Effects of anoxia and aglycaemia on cytosolic calcium regulation in rat sensory neurons

Michael Henrich and Keith J. Buckler

Department of Physiology, Anatomy and Genetics, Sherrington Building, Parks Road, Oxford OX1 3PT, UK

Address correspondence to
Keith J. Buckler
Department of Physiology, Anatomy and Genetics, Sherrington Building Parks Road, Oxford OX1 3PT, UK
Phone: 0044-1865- 272113, Fax: 0044-1865-272469,
E-mail: keith.buckler@physiol. ox.ac.uk

Running head: Effects of anoxia and aglycaemia on neuronal [Ca^{2+}]i.
ABSTRACT

Nociceptive neurons play an important role in ischemia by sensing and transmitting information to the CNS and by secreting peptides and nitric oxide which can have local effects. Whilst these responses are probably primarily mediated by acid sensing channels other events occurring in ischemia may also influence neuron function. In this study we have investigated the effects of anoxia and anoxic aglycaemia on Ca$^{2+}$ regulation in sensory neurons from rat dorsal root ganglia. Anoxia increased [Ca$^{2+}$]$\text{_{i}}$ by evoking Ca$^{2+}$ release from two distinct internal stores one sensitive to FCCP and one sensitive to caffeine, CPA and ryanodine (assumed to be the endoplasmic reticulum (ER)). Anoxia also promoted progressive decline in ER Ca$^{2+}$ content. Despite partially depolarising mitochondria, anoxia had relatively little effect upon mitochondrial Ca$^{2+}$ uptake when neurons were depolarised but substantively delayed mitochondrial Ca$^{2+}$ release and subsequent Ca$^{2+}$ clearance from the cytosol upon repolarisation. Anoxia also reduced both SERCA activity and Ca$^{2+}$ extrusion (probably via PMCA). Thus anoxia has multiple effects upon [Ca$^{2+}$]$\text{_{i}}$, homeostasis in sensory neurons involving internal stores, mitochondrial buffering, and Ca$^{2+}$ pumps. Under conditions of anoxic aglycaemia there was a biphasic and more profound elevation of [Ca$^{2+}$]$\text{_{i}}$, which was associated with complete ER Ca$^{2+}$ store emptying and progressive, and eventually complete, inhibition of Ca$^{2+}$ clearance by PMCA and SERCA. These data clearly show that loss of oxygen, and exhaustion of glycolytic substrates, can profoundly affect many aspects of cell Ca$^{2+}$ regulation and this may play an important role in modulating neuronal responses to ischemia.

Keywords: Calcium, sensory neurons, anoxia, mitochondria.
INTRODUCTION

Nociceptive neurons play an important role in mediating responses to tissue ischemia (Armour 1999; Longhurst et al. 2001). Not only do they sense and transmit information that can elicit central responses but they also secrete substance P (SP), calcitonin gene related peptide (CGRP) and nitric oxide which have local effects. The importance of these responses in ischemia is clearly illustrated in the heart where sensory neurons are reported to be involved in: i) the transmission of myocardial pain (Armour et al. 1994; Benson et al. 1999; Brown 1967; Huang et al. 1996; White 1957), ii) evoking cardio-cardiac and sympathetic autonomic reflexes (Longhurst et al. 2001; Malliani 1990), iii) mediating coronary vasodilatation through the release of NO, CGRP & SP (Ledda et al. 1993; Owman 1990; Yamamoto et al. 2003), iv) CGRP evoked chronotropic & ionotropic responses (Ledda et al. 1993) and v) CGRP mediated preconditioning (Li and Peng 2002).

Both excitatory and secretory responses to ischemia are thought to be mediated primarily by the activation of acid sensing channels in the cell membrane which causes membrane depolarisation and calcium entry (Benson et al. 1999; Caterina et al. 1997; Davies et al. 1988; Immke and McCleskey 2001a, 2001b; Konnerth et al. 1987; Krishtal and Pidoplichko 1981, 1980; Longhurst et al. 2001; Pan et al. 1999), (Caterina et al. 1997; Liu et al. 2004; Waldmann and Lazdunski 1998) (Bevan and Geppetti 1994; Bevan and Yeats 1991). Acidosis however is not the only consequence of ischemia. In animal models of myocardial ischemia oxygen levels at the ischemic focus fall rapidly to zero with a surrounding hypoxic border zone (Rumsey et al. 1994; Walfridsson and Lewis 1987). Indeed it is partly the lack of oxygen that causes tissue acidosis by necessitating anaerobic respiration. In addition to anoxia/hypoxia ischemia also leads to loss of other metabolic substrates, ionic redistribution between intracellular and extracellular compartments and the release of various substances from other cellular elements (see e.g. (Opie 1991). Many of these factors are likely to influence neuronal function. In particular the consequences of anoxia and hypoxia are potentially far reaching since, i) there are wide spread reports of ion channels being sensitive to oxygen (Lopez Barneo 1994; Patel and Honoré 2001; Peers 1997; Weir et al. 2005; Yuan 2001), ii) the mitochondrion plays a major role in cell calcium homeostasis (Nicholls 2005), and iii) ATP depletion can influence the activity of many ion channels & transporters (Hilgemann 1997). Indeed in the CNS ischemia has profound effects upon neuronal ion homeostasis, electrical signalling and Ca$^{2+}$ signalling (Erecinska and Silver 1994; Hansen...
1985) (Budd and Nicholls 1996a; Sims 1995; Yao and Haddad 2004). There have been relatively few studies on the effects of hypoxia, anoxia or aglycaemia on sensory neuron function but inhibitors of oxidative phosphorylation are reported to alter cell Ca\(^{2+}\) homeostasis (Duchen et al. 1990) and a few recent studies suggest that ion channels may also be regulated by hypoxia in these neurons (Gruss et al. 2006; Lukyanetz et al. 2003).

In this study we have investigated the effects of anoxia and of anoxic aglycaemia on Ca\(^{2+}\) regulation in small capsaicin sensitive neurons isolated from rat cervico-thoracic dorsal root ganglia (DRG). Our results show that anoxia has multiple effects upon cellular [Ca\(^{2+}\)], homeostasis involving store release, mitochondrial buffering, and inhibition of Ca\(^{2+}\) pumps, but no obvious activation of Ca\(^{2+}\) influx pathways. Despite these events Ca\(^{2+}\) signalling was not grossly abnormal in anoxia alone. In contrast prolonged anoxic aglycaemia lead to a substantive rise in [Ca\(^{2+}\)], and total loss of Ca\(^{2+}\) store and Ca\(^{2+}\) pump function. These data indicate that altered calcium signalling in response to anoxia may well play an important role in modulating sensory neuron responses to ischemia.
MATERIAL AND METHODS

Neuron dissociation.
Adult Wistar rats of either sex aged between 6 and 8 weeks (130-170 g) were sacrificed by an overdose of halothane (4 %) followed by exsanguination in accordance with schedule 1 of the UK Animals (Scientific Procedures) Act 1986. Cervico-thoracic DRG (C4-Th6) were removed under sterile conditions and were immediately transferred into cooled Ca\(^{2+}\) and Mg\(^{2+}\)-free phosphate-buffered saline (PBS), pH 7.4. After cleaning the ganglia were incubated in a medium comprising 10 mg collagenase type I (208 U/mg, Worthington, CLS-1, MON4393), 1 mg trypsin (9.3 U/mg, Sigma, T-4665), in PBS and with 60 µM CaCl\(_2\) and 33 µM MgCl\(_2\) for 35 min at 37°C. Following enzyme treatment ganglia were washed once in PBS (Ca\(^{2+}\)- and Mg\(^{2+}\)-free) and once in DMEM (containing 10% FBS, 1.2 mM L-Glutamine), before mechanical trituration in 1.5 ml of DMEM. The dissociated cells were then washed twice by centrifugation (at 1000g for 5 min) followed by resuspension in fresh DMEM. Following the final wash the cell pellet was resuspended in 500 µl Basal TNB-100 culture medium containing Protein-Lipid-Complex (Biochrom, Berlin, Germany), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10 µM/ml NGF. Following a second trituration the neurons were seeded onto poly-L-lysine and laminin coated coverslips and incubated in sterile culture dishes in a humidified chamber at 37°C and 5% CO\(_2\) / 95% air for 2 hrs. After this incubation period, a further 3 ml TNB was added to each culture dish. Neurons were kept in the incubator for 30 min -24 hrs before use.

Fluorescence measurements.
Fluorescence measurements were performed using a microspectrofluorimeter based on a Nikon Diaphot 200 (Japan) equipped with a monochromator (Cairn Instruments, Kent) and a xenon lamp to provide an excitation light source and cooled (-20°C) photomultiplier tubes (PMT; Thorn EMI) to detect emitted fluorescence. Fura-2 was excited alternately at 340 nm and 380 nm (± 8 nm) for 250 msec. at each wavelength with the cycle repeated at 1 Hz. Fura-2 fluorescence was filtered at 510 nm (± 20 nm). Rhodamine 123 was excited continuously at 495 nm and its emitted fluorescence filtered at 525 ± 10 nm. The output from the PMT’s was integrated over each illumination period (Fura-2) or just averaged over 500 msec. (Rh123) and recorded on a microcomputer using a micro 1401 and Spike 4 software (Cambridge Electronic Design). For Fura-2 the fluorescence ratio (340 nm/ 380 nm) was also calculated and recorded using Spike 4 software.
**Selection and superfusion of neurons.**

Neurons were placed in a recording chamber with a volume of approximately 100 µl. This chamber was perfused at approximately 2 ml / min. Solutions were delivered from reservoirs kept in a water bath to the recording chamber via medical grade stainless steel tubing articulated by short sections of pharmed tubing (Norton performance plastics, UK). A mechanically driven two-way tap was placed within a few inches of the recording chamber. A heating coil was placed around a short section of tubing between the tap and the chamber to ensure solutions remained at 37°C. This arrangement allowed rapid solution exchange and tight control over solution gas content and temperature.

Sensory neurons were selected initially on the basis of soma size (15-30 µm) and, in experiments utilising Fura-2, their response to capsaicin (10-100 nM for 10 sec.) was tested at the end of each experiment. In these studies >80% of neurons selected on these size criteria proved to be capsaicin positive (i.e. capsaicin evoked a robust increase in [Ca²⁺]). Only these neurons were included in our studies on [Ca²⁺] regulation.

**Fura-2 in-vitro calibration.**

The effects of Mg²⁺ on Fura-2 fluorescence were investigated *in vitro*. Fura-2 (Molecular Probes, Leiden, NL) was dissolved at a final concentration of 5 µM (prepared from a stock solution in DMSO), in a calibration buffer containing (in mM): KCl: 150, NaCl: 5, HEPES: 20, and 1 mM EGTA. To this varying amounts of CaCl₂ and MgCl₂ were added to achieve a final free Ca²⁺ concentration of 100 nM and free Mg²⁺ concentrations of (in mM) 0, 0.3, 1, 3, 10, 30 (as calculated using WINMAXC version 2.05). Calibration solution pH was 7.2 at room temperature (21-23°C). 20 µl of each calibration solution was placed in a small Petri-dish and the Fura-2 fluorescence ratio determined using the above microspectrofluorimeter.

**Loading and calibration of Fura-2.**

Neurons were loaded with Fura-2 by incubating them in either a HEPES buffered saline (for *in vivo* calibrations) or a bicarbonate buffered saline (for experiments) containing 5 µM Fura-2-AM (Molecular Probes, Leiden, NL) at room temperature for 25 min in a dark chamber. The HEPES buffered saline used comprised (in mM): HEPES: 20, Glucose: 11, KCl: 4.5, MgCl₂: 1, CaCl₂: 2.5, NaCl: 117, pH 7.4 at RT.
In vivo calibrations were performed by incubating Fura-2 loaded neurons in a zero-Ca\(^{2+}\) calibration medium containing 150 mM KCl, 5 mM NaCl, 1 mM EDTA, 1 mM EGTA and 10 µM Ionomycin (Sigma, Dorset, UK), Ca\(^{2+}\) for 10-20 min at room temperature. After this pre-incubation the neurons were placed in the perfusion chamber of the microspectrofluorometer and perfused with zero-Ca\(^{2+}\) medium (+1 µM Ionomycin) at 37°C. After a 5 min perfusion Fura-2 fluorescence was recorded in 5 identified sensory neurons. The ratio of fluorescence obtained under these conditions was deemed equivalent to the calibration constant \(R_{\text{min}}\) (Grynkiewicz et al. 1985). The perfusate was then changed to a high-Ca\(^{2+}\) calibration medium containing 150 mM KCl, 5 mM NaCl, 10 mM CaCl\(_2\) and 1 µM Ionomycin. The change in fluorescence ratio was followed in one of the 5 identified neurons until it reached a new stable value and then the fluorescence ratio in it, and in the other 4 identified neurons, was recorded and deemed to be equivalent to the calibration constant \(R_{\text{max}}\). The ratio of fluorescence at 380 nm in zero-Ca\(^{2+}\) medium divided by that obtained in high Ca\(^{2+}\) medium (\(S_{f2}/S_{b2}\)) was also calculated for each neuron. The mean values obtained for \(R_{\text{min}}\), \(R_{\text{max}}\) and \(S_{f2}/S_{b2}\) were then used to calibrate measurements of the fluorescence ratio in subsequent experiments using the equation \([\text{Ca}^{2+}] = (R-R_{\text{min}})/(R_{\text{max}}-R) \times S_{f2}/S_{b2} \times K_d\) (Grynkiewicz et al. 1985).

Measurement of mitochondrial membrane potential with Rhodamine 123.

Changes in mitochondrial membrane potential were detected using Rhodamine 123 (Rh123). This is a membrane permeant cation that is strongly sequestered in mitochondria due to their negative membrane potential. In concentrated solutions, as occur within the mitochondrial, Rh123 fluorescence is quenched. If the mitochondria become depolarised Rh123 is redistributed from the mitochondrion to the cytosol where it becomes diluted and as a consequence fluorescence increases. Measurements of total Rh123 fluorescence from an intact cell can therefore be used to follow changes in mitochondrial membrane potential (\(\Psi_m\)) (Toescu and Verkhratsky 2000). Neurons were loaded with Rh123 (5 µM, Sigma, Dorset, UK) in bicarbonate buffered medium at room temperature for 12 min. The cells were then transferred to the perfusion chamber described above. Because Rh123 is relatively poorly retained by cells a correction for both baseline drift and decline in maximum signal amplitude was carried out. This consisted of taking measurements of baseline fluorescence under control conditions and peak signal amplitude measured during brief application of 1 µM FCCP (an uncoupler which fully depolarises the mitochondria) at the beginning and end of each recording. Each recording was limited to a maximum of 20 min duration. The time dependent
decline in baseline fluorescence was modelled as a linear process and was subtracted from the recording. The decline in maximum signal amplitude (measured in presence of FCCP) was also modelled as a linear process. The baseline subtracted signal was then divided by the time dependent maximum signal and multiplied by 100 to convert the recorded signal to a % of the maximum signal attainable by full mitochondrial depolarisation (%ΔΨm).

Solutions
Standard bicarbonate buffered Tyrode solutions contained (in mM): NaCl: 117, KCl: 4.5, CaCl2: 2.5, MgCl2: 1, HCO3⁻: 23, Glucose: 11. Glucose-free Tyrode solution was prepared by replacing glucose with 11 mM sucrose. In Ca²⁺-free solutions CaCl2 was omitted and 1 mM EGTA added. High K⁺ Tyrode contained 50 mM KCl and 71.5 mM NaCl, all other constituents remained the same. Equilibration of these solutions with 5% CO₂ and 95% air, achieved normoxic conditions with pH 7.4 at 37°C. Hypoxic solutions were generated by equilibration with 5% CO₂ and 95% N₂ (PO₂=2 torr). Anoxic solutions were obtained by the further addition of 0.5 mM Na₂S₂O₄ (Sato et al. 1991) following 15-30 min prior equilibration with 5% CO₂ /95% N₂ (pH: 7.39 ± 0.2, n = 23). All solutions were equilibrated with appropriate gas mixes at 37°C in a water bath for at least 30 min before use.

Drugs
Ryanodine was from Tocris (Avonmouth, UK). All other chemicals were from either Sigma (Poole, UK) or VWR/BDH (Nottingham, UK). Cyanide containing solutions were prepared by adding solid NaCN to pre equilibrated Tyrode solution immediately before use. CN⁻ solutions were not used for longer than 30 min (CN⁻ in buffered solutions is volatile and can be very rapidly lost from solution see (Wyatt and Buckler 2004). Cyclopiazonic acid (CPA) and Thapsigargin containing solutions were prepared from stock solutions in DMSO. Capsaicin and FCCP were added from stock solutions in ethanol. The maximum concentration of solvent in Tyrodes were; 50 µM DMSO, 10 µM ethanol.

Statistics
Values are expressed as mean ± standard error of mean (S.E.M.). Statistical significance was tested using the paired Students t-test, or Wilcoxon ranks signed test for experiments with non Gaussian distribution. Statistical testing of in vitro calibration data was performed using one-
way ANOVA and post hoc analyses were carried out using Bonferroni’s multiple comparison, calculated by SPSS 12.0 software for windows. Level of significance was set at p < 0.05.
RESULTS

Effects of Mg$^{2+}$ on Fura-2.
Exposure of sensory neurons to anoxia, aglycaemia or metabolic poisons can increase [Mg$^{2+}$], from a resting level of 1.39 ± 0.1 mM up to 3.48 ± 0.35 mM (due to MgATP hydrolysis, (Henrich and Buckler 2007)). In order to confirm that changes in intracellular magnesium of this magnitude would not interfere with the measurement of [Ca$^{2+}$], using Fura-2 we conducted an analysis of the effects of [Mg$^{2+}$], on Fura-2 fluorescence in vitro. In calibration solutions containing 100 nM Ca$^{2+}$ Fura-2 fluorescence was not significantly enhanced by Mg$^{2+}$ in the range between 0.3 and 5 mM but above this concentration a significant increase in fluorescence ratio was observed (one way ANOVA, Post Hoc, Bonferroni test, see Figure 1). Thus at intracellular calcium levels similar to those found in these cells under resting conditions changes in [Mg$^{2+}$], should not significantly interfere with the measurement of [Ca$^{2+}$], using Fura-2. This was further confirmed by testing the effects of the uncoupler FCCP (which also increases cytosolic [Mg$^{2+}$],) upon Fura-2 fluorescence under conditions in which cytosolic Ca$^{2+}$ was heavily buffered using BAPTA. Under these conditions FCCP had negligible effects on measured [Ca$^{2+}$], (Δ[Ca$^{2+}$] = 1.2 ± 0.2 nM, n = 4, Figure 1B).

Effects of anoxia on resting [Ca$^{2+}$], in DRG neurons.
Capsaicin sensitive neurons had a resting [Ca$^{2+}$], of 163 ± 5 nM (n = 79). Hypoxia (pO$_2$: 2 Torr) had no significant effect upon [Ca$^{2+}$], (n = 16) but anoxia caused an abrupt rise in [Ca$^{2+}$], of 66 ± 5 nM to a stable level of 239 ± 9 nM, p < 0.001, n = 49 (see e.g. Figure 2). This rise in [Ca$^{2+}$], was usually complete within one minute. When anoxia was applied for prolonged periods the rise in [Ca$^{2+}$], measured after 18 min (56 ± 5 nM) was not different to that observed after the first 3 min (60 ± 6 nM, n = 6, p = 0.595) indicating that following an initial rapid rise [Ca$^{2+}$], remains relatively stable under anoxic conditions. [Ca$^{2+}$], recovered immediately back to baseline upon reoxygenation (see Figure 2). This [Ca$^{2+}$], response to anoxia was repeatable (Figure 2).

The anoxia induced rise in [Ca$^{2+}$], was not reduced in Ca$^{2+}$-free solution (Δ[Ca$^{2+}$] = 64 ± 4 nM, n = 28, Figure 2C) or in the presence of 1 mM Ni$^{2+}$ (Δ [Ca$^{2+}$] = 90 ± 23 nM + 1 mM Ni$^{2+}$ vs. 103 ± 21 nM, control, n = 6, p = 0.463) indicating that this rise in [Ca$^{2+}$], was not mediated by enhanced Ca$^{2+}$ influx.
Intracellular Calcium stores contribute to the anoxic evoked rise in $[\text{Ca}^{2+}]_i$.

Having excluded $\text{Ca}^{2+}$ influx as a potential cause of the anoxia induced rise in $[\text{Ca}^{2+}]_i$, we sought to determine the role of intracellular $\text{Ca}^{2+}$ stores. The $[\text{Ca}^{2+}]_i$ response to anoxia was therefore measured under control conditions and following depletion of specific $\text{Ca}^{2+}$ stores. All of these following experiments were conducted in a $\text{Ca}^{2+}$-free Tyrode solution.

We investigated a role for mitochondria by using the uncoupler FCCP to depolarise the mitochondria and thus release any $\text{Ca}^{2+}$ stored within (Shishkin et al. 2002; Thayer and Miller 1990). In experiments using Rh123, 1 $\mu$M FCCP appeared to produce a maximal mitochondrial depolarisation ($n = 3$ data not shown). Application of 1 $\mu$M FCCP alone caused a significant rise in $[\text{Ca}^{2+}]_i$ from $174 \pm 13$ to $214 \pm 18$ nM ($n = 11$, $p < 0.001$). Subsequent exposure to anoxia, in the continued presence of FCCP, caused a further rise in $[\text{Ca}^{2+}]_i$ of $36 \pm 4.5$ nM ($n = 11$, $p < 0.01$, Figure 3A & D). This *anoxia induced* increase in $[\text{Ca}^{2+}]_i$ was significantly less than that seen in the absence of FCCP ($p < 0.001$, Figure 3). The *combination* of FCCP and anoxia however increased $[\text{Ca}^{2+}]_i$, to a final level similar to that observed in anoxia alone ($[\text{Ca}^{2+}]_i = 243 \pm 19$ nM, anoxia + FCCP vs. $238 \pm 13$ nM, anoxia alone, $n = 11$, $p = 0.949$). Therefore prior depletion of mitochondrial stores with FCCP diminishes the response to anoxia by an amount equivalent to the rise in $[\text{Ca}^{2+}]_i$, caused by FCCP alone. Consequently part of the $\text{Ca}^{2+}$ response to anoxia seems to be dependent upon some aspect of mitochondrial function but there is also another component.

DRG sensory neurons contain caffeine sensitive stores (Usachev et al. 1993). Depletion of these stores with 30 mM caffeine did not abolish the $[\text{Ca}^{2+}]_i$ response to anoxia but did significantly reduce it compared to that seen when stores were not depleted ($\Delta[\text{Ca}^{2+}]_i = 22 \pm 3.5$ nM, $n = 21$ post caffeine, $p < 0.001$, see Figure 3D). Similar results were also obtained when both CPA, an inhibitor of the endoplasmic reticulum $\text{Ca}^{2+}$-pump (SERCA), and caffeine were used together to deplete $\text{Ca}^{2+}$ stores; i.e. the rise in $[\text{Ca}^{2+}]_i$ in response to anoxia was reduced (to $21 \pm 3.4$ nM, $n = 7$, $p < 0.001$), but was not abolished (Figure 3).

The above data suggested that part of the response to anoxia could be mediated by $\text{Ca}^{2+}$ release from caffeine sensitive internal stores. To determine whether any other internal stores could be involved we depleted both caffeine/CPA sensitive stores and mitochondrial stores. In this series of experiments FCCP and caffeine (and/or CPA) were applied sequentially but in a random order. Irrespective of the order of addition the combination of FCCP with caffeine
and or CPA completely prevented any anoxia induced rise in $[Ca^{2+}]_i$, ($\Delta[Ca^{2+}]_i = 9 \pm 3.4 \text{nM}$, $n = 12$, n.s. see Figure 4). The anoxia induced rise in calcium could also be inhibited by a combination of FCCP plus Thapsigargin (100 nM), an irreversible inhibitor of SERCA ($n= 4$, Figure 4B), and by FCCP in combination with 25 µM ryanodine ($n = 4$, Figure 4D). Note that ryanodine did not deplete ER stores of $Ca^{2+}$ (as evidenced by the observation that following removal of ryanodine caffeine evoked a large rise in $[Ca^{2+}]_i$, Figure 4D) but none the less reduced $Ca^{2+}$ release in response to anoxia.

**Mitochondrial function and Ca release.**

The observation that part of the $[Ca^{2+}]_i$ response to anoxia was abolished by application of FCCP led us to further investigate the role of mitochondria in mediating $Ca^{2+}$ release. Application of the electron transport inhibitor cyanide (2 mM) in a Ca$_{o}$-free medium also led to an abrupt increase in $[Ca^{2+}]_i$ ($63 \pm 9 \text{nM}$, $n = 6$, $p < 0.001$) similar to that observed for anoxia (Figure 5 A). The effect of cyanide was partially inhibited (reduced to $32 \pm 4 \text{nM}$, $n = 6$, $p < 0.05$), by prior mitochondrial depolarisation with FCCP (Figure 5 B). These data suggest that both anoxia and cyanide might induce some $Ca^{2+}$ release through mitochondrial depolarisation. The effects of anoxia and cyanide upon mitochondrial membrane potential ($\psi_m$) were therefore assessed using Rh123 (see methods). FCCP (1 µM) was used as a reference to evoke full mitochondrial depolarisation and thus a maximal increase in Rh123 fluorescence (see methods). Anoxia evoked a rapid increase in Rh123 fluorescence but only to $25 \pm 7\%$ of the maximum seen with FCCP ($n = 6$). CN$^-$ (2.5 mM) similarly increased Rh123 fluorescence by $24 \pm 5\%$ ($n = 8$) of maximum. These effects were rapidly reversible upon reoxygenation or removal of CN$^-$. Thus both anoxia and CN$^-$ induce rapid but only partial depolarisation of $\psi_m$ which may be sufficient to evoke some mitochondrial $Ca^{2+}$ release (but see discussion).

The above studies with cyanide also indicate that inhibition of electron transport can promote $Ca^{2+}$ release via another pathway (i.e. independent of mitochondrial depolarisation). In pulmonary vascular smooth muscle it has been suggested that hypoxia induced increase in NADH might stimulate cyclic-ADP-ribose (cADPR) production (Dipp and Evans 2001; Evans and Dipp 2002) and thus evoke $Ca^{2+}$ release from the sarcoplasmic/endoplasmic reticulum. Since a significant fraction of the $[Ca^{2+}]_i$ response to anoxia in DRG neurons appears to be dependent upon ER function (see above & discussion) and since the effects of anoxia are mimicked by electron transport inhibition using cyanide, we sought to test for the
possible involvement of cADPR in mediating the Ca^{2+} response to anoxia. Pre-treatment of sensory neurons with the cADPR antagonist 8-bromo-cyclic-ADP-ribose (8-Br-cADPR, 100µM) for 10 min significantly reduced [Ca^{2+}]_i responses to anoxia both in the absence and presence of FCCP (Figure 7) when compared to [Ca^{2+}]_i responses in untreated neurons.

**Effects of anoxia on mitochondrial Ca^{2+} buffering.**

Given that anoxia depolarises mitochondria, albeit only partially, we also investigated the effects of anoxia on mitochondrial Ca^{2+} buffering. Voltage gated Ca^{2+} entry was triggered by a brief (5 sec.) application of a high K^+ Tyrode (50 mM), containing normal levels of extracellular Ca^{2+} (2.5 mM). The peak [Ca^{2+}]_i attained under these conditions is determined by the rate of voltage-gated Ca^{2+} influx, Ca^{2+} efflux, Ca^{2+} buffering and Ca^{2+} uptake by mitochondria. Upon subsequent removal of the high K^+-medium there is initially a very rapid decline in [Ca^{2+}]_i. This is followed a few seconds later, and after [Ca^{2+}]_i has fallen to 357 ± 128 nM, by a secondary slow rise in [Ca^{2+}]_i to a plateau and then a much slower decline in [Ca^{2+}]_i (see e.g. Figure 8A & B). This secondary phase, or shoulder, in [Ca^{2+}]_i, has been attributed to the release of Ca^{2+} from mitochondria that had previously been accumulated during exposure to high K^+ when [Ca^{2+}]_i is very high (Werth and Thayer 1994). The role of mitochondrial Ca^{2+} buffering in these events is most readily demonstrated by depolarising the mitochondria using an uncoupler and thus preventing Ca^{2+} uptake (Thayer and Miller 1990). Figure 8B confirms that brief application of FCCP prior to and during application of high K^+ Tyrode caused a substantial increase in the [Ca^{2+}]_i response to depolarisation (by 224 ± 50%, n = 5, p < 0.05) and the abolition of the mitochondrial Ca^{2+} release phase during recovery of [Ca^{2+}]_i. These observations are consistent with the mitochondrion normally buffering large amounts of Ca^{2+} during voltage-gated Ca^{2+} influx in these neurons.

When we depolarised neurons under anoxic conditions the initial Ca^{2+} transient was slightly enhanced compared to that obtained under normoxic conditions to 117 ± 4% (n = 10, p < 0.05, Figure 8). The level of [Ca^{2+}]_i attained during the second slow phase (shoulder) when calculated as a rise in [Ca^{2+}]_i, relative to baseline was reduced to 83 ± 5.5% of control (n = 10, p < 0.01); but when expressed as an absolute level was not significantly altered (89 ± 4% of control, p = 0.07). These observations were consistent with our expectations that anoxia might impair mitochondrial Ca^{2+} buffering but were notably rather minor. The most obvious effect of anoxia however was a near doubling of the duration of the mitochondrial Ca^{2+} release.
phase from 119 ± 22 sec. under control conditions to 235 ± 64 sec. under anoxic conditions (n = 8, p < 0.05, measured at half height, see Figure 8). These data could indicate either a paradoxical increase in the Ca\textsuperscript{2+} load buffered by the mitochondria during anoxia or slowed Ca\textsuperscript{2+} clearance.

In order to semi-quantify the amount of Ca\textsuperscript{2+} taken up by mitochondria we used uncouplers to induce rapid mitochondrial Ca\textsuperscript{2+} release. Cells were briefly exposed to a high K\textsuperscript{+} solution (containing 2.5 mM Ca\textsuperscript{2+}) and then returned to a normal Ca\textsuperscript{2+}-free Tyrode and [Ca\textsuperscript{2+}]\textsubscript{i} allowed to recover until the onset of the second phase (shoulder) at which point 1 µM FCCP was applied to rapidly dump any Ca\textsuperscript{2+} stored in the mitochondrion back into the cytosol (see Figure 8C). This protocol results in a substantial rise in [Ca\textsuperscript{2+}]\textsubscript{i}, upon FCCP application which was quantified, relative to base line [Ca\textsuperscript{2+}]\textsubscript{i}, as an index of the amount of Ca\textsuperscript{2+} stored in the mitochondrion. Anoxia had no significant effect upon this measure of mitochondrial Ca\textsuperscript{2+} load (n = 5, p = 0.293, see Figure 8E). We could not therefore find any corroborating evidence that anoxia increased the amount of Ca\textsuperscript{2+} stored within the mitochondria.

**Effects of anoxia on ER Ca\textsuperscript{2+} stores.**

In view of the fact that anoxia appears to induce Ca\textsuperscript{2+} release from the ER we sought to determine the extent to which anoxia might deplete those stores of Ca\textsuperscript{2+}. To assess store content we applied 30 mM caffeine and measured the resultant rise in [Ca\textsuperscript{2+}]\textsubscript{i}, which is caused by caffeine induced Ca\textsuperscript{2+} release. These experiments were carried out in Ca\textsuperscript{2+}-free Tyrode to exclude any contribution from capacitive Ca\textsuperscript{2+} entry. Store filling before, and between, caffeine exposures was facilitated by brief (5 sec.) application of a high K\textsuperscript{+} Tyrode containing normal levels of [Ca\textsuperscript{2+}]\textsubscript{o}. Following 3 min of anoxia the maximum amplitude of the caffeine response was significantly reduced to 78 ± 5% (n = 6, p < 0.01) of control. Following re-oxygenation the response to caffeine recovered to 120 ± 11% (n = 6, p = 0.128) compared to control (Figure 9A & D). Protracted exposure to anoxia (18 min) resulted in a further reduction in the Ca\textsuperscript{2+} response to caffeine to 41 ± 11% (n = 6, p < 0.01) of control (exposure of neurons to Ca\textsuperscript{2+}-free medium under normoxic conditions for 20 min only reduced the response to caffeine to 82 ± 6%, n = 6). These data indicate that anoxia causes a slow depletion of ER Ca\textsuperscript{2+} store content. This effect of anoxia was reversible, i.e. even after extended exposure to anoxia upon re-oxygenation and re-loading in high K\textsuperscript{+} Tyrode the caffeine releasable Ca\textsuperscript{2+} store recovered fully (to 93 ± 17% of control, n = 6, p = 0.693, Figure
9B & D). Indeed even during prolonged anoxia, caffeine sensitive stores could be almost completely refilled (to 92 ± 18% of control, n = 5, n.s.) by high K+ induced voltage-gated Ca2+ entry (Figure 10). Thus although anoxia evokes Ca2+ release or leak from ER stores it does not cause major disruption of store function.

**Mitochondrial buffering assists the refilling of caffeine sensitive Ca2+ stores.**

We briefly investigated whether mitochondrial Ca2+ buffering could influence ER store loading by looking at the effects of FCCP upon store loading and caffeine evoked Ca2+ release. ER stores were depleted by repetitive caffeine applications (Figure 11) and then FCCP (1 µM) was applied and voltage-gated Ca2+ entry triggered by a high K+ pulse (50 mM, 5 sec.). The following response to caffeine (30 mM, still in the presence of FCCP), was substantively reduced to 33 ± 6% (n = 5, p < 0.05) of control. Following subsequent removal of FCCP, a rest period of 3 min, a further high K+ pulse (50 mM, 5 sec.), the caffeine response recovered to 95 ± 6% (n = 5, Figure 11) of control. Since store refilling was near normal under anoxic conditions (see above) these data suggest a specific role for mitochondrial Ca2+ buffering in facilitating store loading.

**Effects of anoxia on cytosolic Ca2+ clearance.**

During the above studies it was noted that [Ca2+]i recovery following high K+ depolarisation (i.e. during the mitochondrial Ca2+ release phase) was slower under anoxic conditions (Figure 8A). Since this could not be attributed to an increased mitochondrial Ca2+ load, it must reflect some effect of anoxia upon mitochondrial Ca2+ release or cytosolic Ca2+ clearance/extrusion mechanisms. We therefore sought to determine the effects of anoxia upon cytosolic Ca2+ clearance under conditions in which mitochondrial Ca2+ buffering is thought to be insignificant. To achieve this cells were depolarised in a high K+ (50 mM) Tyrode with extracellular calcium concentration reduced to 250 µM (high-K+-low-Ca2+). Brief (5 sec.) application of this solution induced a rapid rise in [Ca2+]i, which was limited to a peak of < 500 nM (mean = 274 ± 8 nM, n = 9). [Ca2+]i, recovery following removal of high-K+-low-Ca2+ Tyrode was monophasic (Figure 12A) confirming that the threshold for significant mitochondrial Ca2+ buffering was not breached (Nicholls and Budd 2000). Cytosolic Ca2+ clearance was quantified during this recovery phase as the rate of change in [Ca2+]i, measured over a small range of [Ca2+]i, between 150 and 200 nM or 200 & 250 nM (see legend to Figure 12). Repeated exposures to this solution under control conditions evoked similar [Ca2+]i, responses with reproducible recovery / Ca2+ clearance rates (Figure 12D) Anoxia reduced the
amplitude of these high-K⁺-low-Ca²⁺ induced [Ca²⁺] transients to 78 ± 5% (n = 9, p < 0.01) of control and significantly slowed Ca²⁺ clearance from 14.8 ± 1.34 nM/sec. (n = 23) under control conditions, to 8.38 ± 1.1 nM/sec. (n = 12, p < 0.001) after 3 min of anoxia and 2.98 ± 0.6 nM/sec. after 25 min of anoxia (see Figure 12 B & D).

Since Ca²⁺ clearance can occur via the plasma membrane and/or uptake into the ER we sought to determine which of these pathways was being inhibited by anoxia. Inhibition of SERCA under normoxic conditions with either Thapsigargin (100 nM) or CPA (10 µM) reduced Ca²⁺ clearance rates from 14.8 ± 1.3 nM/sec. to 4.62 ± 0.85 nM/sec. (n = 11, p < 0.01, Figure 13) confirming that ER Ca²⁺ uptake contributes significantly to Ca²⁺ clearance under these experimental conditions (see Figure 13 & discussion). The remaining Ca²⁺ clearance presumably represents Ca²⁺ efflux across the plasma membrane. In the presence of Thapsigargin or CPA, anoxia (3 min) further reduced Ca²⁺ clearance to 2.2 ± 0.48 nM/sec. (n = 11 p < 0.001, Figure 13 & 14). Thus anoxia inhibits plasma membrane Ca²⁺ clearance by about 53%.

To estimate Ca²⁺ clearance by SERCA we subtracted Ca²⁺ clearance rates measured in the presence of CPA or thapsigargin from total cytosolic Ca²⁺ clearance rates measured in the absence of CPA or thapsigargin (see Figure 14). Ca²⁺ clearance via SERCA under normoxic conditions was thus estimated to be 10.2 nM/sec. Anoxia (3 min) reduced this SERCA mediated Ca²⁺ clearance to 6.2 nM/sec. or about 60% of control. Thus anoxia appears to inhibit both SERCA and PMCA to roughly the same extent (Figure 14).

We also attempted to assay SERCA mediated Ca²⁺ clearance by inhibiting PMCA activity. It has been reported that as PMCA mediates exchange of [Ca²⁺] for H⁺ it can be inhibited by extracellular alkalosis (Benham et al. 1992). We found that whilst raising external pH to 8.8 caused a 90% inhibition of Ca²⁺ clearance in Ca²⁺-containing medium, the effects of alkalisation in Ca²⁺-free medium were much less pronounced and significant Ca²⁺ clearance remained even when the SERCA was also inhibited (Buckler & Henrich unpublished). One possible explanation for these observations is that the inhibitory effect of external alkalosis derive not simply from kinetic restrictions imposed by reduced H⁺ availability but also from thermodynamic constraints i.e. at very low [H⁺]o Ca²⁺ extrusion may become energetically unfavourable in a normal Ca²⁺ medium (but not in a Ca²⁺-free medium). We could not
therefore block PMCA in order to directly assess the effects of anoxia on SERCA mediated cytosolic Ca\textsuperscript{2+} clearance.

**Effects of anoxic aglycaemia on Ca\textsuperscript{2+} regulation.**
In the preceding studies it is clear that although anoxia induces significant changes, Ca\textsuperscript{2+} regulation does not become totally dysfunctional. This probably reflects the fact that whilst cellular ATP levels are reduced by anoxia they are not exhausted (see discussion). In order to determine how more complete ATP depletion affects [Ca\textsuperscript{2+}]\textsubscript{i}, regulation we exposed some neurons to a combination of both anoxia and aglycaemia. Under these conditions the rise in [Ca\textsuperscript{2+}]\textsubscript{i} was biphasic (see Figure 9C). During the first phase [Ca\textsuperscript{2+}]\textsubscript{i} rose rapidly (within 1 min) to 80 ± 12 nM (n = 9) and then remained stable for 8-12 min. This initial rise in [Ca\textsuperscript{2+}]\textsubscript{i} was not significantly different to that observed in response to anoxia alone (p = 0.35). This was then followed by a second more profound increase in [Ca\textsuperscript{2+}]\textsubscript{i} by up to 187 ± 32 nM (n = 9). During this second phase responses to caffeine (30 mM for 2 min) were effectively abolished (2 ± 2% of control, n = 6, p < 0.0001, Figure 9C & D) indicating complete depletion of ER Ca\textsuperscript{2+} stores. Ca\textsuperscript{2+} clearance rates (measured using the protocol described above) were also greatly reduced after about 18 min of continued anoxic aglycaemia (both in the presence and absence of CPA, see Figure 12 & 13) indicating an almost complete inhibition of both SERCA and PMCA. Ca\textsuperscript{2+} influx in response to high-K\textsuperscript{+}-low-Ca\textsuperscript{2+} medium was also much reduced after prolonged anoxic aglycaemia suggesting that voltage-gated Ca\textsuperscript{2+} channels may also become inhibited under these more extreme conditions.
DISCUSSION

In this study we have primarily investigated the effects of anoxia upon sensory neuron Ca\(^{2+}\) regulation. In animal models of cardiac ischemia (blood vessel occlusion) oxygen levels at the ischemic focus fall rapidly to zero (Rumsey et al. 1994; Walfridsson and Lewis 1987). Lack of oxygen delivery results in inhibition of mitochondrial respiration in the surrounding tissue as assessed by NADH accumulation (Barlow and Chance 1976) and depletion of phosphocreatine & ATP (Elliott et al. 1992; Zhang et al. 2001). Surrounding this core zone there may also be a hypoxic border zone. Our anoxia data however is most relevant to events in the “core region” of ischemic tissue where oxygen delivery is so low as to be unable to support oxidative phosphorylation.

In our isolated sensory neuron model anoxia alone results in a rapid but only partial depletion of MgATP as assessed by increase in [Mg\(^{2+}\)]. In contrast anoxic aglycaemia causes a biphasic fall in MgATP the first phase, which lasts approximately 10 min, is similar to that observed in anoxia alone. The second phase of the MgATP response appears to culminate in near complete exhaustion of MgATP (see (Henrich and Buckler 2007)). The anoxia model may therefore only simulate the early stages of no-flow ischemia where there is still glycolytic substrate available. In later stages of ischemia, with exhaustion of glycolytic substrates, prolonged anoxic aglycaemia would probably be a better model.

Exposure of neurons to acute anoxia lead to a rapid rise in [Ca\(^{2+}\)], that appeared to result from Ca\(^{2+}\) release from internal stores. This observation is similar to a previous study in sensory neurons in which inhibitors of oxidative metabolism (cyanide) caused Ca\(^{2+}\) release from internal stores (Duchen et al. 1990). They are also consistent with reports of hypoxia evoked Ca\(^{2+}\) release from internal stores in hippocampal neurons (Dubinsky and Rothman 1991; Grondahl et al. 1998), astrocytes (Aley et al. 2006) and pulmonary vascular smooth muscle (Jabr et al. 1997; Kang et al. 2002). The effects of anoxia on DRG neurons were not limited to Ca\(^{2+}\) store release however it also had multiple other effects upon [Ca\(^{2+}\)] regulation (see below).

We were unable to corroborate a recent study claiming that hypoxia (10-40 Torr) evokes voltage-gated Ca\(^{2+}\) influx in DRG neurons (Lukyanetz et al. 2003). In our study hypoxia had little effect upon [Ca\(^{2+}\)] and the Ca\(^{2+}\) responses to anoxia were not diminished by either Ca\(^{2+}\)
channel blocker (Ni\textsuperscript{2+}) or removal of extracellular Ca\textsuperscript{2+}. The difference between our results and those of Lukyanetz et al. cannot be accounted for by our selective use of capsaicin sensitive neurons because we found that capsaicin negative neurons also failed to respond to hypoxia (data not shown). We note however that the study of Lukyanetz at al. was conducted in medium apparently lacking any metabolic substrate and it is unclear how long neurons were maintained in this medium.

**Does anoxia promote mitochondrial Ca\textsuperscript{2+} release?**

Mitochondria play an important role in Ca\textsuperscript{2+} homeostasis in neurons by acting as a dynamic Ca\textsuperscript{2+} buffering system. It is however thought that under resting conditions [Ca\textsuperscript{2+}]\textsubscript{i} is below the set point for mitochondrial Ca\textsuperscript{2+} uptake and consequently the mitochondrion contains relatively little Ca\textsuperscript{2+} (Budd and Nicholls 1996b; Chalmers and Nicholls 2003; Nicholls 1978; Nicholls and Budd 2000; Nicholls and Scott 1980; Somlyo et al. 1985; Thayer and Miller 1990; Thayer and Wang 1995; Zoccarato and Nicholls 1982). In order to determine whether mitochondria contain enough Ca\textsuperscript{2+} under resting conditions to enhance [Ca\textsuperscript{2+}]\textsubscript{i}, we investigated the effects of releasing mitochondrial Ca\textsuperscript{2+} with FCCP (Babcock et al. 1997; Duchen 1999; Friel and Tsien 1994). The application of FCCP both enhanced [Ca\textsuperscript{2+}]\textsubscript{i} and diminished any subsequent [Ca\textsuperscript{2+}]\textsubscript{i} response to anoxia or cyanide. These data therefore suggest that part of the [Ca\textsuperscript{2+}]\textsubscript{i} response to anoxia is dependent upon \(\psi_m\).

In analysing these data our first concern was whether the \(\psi_m\) dependent Ca\textsuperscript{2+} response could be an artefact caused by MgATP hydrolysis and elevation of cytosolic Mg\textsuperscript{2+} interfering with [Ca\textsuperscript{2+}]\textsubscript{i} measurement. This seems unlikely for the following reasons i) anoxia and FCCP cause only a small increase in [Mg\textsuperscript{2+}]\textsubscript{i}, of about 0.5 mM from a basal level of 1.5 mM (Henrich and Buckler 2007), ii) the selectivity ratio for Fura-2 (K\textsubscript{dMg}/K\textsubscript{dCa}) is around 53,000 (Lattanzio and Bartschat 1991) which suggests that an elevation of [Mg\textsuperscript{2+}]\textsubscript{i} of 0.5 mM would be equivalent to a rise in [Ca\textsuperscript{2+}]\textsubscript{i} of only 9 nM, iii) in vitro calibration of Fura-2 shows that there is no major effect of Mg\textsuperscript{2+} between 0.3 & 3 mM (Figure 1), and iv) loading neurons with BAPTA almost completely abolishes the [Ca\textsuperscript{2+}]\textsubscript{i} response to FCCP (Figure 1B).

Having excluded significant interference from [Mg\textsuperscript{2+}]\textsubscript{i}, the cause of the sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in response to FCCP (& the FCCP inhibitable component of responses to anoxia and cyanide) is not immediately obvious. If it were due simply to Ca\textsuperscript{2+} release from mitochondria that release would either have to be sustained for several minutes without decrement or other Ca\textsuperscript{2+} clearance pathways would have to be inhibited (otherwise any rise in [Ca\textsuperscript{2+}]\textsubscript{i} would be
transient because of the ongoing activity of Ca\(^{2+}\) pumps). Another possible source of Ca\(^{2+}\) could be displacement from internal buffers due to competition from H\(^+\) and Mg\(^{2+}\) both of which increase in response to anoxia and FCCP (Henrich and Buckler 2007). The fall in pH\(_i\) and the rise in [Mg\(^{2+}\)], however both reach stable levels within a few 10 s of seconds of exposure to anoxia. Thus for a rise in [Ca\(^{2+}\)], to be sustained there would again have to be a co-incident impairment of Ca\(^{2+}\) clearance from the cytosol. Thus whilst Ca\(^{2+}\) release from mitochondria and/or displacement from internal buffers are attractive sources for the FCCP inhibitable part of the anoxia induced rise in [Ca\(^{2+}\)], it is unlikely that either could fully account for a sustained rise in [Ca\(^{2+}\)].

**Effects of anoxia on mitochondrial Ca\(^{2+}\) buffering.**

The current concept of the role of mitochondria in cellular Ca\(^{2+}\) homeostasis is that under conditions in which cytosolic [Ca\(^{2+}\)] is substantially elevated the mitochondrion avidly takes up Ca\(^{2+}\) via the uniporter driven by both the calcium gradient and the mitochondrial transmembrane potential (\(\psi_m\)). This Ca\(^{2+}\) load is then buffered within the inner matrix by complexing with phosphate (Chalmers and Nicholls 2003; Nicholls and Akerman 1982). If [Ca\(^{2+}\)], subsequently declines stored Ca\(^{2+}\) is released back into the cytosol via a Na\(^+\):Ca\(^{2+}\) exchange. This process is clearly demonstrated by evoking voltage gated Ca\(^{2+}\) influx into the cytosol (see e.g. Figure 8). Under these conditions mitochondria take up Ca\(^{2+}\) thus constraining any rise in cytosolic [Ca\(^{2+}\)]. Upon repolarisation, and cessation of Ca\(^{2+}\) influx, [Ca\(^{2+}\)], rapidly falls as Ca\(^{2+}\) continues to be taken up into the mitochondrion until it reaches a “set point” (typically around 0.5 µM (Nicholls 2005, 1978; Nicholls and Scott 1980)) whereupon the mitochondrion ceases to take up Ca\(^{2+}\) and instead begins to release it. From this point onwards the decline in [Ca\(^{2+}\)], is halted whilst Ca\(^{2+}\) is released back into the cytosol and then pumped out of the cell or into other internal stores. This process gives rise to a characteristic “plateau”, or “shoulder” during [Ca\(^{2+}\)], recovery.

Given our observation that anoxia caused partial depolarisation of mitochondria and appeared to induce Ca\(^{2+}\) release under resting conditions, we anticipated that it would have a major impact upon mitochondrial Ca\(^{2+}\) buffering. Surprisingly however anoxia had very little effect on mitochondrial Ca\(^{2+}\) buffering during voltage gated Ca\(^{2+}\) entry as assessed either by the resultant rise in [Ca\(^{2+}\)], or by the amount of Ca\(^{2+}\) stored in the mitochondria (see Figure 8 and results). We are therefore forced to conclude that mitochondrial Ca\(^{2+}\) buffering capacity is relatively insensitive to moderate changes in \(\psi_m\). The dynamics of the mitochondrial Ca\(^{2+}\)
buffering process, specifically the Ca\textsuperscript{2+} release phase, were however substantially slowed by anoxia. Whilst this might indicate that anoxia inhibits mitochondrial Ca\textsuperscript{2+} release, there are other possible explanations (see below).

**Effects of anoxia upon ER Ca\textsuperscript{2+} release.**

Sensory neurons possess significant intracellular Ca\textsuperscript{2+} stores (Verkhratsky 2005). Following store depletion the [Ca\textsuperscript{2+}] response to anoxia was reduced by about 60\% (Figure 3). We also noted that during exposure to anoxia emptying of ER Ca\textsuperscript{2+} stores lowered basal [Ca\textsuperscript{2+}], and refilling the stores raised it again (see Figure 10A). These data strongly suggest that ER Ca\textsuperscript{2+} stores play a major role in mediating the [Ca\textsuperscript{2+}] response to anoxia. These data alone however do not enable us to determine how, or indeed if, anoxia alters ER function. Resting [Ca\textsuperscript{2+}], represents a balance of Ca\textsuperscript{2+} fluxes into and out of the cytosol. Under control conditions CPA also caused a small rise in [Ca\textsuperscript{2+}]. This observation indicates that under resting conditions there is significant cycling of Ca\textsuperscript{2+} into and out of the ER such that upon SERCA inhibition there is a net Ca\textsuperscript{2+} efflux. Studies looking at the effects of inhibition of SERCA upon [Ca\textsuperscript{2+}], levels in the ER itself also reveal a significant background Ca\textsuperscript{2+} leak from those stores (Solovyova and Verkhratsky 2003). Consequently the anoxia induced elevation of [Ca\textsuperscript{2+}], that appears to be dependent upon ER Ca\textsuperscript{2+} stores could result from increased store release, decreased store uptake or simply decreased Ca\textsuperscript{2+} efflux across the plasma membrane (or indeed any combination of these events). Evidence that anoxia enhances net Ca\textsuperscript{2+} release (or leak) from the ER comes primarily from our observation that the caffeine releasable pool of Ca\textsuperscript{2+} progressively declines during anoxia (Figure 9A & B). Moreover, the observation that ryanodine prevented anoxia-induced release of Ca\textsuperscript{2+} from the ER suggests that Ca\textsuperscript{2+} efflux via ryanodine receptors plays an important role in mediating this slow ER Ca\textsuperscript{2+} release. Decreased Ca\textsuperscript{2+} uptake by SERCA must however also be an important factor (see below).

Although we have not investigated possible links between anoxia and ER function in any detail, it is notable that the anoxia induced ER Ca\textsuperscript{2+} release persists in the presence of FCCP. Since the level of FCCP (1 µM) used in this experiment is sufficient to cause near maximal mitochondrial depolarisation (as assessed using Rh123 fluorescence Henrich & Buckler unpublished) it is reasonable to assume that FCCP also fully inhibits mitochondrial ATP synthesis. The ER Ca\textsuperscript{2+} release response to anoxia cannot therefore be due to changes in energy metabolism. We have however noted a very similar response to the electron transport inhibitor cyanide i.e. an abrupt increase in intracellular [Ca\textsuperscript{2+}], that is only partially occluded
by FCCP (see Figure 5B). These observations suggest that inhibition of electron transport might be the key factor in promoting ER Ca\textsuperscript{2+} store release in response to anoxia and cyanide.

A model that has been advanced to explain hypoxic pulmonary vasoconstriction suggests that hypoxia induced increase NADH could stimulate cyclic-ADP-ribose (cADPR) production (Dipp and Evans 2001; Evans and Dipp 2002). cADPR promotes Ca\textsuperscript{2+} release from the endoplasmic reticulum via ryanodine receptors (Galione 1993; Tanaka and Tashjian 1995; Thorn et al. 1994). Hypoxia evoked ER Ca\textsuperscript{2+} release in astrocytes has also recently been attributed to cADPR mediated activation of ryanodine receptors (Aley et al. 2006). In our studies pre-treatment of sensory neurons with 8-Br-cADPR for 10 min significantly reduced [Ca\textsuperscript{2+}]\textsubscript{i} responses to anoxia both in the absence and presence of FCCP (Figure 7) but did not abolish them. This data suggests that at least part of the FCCP resistant [Ca\textsuperscript{2+}]\textsubscript{i} response to anoxia may be due to cADPR mediated Ca\textsuperscript{2+} release via ryanodine receptors. We cannot however exclude the possibility that other mechanisms also contribute to ER Ca\textsuperscript{2+} release. For example it has been suggested that ryanodine receptors can act as an O\textsubscript{2} sensors to evoke Ca\textsuperscript{2+} efflux from the SR in skeletal muscle during severe hypoxia (Eu 2000). Although this has been disputed (Cheong et al. 2005).

**Effects of anoxia on Ca\textsuperscript{2+} clearance.**

Ca\textsuperscript{2+} extrusion from the cytosol following a modest increase in [Ca\textsuperscript{2+}]\textsubscript{i} was significantly and progressively inhibited in the presence of anoxia. Slowing of Ca\textsuperscript{2+} recovery during metabolic inhibition has also been observed in mouse sensory neurons (Duchen et al. 1990).

The PMCA has been reported to be the principal (non-mitochondrial) Ca\textsuperscript{2+} clearance mechanism in DRG (Lu et al. 2006; Usachev et al. 2002). Our studies however indicate that the SERCA also plays a major role in Ca\textsuperscript{2+} clearance (see Figure 13) both in Ca\textsuperscript{2+}-free medium and in normal Ca\textsuperscript{2+}-containing medium (data not shown). Although our data appear at variance with that of others (Usachev et al. 2002) the contribution made by SERCA to Ca\textsuperscript{2+} clearance must depend upon the extent to which ER Ca\textsuperscript{2+} stores are already full. The relative contributions of SERCA vs. PMCA to Ca\textsuperscript{2+} clearance must therefore depend upon the conditions in which such measurements are made. We found no evidence for any Na\textsuperscript{+}\textsubscript{o} dependent Ca\textsuperscript{2+} extrusion (Henrich & Buckler unpublished) and so presume that all CPA insensitive Ca\textsuperscript{2+} extrusion is mediated by the PMCA.
Anoxia appeared to inhibit both SERCA and PMCA mediated Ca\(^{2+}\) clearance equally. This inhibition of Ca\(^{2+}\) clearance may help explain why the actions of anoxia upon \([\text{Ca}^{2+}]\), are relatively sustained. The most likely cause of the inhibition of SERCA and PMCA is impaired cellular energy metabolism. In a parallel study to this we have estimated that in anoxia cytoplasmic [MgATP] falls by about 30% (Henrich and Buckler 2007). Thus changes in ATP concentration and/or phosphorylation potential could influence the activity of Ca\(^{2+}\) pumps. We note that oligomycin, an inhibitor of mitochondrial ATP synthase, has recently been reported to change the dynamics of caffeine induced Ca\(^{2+}\) oscillations in sensory neurons. This effect was also attributed to inhibition of SERCA activity by ATP depletion (Jackson and Thayer 2006). At this stage however we cannot exclude the possibility that other oxygen dependent signalling pathways might also be involved.

**Interactions between the effects of anoxia upon Ca\(^{2+}\) clearance, mitochondrial Ca\(^{2+}\) buffering and ER Ca\(^{2+}\) uptake.**

The effects of anoxia upon Ca\(^{2+}\) clearance lead to some interesting interactions between the various sources & sinks of Ca\(^{2+}\) within the cell. We noted that despite the fact that anoxia appeared to have no major effect upon mitochondrial Ca\(^{2+}\) buffering the time taken for the buffered Ca\(^{2+}\) load to be removed was much increased (see e.g. Figure 8A). Whilst this could be a due to a direct inhibition of mitochondrial Ca\(^{2+}\) release it is also possible that it is simply an indirect consequence of reduced cytosolic Ca\(^{2+}\) clearance via PMCA & SERCA. In support of this hypothesis we noted that when SERCA is inhibited with CPA (10 µM) or Thapsigargin (100 nM) the duration of the mitochondrial Ca\(^{2+}\) release phase following voltage-gated Ca\(^{2+}\) entry was also increased to 228 ± 95% (n = 5, p < 0.05) compared to control (data not shown).

We also noted that despite anoxia inhibiting SERCA activity, ER store loading was as effective under anoxic conditions as under control conditions (Figure 10). One possible explanation for this is that store filling takes place under conditions in which PMCA and SERCA compete for the Ca\(^{2+}\) released from the mitochondrion. With both pumps inhibited to an equivalent degree the dynamic balance between Ca\(^{2+}\) extrusion and ER Ca\(^{2+}\) uptake is probably not significantly altered. The only functional difference therefore is that redistribution of Ca\(^{2+}\) from the mitochondrion to the ER and external milieu takes longer under anoxic conditions. This diversion of Ca\(^{2+}\) entering through voltage-gated Ca\(^{2+}\) channels via the mitochondria seems to be important to achieving efficient ER Ca\(^{2+}\) loading since
inhibition of mitochondrial Ca\textsuperscript{2+} buffering with FCCP substantially reduced caffeine releasable Ca\textsuperscript{2+} stores (Figure 11).

**Conclusions.**

Our studies indicate that anoxia of short to medium term duration, sufficient to partially compromise cellular energy metabolism, has a number of effects upon Ca\textsuperscript{2+} homeostasis in DRG neurons. These include a small $\psi_m$ dependent release of Ca\textsuperscript{2+} from either the mitochondrion itself or other internal Ca\textsuperscript{2+} buffers / stores and a rather more significant Ca\textsuperscript{2+} release from the ER. Anoxia also significantly reduced Ca\textsuperscript{2+} clearance via both the SERCA and PMCA and substantively slowed Ca\textsuperscript{2+} recycling from the mitochondrion (probably as a consequence of inhibition of SERCA and PMCA). Collectively these events cause a sustained rise in $[\text{Ca}^{2+}]_i$. We have also investigated, albeit only relatively perfunctorily, the effects of more severe energy depletion upon Ca\textsuperscript{2+} homeostasis. Removal of both oxygen and glucose leads to a biphasic decline in cellular ATP (Henrich and Buckler 2007). This is reflected in a biphasic rise in cytosolic $[\text{Ca}^{2+}]_i$ and, (after about 15 min in anoxic aglycaemia), profound disturbance of Ca\textsuperscript{2+} homeostasis with near complete failure of Ca\textsuperscript{2+} clearance by both SERCA & PMCA and complete depletion of the ER Ca\textsuperscript{2+} stores.

Assuming that the basic mechanisms of Ca\textsuperscript{2+} regulation in dendrites are similar to those in the soma, we would predict a sequence of changes in Ca\textsuperscript{2+} metabolism in sensory nerve endings during ischemia. With the initial loss of oxygen we would expect Ca\textsuperscript{2+} metabolism to be significantly altered with a sustained elevation in basal $[\text{Ca}^{2+}]_i$ due to store release and with reduced capacity for Ca\textsuperscript{2+} clearance possibly enhancing and/or prolonging $[\text{Ca}^{2+}]_i$, responses to Ca\textsuperscript{2+} entry. The fundamental components of Ca\textsuperscript{2+} regulation however would probably retain basic functionality. In contrast, once cellular reserves of anaerobic sources of energy are used up, there would be a failure of key regulatory mechanisms and consequent further elevation in $[\text{Ca}^{2+}]_i$.

In addition to the above direct effects anoxia upon $[\text{Ca}^{2+}]_i$ regulation we would also anticipate that these events could significantly affect neuronal responses to other ischemic conditions/stimuli. Of particular interest will be to determine the extent to which anoxia influences responses to acidosis since this is thought to be a key stimulus in mediating sensory neuron response to ischemia.
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Disclosures.

None.
FIGURE LEGENDS

Figure 1 Effects of Mg\(^{2+}\) on Fura2-fluorescence in vitro
A, effects of increasing Mg\(^{2+}\) ion concentration on Fura-2 fluorescence ratio in vitro. Ordinate shows both fluorescence ratio and equivalent Ca\(^{2+}\) ion concentration (calculated using standard calibration equation see methods). Data (mean ± S.E.M., n = 3) was obtained from a calibration buffer containing 100 nM free Ca\(^{2+}\) and varying levels of Mg\(^{2+}\). Note that increasing [Mg\(^{2+}\)] to 10 mM and above significantly increases the Fura-2 fluorescence ratio (and thus estimated [Ca\(^{2+}\)]) but at concentrations below this Mg\(^{2+}\) has no significant effect (*p < 0.05; one way ANOVA followed by Post Hoc Bonferroni test). B, effects of elevation of cytosolic [Mg\(^{2+}\)] on [Ca\(^{2+}\)], estimation in an isolated neuron co-loaded with Fura-2-AM and the calcium chelator BAPTA-AM. BAPTA greatly attenuated the effects of depolarisation (50 mM KCl; 10 sec.) induced Ca\(^{2+}\) influx and abolished the effects of caffeine (30 mM) on [Ca\(^{2+}\)], confirming that BAPTA loading was extremely effective in buffering changes in [Ca\(^{2+}\)], (compare with Figures 2,3,8,9,10 & 11). Under these conditions application of FCCP (1 µM) which causes an abrupt increase in cytosolic [Mg\(^{2+}\)] of around 0.4 mM (Henrich & Buckler, under submission) caused only a tiny apparent rise in [Ca\(^{2+}\)], of 7 ± 2 nM (n = 4). The response to capsaicin (100 nM for 10 sec.) confirms the identity of this cell as a sensory neuron.

Figure 2 Effects of anoxia on [Ca\(^{2+}\)]
A, effects of hypoxia and anoxia on [Ca\(^{2+}\)], in a sensory neurons. B, effects of Ca\(^{2+}\)-free medium on the [Ca\(^{2+}\)], response to anoxia. The anoxia induced rise in [Ca\(^{2+}\)], was measured as the difference between baseline [Ca\(^{2+}\)], measured before exposure to anoxia and the [Ca\(^{2+}\)], attained during exposure to anoxia. C, effects of Ni\(^{2+}\) (2.5 mM) on the anoxic evoked rise in [Ca\(^{2+}\)]. D, summary of effects of anoxia on Δ[Ca\(^{2+}\)], defined as the difference between baseline [Ca\(^{2+}\)], and that attained at the end of the anoxic exposure (e.g. as in B), under control conditions, in Ca\(^{2+}\)-free medium and in the presence of 1 mM Ni\(^{2+}\). Time scale bars are 200 sec.
Figure 3 Intracellular Ca\textsuperscript{2+} stores contribute to anoxic rise in [Ca\textsuperscript{2+}]\textsubscript{i}

A, effects of mitochondrial calcium depletion using FCCP (1 \(\mu\)M), on anoxia induced rise in [Ca\textsuperscript{2+}]\textsubscript{i}. FCCP alone induces first a transitory and then a sustained rise in [Ca\textsuperscript{2+}]\textsubscript{i}, (\(\Delta\)FCCP). Following FCCP treatment anoxia induced a further rise in [Ca\textsuperscript{2+}]\textsubscript{i}, to approximately the same level as under control conditions. Note however that the incremental rise in [Ca\textsuperscript{2+}]\textsubscript{i} in response to anoxia (\(\Delta\) anoxia) is reduced in the presence of FCCP (\(n = 11, **p < 0.001\)).

B & C, effects of ER calcium store depletion and reloading on the [Ca\textsuperscript{2+}]\textsubscript{i} response to anoxia. ER Ca\textsuperscript{2+} stores were depleted either by repeated exposure to caffeine (30 mM), or caffeine and CPA (10 \(\mu\)M), in a Ca\textsuperscript{2+}-free medium. Following removal of caffeine and CPA stores were refilled by brief (approx. 5 sec.) exposure to 50 mM extracellular K\textsuperscript{+} in a normal Ca\textsuperscript{2+}-containing medium before obtaining a control response to anoxia. Note that the [Ca\textsuperscript{2+}]\textsubscript{i} response to anoxia is diminished following store depletion compared to that following store reloading.

D, summary of anoxia induced rise in [Ca\textsuperscript{2+}]\textsubscript{i} measured in normal Ca\textsuperscript{2+}-containing medium, in Ca\textsuperscript{2+}-free medium, following mitochondrial Ca\textsuperscript{2+} store depletion (FCCP), and following ER Ca\textsuperscript{2+} store depletion (caffeine or caffeine and CPA). Note that both ER & mitochondrial store depletion significantly (**p < 0.001, Mann-Whitney U-test) diminish the anoxia induced rise in [Ca\textsuperscript{2+}]\textsubscript{i}, but neither fully inhibit it. Time scale bars in A, B & C are 200 sec.

Figure 4 Effects of Ca\textsuperscript{2+} store depletion on anoxia induced [Ca\textsuperscript{2+}]\textsubscript{i} rise

A, B & C, neurons were superfused with a Ca\textsuperscript{2+}-free buffer and then mitochondrial Ca\textsuperscript{2+} stores were depleted by application of FCCP (1 \(\mu\)M), followed by the depletion of ER Ca\textsuperscript{2+} stores using; A, CPA (10 \(\mu\)M); B, Thapsigargin (100 nM); or C, caffeine (30 mM). D, Effects of FCCP and ryanodine (25 \(\mu\)M) on [Ca\textsuperscript{2+}]\textsubscript{i} response to anoxia. E, summary of effects of combining mitochondrial store depletion with either ER store depletion or inhibition of ryanodine receptors on the [Ca\textsuperscript{2+}]\textsubscript{i} response to anoxia. Note that whilst depletion of mitochondrial stores or ER stores alone inhibits the Ca\textsuperscript{2+} response to anoxia by 40-60\%, the combination of mitochondrial store depletion and ER store depletion (or RyR-inhibition) blocks over 80\% of the response to anoxia. Data are means + S.E.M. with number of experiments in parenthesis. Significance was assessed by reference to control data in the same neuron for each individual experiment using Students paired t-test (*p < 0.05, **p < 0.01, ***p < 0.001). Pooled data (all exp.) for FCCP + caffeine, FCCP + CPA, FCCP + caffeine + CPA, FCCP + Thapsigargin and FCCP + ryanodine, was compared against 1) control, 2) FCCP alone, 3) caffeine alone & 4) caffeine + CPA, using Students unpaired t-test. Time scale bars in A-D: 200 sec.
Figure 5. Effects of cyanide on \([Ca^{2+}]_i\)

A & B Effects of 2 mM CN\(^-\) on \([Ca^{2+}]_i\) in a Ca\(^{2+}\)-free medium both in the absence and presence of FCCP (1 µM). CN\(^-\) produces an abrupt increase in \([Ca^{2+}]_i\), similar to that seen with anoxia, indicating Ca\(^{2+}\) release from internal stores. Note that this response is only partially occluded by prior treatment with FCCP. Data on the effects of anoxia alone and anoxia in the presence of FCCP (taken from Figure 3) are included for comparison. Values are mean + S.E.M. with numbers of observations in parenthesis. Statistical significance was assessed using Students t-test (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 6 Effects of anoxia and cyanide on mitochondrial membrane potential

The effects of anoxia and cyanide upon mitochondrial membrane potential were assessed using the fluorescent compound rhodamine 123 (Rh123, see methods). Rh123 fluorescence intensity in A and B is expressed simply as a % of the initial baseline value. In order to quantify the effects of anoxia and cyanide on mitochondrial membrane potential FCCP (1 µM) was applied to obtain reference values for Rh123 fluorescence corresponding to full mitochondrial depolarisation (see methods). The application of either CN\(^-\) (2.5 mM), A, or anoxia B, evoked an increase in Rh123 fluorescence (mitochondrial depolarisation) equivalent to approximately 25% of the maximal FCCP evoked response. Depolarisation in response to both cyanide and anoxia was rapid in onset but reached a stable level within a minute that was sustained throughout the exposure period (approx. 200 sec.).

Figure 7. Effects of anoxia and 8-Br-cADPr on \([Ca^{2+}]_i\)

A & B, effects of pretreatment with 100 µM 8-Br-cADPr (for 10 min prior to experimentation) on \([Ca^{2+}]_i\) response to anoxia and anoxia in the presence of FCCP (experiments conducted in Ca\(^{2+}\)-free Tyrode). Data on the effects of anoxia alone and anoxia in the presence of FCCP (taken from Figure 3) are included for comparison. Values are mean + S.E.M. with number of observations in parenthesis. Statistical significance was assessed using Students t-test (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 8 Effects of anoxia on mitochondrial Ca\(^{2+}\) buffering

A, mitochondrial Ca\(^{2+}\) buffering was assessed by evoking voltage-gated Ca\(^{2+}\) entry with a 5 sec. pulse of a 50 mM potassium Tyrode containing 2.5 mM \([Ca^{2+}]_o\). The initial Ca\(^{2+}\) response was quantified as the difference between resting \([Ca^{2+}]_i\) immediately before, and the maximum \([Ca^{2+}]_i\) attained during, high K\(^+\) exposure. Upon high K\(^+\) removal there is an initial
rapid decline in [Ca\(^{2+}\)], followed by a secondary slow rise and/or plateau in [Ca\(^{2+}\)], that was sustained for several minutes. The amplitude of this “shoulder” was also quantified relative to resting [Ca\(^{2+}\)], immediately before high K\(^+\) exposure. B, effects of abolishing mitochondrial Ca\(^{2+}\) buffering with FCCP (1 µM). C, estimation of mitochondrial Ca\(^{2+}\) uptake during high K\(^+\) exposure. Cells were first exposed to a high K\(^+\) solution containing normal Ca\(^{2+}\) for 5 sec. and then returned to Ca\(^{2+}\)-free medium, immediately following the initial decline in [Ca\(^{2+}\)], i.e. at the onset of the “shoulder”, FCCP was rapidly applied. Mitochondrial Ca\(^{2+}\) uptake/FCCP evoked Ca\(^{2+}\) release was then quantified as the difference between the maximal value of [Ca\(^{2+}\)], attained during FCCP exposure minus baseline [Ca\(^{2+}\)], immediately before application of the high K\(^+\) solution. D, summary of effects of both anoxia and FCCP on initial [Ca\(^{2+}\)], transient amplitude in high K\(^+\) medium. Data are normalised to the control transient amplitude. E, summary of the effects of anoxia on mitochondrial Ca\(^{2+}\) uptake during high K\(^+\) exposure (experimental protocol as in C). All statistical tests were conducted using Paired Students t-test, *p < 0.05. Time scale bar: 200 sec.

**Figure 9 Anoxia and anoxic aglycaemia deplete ER Ca\(^{2+}\) stores**

ER Ca\(^{2+}\) store content was estimated by measuring the amplitude of the [Ca\(^{2+}\)], response to caffeine application (30 mM for 2 min) in a Ca\(^{2+}\)-free medium. When required, ER Ca\(^{2+}\) store loading was facilitated by brief exposure to a high K\(^+\) (50 mM) solution containing normal extracellular [Ca\(^{2+}\)]. A, effects of brief (3 min) anoxia on ER Ca\(^{2+}\) content. B, effects of prolonged anoxia (18 min) on ER Ca\(^{2+}\) content. Note peak response to caffeine was significantly reduced under anoxic conditions compared to that observed before or after anoxia. C, effects of prolonged (18 min) anoxia and aglycaemia on [Ca\(^{2+}\)], and ER Ca\(^{2+}\) content. Note that anoxic aglycemia causes a biphasic rise in [Ca\(^{2+}\)]; following the second phase rise in [Ca\(^{2+}\)], application of caffeine failed to elicit any further rise in [Ca\(^{2+}\)]. The response to caffeine recovered upon reintroduction of both oxygen and glucose. D, summary of effects of anoxia and anoxic aglycaemia on the response to caffeine expressed as a % of the pre-intervention control. Data are means + S.E.M. with number of observations in parenthesis. Statistical significance was assessed using Students paired t-test (**p < 0.01, ***p < 0.0001). Abbreviations; caff. = caffeine. Time bars in all recordings: 200 sec.
Figure 10 Refilling of the ER Ca\textsuperscript{2+} store during anoxia

A, the amplitude of the [Ca\textsuperscript{2+}]\textsubscript{i} response to caffeine application (30 mM for 2 min) in a Ca\textsuperscript{2+}-free medium was used as an assay of the extent of ER Ca\textsuperscript{2+} store filling. ER store loading was facilitated by brief exposure to a high K\textsuperscript{+} solution containing normal extracellular [Ca\textsuperscript{2+}]. A control caffeine response was first determined under normoxic conditions. Stores were then refilled in high K\textsuperscript{+} and the neuron transferred to an anoxic solution. Ca\textsuperscript{2+} release was then evoked a second time by application of caffeine in a Ca\textsuperscript{2+}-free medium. A third pulse of caffeine confirmed that the stores were effectively empty. The neuron was then exposed to high K\textsuperscript{+} solution (containing Ca\textsuperscript{2+}) whilst still in anoxia followed by a fourth exposure to caffeine. Note that this fourth exposure to caffeine evoked a robust [Ca\textsuperscript{2+}]\textsubscript{i} response, similar to first control response demonstrating that the ER Ca\textsuperscript{2+} store can be effectively refilled under anoxic conditions. Finally the cell was returned to normoxia and stores reloaded and then discharged again by sequential application of high K\textsuperscript{+} followed by caffeine. B) summary of effects of anoxia on caffeine response after 3 min of anoxia, following ER store refilling under anoxic conditions and following store refilling post anoxia. Histograms depict the normalised mean values as [%] + S.E.M. (**p < 0.01, Students paired t-test). Numbers of experiments are given in parenthesis.

Figure 11 Refilling of caffeine sensitive Ca\textsuperscript{2+} stores depends partly on mitochondrial function

A, an initial high K\textsuperscript{+} pulse (50 mM for 2 sec.) was applied to facilitate ER Ca\textsuperscript{2+} store filling. The extent of store filling was then assessed by application of caffeine (30 mM) in Ca\textsuperscript{2+}-free medium. A second and third application of caffeine confirmed that the ER stores were now empty. FCCP (1 µM) was then applied to inhibit mitochondrial Ca\textsuperscript{2+} buffering and another a high K\textsuperscript{+} pulse (50 mM in normal Ca\textsuperscript{2+} medium) followed by caffeine was applied to refill and then empty ER Ca\textsuperscript{2+} stores. Finally exposure to FCCP was terminated and ER Ca\textsuperscript{2+} stores again refilled and then discharged by sequential exposure to high K\textsuperscript{+} followed by caffeine. Note reduction in the caffeine response during exposure to FCCP. B, summary of the effects of FCCP on ER store filling/caffeine response obtained using the protocol in A. Responses to caffeine are expressed as a % of the initial control response to caffeine. Values are mean + S.E.M. with number of observations in parenthesis. Statistical significance was assessed using Students paired t-test (*p < 0.05).
Figure 12 Ca$^{2+}$ extrusion during anoxia and anoxic aglycaemia

In order to measure Ca$^{2+}$ extrusion/clearance from the cytosol, cells were subject to a moderate [Ca$^{2+}$]$_i$ increase by brief exposure to a depolarising solution containing 50 mM K$^+$ and low extracellular [Ca$^{2+}$] (250 µM). [Ca$^{2+}$]$_i$ recovery was then observed in a Ca$^{2+}$-free solution as an index of Ca$^{2+}$ extrusion. A, control response to repeated application of high K$^+$, low Ca$^{2+}$ medium. B, effects of anoxia on [Ca$^{2+}$]$_i$ recovery. C, effects of anoxic aglycaemia on [Ca$^{2+}$]$_i$ recovery. D, time dependence of the effects of; ★ control conditions, ▲ anoxia and ○ anoxic aglycaemia on Ca$^{2+}$ extrusion. Data points are mean ± S.E.M. of [Ca$^{2+}$]$_i$ recovery rates calculated between 150 and 200 nM [Ca$^{2+}$]$_i$ (★ control conditions and ▲ anoxia) or between 200 & 250 nM [Ca$^{2+}$]$_i$ (○ anoxic aglycaemia) measured under control conditions and at various time points following introduction of anoxia or anoxic aglycaemia. Statistical significance, relative to control (initial) recovery rates, were determined by one way ANOVA (★★★p < 0.001, ★★p < 0.01, ★p < 0.05).

Figure 13 Ca$^{2+}$ extrusion during CPA application

Neurons were depolarised with a brief (5 sec.) application of 50 mM K$^+$ low Ca$^{2+}$ (250 µM) solution and cytosolic Ca$^{2+}$ clearance was observed in a Ca$^{2+}$-free medium. Ca$^{2+}$ uptake by SERCA was then inhibited with CPA (10 µM). The remaining Ca$^{2+}$ clearance was assumed to represent efflux across the plasma membrane. A, effects of anoxia on CPA resistant Ca$^{2+}$ clearance. B, effects of anoxic aglycaemia on CPA resistant Ca$^{2+}$ clearance. C, effects of anoxia on Ca$^{2+}$ extrusion. Data are mean ± S.E.M. of [Ca$^{2+}$]$_i$ recovery rates (calculated between 150 & 200 nM [Ca$^{2+}$]$_i$) measured under control conditions, in the presence of CPA and at various time points following introduction of anoxia. D, effects of anoxic aglycaemia on Ca$^{2+}$ extrusion. Data are mean ± S.E.M. of [Ca$^{2+}$]$_i$ recovery rates (calculated between 200 & 250 nM [Ca$^{2+}$]$_i$) under control conditions, in the presence of CPA and at various time points following the introduction of anoxic aglycaemia. Each point depicts the mean ± S.E.M. of six (C) or five (D) individual experiments.

Figure 14 Effects of anoxia on PMCA and SERCA mediated Ca$^{2+}$ clearance

Summary of data from Figures 12 & 13. The left bar displays total Ca$^{2+}$ clearance ([Ca$^{2+}$]$_i$ recovery rate) in Ca$^{2+}$-free medium under normoxic conditions (control; 14.8 ± 1.34 nM / sec.; n = 23). Ca$^{2+}$ clearance was reduced to 4.62 nM / sec. by the SERCA inhibitors CPA and Thapsigargin (★★p < 0.01). Exposure of neurons to anoxia (3 min) without CPA or Thapsigargin treatment reduced the total neuronal Ca$^{2+}$ clearance to 8.38 ± 1.1 nM / sec. (n =
CPA resistant Ca$^{2+}$ clearance was also reduced after 3 min exposure to anoxia to 2.2 ± 0.48 nM/sec. (n = 11, ***p < 0.001). Ca$^{2+}$ clearance in the presence of CPA (i.e. CPA resistant) was assumed to correspond to that mediated by the PMCA whereas the CPA sensitive component (control – CPA) was assumed to be mediated by SERCA. Arrows indicate the contribution of each individual Ca$^{2+}$ clearance pathway to the total Ca$^{2+}$ clearance under both normoxic and anoxic conditions. Numbers of experiments are given in parenthesis. Statistical significance was assessed using Students paired t-test, (***p < 0.001, **p < 0.01, *p < 0.05) ¹compared to control (normoxia); ²compared to normoxia / CPA; ³compared to control (normoxia)).
REFERENCES


Figure 1
Figure 2
Figure 3
Figure 4

A) 

B) 

C) 

D) 

E)

Figure 4
Figure 5

A) 

B)
Figure 6

A) Rhodamine 123-fluorescence [%]

B) Rhodamine 123-fluorescence [%]

FCCP, CN⁻, anoxia
Figure 7

A)

B)

**Figure 7**
Figure 8
Figure 9
Figure 10

A) Graph showing changes in $	ext{[Ca}^{2+}]_i$ with different conditions: control, anoxia, and post high K+ recovery.

B) Bar graph showing caffeine response percentages under different conditions: control, anoxia (3 min), anoxia post high K+, and recovery (n = 5).
Figure 11

A) 

B) 

Figure 11
Figure 12
Figure 13
Figure 14