Effects of anoxia, aglycemia, and acidosis on cytosolic Mg\(^{2+}\), ATP, and pH in rat sensory neurons

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Submitted 3 August 2007; accepted in final form 26 October 2007

Henrich M, Buckler KJ. Effects of anoxia, aglycemia, and acidosis on cytosolic Mg\(^{2+}\), ATP, and pH in rat sensory neurons. Am J Physiol Cell Physiol 294: C280–C294, 2008.—Sensory neurons can detect ischemia and transmit pain from various organs. Whereas the primary stimulus in ischemia is assumed to be acidosis, little is known about how the inevitable metabolic challenge influences neuron function. In this study, we have investigated the effects of anoxia, aglycemia, and acidosis on intracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_i\)), and intracellular pH (pHi) in isolated sensory neurons. Anoxia, anoxic aglycemia, and acidosis caused a rise in [Mg\(^{2+}\)]\(_i\) and a fall in pH\(_i\). The rise in [Mg\(^{2+}\)]\(_i\) and a fall in pH\(_i\) appears to be due to cytosolic [Mg\(^{2+}\)]\(_i\) competition for intracellular Mg\(^{2+}\) binding sites. The effects of anoxia and aglycemia were mimicked by metabolic inhibition and, in a dorsal root ganglion (DRG)-derived cell line, the rise in [Mg\(^{2+}\)]\(_i\) during metabolic blockade was closely correlated with fall in intracellular ATP concentration ([ATP]\(_i\)). Increase in [Mg\(^{2+}\)]\(_i\) during anoxia and aglycemia were therefore assumed to be due to MgATP hydrolysis. Even brief periods of anoxia (<3 min) resulted in rapid internal acidosis and a rise in [Mg\(^{2+}\)]\(_i\), equivalent to a decline in MgATP levels of 15–20%. With more prolonged anoxia (20 min) MgATP depletion is estimated to be around 40%. With anoxic aglycemia, the [Mg\(^{2+}\)]\(_i\) rise occurs in two phases: the first beginning almost immediately and the second after an 8–10 min delay. Within 20 min of anoxic aglycemia [Mg\(^{2+}\)]\(_i\) was comparable to that observed following complete metabolic inhibition (dinitrophenol + 2-deoxyglucose, DNP + 2-DG) indicating a near total loss of MgATP. The consequences of these events therefore need to be considered in the context of sensory neuron function in ischemia.

MAGNESIUM is an abundant and essential intracellular ion that can regulate the activity of numerous enzymes, G proteins, ion transporters, and ion channels (62). The majority of magnesium within cells is, however, usually either bound or is located in intracellular organelles (22, 25) such that the concentration of free magnesium ions is relatively low; i.e., 0.3–1.25 mM (29, 44, 65). Moreover, although intracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_i\)) is regulated, transmembrane fluxes of Mg\(^{2+}\) are invariably slow (24, 60, 63). As a consequence, under normal conditions, [Mg\(^{2+}\)]\(_i\), tends to remain fairly constant. One notable exception to this is where there is net ATP hydrolysis (65). ATP has a relatively high affinity for Mg\(^{2+}\) such that almost all ATP within the cell is in the form MgATP\(^{-}\). In contrast, AMP has a low affinity for Mg\(^{2+}\). Thus whenever there is net hydrolysis of MgATP (to ADP and then AMP), magnesium ions are released into the cytosol (39, 50, 65). Thus an increase in cytosolic magnesium is not only invariably a consequence of metabolic compromise but also provides for a simple method with which to monitor changes in cytosolic MgATP (via measurement of [Mg\(^{2+}\)]\(_i\), (32, 43)). Although indirect, this technique has the advantage that one can observe dynamic changes in MgATP levels in real time in a single neuron.

Interference with metabolism can also result in disturbances of cellular pH regulation (11, 14, 58, 77, 78). Changes in intracellular pH (pHi) can have far-reaching consequences for cell function because a great many processes involving enzymes, transporters, ion channels, and signaling pathways are sensitive to pH to varying degrees. In this study we have therefore investigated the effects of ischemia-like conditions, including anoxia, anoxic-aglycemia, and acidosis on [Mg\(^{2+}\)]\(_i\), and pH\(_i\) in sensory neurons from rat dorsal root ganglia (DRG) and nodose ganglia. Our data indicate that a significant rise in [Mg\(^{2+}\)]\(_i\), fall in pH\(_i\), and ATP depletion can occur even during very brief periods of anoxia (up to 3 min), and over longer periods (15–20 min) elevation of [Mg\(^{2+}\)]\(_i\), and decline in ATP can be substantive especially if accompanied by aglycemia.

METHODS

Neuron dissociation. Adult Wistar rats aged between 6 and 8 wk (130–150 g) were euthanized by an overdose of halothane (4%) followed by exsanguination in accordance with schedule 1 of the United Kingdom Animals (Scientific Procedures) act 1986 and United Kingdom Home Office Code of Practice for the Humane Killing of Animals under Schedule 1 of the Animals (Scientific Procedures) Act 1986. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Cervical-thoracic DRG (C2-Th6) and no dose ganglia (NG) were removed under sterile conditions and immediately transferred into ice-cold Ca2+- and Mg2+-free phosphate-buffered saline (PBS), pH 7.4. After being cleaned, the ganglia were incubated at 37°C for 30–35 min in a dissociation media comprising 10 mg collagenase type I (Worthington), 1 mg/trypsin (Sigma), 60 μM CaCl2, and 33 μM MgCl2 in PBS. After the enzyme treatment, ganglia were washed once in PBS (Ca2+- and Mg2+-free) and once in Dulbecco’s modified Eagle’s medium (DMEM, containing 10% fetal bovine serum and 1.2 mM l-glutamine), before mechanical dispersion by trituration in 1.5 ml of DMEM. The dissociated cells were then washed twice by centrifugation (at 1,000 g for 5 min) followed by resuspension in fresh DMEM. After the final wash, the cell pellet was resuspended in 500 μl basal TNB-100 containing Protein-Lipid-Complex (Biochrom, Berlin, Germany), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 1 mM l-glutamine), before mechanical dispersion by trituration in 1.5 ml of DMEM. The dissociated cells were then washed twice by centrifugation at 1,000 g for 5 min followed by resuspension in fresh DMEM. After the final wash, the cell pellet was resuspended in 500 μl basal TNB-100 containing Protein-Lipid-Complex (Biochrom, Berlin, Germany), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10 μM/ml nerve growth factor (NGF). The cell suspension was then seeded onto coverslips coated with poly-l-lysine and laminin and incubated in culture dishes in a humidified chamber at 37°C and 5% CO2-95% air for 2 h. After this incubation period, an additional 3 ml of TNB-100 containing 10 μM/ml NGF was added to each culture dish. The neurons were then kept in the incubator before being used in experiments. Only small-sized neurons with diameters ranging between 15 and 27 μm (from both cervical-thoracic DRG and NG) were used in this study. In parallel studies, >70% of neurons meeting these size criteria responded to brief application of capsaicin (10–100 nM for 10 s) with an increase in intracellular Ca2+ concentration (measured using Fura-2; data not shown).

Culture of F11 cells. To provide a large number of essentially identical cells for the chemical estimation of cellular ATP levels, we used a DRG-derived cell line. The F11 cell line is a mouse neuroblastoma × rat dorsal root ganglia hybridoma (55). The cell line used in these experiments was also stably transfected with TRPV1 (8). F11 cells were the kind gift of Mathias Dreger (Dept. of Physiology Anatomy and Genetics, University of Oxford). F11 cells were cultured in Ham’s F12 media supplemented with Glutamax-1 (Life Technologies), 20% FBS (GIBCO-BRL), 2% hypoxanthine-thymidine-aminopterin supplement (Biochrom KG), 100 μg/ml streptomycin, and 100 μg/ml penicillin at 37°C and 5% CO2 in plastic tissue culture flasks (Nunc, DK).

Fluorescence recording. Fluorescence measurements were performed using an inverted microscope (Nikon) equipped for multi-wavelength epifluorescence microscopy. The excitation light was provided by a 75-Watt xenon lamp plus an appropriate band-pass filter. Mag-indo-1 was illuminated continuously at 340 ± 5 nm (plus >64 × neutral density filtration), and emitted fluorescence was filtered and measured at 405 ± 10 nm and 495 ± 12 nm. Carboxyseminaphthorhodafluor-1 (SNARF-1) was illuminated continuously at 540 ± 12 nm (plus 512 × neutral density filtration), and emitted fluorescence was filtered and measured at 590 ± 5 nm and 640 ± 5 nm. Emitted fluorescence was detected by tri-alkali photomultiplier tubes (PMT Thorn, EMI, UK) that were air-cooled to −20°C. The output from each PMT was fed through a current-to-voltage converter, filtered at 10 Hz, digitized at 250 Hz, and then averaged over 500-ms intervals. Data acquisition and analysis was performed using a CED 1401, or micro 1401, and a PC running Spike 2 software (Cambridge Electronic Design).

Calibration of Mag-indo-1 in saline. Calibration of Mag-indo-1 (Sigma, Dorset, UK) in simple saline calibration solutions was performed at a final concentration of 5 μM (prepared from a stock solution of 1 mg/ml in dimethyl sulfoxide, DMSO). The calibration solutions contained (in mM) 0–150 KCl, 0–100 MgCl2, 5 NaCl, 20 HEPES, and 1 EGTA, pH 7.2, at room temperature (21–23°C or 37°C). MgCl2 and KCl concentrations in these solutions were varied as follows (MgCl2/KCl, in mM), 0:150, 0.3:149.5, 1:148.5, 5:142.5, 10:135, 30:105, and 100:0. The pH sensitivity of Mag-indo-1 was determined by titrating the above solutions to alternative pH values, between 8.0 and 5.5, using KOH or HCl. Fluorescence measurements were obtained from 20- to 200-μl aliquots of the above solutions placed on a glass bottomed culture dish situated on the stage of an inverted microscope. For measurements at 37°C the culture dish was heated, and the temperature of the Mag-indo-1 solution was continuously monitored. Correction for background fluorescence was performed by taking measurements from an aliquot of HEPES solution (pH 7.2) lacking Mag-indo-1.

The calibration equation [Mg2+]i = (R − Rmin)/(Rmax − R) · Ka · Fb/Fa, where R is emission fluorescence ratio F405/F495, was fitted to calibration data using a Marquardt-Levenberg algorithm (Sigma-pplot) to determine all three parameters (i.e., Rmin, Rmax, and Ka · Fb/Fa). These parameters correspond to the fluorescence ratio in the absence of Mg2+ (Rmin), the fluorescence ratio saturating Mg2+ (Rmax), and the product of the dissociation constant for Mg2+ and the ratio of fluorescence at 495 nm for the Mg2+-free form of the dye divided by that for the Mg2+-bound form of the dye (Ka · Fb/Fa).

Dye loading. For in vivo measurements of [Mg2+], or pH, cells were incubated with the acetoxymethyl ester forms of Mag-indo-1 or carboxy-SNARF-1 (all from Molecular Probes, Leiden, NL) at a concentration of 5 μM in either HEPES-buffered Tyrode or bicarbonate-buffered Tyrode solution. Dye loading was carried out at room temperature in a dark chamber for 20 min (Mag-indo-1-AM) or 15 min (SNARF-1-AM).

In situ calibration of Mag-indo-1. In situ calibrations, i.e., in the intracellular environment, were attempted using Mg2+-calibration solutions containing 0–150 mM KCl, 0–100 mM MgCl2, 5 mM NaCl, 1 mM EGTA, 20 mM HEPES, and 1 μM ionomycin (plus other constituents where indicated), pH 7.2. To quench Mag-indo-1 fluorescence, cells were incubated with a solution containing 150 mM KCl, 5 mM NaCl, 1 mM MnCl2 20 mM HEPES, and 1 μM ionomycin, pH 7.2.

In situ calibration of carboxy-SNARF. In the situ calibration of carboxy-SNARF-1 was carried out using the nigericin method as described previously (8). pH calibration solutions contained 140 mM KCl and 10 μM nigericin plus one of the following buffers: 20 mM 2-(N-morpholino)ethane sulfonic acid or 20 mM HEPES or 20 mM 3-cyclohexylaminol-2-hydroxy-1-propanesulfonic acid. The pH of these three solutions was adjusted to 5.0, 7.0, and 9.0, respectively, using HCl or KOH. Neurons were exposed to each of these solutions in order of ascending pH value to determine the calibration constants Rmin, pKd, and Rmax (see Ref. 8).

ATP measurement. For measurement of cellular ATP levels F11 cells were resuspended in a Tyrode solution preequilibrated with 5% CO2 containing either 11 mM glucose (control) or omitting glucose and containing 2–4 dinitrophenol (DNP, 250 μM) and 2-deoxyglucose (2-DOG, 10 mM). Aliquots of 100 μl of this cell suspension were placed in a 96-well plate and then incubated at 37°C in a humidified chamber containing 5% CO2 in the gas phase for 0 (control), 0.5, 1, 2, 3, 5, 8, 11, 15, 18, and 25 min (+DNP and 2-DOG). ATP was measured using a modified luciferase assay (ATPlite, PerkinElmer, Wellesley, MA) and a Wallac 1450 MicroBeta TriLux luminometer according to the manufacturer’s instructions.

Physiological saline solutions. Standard bicarbonate buffered Tyrode solution (in mM) 117 NaCl, 4.5 KCl, 2.5 CaCl2, 1 MgCl2, 24 HCO3, and 11 glucose and was equilibrated with 5% CO2, pH 7.4. Ca2+-free bicarbonate-buffered Tyrode solutions were devoid of CaCl2 and contained 1 mM EGTA. Glucose was replaced in glucose-free solutions by an equimolar amount of sucrose.

Normoxic and normocapnic solutions were prepared by bubbling bicarbonate-buffered Tyrode solution with 5% CO2-95% air. Anoxic solutions were prepared by bubbling Tyrode with 5% CO2-95% N2 for at least 30 min followed by the addition of 0.5 mM Na2S2O4 (64) ~3 min before use. Measurement of bath PO2 using optical oxygen meter (Microx TX2, PreSens, Germany) did not reveal any detectable difference between anoxic solutions in the bath and the zero-point
calibration. All normoxic and anoxic bicarbonate-buffered Tyrode solutions were maintained at 37°C (in a water bath) and a constant pH of 7.4.

Hypercapnic acidosis was achieved by bubbling standard bicarbonate-buffered Tyrode solutions with 20% CO2:80% air (pH 6.8). Metabolic acidosis (pH 7.0) was achieved by reducing NaHCO3 to 10 mM (NaCl was increased to 130 mM to maintain osmolarity) and bubbling with 5% CO2-95% air. To achieve a combined respiratory and metabolic acidosis Tyrode solution with reduced NaHCO3 (10 mM) was bubbled with 20% CO2-80% air (pH 6.2).

Physiological salines were continuously bubbled with gasses in glass bottles in a water bath (at 37°C) and were delivered to the experimental chamber via medical grade stainless steel tubing articulated with short sections of Pharmed tubing (Norton Performance plastics) to ensure the stability of CO2 and O2 levels in these solutions. A zero dead space two-way tap was placed just upstream of the recording chamber to allow change of solutions entering the chamber, and a heater was placed between the tap and the recording chamber to compensate for any temperature loss between solution reservoirs and a heater was placed between the tap and the recording chamber to compensate for any temperature loss between solution reservoirs in the water bath and solutions entering the chamber (thus maintaining the temperature at 37°C in the recording chamber). The recording chamber itself had a low volume (~100 µl) and solution flow rate was ~2 ml/min.

**Drugs.** Carbonyl cyanide p-(trifluromethoxy) phenylhydrazone (FCCP, 1 µM) was added to the experimental buffer from a 10 mM stock solution in DMSO. DNP (250 µM) was added from a stock solution of 250 mM in water. 2-DOG (10 mM) and propionate (10, 15, and 20 mM) were directly added to the experimental buffer solutions (all reagents from Sigma, Dorset, UK).

**Statistics.** Values were expressed as means ± SE. Statistical significance was tested using paired Student’s t-test for cell experiments. Statistical tests on calibration data were performed using one-way analysis of variance, and post hoc analyses were carried out using Bonferroni’s multiple comparison, calculated by *Statistical Package for the Social Sciences* (SPSS) 12.0 software for windows.

## RESULTS

**Mag-indo-1 calibration in situ.** Some calcium indicators exhibit different properties in the intracellular environment to those in simple saline (69). We therefore attempted an in situ calibration in neurons loaded with Mag-indo-1-AM. Exposure to a Ca2+-free depolarizing solution (50 mM K+ containing 1 mM Mn2+ (to promote Mn2+ influx via voltage-gated Ca2+ channels) caused a rapid quenching of the Mag-indo-1 fluorescence signal at 495 nm to 15.5 ± 1.2% of control (n = 4).

Addition of ionomycin (1 µM) caused a slight further quenching of fluorescence at 495 nm to 12.1 ± 2.5% of control (Fig. 2A). Permeabilization of Mag-indo-1-loaded neurons with digitonin also dramatically reduced fluorescence at 405 and 495 nm to 8 ± 2.3% and 1 ± 0.3% (n = 4) of control. These data indicate that there is little interference from cellular autofluorescence or the presence of any form of Mag-indo-1 that is not readily able to bind divalent cations (by virtue of being only partially hydrolyzed, degraded, bound to another molecule, or sequestered in some inaccessible space).

Attempts to estimate Rmax in situ were partially successful. Incubation of Mag-indo-1-loaded neurons in a calibration solution containing 1 µM ionomycin and 100 mM Mg2+ resulted in a progressive increase in fluorescence ratio that failed to reach a steady state within 15 min (Fig. 2D). This situation could not be improved by the use of higher concentrations of ionomycin (65) or by using another ionophore 4-Br-A23187 (59) either alone or in combination with monesin and nigericin. The most likely explanation for this observation is that neither ionophore allows a sufficiently rapid equilibration of large amounts of Mg2+ across the cell membrane. Similar problems have been encountered by others; e.g., Tashiro and Konishi (66) used incubation times of between 5 and 14 h to try to calibrate Mag-fura using 4-Br-A23187, monesin, and nigericin. For our purposes incubation times of this duration were considered too long as both dye leakage and cell swelling would perturb measurements. Switching from a magnesium-containing solution to one containing 10 mM Ca2+, however, resulted in a very rapid increase in fluorescence ratio (Fig. 2D), which stabilized after a few seconds. This reflects the fact that Mag-indo-1 has a much higher affinity for Ca2+ than for Mg2+ (42), and consequently less Ca2+ needs to enter the cell to saturate the Mag-Indo-1. We were therefore able to obtain an in situ value for Rmax with Ca2+ (Rmax, Ca = 1.08 ± 0.14, n = 4) from which we estimated the in situ Rmax for Mg2+ (Rmax,Mg) by multiplying by a small conversion factor (~1.2) derived from the ratio of the values for Rmax,Ca and Rmax,Mg obtained in saline. Using this approach gives a value for Rmax,Mg in situ of ~1.3, which is essentially the same as that obtained in saline (1.3 at pH 7.2). Intracellular calibration of Mag-indo-1 or the related probe furaptra (Mag-fura-2) in cardiac and skeletal muscle has also been shown to yield similar values to those obtained in saline (23, 65, 74).

Attempts to obtain a value for Rmin by incubation in Mg2+- (and Ca2+)-free solutions containing 1 µM ionomycin failed. Although the fluorescence ratio declined significantly in this
calibration solution, a steady-state value was not attained even after 40 min incubation (Fig. 2C), suggesting a failure to completely deplete the cell of Mg$^{2+}$. Consequently, as there was good agreement between $R_{\text{max}}$ values obtained in situ and in saline, we used calibration data obtained in saline, pH adjusted where necessary, for the estimation of [Mg$^{2+}$]$_i$ in this study.

Mag-indo-1 calibration: possible interference from intracellular calcium. Magnesium-sensitive indicators invariably have a higher affinity for calcium than that for magnesium. For Mag-indo-1 the $K_d$ for Ca$^{2+}$ is $\sim$34 $\mu$M, whereas that for Mg$^{2+}$ is 2.03 mM (42). However, [Mg$^{2+}$], is approximately four orders of magnitude greater than intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) (resting [Ca$^{2+}$] $\approx$160 nM in both NG and DRG neurons, Henrich and Buckler, unpublished observations). Thus, under resting conditions, the intracellular concentration of Ca$^{2+}$-bound Mag-indo-1 is approximately 1/100th of that of the Mg$^{2+}$-bound form. Under conditions of metabolic inhibition or anoxia, [Ca$^{2+}$], is increased in these neurons but by no more than about 150 nM in Ca$^{2+}$-o-free conditions (Henrich and Buckler unpublished observations), which would be equivalent to a rise in [Mg$^{2+}$], of $\sim$10 $\mu$M. Thus under the conditions employed in this study, changes in [Ca$^{2+}$], will have no significant effect upon the estimation of [Mg$^{2+}$],.

Correlation between [Mg$^{2+}$], and ATP depletion. To confirm the correlation between depletion of cellular MgATP and rise in [Mg$^{2+}$], we compared a luciferase-based assay of MgATP with measurement of [Mg$^{2+}$], in F11 cells. A neuronal (DRG) cell line was chosen to ensure, as far as possible, that a uniform population of cells would be used for both measurements. F11 cells were exposed to a combination of the uncoupler DNP and the glycolytic inhibitor 2-DOG, and then either [Mg$^{2+}$], was measured continuously by fluorescence microscopy in F11 cells growing on a glass coverslip or ATP content...
was analyzed at specific time points in aliquots of an F11 cell suspension. As can be seen from the results (Fig. 3), there was a strong correlation between ATP depletion and elevation of \([\text{Mg}^{2+}]_i\). Moreover, these data show that exposure to a combination of both uncoupler and 2-DOG results in near-complete (\(\geq 90\%\)) ATP depletion within a relatively short period of time (i.e., \(\geq 11\) min).

Resting levels of free magnesium and \(p\text{Hi}\) in sensory neurons. Under control conditions (bicarbonate-buffered media + 5% \(\text{CO}_2\), \(p\text{H}_0 = 7.4\) at 37°C), \(p\text{Hi}\) for DRG and NG neurons was \(7.13 \pm 0.04\) and \(7.24 \pm 0.05\), respectively. Resting \([\text{Mg}^{2+}]_i\), estimated using \(p\text{H} 7.2\) calibration data obtained in saline, was about 1.5 mM in both types of neuron (Table 1). The removal of extracellular \(\text{Ca}^{2+}\) had no effect upon \([\text{Mg}^{2+}]_i\). The removal of both extracellular \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) caused a slight reduction in \([\text{Mg}^{2+}]_i\), in NG neurons but had no effect in DRG neurons (Table 1), suggesting that at rest transmembrane \(\text{Mg}^{2+}\) fluxes were sufficiently slow as to be unable to rapidly modulate \([\text{Mg}^{2+}]_i\). All of the following experiments are therefore conducted in \(\text{Mg}^{2+}\)- and \(\text{Ca}^{2+}\)-free media to exclude \(\text{Mg}^{2+}\) or \(\text{Ca}^{2+}\) influx from interfering with our measurement of \([\text{Mg}^{2+}]_i\).

Effects of brief anoxia on \([\text{Mg}^{2+}]_i\) and \(p\text{Hi}\). Exposure to brief anoxia led to a rapid increase in Mag-indo-1 fluorescence ratio to an apparent plateau that was sustained throughout the exposure period (3 min, Fig. 4). The mean rise in \([\text{Mg}^{2+}]_i\), at the end of this 3-min anoxic period was \(0.21 \pm 0.04\) mM in NG neurons and \(0.28 \pm 0.02\) mM in DRG neurons. On return to normoxic conditions there was a slow recovery of \([\text{Mg}^{2+}]_i\) toward preanoxic levels, although in 30% of neurons this recovery was incomplete. Brief anoxia also caused a fall in \(p\text{Hi}\) of \(<0.1\) (Fig. 5, Table 2). In about half of the neurons studied, there was a partial recovery of \(p\text{Hi}\) following this initial acidosis, whereas in others it was sustained throughout the anoxic period (see Fig. 5, A–D). To completely exclude the possibility that the observed rise in \(\text{Mg}^{2+}\) during anoxia was an artifact caused by an effect of \(p\text{H}\) upon Mag-indo-1, we recalculated the change in \(\text{Mg}^{2+}\) using calibration data obtained in saline at \(p\text{H} 7.0\) for anoxic conditions and \(p\text{H} 7.2\) for control conditions. This did not substantially change our estimates of the rise in \([\text{Mg}^{2+}]_i\) during anoxia (\(\Delta[\text{Mg}^{2+}]_i = 0.23\) mM in NG neurons and 0.3 mM in DRG neurons see Table 3). Thus even overcompensation for any possible direct \(p\text{H}\) effects on
EFFECTS OF ANOXIA, AGLYCEMIA, AND ACIDOSIS ON [Mg2+]i

Mag- indo-1 did not affect our observation that brief anoxia causes an ~20% increase in intracellular free magnesium.

Effects of prolonged anoxia and anoxic aglycemia on [Mg2+]i and pH. When neurons were exposed to longer periods of anoxia (20 min), we observed a further, slower increase in [Mg2+]i (see, e.g., Fig. 4B). Prolonged anoxia caused little further acidification, compared with brief anoxia, in either NG or DRG neurons (Fig. 5 and Table 2). After 20 min of anoxia, the rise in [Mg2+]i, (calculated using pH 7.0 calibration data) was 0.43 mM in NG neurons and 0.69 mM in DRG neurons (Table 3). Exposure to prolonged anoxia in the absence of glucose (anoxic aglycemia) resulted in an increase in [Mg2+]i, (by 1.35 mM in DRG and 0.67 mM in NG, Table 3) that was larger than that seen in anoxia alone (P < 0.05 for both DRG and NG neurons). Under these conditions, the rise in [Mg2+]i was often biphasic (Fig. 4, C and F). Anoxic aglycemia did not, however, have a greater effect on pH than anoxia alone (Fig. 5, C and F, and Table 2).

Effects of metabolic inhibition on [Mg2+]i, and pH. Since the above experiments were conducted in Mg2+-free media, the rise in [Mg2+]i must derive from internal sources. The most likely source is release of Mg2+ formerly bound to ATP into the cytosol as a consequence of metabolic inhibition and net ATP hydrolysis. To confirm that inhibition of energy metabolism would indeed cause a rise in [Mg2+]i in these cells, we tested the effects of two uncouplers. FCCP induced a rapid increase in Mag-indo-1 fluorescence ratio (Fig. 6D) and a substantial acidification of ~0.5 pH units (Fig. 6A and Table 2). With the use of calibration data obtained at pH 6.5 in saline for Mag-indo-1 (to compensate for the fall in pH), the FCCP-induced rise in [Mg2+]i was ~0.4 mM (Table 3). This rapid rise in [Mg2+]i, is consistent with the expectation that FCCP would not only halt oxidative phosphorylation but probably also accelerate ATP hydrolysis via the mitochondrial ATP synthase. In view of the acidification caused by FCCP, however, we sought to confirm this result using another mitochondrial uncoupler 2,4-DNP. DNP had a smaller effect on pH than FCCP (Fig. 6B and Table 2) but also caused a rapid rise in [Mg2+]i of 0.65 and 0.37 mM in DRG and NG, respectively (Fig. 6E and Table 3). At the concentrations used both uncouplers appeared equally effective in depolarizing sensory neuron mitochondria (as assessed using Rhodamine-123, Buckler and Henrich, unpublished observations), but FCCP appeared to be a more effective protonophore at the cell membrane causing a greater intracellular acidification.

To further assess the effects of inhibition of energy metabolism on [Mg2+]i, without unnecessary complications from changes in pH, we therefore chose to use DNP rather than FCCP as an inhibitor of oxidative phosphorylation. Combining DNP with glucose removal and 2-DOG to inhibit both oxidative phosphorylation and glycolysis caused a similar rise in [Mg2+]i during brief exposure (3 min) to that observed in response to DNP alone (Fig. 6, E and F, and Table 3). The fall in pH within a 3-min exposure to DNP + 2-DOG was slightly smaller than that seen with DNP alone (Table 2). This might reflect a reduction in the production of acid by anaerobic metabolism. Longer exposure (20 min) to this solution caused a much larger increase in [Mg2+]i (1.2 and 0.65 mM in DRG and NG neurons, respectively, see Tables 2 and 3).

Effects of anoxia and metabolic inhibition on [Mg2+]i in Na+-free conditions. Intracellular Mg2+ concentration in most cells is normally regulated via a plasma membrane Na+/ Mg2+ exchanger (63, 68) albeit slowly. To determine the maximum rise in [Mg2+]i, attainable with complete ATP depletion in the absence of any Mg2+ extrusion, we exposed neurons to a combination of DNP, aglycemia and 2-DOG in Na+-free solution until the rise in [Mg2+]i reached a maximum (~20 min, see Fig. 7C). Under these conditions free [Mg2+]i, rose by 2.1 and 1.1 mM in DRG and NG neurons, respectively (see Table 3). This rise in [Mg2+]i, was significantly greater than that seen in Na+-containing saline (P < 0.05 for both DRG and NG neurons). In contrast, the rise in [Mg2+]i following 20 min of anoxia in Na+-free conditions was smaller (0.5 mM in DRG and 0.3 mM in NG, see Table 3 and Fig. 7D). Na+-free conditions alone had no significant affect upon resting [Mg2+]i.

Effects of Na+-removal on intracellular pH. The removal of sodium from the extracellular solution under normoxic conditions caused only a very small and relatively slow cellular acidification; in DRG neurons pH fell from 7.17 (~0.02) to 7.15 (~0.02, n = 7, P < 0.05) and in NG neurons pH fell from 7.15 (~0.02) to 7.12 (~0.02, ns) over a 3-min interval. The
degree of acidosis caused by anoxia was little affected by removal of extracellular sodium (Fig. 7 B), but the acidification caused by DNP + 2-Dog was significantly greater in Na/H-free Tyrode compared with normal Tyrode (P < 0.001 in DRG, P < 0.05 in NG, see Table 2).

Effects of intracellular acidosis on [Mg2+]i. The above data clearly suggest that a major source of rise in [Mg2+]i in response to anoxia is likely to be due to release from hydrolyzed ATP. In all of our experiments involving anoxia or metabolic inhibitors we also observed a fall in pHi albeit, in most cases, by only a small amount (0.1–0.3 pH units). Although such a change in pHi is unlikely to have a significant impact upon the accuracy of our measurement of [Mg2+]i, acidosis could directly alter [Mg2+]i in these cells through actions on Mg2+ buffering or transport. We therefore investigated the effects of intracellular acidification alone upon [Mg2+]i in magnesium-free media by the addition of the weak acid propionate at 10, 15, and 20 mM. In addition, we also subjected neurons to physiological acid challenges, such as might be encountered during ischemia, using hypercapnic acidosis (high CO2, pH 6.8), isocapnic acidosis (low HCO3−, pH 7.0), mixed acidosis (high CO2 and low HCO3−, pH 6.2). All of these maneuvers induced a fall in pHi (Fig. 8, A–D). Intracellular acidosis was most marked with the hypercapnic and mixed acidosis, which caused a rapid fall in pHi of ~0.4 (see Table 4). This effect is probably due to the rapid permeation and hydration of CO2. Any subsequent pHi recovery during these experiments was notably slow (see Fig. 8, C and D). In each case, we then used the measured pHi (Table 4) to determine the most appropriate pH-corrected Mag-indo-1 calibration data to use to estimate corresponding changes in [Mg2+]i (Table 5). With all methods used to induce intracellular acidification, we observed a small rise in [Mg2+]i (Fig. 8, E–H, and Table 5), the magnitude of which was closely correlated with the size of the intracellular acidosis (Fig. 9). Thus internal acidification alone appears to also have a direct effect upon [Mg2+]i (see DISCUSSION).

DISCUSSION

In the present study we investigated the effects of anoxia and aglycemia on [Mg2+]i, as an index of ATP depletion in sensory neurons, and upon pHi. Evaluation of the suitability of Mag-indo-1 revealed potential limitations to its use under very acid conditions but not within the range of pHi encountered in this study. We observed rapid changes in both [Mg2+]i, and pHi in response to anoxia and anoxic aglycemia. In addition we found that intracellular acidosis alone increases cytosolic magnesium. The significance of these observations is discussed below.

Resting intracellular magnesium concentration and effects of removal of extracellular magnesium. Resting [Mg2+]i, for both types of neuron was ~1.5 mM, which is higher than previously reported in DRG neurons from newborn rats, between 0.26 and 0.48 mM; (21, 35). In these latter studies neurons were cultured for 3–7 days, and [Mg2+]i was determined at room temperature using Mag-fura-2. The disparity between our and other studies could therefore reflect differences in age, culture conditions, temperature, or the indicator used. Similar differences between [Mg2+]i, measurements made using Mag-indo-1 and Mag-fura-2 have also been observed in cardiac myocytes (65, 67).
Removal of extracellular magnesium has little effect on 
\([\text{Mg}^{2+}]_\text{i}\) (Table 1) suggesting that resting transmembrane 
\(\text{Mg}^{2+}\) fluxes in these neurons are relatively slow. Similar 
observations have been reported in smooth muscle (56) and 
cardiac myocytes (65).

**Interaction between neuronal pH and \([\text{Mg}^{2+}]_\text{i}\).** Exposure of 
sensory neurons to hypercapnic acidosis, isocapnic acidosis, 
or the weak acid propionate caused an intracellular acidification 
with concomitant elevation of \([\text{Mg}^{2+}]_\text{i}\) (Fig. 8, A–H, and 
Tables 4 and 5). Compensation (or even over compensation) 
for any minor effects of \(\text{pH}\) upon \(\text{Mag}-\text{indo-1}\) did not signifi-
cantly alter our estimates of this rise in \([\text{Mg}^{2+}]_\text{i}\). We therefore 
believe this rise in \([\text{Mg}^{2+}]_\text{i}\), to be a genuine effect of intracell-
ular acidosis. It was nevertheless noted that the time course of 
change in \([\text{Mg}^{2+}]_\text{i}\) was exceptionally closely correlated with 
the change of \(\text{pH}_\text{i}\) during both the application of an acid 
challenge and upon its removal. This is particularly apparent 
when comparing the effects of isocapnic acidosis, where 
changes in \(\text{pH}_\text{i}\) and \([\text{Mg}^{2+}]_\text{i}\) were relatively slow (Fig. 8, B and 
F), with hypercapnic acidosis where change in \(\text{pH}_\text{i}\) and 
\([\text{Mg}^{2+}]_\text{i}\) occur very rapidly (Fig. 8, C, D, G, and H). Indeed 
during hypercapnic acidosis the temporal correlation between 
the anticipated rapid change in \(\text{pH}_\text{i}\) and the change in \([\text{Mg}^{2+}]_\text{i}\), 
is striking (see Fig. 9A). We also noted a simple linear relationship 
between change in \(\text{pH}_\text{i}\) and \([\text{Mg}^{2+}]_\text{i}\), in both neuron 
groups (Fig. 9, D and E). Because these experiments were 
conducted in the absence of external magnesium (and calcium), 
the rise in \([\text{Mg}^{2+}]_\text{i}\) cannot be attributed to magnesium 
(or calcium) influx and must therefore originate from some 
internal source. From the extreme rapidity of the changes in 
\([\text{Mg}^{2+}]_\text{i}\), that occur upon addition and removal of weak acids 
\(\text{CO}_2\) and propionate see Fig. 8, E, G, H, and Fig. 9A), the most 
likely explanation is that protons compete for, and displace 
\(\text{Mg}^{2+}\) ions from, cytosolic binding sites/buffers. A similar 

close association between \(\text{pH}_\text{i}\) and \([\text{Mg}^{2+}]_\text{i}\), has also been 
reported in leech neurons and was attributed largely to \(\text{H}^+\) 
displacing \(\text{Mg}^{2+}\) from \(\text{MgATP}\) (26). In our study, however, 
even if \([\text{MgATP}]_\text{i}\) were 10 mM, a fall in \(\text{pH}_\text{i}\) from 7.2 to 6.8 
would release <0.2 mM \(\text{Mg}^{2+}\) (see Ref. 47), this is half the 
observed rise in \([\text{Mg}^{2+}]_\text{i}\), in response to a hypercapnic acidosis. 
Rise in intracellular \(\text{H}^+\) must therefore cause displacement of 
\(\text{Mg}^{2+}\) from other binding sites not just \(\text{MgATP}\) (see below).

**Effect of metabolic inhibition and anaerobiosis upon \(\text{pH}_\text{i}\).** When 
sensory neurons were exposed to anoxia or DNP (or cyanide, 
data not shown), there was a small reversible fall in \(\text{pH}_\text{i}\), similar 
in magnitude to that reported in carotid body type I cells (9), 
brainstem neurons of the nucleus tractus solitari, ventrolateral 
medulla, inferior olive and hypoglossal neurons (11), and 
hippocampal neurons (14, 78) but smaller than in dorsal vagal 
neurons (58) and cardiac myocytes (48). There are a number of 
possible causes for such a fall in \(\text{pH}_\text{i}\) including: 1) the gener-
ation of \(\text{H}^+\) from net hydrolysis of ATP, 2) anaerobic metab-
olism and lactate production, 3) enhanced acid influx, and 
4) the inhibition of acid extruding pathways, e.g., as described 
for hippocampal neurons (77, 78). In addition, from the obser-
vation that acidosis can displace \(\text{Mg}^{2+}\) from internal buffering 
sites, the converse must also occur; i.e., a rise in magnesium 
should displace protons from these same buffer sites.

Although we have not explicitly investigated these mecha-
nisms, we have made a number of observations that may shed 
some light on this issue. We noted that upon exposure to 
\(\text{Na}^+\)-free media, the fall in \(\text{pH}_\text{i}\) was very slow (see RESULTS). As 
\(\text{Na}^+\)-removal leads to the inevitable inhibition of \(\text{Na}^+\)-depend-
ent acid efflux, this maneuver gives an approximate measure.

Fig. 5. Change in intracellular (\(\text{pH}_\text{i}\)) under anoxic and anoxic aglycemic conditions. A–C: recordings of \(\text{pH}_\text{i}\) using 
carboxyseminalaphthorhodafluor-1 (SNARF-1) in DRG neurons in response to brief anoxia (A), prolonged anoxia (B), and anoxic aglycemia (C). D–F: recording of \(\text{pH}_\text{i}\) in NG neurons exposed to brief anoxia (D), prolonged anoxia (E), and anoxic aglycemia (F). Note that even brief anoxia induced a fall in intracellular \(\text{pH}\) in both neuron types. Time scale bars in all recordings: 200 s.
Table 2. Effects of anoxia, anoxic aglycemia, and metabolic inhibitors on neuronal pH_i

<table>
<thead>
<tr>
<th></th>
<th>Anoxia (3 min)</th>
<th>Anoxia (20 min)</th>
<th>Anoxia Na⁺ Glucose (20 min)</th>
<th>Anoxia 0 Glucose (20 min)</th>
<th>FCCP (3 min)</th>
<th>DNP (3 min)</th>
<th>DNP + 2-DOG (3 min)</th>
<th>DNP + 2-DOG (20 min)</th>
<th>DNP + 2-DOG Na Free (20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG</td>
<td>-0.08±0.01‡ (11)</td>
<td>-0.13±0.07* (5)</td>
<td>-0.12±0.03* (8)</td>
<td>-0.44±0.1† (5)</td>
<td>-0.25±0.04† (5)</td>
<td>-0.05±0.01† (6)</td>
<td>-0.07±0.01† (6)</td>
<td>-0.25±0.01‡ (6)</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>-0.09±0.01† (6)</td>
<td>-0.09±0.02* (5)</td>
<td>-0.12±0.02† (6)</td>
<td>-0.03±0.04† (6)</td>
<td>-0.23±0.04† (5)</td>
<td>-0.14±0.01† (6)</td>
<td>-0.02±0.01† (6)</td>
<td>-0.29±0.05† (6)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE, with n given in parentheses. Change in intracellular pH (pHi) (ΔpHi) is measured as the difference between a steady-state (control) pH value obtained before any experimental intervention and the minimum pH level attained during exposure to anoxia, anoxia + 0 glucose, or metabolic inhibitors. Statistical comparison between control (preintervention) pH and the minimum pH obtained during the intervention was performed using a paired Student’s t-test. *P < 0.05, †P < 0.01, ‡P < 0.001.

Table 3. Effects of anoxia, anoxia + 0 glucose, and metabolic inhibitors on neuronal [Mg²⁺]_i

<table>
<thead>
<tr>
<th></th>
<th>Anoxia (3 min)</th>
<th>Anoxia (20 min)</th>
<th>Anoxia Na⁺ Free (20 min)</th>
<th>Anoxia 0 Glucose (20 min)</th>
<th>FCCP (3 min)</th>
<th>DNP (3 min)</th>
<th>DNP + 2-DOG (3 min)</th>
<th>DNP + 2-DOG (20 min)</th>
<th>DNP + 2-DOG Na Free (20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG</td>
<td>+0.30±0.02† (21)</td>
<td>+0.69±0.11† (5)</td>
<td>+0.52±0.12† (6)</td>
<td>+1.35±0.31* (6)</td>
<td>+0.39±0.08‡ (29)</td>
<td>+0.65±0.06† (6)</td>
<td>+0.66±0.12† (6)</td>
<td>+1.26±0.14† (6)</td>
<td>+2.08±0.35† (6)</td>
</tr>
<tr>
<td>NG</td>
<td>+0.23±0.05† (19)</td>
<td>+0.43±0.10* (6)</td>
<td>+0.33±0.03* (6)</td>
<td>+0.67±0.03 (6)</td>
<td>+0.36±0.05‡ (18)</td>
<td>+0.37±0.07† (6)</td>
<td>+0.37±0.06† (6)</td>
<td>+0.65±0.05‡ (6)</td>
<td>+1.07±0.15† (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE, with n given in parentheses. Change in intracellular Mg²⁺ ([Mg²⁺]_i) is calculated as the difference between a steady-state (control) value obtained before any experimental intervention and the maximum (or steady state) level attained during exposure to anoxia, anoxia + 0 glucose, or metabolic inhibitors. [Mg²⁺]_i was calculated using calibration data obtained in saline at 37°C and at the following pH: pH 7.2, control; pH 7.0, anoxia (3 and 20 min), and anoxia 0 glucose; pH 6.5, FCCP, DNP, and DNP + 2-DOG. Statistical comparison between control (preintervention) and the maximal value obtained during the intervention was performed using a paired Student’s t-test. *P < 0.05, †P < 0.01, ‡P < 0.001.
of background acid loading due to acid-equivalent influx and metabolic acid generation under normal control conditions. The observation that the application of anoxia (Fig. 7B) causes a much more rapid cellular acidification than does Na\(^+\) removal (see e.g., Fig. 5) clearly indicates that anoxia must increase acid influx or acid generation or H\(^+\) displacement from internal buffers (or a combination thereof). This rapid initial acidification soon slows, however, and a near steady-state acidosis is then attained (the exact profile of pH change was variable between neurons some showing a partial recovery following the initial acidosis; see Fig. 5). Although this could reflect activation of acid efflux pathways by the fall in pHi (70),

Fig. 6. Effects of metabolic inhibitors on [Mg\(^{2+}\)]\(_i\) and pH\(_i\) in sensory neurons. A and D: effect of the mitochondrial uncoupler FCCP (1 \(\mu\)M, for 3 min) on pH\(_i\) and intracellular Mg\(^{2+}\). B and E: effect of mitochondrial uncoupler DNP (250 \(\mu\)M) on pH\(_i\) and [Mg\(^{2+}\)]. C and F: effects of combined application of the mitochondrial uncoupler DNP (250 \(\mu\)M) and the inhibitor of glycolysis 2-DOG (10 mM) on pH\(_i\) and [Mg\(^{2+}\)]. All traces were recorded in DRG neurons; similar results were also obtained for NG neurons. Time scale bars: 100 s. [Mg\(^{2+}\)]\(_i\) was determined using calibration data obtained in saline at 37°C and pH 6.5.

Fig. 7. Effects of anoxia and metabolic inhibition on pH\(_i\) and [Mg\(^{2+}\)]\(_i\) in Na\(^+\)-free solution. A and C: effects of DNP (250 \(\mu\)M) plus 2-DOG (10 mM) on pH\(_i\) and [Mg\(^{2+}\)]\(_i\) in Na\(^+\)-free media. B and D: effects of anoxia on pH\(_i\) and [Mg\(^{2+}\)]\(_i\) in Na\(^+\)-free media. Traces are examples for neurons from both ganglion groups; all the traces are taken from DRG neurons. Time scale bars: 200 s. [Mg\(^{2+}\)]\(_i\) was calculated using calibration data obtained in saline at 37°C and pH 7.0.
thus countering the enhanced acid load, the observation that
the (20 min) anoxia-induced acidosis was not significantly
enhanced in Na+-free medium suggests that this is unlikely.
Our preferred explanation at present is that the initial
acidification in response to anoxia represents a predominantly
transient acid load. After this event any sustained acidosis may
be due to a much lesser continuing acid loading process and/or
inhibition of acid extrusion. A rapid transient acid load in
response to anoxia would also help explain why a partial pH
recovery was observed in some neurons (e.g., Fig. 9B).

All of the above pathways may therefore be involved in
mediating the observed changes in pH, in response to pro-
longed anoxia. Clearly, however, further studies are required
to evaluate the precise causes and duration of the acid load and
to evaluate the effects of anoxia upon Na+-dependent acid extru-
}

**Effects of metabolic inhibition on [Mg2+]i.** Exposure to
conditions that are likely to cause energy depletion lead to an
increase in [Mg2+]i. As these experiments were conducted in
Mg2+-free media, this must reflect Mg2+ release from internal
sources and is believed to be due to the hydrolysis of MgATP
to Mg2+ + AMP.

The association between decline in ATP levels and elevation
in [Mg2+]i, during metabolic inhibition was confirmed in the

**Table 4. Effect of acidosis and weak acids on pH, in neurons from DRG and NG**

<table>
<thead>
<tr>
<th>Source</th>
<th>ΔpHΔ</th>
<th>ΔpHΔ</th>
<th>ΔpHΔ</th>
<th>ΔpHΔ</th>
<th>ΔpHΔ</th>
<th>ΔpHΔ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate 10 mM</td>
<td>-0.06 ± 0.01 † (6)</td>
<td>-0.07 ± 0.01 † (6)</td>
<td>-0.11 ± 0.01 † (6)</td>
<td>-0.04 ± 0.01 † (5)</td>
<td>-0.39 ± 0.07 † (5)</td>
<td>-0.42 ± 0.08 † (5)</td>
</tr>
<tr>
<td>Propionate 15 mM</td>
<td>-0.09 ± 0.01 † (6)</td>
<td>-0.11 ± 0.01 † (6)</td>
<td>-0.15 ± 0.008 † (6)</td>
<td>-0.08 ± 0.02 † (5)</td>
<td>-0.37 ± 0.01 † (5)</td>
<td>-0.44 ± 0.05 † (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE, with n in parentheses. ΔpH, in response to acidifying conditions was determined as the difference between the steady-state pH, before exposure to acidifying conditions and the minimal value for pH, attained during the application of acidifying conditions. Weak acids were applied in Tyrode solutions at pH 7.4 (as for control), pH7.0 solutions were prepared by lowering [HCO3-], pH6.8 solutions were prepared by raising CO2 (to 20%) and, pH6.2 solutions were prepared by both lowering [HCO3-], and raising CO2 (see Materials and Methods). Statistical comparison between control (preintervention) pH, and the minimum pH, obtained during the intervention was performed using a paired Student’s t-test. *P < 0.05, †P < 0.01, ‡P < 0.001.
Table 5. Effect of acidosis and weak acids on [Mg^{2+}], in neurons from DRG and NG

<table>
<thead>
<tr>
<th></th>
<th>Propionate 10 mM</th>
<th>Propionate 15 mM</th>
<th>Propionate 20 mM</th>
<th>pH 7.0</th>
<th>pH 6.8</th>
<th>pH 6.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG</td>
<td>+0.11±0.02 (6)</td>
<td>+0.12±0.02 (6)</td>
<td>+0.18±0.03 (6)</td>
<td>+0.10±0.03* (5)</td>
<td>+0.48±0.11* (7)</td>
<td>+0.55±0.11* (7)</td>
</tr>
<tr>
<td>NG</td>
<td>+0.06±0.01 (6)</td>
<td>+0.08±0.01 (6)</td>
<td>+0.09±0.004 (6)</td>
<td>+0.12±0.03 (8)</td>
<td>+0.37±0.05 (6)</td>
<td>+0.61±0.101 (7)</td>
</tr>
</tbody>
</table>

Values are means ± SE, with n given in parentheses. △[Mg^{2+}] as a response to extracellular acidosis or weak acids was determined as the difference between the control preintervention [Mg^{2+}], and the maximal value of [Mg^{2+}], attained during exposure to acidosis or weak acids. Control [Mg^{2+}], was calculated using calibration data obtained in saline at 37°C and pH 7.2. [Mg^{2+}], levels in the presence of propionate (10, 15, and 20 mM) and pH = 7.0 were calculated using calibration data obtained in saline at pH 7.0. [Mg^{2+}], levels in the presence of pH = 6.8 and 6.2 were calculated using calibration data obtained in saline at pH 6.5. Statistical comparison between control (preintervention) pH and the minimum pH obtained during the intervention was performed using a paired Student’s t-test. *P < 0.05, †P < 0.01, ‡P < 0.001.

The maximum rise in [Mg^{2+}], observed with total metabolic blockade (DNP + 2-DOG) was increased in Na^{+}-free media, whereas the small rise caused by anoxia was unaffected by sodium removal. These data suggest that a large increase in [Mg^{2+}], leads to the activation of a Na^{+}-dependent Mg^{2+}-extrusion. This is consistent with the view that Mg^{2+}-extrusion in mammalian cells is J mediated via a Na^{+}/Mg^{2+} exchanger (63, 68) and 2) highly [Mg^{2+}], dependent (71).

[Mg^{2+}], and magnesium buffering. As previously discussed, the effects of acidosis upon [Mg^{2+}], can only be accounted for if a significant proportion of intracellular Mg^{2+} is bound to intracellular buffers. Magnesium buffering also appears to occur when Mg^{2+} is released from MgATP. Even when Na^{+}-dependent Mg^{2+} extrusion was blocked the maximum rise in [Mg^{2+}], seen with complete metabolic blockade (DNP + 2-DOG) was only 1 and 2 mM for NG and DRG neurons, respectively. This increase in [Mg^{2+}], is less than would be expected given normal neuronal [MgATP] of around 3 mM (16, 19). Whereas it is conceivable that ATP levels may be lower in sensory neurons than in CNS neurons, it is more likely that the rise in [Mg^{2+}], is simply constrained by Mg^{2+}-buffering. Similarly, in cardiac myocytes the maximum increase in [Mg^{2+}], following metabolic inhibition is limited to around 1.5–2 mM (65). In chick ventricular myocytes, the apparent magnesium-buffering power during metabolic inhibition is 2.5; i.e., for every 2.5 Mg^{2+} ions released by MgATP hydrolysis, only 1 is free in solution and the other 1.5 ions bind to other buffers (39). If we assume the Mg^{2+}-buffering capacity in sensory neurons to be the same, we can estimate [MgATP], to be between 2.5 and 5 mM for nodule and DRG neurons respectively.

Magnesium buffering has only been extensively characterized in erythrocytes (18) where it is mediated primarily by ATP, 2,3 DPG, and hemoglobin (57). Although the nature of magnesium buffers in other cells has yet to be established, a number of binding sites for magnesium are known. In myocytes, creatine phosphate binds Mg^{2+} (20, 27), studies in brain suggest that phospholipids are also major binding sites for Mg^{2+} (49) and some cytosolic proteins e.g., troponin C, calmodulin, S100 protein, and actin [all of which are expressed in sensory neurons (54, 61, 76)], can also bind Mg^{2+}. Many of these also bind calcium so their contribution to Mg^{2+} buffering may well depend on [Ca^{2+}], (10, 51, 52, 73). In this respect it is noteworthy that Mg^{2+} buffering in chick myocytes was lower in the presence of external calcium than in its absence (39).

Time course of ATP depletion in sensory neurons during anoxia and anoxic aglycemia as assessed by change in [Mg^{2+}], It is notable that in response to anoxia the rise in [Mg^{2+}], begins almost immediately. This is in marked contrast to resting cardiac myocytes where there is a delay of several min between exposure to hypoxia or metabolic inhibitors and rise in [Mg^{2+}], (6, 37, 65). This suggests that energy reserves, probably in the form of phosphocreatine, are much greater in muscle than in sensory neurons.

In response to prolonged anoxia or metabolic blockade, the rise in [Mg^{2+}], was mostly monophasic but in aglycemic anoxia there were often two distinct phases the first immediate the second beginning after a delay (Fig. 4, C and F). The maximum rise in [Mg^{2+}], was also greater in aglycemic anoxia than in anoxia alone (Table 3) but equivalent to that seen with metabolic blockade (compare Table 3 anoxia 0 glucose with DNP/2-DOG + Na^{+}). These observations suggest that under anoxic conditions the fall in ATP is probably limited by anaerobic glycolysis. In the absence of exogenous glucose, however, decline in ATP levels can only be constrained for a brief period, presumably while utilizing endogenous carboxylate reserves, before there is a further dramatic fall in ATP. The mean delay between applying anoxic aglycemia and the secondary rise in [Mg^{2+}], was 8.8 (±0.3; n = 6) min and 10.6 (±1; n = 6) min for DRG and NG neurons, respectively. These values are comparable to measurements in the hippocampus, wherein endogenous glucose, glycogen, and other glycolytic intermediates can support basal metabolic requirements for around 5.5 min after metabolic inhibition and removal of glucose (1).

If we assume J that ATP depletion is near complete following prolonged exposure to DNP + 2-DOG (as in the F11 cell line, see Fig. 3), 2) that Mg^{2+} buffering is relatively independent of [Mg^{2+}], and 3) that Mg^{2+} extrusion is minimal, we can derive a relative scale for ATP depletion by expressing rise in [Mg^{2+}], as a percentage of that seen with DNP + 2-DOG (measured under Na^{+}-free conditions). Using this approach, we obtained the following estimates: after 3 min of anoxia ATP levels fall by 14% and 21% in DRG and NG, respectively, and after 20 min, anoxia ATP levels fall by about 33% and 40%. We performed these calculations using data obtained for anoxia in normal Na^{+}-containing media because Na^{+}/K^{+}-pump activity is a major consumer of ATP (2). Arguably these measurements could underestimate ATP depletion if there is loss of Mg^{2+} due to Na^{+}/Mg^{2+} exchange. The rise in [Mg^{2+}], caused by anoxia in Na^{+}-free conditions was
not, however, any greater than that in Na\(^+/\)H\(^{-}\)-containing solutions; indeed it was slightly less (which is consistent with reduced ATP consumption in Na\(^+/\)H\(^{-}\)-free conditions).

In summary, we have utilized the magnesium indicator Mag-indo-1 and the pH indicator SNARF-1 to investigate the effects of ischemia-like conditions upon [Mg\(^{2+}\)]\(_i\), [H\(^+\)]\(_i\), and ATP levels in isolated sensory neurons. Our data indicate that in response to anoxia there is a rapid initial rise in [Mg\(^{2+}\)]\(_i\) that most probably results from a fall in MgATP to 75–80% of control within minutes. This is followed by a slower rise in [Mg\(^{2+}\)]\(_i\) suggesting that after 20 min, MgATP levels are reduced to around 60–70% of control. The consequences of short periods of aglycemic anoxia are initially comparable to those of anoxia alone, but after 8–10 min there is another abrupt and dramatic rise in [Mg\(^{2+}\)]\(_i\), similar to that seen with complete metabolic blockade suggesting near-total MgATP depletion. Thus with concomitant loss of both oxygen and glucose endogenous energy reserves can partially defend neuronal ATP levels but only for a relatively short period of time. These changes in [Mg\(^{2+}\)]\(_i\) suggest that there may be two distinct phases to changes in neuronal energy metabolism in response to ischemia: the first occurring almost immediately upon oxygen depletion in which there is a fall in [MgATP] and is then maintained until anaerobic substrates are used up at which point the remaining MgATP is rapidly exhausted. In addition to changes in [Mg\(^{2+}\)]\(_i\), there was a small, but very rapid, cellular acidification that suggests a transient generation of acid from within the cell upon loss of oxygen. We also

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Fig. 9. Interaction between pH\(_i\) and [Mg\(^{2+}\)]\(_i\). A: overlay of pH\(_i\) (shaded lines) and [Mg\(^{2+}\)]\(_i\), (solid lines) responses to the application and subsequent removal of a hypercapnic acidosis (20% CO\(_2\), pH 6.8). B: overlay of pH\(_i\) and [Mg\(^{2+}\)]\(_i\), recordings during brief application of anoxia. Note that although the onset of change in both [Mg\(^{2+}\)]\(_i\) and pH\(_i\) is rapid, the fall in pH\(_i\) soon reaches a maximum (and in some cases subsequently recovers slightly), whereas the rise in [Mg\(^{2+}\)]\(_i\) continues. C and D: correlation between the fall in pH\(_i\) and rise in [Mg\(^{2+}\)]\(_i\) (determined using pH-corrected calibration data; see Tables 3 and 5) for acidosis (○) and anoxia and metabolic inhibitors (×) in DRG neurons (C) and NG neurons (D). Solid line represents linear regression to acidosis (○) data alone. Note that there is a very strong correlation between the extent of the fall in pH\(_i\) and the rise in [Mg\(^{2+}\)]\(_i\), under conditions of acidosis but not during anoxia or metabolic inhibition. Acidosis data (○) were obtained using propionate (10, 15, and 20 mM) isocapnic acidosis pH\(_o\) = 7.0, hypercapnic acidosis pH\(_o\) = 6.8, and a mixed acidosis pH\(_o\) = 6.2. Metabolic inhibition and anoxia data were obtained using DNP (250 μM) alone for ~3 min, DNP + 2-DOG (10 mM) for 20 min, anoxia for 3 min, anoxia for 20 min, and anoxia and aglycemia for 20 min. Interruption in A: 60 s. Recordings in A and B are taken from DRG neurons; the displayed recordings are representative for both ganglion groups. Time scale bar in A: 10 s, in B: 25 s.
observed that acidosis alone caused a small increase in [Mg^{2+}]. Because the effects of anoxia and extracellular acidosis on both cytosolic pH and [Mg^{2+}], are likely to be additive, true ischemic conditions might produce even more profound changes in [Mg^{2+}], and pH. Although the time course of these events may well be different in vivo, even short periods of ischemia could produce the anoxic conditions necessary to initiate these events. Increased [Mg^{2+}], acidosis, and ATP depletion are likely to affect many aspects of cell function, including ion channel activity, cell signaling, and ionic homeostasis. The functional consequences of these metabolic events for neuronal responses to ischemia therefore need to be investigated.

ACKNOWLEDGMENTS
We thank Dr. Robert Wilkins for his kind advice regarding ATP measurement and Dr. Mathias Dreger for the gift of the F11 cell line.

GRANTS
This work was supported by a grant from the Wellcome Trust (to K. J. Buckler) and a DFG research fellowship (HE3678/1-1 to M. Henrich).

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