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Mobilization of Intracellular Calcium by Ryanodine in Cultured, Identified Molluscan Neurons is Cell Specific

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Abstract

- Previously we showed that the calcium mobilizing agent, ryanodine, altered action potential shape in some identified neurons of the mollusc *Lymnaea stagnalis*.
- Here we investigate the actions of ryanodine (1 µM and 5 µM) on intracellular calcium concentration $[Ca^{2+}]_i$ in the same identified neurons in single cell culture.
- The free $[Ca^{2+}]_i$ levels in these neurons were determined using the cell permeable Ca^{2+} indicator Fura-2AM, both in the presence and absence of extracellular calcium (0-Ca^{2+}/EGTA).
- In the presence of extracellular calcium, both concentrations of ryanodine produced a substantial increase in $[Ca^{2+}]_i$ in three neuron types (VV1or 2, RPD1 and RPD2), whereas, in the fourth neuron type (RPeD1) there was a decrease below resting level.
- The free $[Ca^{2+}]_i$ remained elevated even after washing with normal saline, except in one case (VV1or 2) where it was found to be reversible. However, there was a general decline in the level of $[Ca^{2+}]_i$ after addition of ryanodine in the absence of Ca^{2+} in the external medium.
- Therefore, extracellular Ca^{2+} is a prerequisite for any appreciable rise in $[Ca^{2+}]_i$ due to ryanodine.

Keywords: *Ryanodine; Intracellular Calcium Concentration; Lymnaea Neurons; Neuron Specificity*

Introduction

Many cellular functions depend on changes in the free cytosolic Ca^{2+} ion concentration $[Ca^{2+}]_i$. Transient increases in $[Ca^{2+}]_i$ can be initiated either by release from intracellular storage sites associated with the endoplasmic reticulum (ER) [1] and/or due to an increase in the Ca^{2+} permeability of the plasma membrane due to changes in membrane potential or the actions of neurotransmitters [2] and may operate by a calcium-induced calcium release (CICR) mechanism [3,4].

The neutral plant alkaloid ryanodine has a specific action on the sarcoplasmic reticulum (SR) Ca^{2+}-release channels of skeletal and cardiac muscle, the ryanodine receptors RYR1 and RYR2 respectively [5]. Its effects have also been demonstrated via RYR3 receptors of the endoplasmic reticulum of neurons in both vertebrates [1] and invertebrates, including *Lymnaea* [6,7]. The activity of ryanodine receptors (RYRs) is strongly enhanced by adenine nucleotides, caffeine, calcium via CICR and inhibited by ruthenium red or procaine.

In a previous study we showed that low concentrations of caffeine raised $[Ca^{2+}]_i$ and this was dependent on the presence of extracellular calcium [8] in four identified, cultured neurons from the mollusc *Lymnaea stagnalis* (L.) as follows: the giant dopamine containing neuron right pedal dorsal 1 (RPeD1), the identical cells visceral ventral 1 and 2 (VV1/2), right parietal dorsal neurons 1 and 2 (RPD1 and RPD2) whose locations are shown in figure 1. The properties of the neurons are reviewed elsewhere [8,9] as are their responses to applied volatile and intravenous anesthetics [10,11]. RPeD1, VV1 and VV2 are interneurons (which are electrophysiologically indistinguishable from one another) and RPD1 is a putative interneuron, while RPD2 is a motor neuron (see 11 for details) strongly electrically connected to VD1 [12-14]. All these neurons have type 2 action potentials with a marked calcium pseudoplateau on the falling phase [12]. Here, we examine and compare the actions of ryanodine on the level of $[Ca^{2+}]_i$ in the same cultured neuron types from *Lymnaea* as in our previous study, both in the presence and absence of extracellular calcium (0-Ca^{2+}/EGTA).

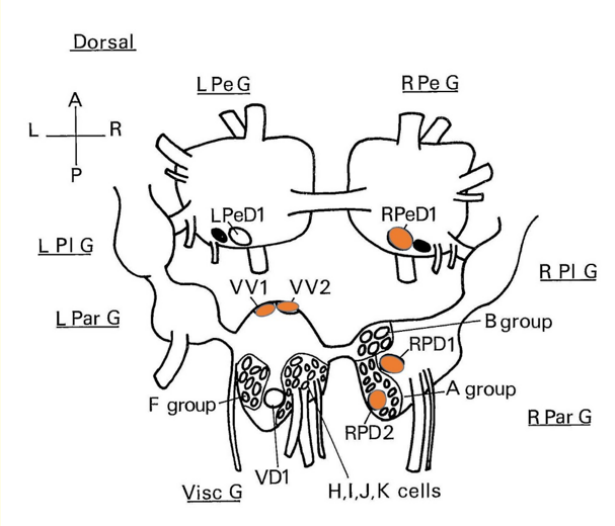

Figure 1: *Dorsal view of the lower nerve ring of the Lymnaea brain showing the different ganglia and the four types of giant identified neurons studied here, identified in orange: RPeD1, RPD1, RPD2 & VV1/2 (for nomenclature see Benjamin and Winlow, 1981). It should be noted that VV1 and VV2 are electrophysiologically indistinguishable from one another. Abbreviations: R Pe G: Right Pedal Ganglion; R Par G: Right Parietal Ganglion; Visc G: Visceral Ganglion; R and L Pl G: Right and Left Pleural Ganglia.*

Materials and Methods

Specimens of *Lymnaea* were supplied by Blades Biological, Kent, kept in a controlled temperature room at 14 - 16C in aerated tap water and fed on lettuce. In the present study, the four types of identified *Lymnaea* neurons were isolated and cultured according to previous methods [8,15].

Individual neurons were loaded with the acetoxymethyl form of the fluorescent calcium indicator Fura 2/AM (Sigma) (4 μM) and intracellular calcium concentration was measured as described by Ahmed., *et al* [8]. Control levels of cytosolic free calcium $[Ca^{2+}]_i$ were

recorded for individual neurons after 2 - 3 hours of incubation in Fura2/AM in standard snail saline [12]. Ratioed images of the emission intensity at 510 nm at the two excitation wavelengths (340 nm/380 nm), from which the background image brightness at each wavelength had been subtracted, were used to calculate $[Ca^{2+}]_i$ according to the formula of Grynkiewicz., *et al* [16]. Experiments were carried out either in standard snail saline or in calcium free saline (0 Ca^{2+}/5 mM EGTA from Sigma). Ryanodine (98% pure, Calbiochem, USA) was dissolved in deionized water and, because it is photosensitive, was protected from light and refrigerated. 100 µM stock solutions were prepared and renewed monthly. These were diluted to give working concentrations of 1 µM and 5 µM in standard snail saline. 1 µM ryanodine solution was first applied to each cell type and measurements of $[Ca^{2+}]_i$ were taken at intervals of 6, 18 and 30 minutes after which 5 µM ryanodine was applied at the same intervals up to 30 min before washout in standard snail saline during which further readings of $[Ca^{2+}]_i$ were taken after 10 minutes and 30 minutes.

Data analysis

The raw data of $[Ca^{2+}]_i$ measurements were initially expressed as mean (± S.E.M.). Analysed data were then normalised and expressed as a mean percentage (± S.E.M.) change in $[Ca^{2+}]_i$ and plotted against time in a series of graphs using "Fig. P" software (Fig. P Software corporation, version 6.0a 91/11). The use of percentage change in $[Ca^{2+}]_i$ allowed us to compare resting $[Ca^{2+}]_i$ levels, despite variations in $[Ca^{2+}]_i$ within the same cell type from one animal to the next.

Results

Actions of ryanodine on specific neuron types in normal saline

There was substantial variability in the baseline resting values of $[Ca^{2+}]_i$ both within and between neurons (Table 1) as well as the temporal effects of ryanodine on each neuron (Figure 2). The progressive changes in $[Ca^{2+}]_i$ taking place after application of ryanodine in RPD1 can be seen in the series ratioflurometric images for an isolated RPD1, using Fura 2AM to measure the changes in $[Ca^{2+}]_i$, is shown in figure 2 at right. Percentage changes in $[Ca^{2+}]_i$ for each neuronal type are presented in figure 3.

Cell Type	Standard Saline	0-Ca^{2+}/EGTA Saline
VV1/2	98 (39 - 156)	79 (48 - 118)
RPD1	154 (149 - 160)	70 (32 - 101)
RPeD1	135(27 - 224)	50 (30 - 70)
RPD2	107 (15 - 200)	63 (43 - 82)

Table 1: *Baseline $[Ca^{2+}]_i$ in the four types of neuron in standard snail saline and snail saline with zero calcium and EGTA. Values are mean and range (nM).*

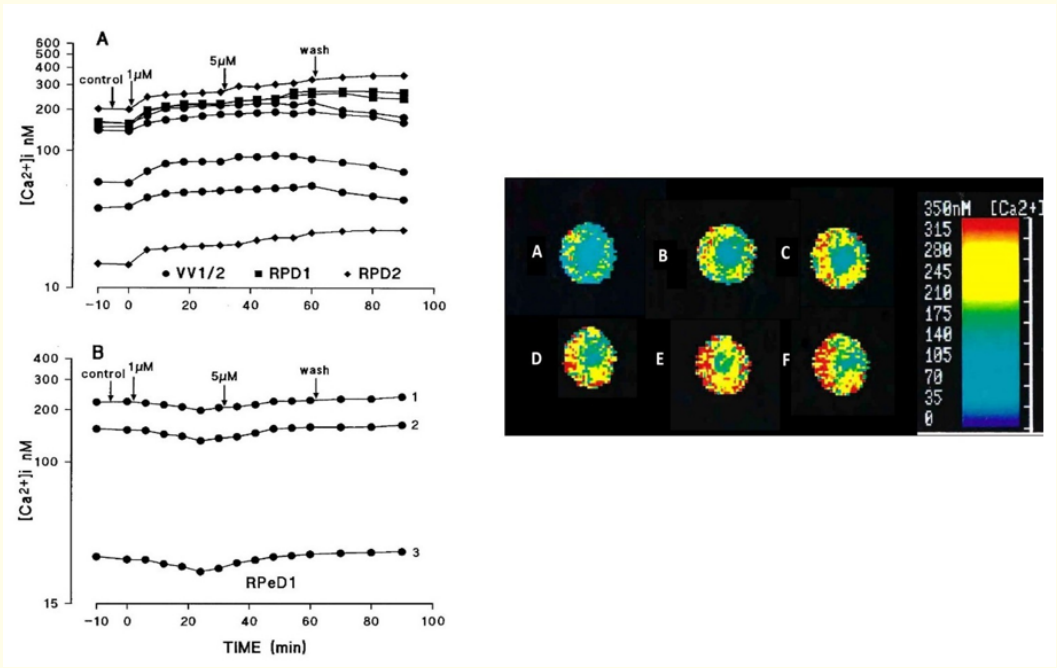

Figure 2: *Comparative effects of ryanodine on different identified neurons. This figure shows the measured values of $[Ca^{2+}]_i$ against time for eleven neurons and also illustrates the variability of $[Ca^{2+}]_i$ both within and between neuron types. A) in VV1/2, RPD1 and RPD2 $[Ca^{2+}]_i$ was substantially increased except for B) RPeD1where there was a decrease in 1µM ryanodine and a weak increase in 5 µM ryanodine. Colour Images at right - Effects of ryanodine on $[Ca^{2+}]_i$ in RPD1. This series of ratiofluorimetric images (A-F) shows the time course of action of low and high doses of ryanodine (1 µM and 5 µM) on $[Ca^{2+}]_i$ in RPD1 neuron. The calcium concentration scale bar is at right. A) Control level of $[Ca^{2+}]_i$ - 155 nM - before addition of ryanodine; B) after 6 minutes in 1 µM ryanodine (Ca^{2+} 198 nM); C) 30 min after 1 µM ryanodine (Ca^{2+} 221 nM); D) 6 min after addition of 5 µM ryanodine (Ca^{2+} 230 nm); E) 30 min after addition of 5 µM ryanodine (Ca^{2+} 266 nm); F) 30 min after washout with normal saline (Ca^{2+} 248 nm). There was little reduction after 30 min of washout when the $[Ca^{2+}]_i$ remained 60% above resting level. Note also the progressive spread of the free intracellular Ca^{2+} ions from a localized region near the plasma membrane towards the centre of the cell.*

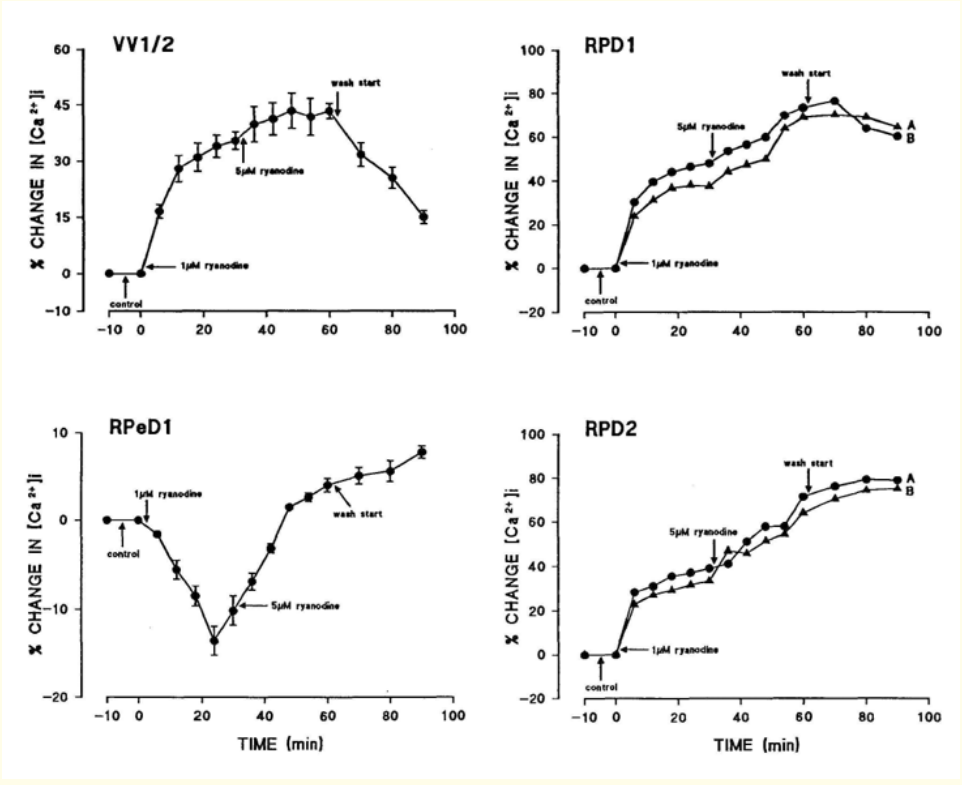

Figure 3: *Effects of 1 μM and 5μM ryanodine on percentage change in $[Ca^{2+}]_i$ in different neuronal types in the presence of extracellular calcium. Data points are expressed as mean ± S.E.M. in VV1/2 (n=4) and RPeD1 (N=3) and as individual values for RPD1 and RPD2 (n=2 for each neuronal type). $[Ca^{2+}]_i$ was substantially increased in all neuron types except RPeD1, which decreased. $[Ca^{2+}]_i$ remained elevated even after 30 min washout in all neurons except VVI/2 where it was partially reversible.*

As shown in figures 2 and 3, neurons VV1/2, RPD1 and RPD2 demonstrated a marked increase in $[Ca^{2+}]_i$ within 6 minutes of addition of 1 μM ryanodine. The increase continued at a slower rate until the ryanodine was removed by washing. In some of these neurons there appeared to be an increase in the rate of change of $[Ca^{2+}]_i$ in response to adding 5 μM ryanodine, although any conclusions on the nature of the dose-response relationship are not possible from these experiments. The effect of ryanodine was clearly reversed during the wash period of 30 minutes only in VV1/2 neurons and even this reversal was incomplete by the end of the experiment.

The situation in RPeD1was markedly different from the other cells in that 1 μM ryanodine caused a biphasic response in $[Ca^{2+}]_i$, with an initial decline followed by rise. The increase preceded the application of 5 μM ryanodine, which did not appear to have any additional effect.

Actions ryanodine on $[Ca^{2+}]_i$ in specific neuron types in the absence of extracellular calcium

In general application of both low and high concentrations of ryanodine in the absence of extracellular calcium produced a gradual decline in the level of $[Ca^{2+}]_i$ in the studied neurons (Figures 4 and 5), except that in VV1/2 and RPD1 there was a transient increase of 6.5% and 5.6% after 6 minutes of addition of the low concentration of ryanodine respectively and this remained elevated above control even at 18 minutes. In VV1/2, $[Ca^{2+}]_i$ was reduced from a control level of 79 ± 21 nM to a minimum level of 70 ± 18 nM (a reduction of 11.0%) after 30 min of addition of the high dose of ryanodine. In RPD1, there was a maximum reduction in $[Ca^{2+}]_i$ of 9 nM from the resting value (a reduction of 12.8%) after the application of ryanodine. This was then followed by a slight rise in $[Ca^{2+}]_i$ after washing in both neurons. The greatest reduction in $[Ca^{2+}]_i$ following the addition of ryanodine was recorded in RPeD1 and RPD2 with a value of 27.2% and 21.6% respectively and this continued in RPeD1 and RPD2 even after washout.

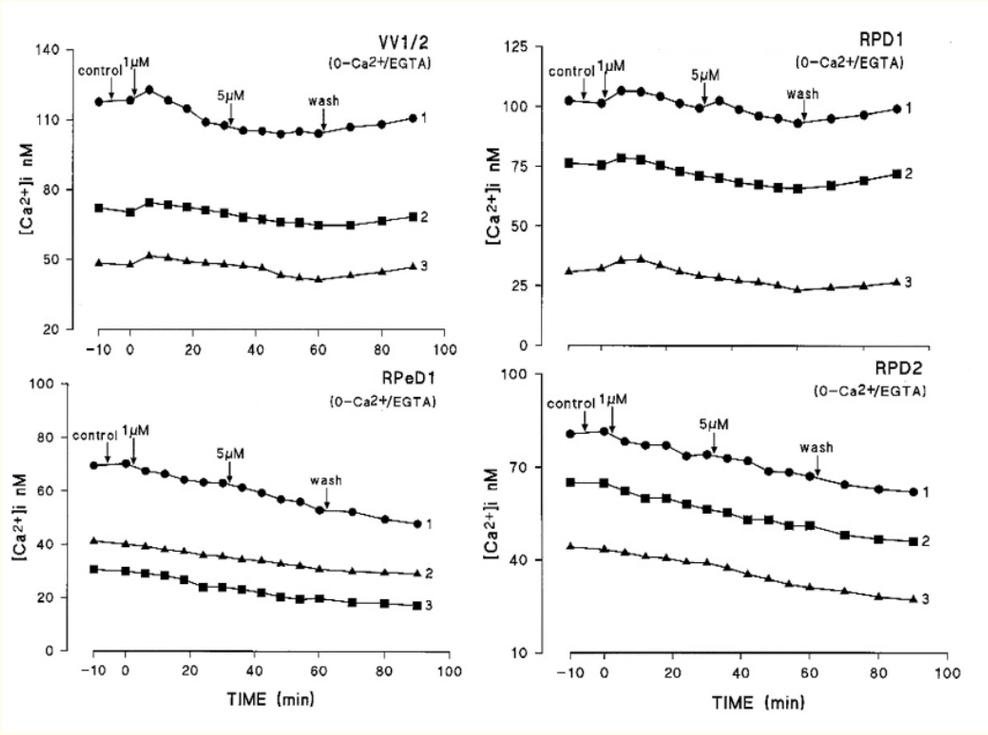

Figure 4: *Effect of ryanodine on $[Ca^{2+}]_i$ (nM) in different neurons in the absence of extracellular calcium (0-Ca^{2+}/EGTA). Individual traces for all neurons are shown to demonstrate both the variability of $[Ca^{2+}]_i$ within and between neuron types and to demonstrate the similarity of responses within neuron types.*

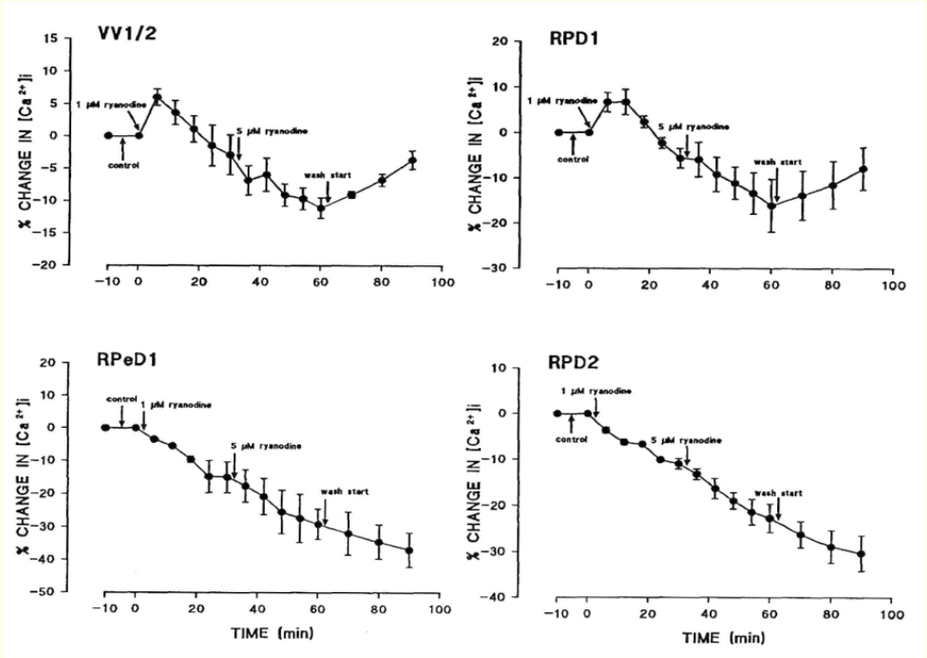

Figure 5: *Effect of 1 μM and 5 μM ryanodine on percentage change in $[Ca^{2+}]_i$ in different neurons in the absence of extracellular calcium (0-Ca^{2+}/EGTA). Percentage change was determined using the data from figure 4. Data points are expressed as mean ± S.E.M (n = 3 for each neuron type). Application of both concentrations of ryanodine produced a gradual decline in $[Ca^{2+}]_i$ to below resting level in all neurone types, except for an initial and weak increase in VV1/2 and RPD1 after the addition of the low concentration. $[Ca^{2+}]_i$ continued to decrease after 30 min washout in RPeD1 and RPD2, whereas an elevation was observed in VV1/2 and RPD1, but none returned to control values.*

Discussion

Our data indicate that ryanodine can raise $[Ca^{2+}]_i$ in identified neurons from *Lymnaea stagnalis*, but the temporal and quantitative dynamics of these responses are cell specific and vary between different neuronal types. For example, RPeD1 responded negatively to 1 μM ryanodine and then weakly to the higher concentration of 5 μM in comparison to all three of the other neurons (Figure 2 and 3). None of the neurons had clear concentration dependent responses to ryanodine and further experiments are required to determine whether this is the case. In all cases the responses to ryanodine were relatively slow (Figure 3). In zero extracellular calcium saline all neurons showed a gradual reduction in $[Ca^{2+}]_i$ over 30 minutes (Figure 5) and only VV1/2 and RPD2 started to recover after washout. Previously we demonstrated that low concentrations of caffeine (100 and 300 μM) induced similar rises in $[Ca^{2+}]_i$ in the same four neuron types studied here [8]. Similarly, low concentrations of caffeine did not produce any appreciable rise in $[Ca^{2+}]_i$ in the absence of external calcium. However, we demonstrated the availability of Ca^{2+} from internal stores under these circumstances because clinical concentrations of halothane were still able to raise $[Ca^{2+}]_i$ substantially in the absence of extracellular Ca^{2+} [8]. Thus, the calcium mobilising effects of both ryanodine and caffeine appear to be dependent on extracellular calcium to induce CICR, although the fact that ryanodine locks the channel open in a subconductance state [17] may well complicate this proposition.

The data we have presented here are consistent with our earlier electrophysiological findings on the same four neuronal types *in situ*, rather than in culture. The same concentrations of ryanodine (1 and 5 μM) produced significant spike-broadening, which is a calcium dependent phenomenon, in three neurones, particularly VV1/2, but had no effect on the action potential of RPeD1 [18,19]. On this basis we postulated the presence of ryanodine receptors in molluscan neurons, which have now been demonstrated elsewhere [6,7]. However, the mechanism of spike broadening was unclear since a rise in free $[Ca^{2+}]_i$ would be expected to reduce the driving force on Ca^{2+} ions into the cell and therefore to diminish spike broadening, which is due to the calcium-dependent pseudoplateau during repolarization in all four cell types described here [20,21]. However, the explanation my lie in the presence of calcium dependent BK channels [22,23] in molluscs [24-26], whose inactivation contributes to spike broadening in vertebrates [27]. This is believed to be due to rising $[Ca^{2+}]_i$ from external sources near the action potential peak and during the falling phase [28] which in many neurons is coupled to activation of the calcium-activated, large conductance, BK channels [29]. According to Bean [29], blocking calcium entry will inhibit the net outward potassium flux, resulting in action potential broadening [30]. This would be particularly true where calcium induced calcium release (CICR) activates BK channels which are in close apposition to RYRs located on subsurface cisterns of the endoplasmic reticulum (ER) as in the mouse dorsal cochlear nucleus [31], which may also be the situation in RPD1 (See figure 2 at right), where the progressive spread of the free intracellular Ca^{2+} ions starts close to the plasma membrane.

Given the different responses of individual neurons, it is conceivable that these effects may be due to differential actions of two types of ryanodine receptor (RyR) on the Ca^{2+} release channels on the ER, which can either increase or decrease the Ca^{2+} conductance of the ER, although only a single type of RyR has so far been described in *Lymnaea* [6,7]. If this were the case, the overall effects would then be due to the proportion of positive and negative RyRs in each neuron. However this hypothesis may be unnecessary, since positive and negative feedback loops are usually involved in cellular signalling pathways [32] and it is more likely that calsequestrin, which is known to inhibit RyR1 receptors in skeletal muscle [33] and calmodulin [34] have a major roles to play in determining cell specific responses to ryanodine [1]. If there is only a single RyR in the *Lymnaea* neurons studied here, the overall responses to ryanodine will also be influenced by local factors, such as the size of ER Ca^{2+} store, proximity of the ER to the plasma membrane as mentioned above, and the concentration of ligands that can affect these Ca^{2+}-release channels (Ca^{2+}, Mg^{2+}, ATP, cAMP).

Conclusion

Application of ryanodine produced a substantial elevation in $[Ca^{2+}]_i$ in three identified *Lymnaea* neurons, but $[Ca^{2+}]_i$ was reduced to well below resting level in one type of neuron (RPeD1). If ryanodine was applied in zero calcium/EGTA saline there was a gradual decline in

$[Ca^{2+}]_i$ in all four neuron types, indicating that calcium mobilization by ryanodine is dependent on the presence of extracellular calcium to trigger CICR. The cell specific actions of ryanodine suggest that the actions of ryanodine, via RyRs, are differentially modulated in a neuron specific manner.

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