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NO and H_2O_2 Modulate Ca^{2+}, Ca^{2+} Dependent K^+ Currents and Intracellular Calcium Concentration of Cultured, Identified *Lymnaea* Neurons

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Abstract

The actions of NO and H_2O_2 on gross K^+ and Ca^{2+} currents were investigated on cultured identified neurons of the mollusc *Lymnaea stagnalis* (L).

Pedal I Cluster (RPeI) neurons were isolated and cultured for 1 - 5 days and K^+ and Ca^{2+} currents were measured in the whole cell patch clamp configuration.

Both H_2O_2 (3 μM to 300 μM) and NO (0.1 mM to 1 mM) substantially decreased L-type Ca^{2+} and K^+ currents in a concentration-dependent manner. These effects were partially reversible.

Both free radicals also increased intracellular calcium concentration ($[Ca^{2+}]_i$) in these neurons, clarifying an earlier study.

Keywords: *Nitric Oxide; Hydrogen Peroxide; Ca^{2+} Dependent K^+ Current; Ca^{2+} Current; $[Ca^{2+}]_i$, Identified Neurons; Cell Culture; Lymnaea stagnalis*

Introduction

Nitric oxide synthase (NOS) was first demonstrated in the CNS of the freshwater snail *Lymnaea stagnalis* (L.) by Moroz., *et al.* [1] in 1994 and various NOS's, of which NADPH diaphorase is an example [2,3], are found throughout the animal kingdom [4], including the Placozoa [5], the simplest known free-living animals. In addition to generating the gas transmitter nitric oxide (NO) [6], NOS also generates the superoxide free radical and H_20_2 [7], which is a free radical generator. Thus, free radicals of oxygen [6,8,9] are products of normal cellular metabolism [10] produced by NO synthase and mitochondrial respiration is believed to be the main source of H_2O_2 generation for dynamic neuronal signalling [11]. Free radicals are involved in physiological and pathophysiological phenomena [12,13] and nitric oxide is known as signalling molecule in nervous systems [14,15]. It may act as a neurotransmitter, although the exact mechanism of action may be contentious, and a neuromodulator [16-19]. Currently, H_2O_2 may be best thought of as a neuromodulator [20-23].

NADPH diaphorase/NO synthase is found in both peripheral and central neurons of *Lymnaea* [1,24] and NO has been shown to activate buccal motor patterns, specifically the central feeding and respiratory programmes in *Lymnaea* [25], later substantiated by Sidorov [26]. H_2O_2 and NO cause differential activation of neurons of the feeding and respiratory networks of *Lymnaea* [27] indicating that the actions of NO and H_2O_2 vary according to cell type as was also demonstrated by endogenously generated NO in the feeding system of the related gastropod *Helisoma trivolvis* [28]. In 1995, Moghadam., *et al.* [28] showed that H_20_2 differentially modulated the activity patterns of a number of different *Lymnaea* neurons and showed various effects of the NO donor sodium nitroprusside (SNP) and H_20_2 on intracellular calcium concentration $[Ca^{2+}]_i$ of several different neuron types. However, the actions of H_20_2 on the right pedal I (RPeI) cluster neurons were not studied at that time and SNP was only found to modulate $[Ca^{2+}]_i$ in 5 out of 19 cells tested.

If we are to fully understand the role of free radical neuromodulators on behaviour in the intact preparation, we must first understand their modes of activity on individual neurons. The RPeI neurons form a distinct lobe of over 60 homogeneous cells, have common excitatory inputs with putative ciliomotoneurons of the foot and project to the periphery via the labial nerves, penis nerve and pedal nerves [29,30]. We have previously demonstrated the presence of an L-type calcium current [31] and several potassium currents [32] in the RPeI cluster neurons and have shown them to be diminished by both volatile and systemic anaesthetics [33-36] which at the same time raise their $[Ca^{2+}]_i$ [35,37].

In this paper we describe for the first time the effects on NO and H_2O_2 on the L-type Ca^{2+} and calcium-dependent potassium currents (IK_{Ca}) currents of isolated cultured RPeI cluster neurons using the whole cell clamp technique. We also clarify their effects on $[Ca^{2+}]_i$ in this cell type. The advantage of isolated RPeI cluster neurons in culture as a model system is that the only calcium channels they express are HVA L-type calcium channels [31] and their calcium-dependent potassium currents can easily be separated from other potassium currents using appropriate recording pipettes [32].

Materials and Methods

Specimens of *Lymnaea stagnalis* (L) were supplied by Blades Biological, Kent, UK, kept in a controlled temperature room at 14 - 16°C in aerated tap water and fed on lettuce. Snails were dissected and the RPeI Cluster neurons, 20 - 30 µm in diameter, were isolated and cultured for 1 - 5 days according to the methods of Walcourt-Ambakederemo and Winlow [38,39]. Neurons were viewed with an Olympus inverted microscope, after being cultured on poly-L-lysine-coated Falcon dishes 3001 for 1 - 5 days.

Ca^{2+} and K_{Ca} currents were measured in the whole cell patch clamp configuration as previously described [31,32]. Pipettes had a resistance of 2 - 5 MΩ. The Pipette solution for Ca^{2+} current recording contained: CsCl, 40; EGTA, 5; $MgCl_2$, 5; HEPES, 10; ATP, 3 mM and that for I_{KCa} recording contained: KCl 50, NaCl 1.6, $MgCl_2$ 1.5 HEPES10, Mannitol 10, EGTA 5, $CaCl_2$ 4.7, ATP 3 mM. Pedal I cluster neurons were clamped using 500 ms pulses at -50 mV and stepped from -90 mV to +90 mV at 10 mV intervals. One minute was allowed between test pulses. Bath and pipette solutions for recording K^+ currents were prepared as previously described [32]. Both the bath and pipette solutions for Ca^{2+} current recordings were prepared according to the method of Yar and Winlow [31]. Note that in the electrophysiological figures presented here error bars are not shown for the sake of clarity.

Intracellular calcium imaging with Fura-2-Individual neurons were loaded with the cell-permeable ratiometric Ca^{2+} indicator Fura-2 acetoxymethyl ester (Fura-2 AM) as previously described [40-42]. Control levels of $[Ca^{2+}]_i$ were recorded for individual neurons after 2 - 3 hours of incubation in Fura-2 AM in standard snail saline. Digital-imaging microscopy of Fura-2 fluorescence from a limited number of cultured identified molluscan neurones was carried out using a "MAGICAL" video-imaging system [43] and "TARDIS" (version 7.35) as previously described [40-42]. The associated software allowed us to record the subcellular alterations in Ca^{2+} concentration $[Ca^{2+}]_i$ that occurs immediately and after a period of exposure to free radicals.

Solutions

HEPES-buffered saline [44] at 20°C, buffered with NaOH with pH was adjusted to 7.9.

Zero Ca^{2+} EGTA saline: NaCl, 12; KCl, 1; $NaH_2PO_4.3H_2O$, 0.05; $MgCl_2.6H_2O$, 1.7; EGTA, 5; NaOH, 35.5; HEPES, 50 (in mM). The pH was adjusted to 7.9 and the $CaCl_2$ of the normal saline was replaced with 5 mM EGTA; Mg and Cd saline this solution was prepared as previously described (Benjamin and Winlow, 1981) and contained (in mM); Na^+ 59.4; K^+ 2.0; Mg^{2+} 3.79; Cd^{2+} 0.5; Cl^- 36.58; HPO_4^- 0.1; Glucose 0.3 and buffered by HEPES 50 mM. Its pH was adjusted to 7.9 with NaOH.

Hydrogen peroxide saline - Hydrogen peroxide (Sigma) 3% was diluted to appropriate concentrations for application to the preparation.

Nitric oxide saline - Nitric oxide was generated using two procedures and then dissolved in the normal saline:

- Pure NO was collected from the Department of anaesthesia St. James University Hospital, and then diluted with normal saline to make 0.3, 3 30, 300 µM NO.
- Sodium nitroprusside saline - SNP is a known NO donor. 30 mg of SNP from powdered stock (SNP; BDH) were dissolved in 100 ml saline to yield 1 mM SNP which was protected from light until used. It was then diluted appropriately prior to application. Measurements performed by Moroz., *et al.* [45] with NOsensitive microelectrodes suggest that the estimated concentrations of free radicals and NO generated from SNP under our experimental conditions could be about 100 times less than the concentration of SNP used. For example, 10^3 M SNP could generate up to 10^5 M NO. Light induces the release of NO from SNP. The amount of the NO generated strongly depends on the intensity of illumination.

Statistics Data are expressed as mean ± S.E.M.

Results

In the experiments described below, both free radical generators of H_2O_2 (n = 5) (Figure 1 and 3) and NO (n = 6) (Figure 2 and 4) decreased L-type Ca^{2+} currents and I_{KCa} respectively in RPeI cluster neurons in a concentration-dependent manner.

Actions of H_2O_2 and NO on Ca^{2+} currents of RPeI neurons

Calcium currents in RPeI cluster neurons of *Lymnaea* have been previously characterized [31] and are dihydropyridine sensitive, high voltage activated channels similar to L-type calcium currents described in vertebrates and to those characterized by Dreijer., *et al.* [46,47] in the neuroendocrine caudo-dorsal cells of *Lymnaea*.

H_2O_2 produces partial blockade of the L type Ca^{2+} currents as shown in figure 1. No significant difference was found between control and the effect of 0.3 µM H_2O_2, except at command potentials between 0 mV to +60 mV (P value of ≤ 0.005). At the peak potential (+30 mV), 0.3 µM H_2O_2 decreased Ca^{2+} current, by 23%. Other concentrations of H_2O_2 blocked Ca^{2+} currents significantly and at the peak potential Ca^{2+} currents were blocked by 57%, 62% and 63% by concentrations of 3, 30 and 300 µM of H_2O_2 respectively. No significant differences in the reduction of Ca^{2+} current were found between application of 3, 30 and 300 µM of H_2O_2. Low concentrations of H_2O_2 (0.3 µM) partially blocked voltage activated Ca^{2+} currents at command potentials above 10 mV, whereas higher concentrations (3, 30 and 300 µM) caused significant-blockade of Ca^{2+} currents at all command potentials. The effects of H_2O_2 were partially reversible after continuous washout for 10 minutes. The effect of NO application on Ca^{2+} current on pedal I cluster neurons was also partially reversible (Figure 2). Concentrations of 0.1 mM NO significantly decrease Ca^{2+} currents in a concentration-dependent manner and these effects are partially reversible with continuous washout. At a command potential, of -20 mV, NO decreased Ca^{2+} current by 24% at low concentrations of NO, whereas with a higher concentration (0.25 mM) 80% of the current was blocked. At the peak potential (0 mV), Ca^{2+} currents were reduced by 54% and 95% respectively by low (0.1 mM) and high concentrations (0.25 mM) of NO. Significant differences have been found between the extent of blockade between low (0.1 mM) and high concentrations (0.25, 0.5 and 1 mM) of NO, but no significant differences were found between the higher concentrations (P value of ≤ 0.005). With continuous wash out Ca^{2+} current increased but did not return to the normal level (P value of ≤ 0.005) within 10 min.

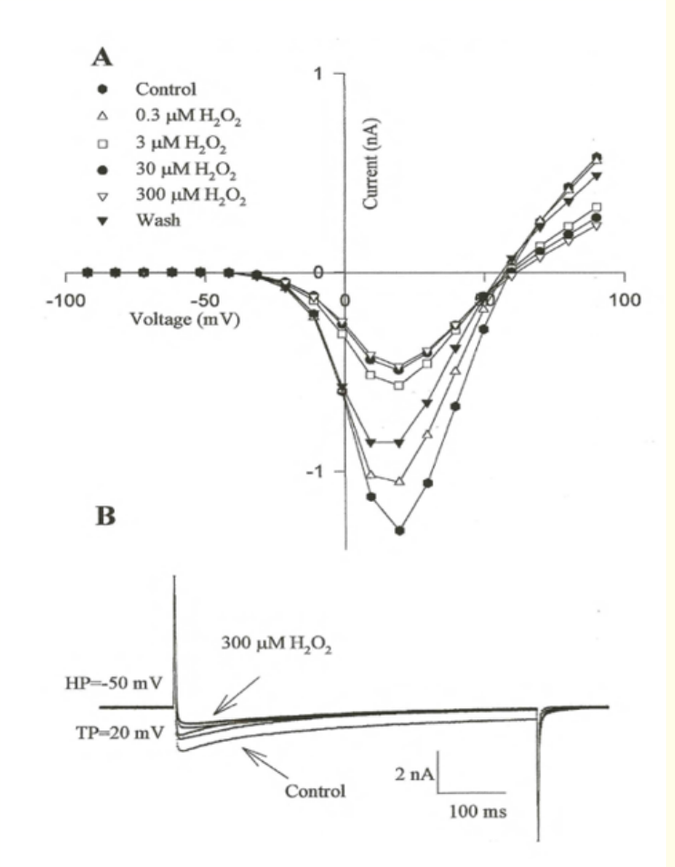

Figure 1: *Effects of H_2O_2 on I_{Ca} of RPeI neurons. A) Concentration-dependent decline in calcium current due to increasing concentrations of H2O2. Ca^{2+} currents were recorded in the presence of extracellular 0.3, 3, 30, 300 µM of H_2O_2. Data averaged from 5 cells with leakage subtraction. Note that only partial recovery occurs on washing in control solution. Bath solution contained (mM): TEA Cl, 40; 4 AP, 10; $MgCl_2$, 1.5; Glucose, 10; HEPES, 10; $CaCl_2$, 4. Sequence of application of solutions: 1) Control in normal bath solution; 2) 0.3 µM H_2O_2; 3) 3 µM H_2O_2; 4) 30 µM H_2O_2; 5) 300 µM H_2O_2; 6) Wash in normal bath solution. The Stimulus protocol showing the amplitude and duration of the command pulse is shown in figure 2. B) The original Ca^{2+} current recording from a pedal I cluster neuron as described above.*

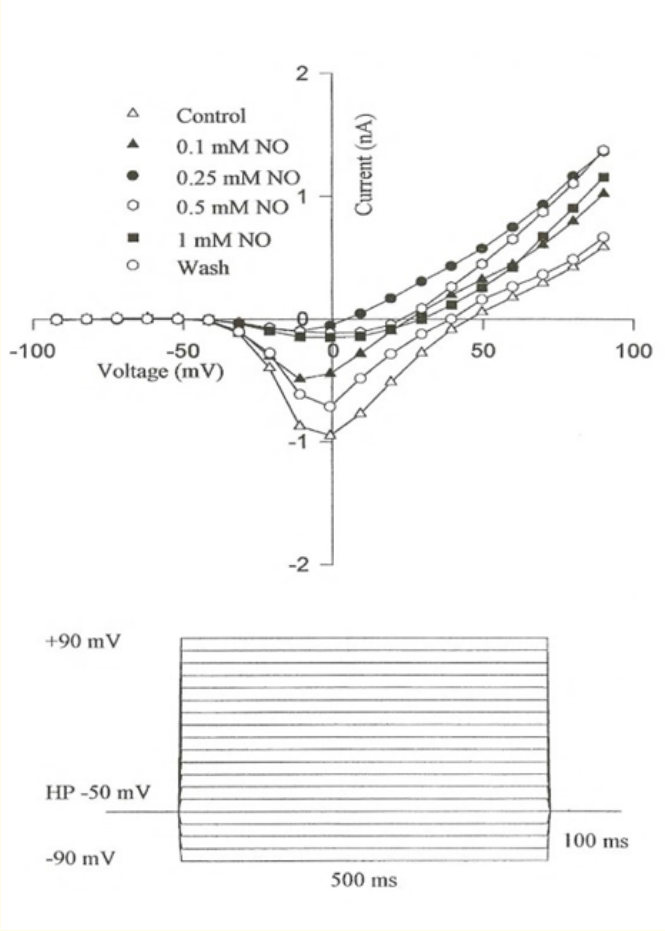

Figure 2: *I-V relationship of I_{Ca} from RPeI Neurons before and after NO application. A) Concentration-dependent decline in L-type I_{Ca} of pedal I cluster neurons due to increasing concentrations of NO. Data averaged from 6 cells after leakage subtraction. Nitric oxide decreases Ca^{2+} current in cultured pedal I neurons of Lymnaea. The control Ca^{2+} current record was elicited from a holding potential of -50 mV by 500 mS depolarization steps to the indicated test potentials (mV). Pipette and bath solutions were similar to those have used in figure 2. Ca^{2+} currents recorded in the presence of extracellular 0.1, 0.25, 0.5, 1 mM of NO. Sequence of application of solutions: 1) Control in normal bath solution; 2) 0.1 mM NO; 3) 0.25 mM NO; 4) 0.5 mM NO; 5) 1 mM NO; 6) Wash in normal bath solution. B) The Stimulus protocol showing the amplitude and duration of the command pulse for this figure, figure 1 and all subsequent figures.*

Actions of H_2O_2 and NO on I_{KCa} of RPeI neurons

In a previous paper we pharmacologically dissected the potassium currents of the RPeI neurons [32] and were able to separate I_{KCa} from I_{KATP} using appropriate pipette solutions (See figure in reference 32), but it should be noted that the K^+ current shown here also contains much smaller components, the transient or A current (I_A) and the delayed rectifier current (I_K). In the experiments below we consider the effects of NO and H_2O_2 on the I_{KCa} which was recorded using a pipette containing ATP (to minimise run down of I_{KCa}) and Ca^{2+}. Figure 3 shows the effect of

H_2O_2 on K^+ currents. Statistics show that at a concentration of 0.3 µM, H_2O_2 has no significant effect on K^+ currents, but at concentrations of 3, 30 and 300 µM K^+ currents decreased significantly (P value of ≤ 0.005). The effects of H_2O_2 were partially reversible on washout.

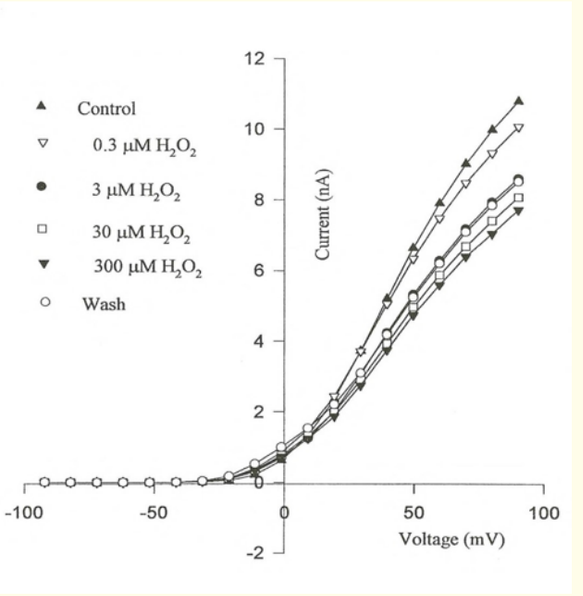

Figure 3: *Concentration-dependent effects of H_2O_2 on I_{KCa} of isolated RPeI cluster neurons (n = 5) of Lymnaea stagnalis. In comparison to the data in figure 5 the effect of H_2O_2 on potassium currents is much more potent than NO, with regard to the concentrations used. The bath solution contained NMDG 51.6, $CaCl_2$ 4, $MgCl_2$ 1.5, HEPES 10 and Glucose 10 mM. Sequence of application of solutions: 1) Control in normal bath solution; 2) 0.3 µM H_2O_2; 3) 3 µM H_2O_2; 4) 30 µM H_2O_2; 5) 300 µM H_2O_2; 6) Wash in normal bath solution.*

The effect of NO on K^+ currents is shown in figure 4. At all concentrations which were used in this study the K^+ current was significantly decreased (P value of 0.005). Significant differences in K^+ currents were found between each drug application compared with the control and also there were significant differences between the data obtained for each drug application (P value of ≤ 0.005) except for the results of application of 0.25 mM and 0.5 mM NO (P value of ≤ 0.025). The effect of NO on K^+ channels was partially reversible with continuous wash out (P value of ≤ 0.005).

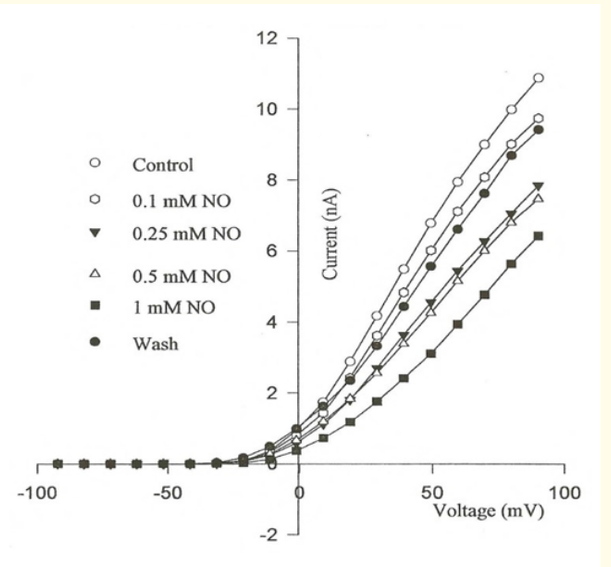

Figure 4: *Effects of NO on I_{KCa} of isolated neurons of isolated pedal I cluster neurons (n = 6). A) NO significantly decreases IKCa in the presence of different concentrations of the NO generator L-cysteine. The bath solution contained NMDG 51.6, $CaCl_2$ 4, $MgCl_2$ 1.5, HEPES 10 and Glucose 10 mM. Sequence of application of solutions: 1) Control in normal bath solution; 2) 0.1 mM NO; 3) 0.25 mM NO; 4) 0.5 mM NO; 5) 1 mM NO; 6) Wash in normal bath solution.*

Actions of NO and H_2O_2 on $[Ca^{2+}]_i$

Although in a previous study [27] we had shown that only a limited proportion (29%) of RPeI cluster neurons responded to the NO generator SNP with a rise in $[Ca^{2+}]_i$, we carried out a further study on a limited number of cells (n = 5) that revealed them all to be responsive to SNP which raised $[Ca^{2+}]_i$ first in darkness, and this effect was enhanced by illumination (Figure 4a). RPeI cluster neurons (n = 5) were also responsive to pure NO (Figure 4b), and to H_2O_2 (n = 4) even in the presence of calcium channel blockers (Figure 6).

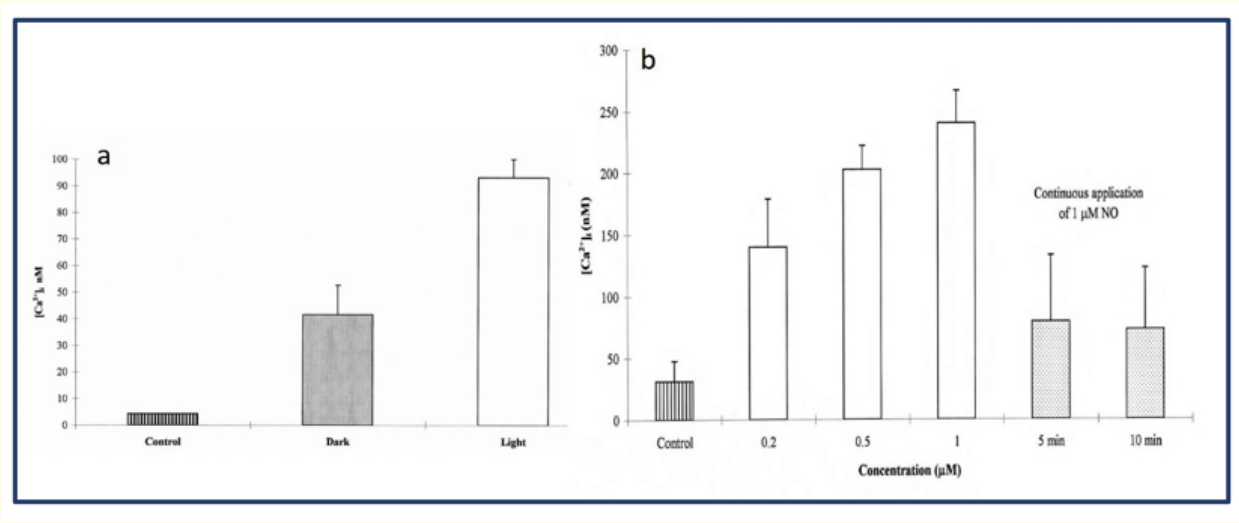

Figure 5: *Both SNP (10^{-4}M) and pure NO increase $[Ca^{2+}]_i$ in RPeI cluster neurons (n = 5). (a) The NO donor SNP (10^{-4} M) increases $[Ca^{2+}]_i$ significantly in Pedal I cluster neurones (n = 5), and illumination of the medium with white light, increases $[Ca^{2+}]_i$ dramatically due to release of NO. Furthermore, (b) application of different concentrations of pure NO, to another group of Pedal I cluster neurones (n = 5) increased $[Ca^{2+}]_i$ in an apparently concentration-dependent manner.*

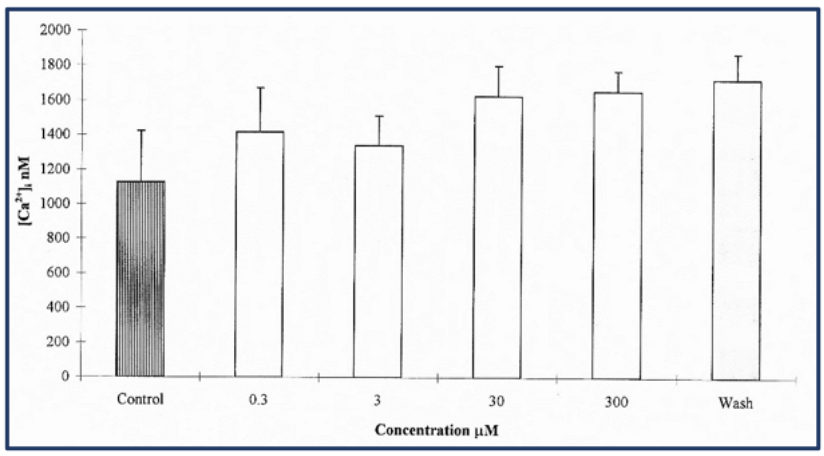

Figure 6: *H_2O_2 increases $[Ca^{2+}]i$ of pedal I cluster neurons in Mg/Cd saline (n = 4). $CdCl_2$ is potent Ca^{2+} channel blocker, but $[Ca^{2+}]_i$ still increases by more than 50% in its presence. This effect was not reversible at washout.*

Discussion and Conclusion

The data we have presented here clearly demonstrate that both H_2O_2 (3 μM to 300 μM) and NO (0.1 mM to 1 mM) substantially decreased L-type Ca^{2+} and K_{Ca} currents in a concentration-dependent manner. These effects were only partially reversible and perhaps could have been fully reversible with longer washout times than the standard timings that we applied. In addition, we have presented some limited data to support the view that both substances increase $[Ca^{2+}]_i$ in these cell types, although we are still unclear as to why only 29% of RPeI cells generated this response to SNP in our earlier paper [27]. Further studies on the interactions between the L-type calcium channels and intracellular concentration in these neurons would be beneficial given the relationship between L-type calcium channel/dihydropyridine receptors and the ryanodine receptors of the endoplasmic reticulum in other preparations [48-50] as discussed by Ahmed., *et al* [41,42].

In guinea pig midbrain slices H_2O_2 regulates neuronal excitability via ATP-sensitive K^+ channels [51] and in *Aplysia*, H_2O_2 has been reported to gate a complex non-selective voltage-dependent cation channel which includes a fast calcium component [52]. It also modulates synaptic transmission in rat ventral horn neurons [23] via Ca^{2+} influx through N-type voltage gated calcium receptors and ryanodine receptors, resulting in inositol triphosphate receptor-mediated calcium release from the endoplasmic reticulum. Similar events may occur in RPeI neurons via the L-type calcium channels under normal circumstances, but these are blocked by H_20_2, which may thus have a direct effect on the endoplasmic reticulum by direct stimulation of ryanodine receptors to induce calcium release.

Different preparations indicate the variable nature of responses to NO. For example, exposure to NO has been shown to inhibit Ca^{2+} currents in chick ciliary ganglion neurons [53], and also to inhibit Ca^{2+} currents and the mobilization of intracellular Ca^{2+} stores in smooth muscle cells [54]. In contrast, in rat sympathetic neurons it has been shown that the NO donors SNP and SNAP (sodium-nitro-N-acetylpenicillamine) enhance Ca^{2+} currents either by extracellular or intracellular application [55]. Similar enhancement of L-type and P/Q-type calcium currents by NO donors have also been observed in the principal neurons of the medial nucleus of the mouse trapezoid body [56]. Méry., *et al.* [57] suggested that the activating or inhibitory effects of NO donors on calcium current are due to "an inhibition of the cGMPinhibited cAMPphosphodiesterase and an activation of the cGMPstimulated cAMPphosphodiesterase, respectively, both linked to the activation of guanylyl cyclase, possibly a membrane form of the enzyme". Thus, NO probably decreases calcium currents (I_{ca}) via the cGMP mechanism and NO donors like SNP and SNAP are known to inhibit Ca^{2+} currents in dorsal root ganglion neurons of the chick embryo, as does the cGMP analogue 8-Br-cGMP (a membrane permeant analogue of cyclic GMP) [58]. Ward., *et al.* [58] have suggested that the Ca^{2+} current of the embryonic chick dorsal root ganglionic neurons is inhibited by NO, possibly via a cGMP mechanism and protein kinase C. Furthermore, it has been shown to have dual effects on rat substantia nigra neurons [59], where it elicits both excitatory and inhibitory effects in a concentration-dependent manner and can induce both analgesic and hyperalgesic effects. However, in *Helisoma trivolvis*, which is closely related to *Lymnaea*, as mentioned above, NO regulates the buccal motor neuron B19 by inhibition of two types of calcium-activated potassium channels [60].

Comparison with other calcium mobilizers

The actions of NO and H_2O_2 on calcium and potassium currents of RPeI cluster neurons are broadly similar to those of the volatile anesthetics on the same cell types, in that all these substances reduce I_{KCa} and the L-type $Ca2^+$ current in a concentration-dependent manner, but raise $[Ca^{2+}]_i$, which may seem to be a contradiction. However, as with NO and H_20_2, we have previously demonstrated that a number of substances can increase $[Ca^{2+}]_i$ in *Lymnaea* neurones; these include volatile anesthetics [35,42], caffeine [40] and ryanodine [41]. The actions of NO and H_2O_2 are reminiscent of the actions of volatile anaesthetics on these cells because they are all gases that pass freely across cell membranes to raise free $[Ca^{2+}]_i$, unlike systemic anaesthetics such as pentobarbital which can also raise $[Ca^{2+}]_i$, but only in the presence of extracellular calcium [36], as is also true for ryanodine [41] and caffeine [40]. Thus, in the presence of extracellular Ca^{2+}, but not in its absence, ryanodine, caffeine and pentobarbital can raise $[Ca^{2+}]_i$ because of their actions on L-type calcium channels/dihydropyridine receptors which are associated with the ryanodine receptors of the endoplasmic reticulum [50] and may induce calcium induced calcium release (CICR) [61].

Why are calcium dependent potassium currents not activated by release of intracellular calcium?

Given the release of intracellular calcium from internal stores, it is surprising that there is no evidence to suggest that I_{KCa} is activated from these stores during application of NO, H_2O_2 or volatile anaesthetics. One explanation is that halothane-induced elevation in $[Ca^{2+}]_i$ may mediate calcium-dependent inactivation of Ca^{2+} channels [62,63], but it is unclear whether this would include K_{Ca} channels. However, it seems likely that intracellular calcium signalling within cells is both localized and compartmentalized [64]. More recently it has been demonstrated that (IP3) inositol-triphosphate-mediated Ca^{2+} release is co-ordinated by localized puffs of calcium, which are generated by stationary clusters of IP3 receptors (IP3Rs) [65] and such receptors are thought to be targets for general anaesthetics [66]. However, H_2O_2 [67], NO [68] and halothane [69] are known to activate Ca^{2+} leak through IP3Rs and ryanodine receptor (RyR) channels. If this is localized release, not associated with K_{Ca} channels, then activation I_{KCa} would not be expected, but this hypothesis needs further elucidation.

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