured RPeI cluster cells (n = 5). Throughout this procedure measurements of $[Ca^{2+}]_i$ were made at 6 minutes intervals. The neurons were then washed in snail saline for 30 minutes and $[Ca^{2+}]_i$ was determined every 10 minutes. All values of $[Ca^{2+}]_i$ were significantly different from control (P = 0.0002).*
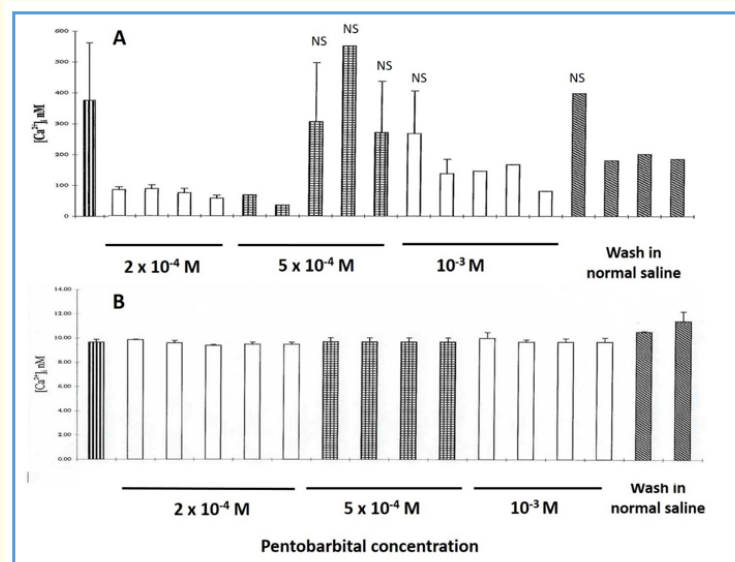

Figure 7: *Actions of pentobarbital on isolated visceral M group motor neurons and pedal A group ciliomotor neurons. A) Visceral M group neurons (n=4) are unaffected by low concentrations of pentobarbital, but a rise in $[Ca^{2+}]_i$ back to control levels eventually occurs in 5 x 10^{-4} pentobarbital which then gradually declines at the highest concentration, followed by rise back to control levels at the beginning of the wash period and then settles to about half the control value. Where no scatter is shown, the values recorded were the same for each cell at that time. B) Pentobarbital has no effect on $[Ca^{2+}]_i$ in neurons from pedal A group (n = 4) which are close to the RPeI neurons. No mean values were significantly different from control. Pentobarbital was delivered as in figure 7. NS = not significantly different from control.*

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Given that the increased calcium level was so high in RPeI cells we conducted further experiments to determine whether it could also release calcium from stores in zero calcium saline as we had previously observed with halothane [10,11]. A repeat of the experiments in the presence of zero calcium/5 mM EGTA saline (Figure 8a) yielded an initial statistically insignificant reduction in $[Ca^{2+}]_i$ in the presence of 0.2 mM pentobarbital followed by a gradual decline of $[Ca^{2+}]_i$ in the presence of the higher concentrations of pentobarbital (n = 8), but did not return to control values after washout in normal saline. A similar effect was also observed in visceral M group neurons. Figure 8b suggesting that this is not a cell specific effect, but may have more to do with calcium leakage from the cells.
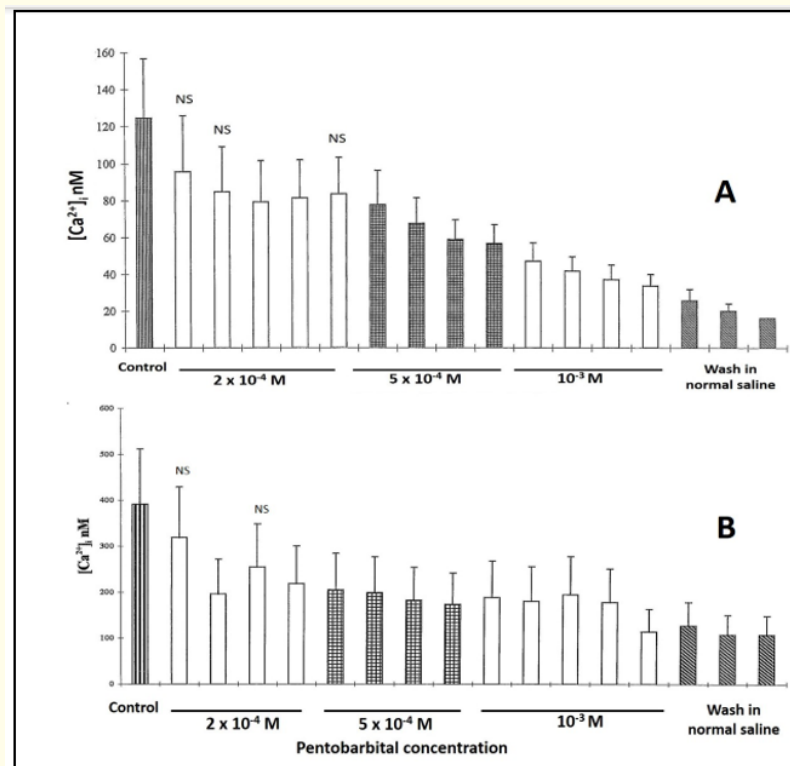

Figure 8: *In zero calcium/5 mM EGTA saline increasing concentrations of pentobarbital are incapable of increasing $[Ca^{2+}]_i$ in neurons in A) RPeI cluster neurons (n = 8); B) Visceral M group neurons (n = 12). Apart from bars labelled NS (not significant) all other values were significantly different from control, P ≤ 0.0022. Readings were taken as in figure 6.*

Discussion

Actions of pentobarbital on potassium currents

Our data indicate that pentobarbital suppresses I_K, I_{KCa} and the unidentified current in a concentration-dependent manner and I_{KATP} is also suppressed. The holding potential was set to -50 mV, in order to inactivate the transient A current [32], which we have previously described in these neurons [3]. Overall, pentobarbital has very similar actions to the volatile agents, halothane and isoflurane in suppressing gross potassium currents in a dose–dependent manner [3]. When the delayed rectifier current is isolated (Figure 1) it is clearly suppressed, but once it is blocked with TEA (Figure 2), a further unidentified current emerges which is partially suppressed by 10^{-5}M pentobarbital and remains to be characterised.

Furthermore, pentobarbital at similar concentrations to those used in this report is known to depress excitatory synaptic transmission in the central nervous system [33], impulse conduction in axons [34] and potassium currents in human SH0SY5Y cells [35]. Pentobarbital

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significantly decreases K^+ current at both lower doses (10^{-9} M) and higher doses (10^{-3} M) than those in clinical use (10^{-6} M) in Pedal I cluster neurons figure 1. These results are different from the previous reports of the effects of pentobarbital on the hippocampal slice of guinea pig [36] or the effect of volatile anesthetics on the novel potassium current, which they activate, in some neurons of the right parietal ganglion of *Lymnaea stagnalis* [37]. These differences may be because we have used cultured neurons without any synaptic influence upon them as compared with those from brain slice or whole brain. The more likely explanation is that different cell types respond differently to the anesthetics as we have demonstrated elsewhere [13]. Identified neurons in the intact brain of *Lymnaea* often have markedly different responses from each other to applied anesthetics, although all neurons eventually become quiescent at high enough anesthetic concentrations [11,13].

Although pentobarbital increases the coupling between the GABA receptor and the chloride channels [38] or activates the GABA receptor [39], the effects of pentobarbital are not via GABA receptors in this study, because we have shown (Figure 5) that GABA has no effects on the K^+ currents (P value 0.005) in isolated RPeI cells. Presumably pedal I cluster neurons have no GABA receptors, since the reversal potential of the outward current is -75 mV and is thus a K^+ current [3] and GABA has no significant effect on it. However, GABA-like (GABAli) immunoreactive neurons were described in *Lymnaea* in 2000 [40] and in related species [41]. GABAli neurons have now been described in many gastropod molluscs [42], including the strongly electrically coupled neurons VD1 and RPD2 [23,43] of *Lymnaea* where GABA is colocalized with multiple neuropeptides [44]. Finally, $GABA_A$ and AMPA-like receptors have been found to modulate the activity of RPeD1 [30], one of the three interneurons comprising the respiratory central pattern generator of *Lymnaea* [45,46].

Actions of pentobarbital on $[Ca^{2+}]_i$

From the data presented above (Figure 6) we conclude that pentobarbital may enhance Ca^{2+} release from intracellular stores, since it has already been shown that it also inhibits Ca^{2+} currents in neurons [47-49]. However, the effects on $[Ca^{2+}]_i$ are cell specific as demonstrated in figure 7 where two different cells types were shown to respond quite differently to the application of pentobarbital. Furthermore, different cell types have very different resting $[Ca^{2+}]_i$ levels. The dramatic rise in $[Ca^{2+}]_i$ in RPeI neurons may be symptomatic of cell damage as it could signal the onset of apoptosis as has been demonstrated under some circumstances with the inhalational anesthetic isoflurane [50], particularly as the $[Ca^{2+}]_i$ does not return to pre-control levels in RPeI cells after washout of pentobarbital. However, in the absence of extracellular calcium there is a gradual decline in intracellular free calcium as the external concentration of pentobarbital increases (Figure 7), which might best be explained by leakage of free calcium across the cell membrane, following release from stores by pentobarbital. NALCN channels (sodium leak channels, non-selective) are known to occur abundantly in *Lymnaea*, particularly in the skeletal muscle, cardiac muscle, glandular tissue and in the brain [51] and are known to be permeable to sodium, potassium and calcium ions. NALCN channels have also been identified in the pacemaker neuron, RPeD1 of *Lymnaea* [52]) and are likely to occur in other neurons. We therefore hypothesise that the reduction in free cytosolic $[Ca^{2+}]_i$ that we have observed in zero calcium/pentobarbital saline is most likely due to pentobarbital modulation of NALCN channels causing free calcium released from stores to leak out of the cells. Although there may have been long-lasting effects on the RPeI neurons, at concentrations up to 10^{-3} M the actions of pentobarbital were reversible on the delayed rectifier current (Figure 1), but not fully reversible on I_{KCa} (Figure 4), suggesting that the membrane was functional for these currents.

Conclusion

I_K, I_{ATP} and K_{Ca} are present in pedal I cluster neurons and all are suppressed by clinical concentrations of pentobarbital. An unidentified K^+ current is also suppressed by this treatment. In addition, pentobarbital increases free intracellular calcium concentration, but this is a cell specific effect.

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