Hypoxic Remodeling of Ca\(^{2+}\) Stores in Type I Cortical Astrocytes*

Ian F. Smith, John P. Boyle, Leigh D. Plant†, Hugh A. Pearson‡, and Chris Peers§

From the Institute for Cardiovascular Research and the §School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom

Prolonged periods of hypoxia are deleterious to higher brain functions and increase the likelihood of developing dementias. Here, we have used fluorimetric techniques to investigate the effects of chronic hypoxia (2.5% O\(_2\), 24 h) on Ca\(^{2+}\) stores in type I cortical astrocytes, because such stores are crucial to various astrocyte functions, including Ca\(^{2+}\)-dependent modulation of neuronal activity. Rises of [Ca\(^{2+}\)]_i, evoked by exposure of astrocytes to bradykinin were enhanced following chronic hypoxia, as were transient increases in [Ca\(^{2+}\)]_i recorded in Ca\(^{2+}\)-free perfusate. The enhanced responses were due partly to impaired plasmalemmal Na\(^+/\)Ca\(^{2+}\) exchange following chronic hypoxia. More importantly, chronic hypoxia increased the Ca\(^{2+}\) content of mitochondria (as determined by exposing cells to mitochondrial inhibitors), such that they were unable to act as Ca\(^{2+}\) buffers following bradykinin-evoked Ca\(^{2+}\) release from the endoplasmic reticulum. Hypoxic enhancement of mitochondrial Ca\(^{2+}\) content was also observed in confocal images of cells loaded with the mitochondrial Ca\(^{2+}\) indicator, Rhod-2. Confocal imaging of cells loaded with tetramethylrhodamine ethyl ester, an indicator of mitochondrial membrane potential, indicated that mitochondria were hyperpolarized in astrocytes following chronic hypoxia. Our findings indicate that hypoxia disturbs Ca\(^{2+}\) signaling in type I astrocytes, primarily by causing mitochondrial Ca\(^{2+}\) overload.

Higher brain functions are susceptible to damage through exposure to the prolonged hypoxia of ischemia or chronic cardiorespiratory disease (1–3). Indeed, there is a well documented increased incidence of dementias in patients who have previously suffered prolonged hypoxic or ischemic episodes arising as a consequence of cardiovascular dysfunction such as stroke or arrhythmia (4–6). Such a clear link between hypoxic/ischemic episodes and increased incidence of dementias strongly suggests that lack of oxygen is a contributory factor in the precipitation of such diseases.

Many non-neuronal cell types (particularly astrocytes) contribute to intercellular signaling in the central nervous system at several levels (7–9). Astrocytes, as well as other glia, are in intimate contact with neurones and have projections that are located at neuronal synapses (10). Indeed, chemical synapses and gap junction connections between astrocytes and neurones have been identified (11, 12). Astrocytes possess receptors for numerous transmitters (e.g. glutamate, γ-aminobutyric acid (GABA), acetylcholine, ATP, bradykinin; reviewed in Ref. 7) and so play important, active roles in synaptic activity. Activation of astrocytes by transmitters released from neurones has been reported at levels of transmitter concentrations found outside (but adjacent to) synaptic clefts (13, 14). Astrocytic activation is usually manifest as a rise of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]_i) due to release of Ca\(^{2+}\) from internal stores as well as Ca\(^{2+}\) uptake from the extracellular space (9, 14–16). This fundamental initial response correlates with neuronal synaptic activity, and a rise of [Ca\(^{2+}\)]_i, in one astrocyte can initiate Ca\(^{2+}\) waves that propagate across significant distances via adjacent astrocytes (17–19). This represents a means of intercellular signaling in the brain that parallels and modulates classical neuronal synaptic communication and, as such, is of fundamental importance to central neuronal activity (8, 20). Within an individual astrocyte, a rise of [Ca\(^{2+}\)]_i, can also initiate important processes. In particular, elevated [Ca\(^{2+}\)]_i, triggers glutamate release which modulates neuronal activity via extrasynaptic metabotropic glutamate receptors (19, 21). Indeed, astrocytes are capable of releasing glutamate via regulated, Ca\(^{2+}\)-dependent exocytosis, in addition to reverse-mode uptake systems (17).

In the present study, we have examined how intracellular Ca\(^{2+}\) stores, key components in astrocyte Ca\(^{2+}\) signaling coupled to receptor activation via generation of inositol trisphosphate, are modulated by prolonged hypoxia. Hypoxia is a key feature of numerous cardiorespiratory diseases associated with disturbance of higher brain functions (see above), and is also a well known regulator of gene expression (22). Our results indicate that hypoxia dramatically modulates intracellular Ca\(^{2+}\) stores, primarily by causing mitochondrial Ca\(^{2+}\) loading.

MATERIALS AND METHODS

Astrocyte Culture—To obtain astrocytes, cerebral cortices were removed from 6–8 day old Wistar rat pups and placed immediately in ice-cold buffer solution consisting of 10 mM NaH\(_2\)PO\(_4\), 2.7 mM KCl, 137 mM NaCl, 14 mM glucose, 1.5 mM MgSO\(_4\), and 3 mg/ml bovine serum albumin. Meninges were removed using fine forceps, and whole cortices were then minced gently with a mechanical tissue chopper (McIlwain) and dispersed into the same buffer containing 0.25 μg/ml trypsin, at 37°C for 15 min. Trypsin digestion was halted by the addition of an equal volume of buffer supplemented with 16 μg/ml soy bean trypsin inhibitor (SBTI, type 1-S; Sigma), 0.5 μg/ml DNase I (EC 3.1.21.1 type II from bovine pancreas; 125 kilounits/ml; Sigma) and 1.5 mM MgSO\(_4\), The tissue was then pelleted by centrifugation at 1300 rpm for 90 s following which the supernatant was removed and the cell pellet resuspended in 2 ml of buffer solution containing 100 μg/ml SBTi, 0.5 μg/ml DNase I, and 1.5 mM MgSO\(_4\). The tissue was subsequently triturated gently with a fire polished Pasteur pipette. After allowing larger pieces of tissue to settle for 5 min, the cell suspension was taken and centrifuged at 1300 rpm for 90 s before resuspension into 60 ml of culture medium (Eagle’s minimal essential medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (GIBCO)). The cell suspension was then aliquoted into 2 × 25 cm\(^2\) flasks and onto glass cover slips in 6- and 24-well tissue culture plates. Cells were then kept

* This work was supported by The Wellcome Trust, The Medical Research Council, and Pfizer Central Research, through a CASE studentship (to I. F. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Inst. for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, UK. Tel.: 0113-343-4174; Fax: 0113-233-4803; E-mail: c.s.peers@leeds.ac.uk.

§ To whom correspondence should be addressed: Inst. for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, UK. Tel.: 0113-343-4174; Fax: 0113-233-4803; E-mail: c.s.peers@leeds.ac.uk.

This paper is available on line at http://www.jbc.org

4875
in a humidified incubator at 37 °C (95% air, 5% CO2). This was designed to passag e 1 and cells were used up to a passage of 2. 4–6 h following plating, cells were washed vigorously several times with fresh medium to remove non-adhered cells. This resulted in a culture of primarily type I cortical astrocytes (as confirmed by positive immunostaining with an anti-GFAP antibody). Culture medium was exchanged every 3–4 days, and cells were grown in culture for up to 14 days. All recordings were made from cells between days 5–12.

Cells exposed to chronic hypoxia were subcultured in the same way as control cells but 24 h prior to experimentation were transferred to a humidified incubator equilibrated with 2.5% O2, 5% CO2 balanced with N2 (termed chronically hypoxic (CH) conditions). Following exposure to hypoxia, cells were kept in room air for no longer than 1 h while microfluorimetric recordings took place. Cells were maintained in a 95% air, 5% CO2 incubator for the same period.

MTT Assay—Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay (23). Absorbance was measured using a spectrophotometer at a test wavelength of 570 nm and reference wavelength of 630 nm. Student’s t test (unpaired) was used to determine the significance of differences in absorbance.

Microfluorimetric Recordings—To measure cytosolic [Ca2+], glass coverslips onto which cells had grown were incubated in 2 ml of control solution containing 4 μM Fura-2-AM for 1 h at 21–24 °C in the dark, as previously described (24). Control solution was composed of NaCl 135 mM, KCl 5 mM, MgSO4 1.2 mM, CaCl2 2.5 mM, Hepes 5 mM, and glucose 10 mM (pH 7.4, osmolarity adjusted to 300 mosM with sucrose, 21–24 °C). Following this incubation period, fragments of coverslips were transferred into an 80-μl recording chamber mounted on the stage of an inverted microscope, where cells were continuously perfused under control and hypoxic conditions. Invert a group of cells using the region of interest (ROI) tool. TMRE (tetra-methylrhodamine ethyl ester; NCX, Na+/Ca2+ exchanger; r.u., relative units; BK, bradykinin; CPZ, cyclopiazonic acid; ER, endoplasmic reticulum; FCCP, p-trifluoromethoxy carbonyl cyanide phenylhydrazone.

RESULTS

Augmentation of [Ca2+], Responses to Bradykinin Caused by Chronic Hypoxia—Fig. 1A shows representative, bright-field images of type I cortical astrocytes cultured under normoxic (upper) or hypoxic (lower) conditions. The cells were typically flat and elongated in appearance, and hypoxia did not noticeably alter their morphology. In order to examine cell viability under hypoxic conditions, we performed colorimetric MTT assays (23), and found that hypoxia (24–48 h) had no significant effect on cell viability as compared with cells cultured under normoxic conditions (Fig. 1B).

Basal Ca2+ levels determined before bradykinin (BK) application were not significantly different between chronically hypoxic (CH) and control cells, being 0.53 ± 0.03 r.u. in control cells (n > 12) and 0.58 ± 0.02 in CH cells (n > 12). When astrocytes were perfused with a solution containing 2.5 mM Ca2+, bath application of 100 nM BK evoked a rapid rise of [Ca2+], due to release of Ca2+ from internal stores mediated by production of inositol trisphosphate, which declined with an extremely slow time course in both control and CH cells (Fig. 1C). This slow decline is likely due to Ca2+ influx (see Fig. 2, below). Full concentration-response relationships are presented in Fig. 1D, which indicates that the EC50 for BK was similar in both groups of cells, at ~5 nM. However, it was notable that peak BK-evoked responses were consistently greater in CH cells. Since the peak of the response reflects Ca2+ release from intracellular stores, rather than subsequent Ca2+ influx, the rest of the study employed Ca2+-free perfusates (containing 1 mM EGTA) to examine release from stores in isolation, and we selected 100 nM BK as a maximally effective agonist concentration.

Chronic Hypoxia Potentiates Liberation of Ca2+ from Intracellular Stores by Bradykinin—In Ca2+-free solution, responses to 100 nM BK were transient in both control (Fig. 2A, left) and CH (Fig. 2A, right) cells. However, a clear enhancement of responses was observed in CH cells. The bar graphs of all measured parameters are presented in Fig. 2B. Thus, as was the case in Ca2+-containing perfusates, basal levels re-
three cells were measured.

...completed stores more rapidly than the irreversible Ca2
...zonic acid (CPZ). We found in these cells that this agent de-
...observed in CH cells, as compared with controls.

...transient response) were both significantly greater in CH
...amount of Ca2
...application of BK following depletion of stores with either CPZ
...a greater store size. To investigate this, we discharged intra-
...could have been due to a more complete discharge of Ca2
...being that modula-
...Chronic Hypoxia Inhibits Plasmalemmal Na+/Ca2+

...chronically hypoxic conditions (open circles) or chronically hypoxic conditions (hatched bars) for the time periods indicated. Data are normalized to control levels of cell viability. C, sample increases of [Ca2+]i, evoked by application of 100 nm BK for the period indicated by the horizontal bars in control (upper) and chronically hypoxic (C.H., lower) type I cortical astrocytes. Scale bars apply to both traces. D, concentration-response relationships indicating peak rises in [Ca2+]i, evoked by varying concentrations of BK in control (open circles) and chronically hypoxic (filled circles) astrocytes. Each point plotted represents the mean ± S.E. response, n = 3–6 experiments in each case, from each of which at least three cells were measured.

...hanced responses could be due to altered buffering of cytosolic Ca2+
...ar from stores. One such mechanism to account for the tran-
...sponse (Fig. 4, A) to release from intracellular stores by bradykinin. A, sample rises in [Ca2+]i, evoked by application of 100 nm BK for the period indicated by the horizontal bars in control (left) and chronically hypoxic (C.H., right) type I cortical astrocytes. For these experiments, the perfusate was nominally Ca2+-free. Scale bars apply to both traces. B, bar graphs indicating mean values of parameters measured from record-
...passenger exemplified in A: basal [Ca2+]i, levels, peak responses to 100 nm BK, the integral of the transient rise of [Ca2+]i, evoked by BK and the time taken for the peak to decline to 50% of its maximal value. Data are means ± S.E. taken from control recordings (open bars) and chronically hypoxic cells (hatched bars). Statistical differences between control and chronically hypoxic cells are indicated by p values above each graph (n = 3–6 experiments in each case, from each of which at least four cells were measured).

...thapsigargin fully discharged intracellular stores.

...thesis data of Fig. 2 suggest that BK-sensitive Ca2+

...evoked by BK and the time course of decay (Fig. 4B). In CH cells, no significant differences were observed in either the amplitude or time course of the response (Fig. 4, A and B). These results indicate that NCX is not the major mechanism for Ca2+ extrusion following release from intracellular stores in astrocytes, and also that modulation of such exchange cannot account for the enhanced responses observed in CH cells. However, perhaps more importantly, the significant influence of NCX in shaping the
responses seen in control cells was not present in CH cells.

**Chronic Hypoxia Potentiates Mitochondrial Ca^{2+} Loading**—Recent studies have indicated that inhibition of NCX can arise due to excessive Ca^{2+} loading of mitochondria (26). We therefore explored the possible involvement of mitochondria in the enhancement of BK-evoked rises in [Ca^{2+}], Fig. 5A (left) illustrates a recording from a control cell, which was firstly exposed to the mitochondrial inhibitor FCCP (10 µM), which was applied together with 2.5 µM/µl oligomycin (to prevent ATP consumption by the F_{1}F_{0}-ATP synthase functioning in reverse mode). This caused a transient rise of cytosolic [Ca^{2+}], evoked by CPZ. In the continued presence of FCCP and oligomycin, application of 100 nM BK evoked rises in control cells that were significantly greater than those evoked without mitochondrial inhibition (Fig. 2). Responses in CH cells (e.g. Fig. 5A, right) differed from those seen in control cells in two important aspects. Firstly, mitochondrial inhibition caused significantly greater rises of [Ca^{2+}], (mean data shown in Fig. 5B) and secondly, the subsequent application of BK evoked rises in [Ca^{2+}], which were not significantly different from those observed in CH cells in the absence of mitochondrial inhibitors. It is also noteworthy that during mitochondrial inhibition, there were no significant differences in the responses to BK observed between control and CH cells, as determined by integration of the transients.

Data presented in Fig. 5 suggest that the cytosolic Ca^{2+} response to BK may be attenuated in control (but not CH) cells due to Ca^{2+} uptake by mitochondria. In further support of this idea, responses to 10 µM CPZ were also significantly potentiated (from 9.15 ± 0.84 r.u. (n = 14) to 19.1 ± 2.56 r.u., p < 0.002, n = 18) during mitochondrial inhibition with FCCP (10 µM) and oligomycin (2.5 µg/ml), as Fig. 6 illustrates.

The enhanced response to FCCP and oligomycin observed in CH cells (Fig. 5A, right) strongly suggested that CH caused excessive mitochondrial Ca^{2+} accumulation, as compared with controls. To investigate this in more detail, we examined focal images of control and CH cells loaded with the mitochondrial Ca^{2+} indicator, Rhod-2, with Mitotracker. Clearly, mitochondria form extensive, complex networks in both control and chronically hypoxic cells, and the mitochondrial density did not appear altered by chronic hypoxia. Fig. 7B shows typical images taken under identically matched exposure conditions in control and CH astrocytes, at two levels of magnification. Mitochondria are evident from their distribution and shape throughout the cell (see for example Ref. 27), although the Rhod-2 images appear more fragmented that the images obtained with Mitotracker. Clearly, fluorescence is greater in the CH cells. The dashed lines in each upper trace show the point at which a line scan was performed, and the corresponding pixel intensities are plotted in Fig. 7C. Peaks in each trace correspond to the line scan crossing mitochondria, and such peaks are consistently greater in CH cells, reflecting a higher mitochondrial Ca^{2+} content. It is noteworthy that while the peaks in the line scans were greater in amplitude in CH astrocytes, the absolute number was similar, indicating that hypoxia did not increase the number or density of mitochondria. These traces were typical of at least six different images of both control and CH astrocytes. While not quantitative, these data further support the Fura-2 data of Fig. 5 indicating that CH causes increased mitochondrial Ca^{2+} loading.

**Chronic Hypoxia Hyperpolarizes Mitochondrial Membrane Potential**—Accumulation of Ca^{2+} by mitochondria is dependent on the maintenance of the mitochondrial membrane potential,
Mitochondrial inhibition enhances CPZ-evoked Ca\textsuperscript{2+} transients. Representative rises in [Ca\textsuperscript{2+}], evoked by bath application of cyclopiazonic acid (CPZ, 10 µM which was present for the period indicated by the horizontal bar) in untreated controls (taken from Fig. 3 for clarity) and in control cells during continued exposure to 10 µM FCCP and 2.5 µg/ml oligomycin (which were applied 1 min before the commencement of the traces), as indicated.

\( (\Psi_m) \). We therefore examined whether \( \Psi_m \) differed between control and CH cells, using the \( \Psi_m \) indicator, TMRE. As can be seen from Fig. 4A, confocal images were consistently brighter in CH cells as compared with control cells. While individual mitochondria could not be resolved with the clarity found using Rhod-2 (Fig. 7), staining appeared more punctate in CH cells, consistent with greater accumulation into specific organelles. Furthermore, the regions of brightest intensity (in both control and CH cells) were found in parts of cells close to nuclei. This is also reflected in the line scan plots of Fig. 8B (made using lines indicated in the corresponding images), where in each case peaks are seen either side of a trough in the plot which represents scanning over the nuclear region of the image.

These peaks are clearly greater in the CH cells, an observation consistently seen in at least six separate experiments.

We also employed TMRE to monitor fluorogenic signals using conventional (non-confocal) imaging, while cells were under continual perfusion. Again, fluorescence observed in CH cells was clearly greater than in control cells, and the brightest regions of each cell were found in cytosolic areas close to the nucleus (Fig. 9A). Despite continual loss of signal, we could also observe an increased rate of signal loss in both cell types when exposed to 10 µM FCCP in the presence of 2.5 µg/ml oligomycin (Fig. 9A, lower traces, and Fig. 9B). These findings strongly suggest that CH causes a hyperpolarization of \( \Delta \Psi_m \), an effect which is most likely responsible for the increased mitochondrial accumulation of Ca\textsuperscript{2+} indicated by experiments described earlier.

**DISCUSSION**

Cellular effects of cerebral hypoxia/ischemia have received intense interest over many years, not only because of the associated neuronal damage and death (28, 29), but also because prolonged, milder hypoxic episodes can lead to deleterious effects on higher brain functions (1–3) and also to an increased likelihood of subsequent development of dementias (4–6). The
majority of studies to date have focused on the effects of hypoxia/ischemia on central neurones, with perhaps less attention given to astrocytes or other, non-neuronal cell types in the central nervous system. The importance of astrocyte function to central intercellular signaling is currently receiving increasing attention (see the Introduction), in part due to emerging physiological regulation of neuronal synaptic transmission (11–14).

Since Ca\(^{2+}\) signaling is one of the major forms of communication between astrocytes and is also a major factor in the physiological activity of individual astrocytes, we have addressed the question of whether prolonged hypoxia can modulate such signaling, using primary cultures of rat type I cortical astrocytes, a well established system for studying astrocyte function, and their robust responses to BK (30–32). Our initial observation was that BK evoked significantly greater release of Ca\(^{2+}\) from intracellular stores in CH cells than was observed in control cells (Fig. 2). This was not due to altered intracellular signaling between BK receptors and Ca\(^{2+}\) stores, since CPZ also liberated more Ca\(^{2+}\) from stores in CH cells (Fig. 3). Likely possibilities to account for this were that the BK-sensitive (endoplasmic reticulum; ER) stores contained greater levels of Ca\(^{2+}\) following chronic hypoxia or that, once liberated from the ER, Ca\(^{2+}\) was less efficiently cleared and so could accumulate in the cytosol to a greater concentration. Mechanisms for cytosolic Ca\(^{2+}\) clearance include re-uptake into ER stores or other organelles, and Ca\(^{2+}\) extrusion via NCX or Ca\(^{2+}\)-ATPase. Re-uptake into the ER was deemed unlikely in the continued presence of agonist, and so we firstly investigated a possible role of NCX. This was also prompted by a recent report indicating that ongoing, acute hypoxia inhibits NCX in vascular smooth muscle (33). Results presented in Fig. 4 indicate that in control cells, NCX plays a significant role in shaping the transient rise of cytosolic [Ca\(^{2+}\)] when ER stores are discharged with BK. By contrast, NCX appeared to be non-functional in CH cells (Fig. 4). Thus, CH somehow appeared to inhibit NCX in cortical astrocytes. Importantly, however, the absence of functional NCX could not account fully for the enhanced cytosolic rise in [Ca\(^{2+}\)] seen in response to BK application in CH cells.

At present, the underlying mechanisms accounting for inhibition of NCX by prolonged hypoxia remain to be determined. However, a recent report has demonstrated that accumulation of Ca\(^{2+}\) by mitochondria specifically inhibits NCX in COS cells transfected with a bovine Na\(^+\)/Ca\(^{2+}\) exchanger (26). On the basis of this report, we investigated the Ca\(^{2+}\) content of mitochondria in astrocytes. Fig. 5 clearly demonstrates that CH leads to excessive Ca\(^{2+}\) loading of mitochondria, as evidenced by the enhanced response to application of FCCP and oligomycin, in cortical astrocytes loaded with the mitochondrial Ca\(^{2+}\) indicator, Rhod-2 (Fig. 7). Additionally, the subsequent exposure to BK (and also to CPZ; Fig. 6) in the continued presence of mitochondrial inhibitors caused a markedly increased rise of cytosolic [Ca\(^{2+}\)] in control cells, while responses in CH cells were unchanged. These data indicate that, in control astrocytes, BK-evoked rises in [Ca\(^{2+}\)] are limited due to Ca\(^{2+}\) buffering into mitochondria. This finding is in accordance with numerous studies, which have documented the close anatomical and functional interactions of mitochondria and the ER (e.g. Refs. 34–36). Importantly, Ca\(^{2+}\) buffering into mitochondria does not appear to occur in CH cells, presumably because the mitochondria in these cells already contain excessive amounts of Ca\(^{2+}\) and so are incapable of acquiring more. Such a suggestion is
supported strongly by the Rhod-2 confocal images and associated pixel intensity lines scans of Fig. 7, which demonstrated marked increases in punctate staining in CH cells. Such mitochondrial accumulation of Ca$^{2+}$ is most likely due to mitochondrial hyperpolarization (upon which mitochondrial Ca$^{2+}$ accumulation is dependent), a view supported by both confocal and conventional images acquired using the indicator TMRE. This dye accumulates in mitochondria in proportion to the $\Psi_m$, the more hyperpolarized $\Psi_m$ is, the more dye accumulates. Clearly, in CH cells, increased (and more punctate) fluorescence was observed which reflects a hyperpolarization of mitochondria in astrocytes following a period of prolonged hypoxia.

Thus, the present study indicates that CH causes mitochondrial hyperpolarization, which is likely to account for excessive accumulation of Ca$^{2+}$, although a possible additional effect of hypoxia to inhibit mitochondrial Na$^+$/Ca$^{2+}$ exchange has not been discounted, and is worthy of future study. Two important consequences arise from this mitochondrial Ca$^{2+}$ overload: firstly, via a mechanism yet to be identified, mitochondrial Ca$^{2+}$-overload inhibiting the plasmalemmal NCX (see also Ref. 26). Secondly, Ca$^{2+}$-overloaded mitochondria are unable to participate in buffering of Ca$^{2+}$ liberated into the cytosol from the ER following agonist application. The mechanism(s) by which prolonged hypoxia leads to hyperpolarization of $\Psi_m$ will be the focus of future work. A recent study has suggested that $\Psi_m$ can be hyperpolarized by Ca$^{2+}$-dependent dephosphorylation of cytochrome c oxidase (37). This “molecular-physiological hypothesis” (see also Ref. 38) is dependent on the mitochondrial ATP:ADP ratio, which may well be altered under hypoxic conditions. Importantly, this hyperpolarization in turn leads to increased formation of reactive oxygen species (ROS), and ROS have been suggested by others to cause irreversible inhibition of the plasmalemmal Na$^+$/Ca$^{2+}$ exchanger (39), a suggestion consistent with our observed lack of NCX function seen in CH astrocytes (Fig. 4).

The concept that cellular ROS levels increase during prolonged periods of hypoxia is not uncontested, but is currently gathering momentum. Recently, a number of groups have suggested that the source of increased cellular ROS during hypoxia is mitochondrial (37, 38, 40–43). The present study is also in accordance with these findings, and indicates that hypoxia may increase ROS production via mitochondrial hyperpolarization. Our findings are likely to have important implications for the understanding of cellular damage and death in the central nervous system following periods of hypoxia or ischemia (44, 45).

REFERENCES
1. Incalzi, R. A., Gemma, A., Marra, C., Muzzolson, R., Capparella, O., and Carbone, P. (1995) Am. Rev. Respir. Dis. 151, 418–424
2. Kogure, K., and Kato, H. (1993) Stroke 24, 2121–2127
3. Kostinatoh, J., Pyrykonen, I., Keinanen, R., and Hokfelt, T. (1996) Neuroreport 7, 2727–2731
4. Tatetmichi, T. K., Paik, M., Bagiella, E., Desmond, D. W., Stern, Y., Sano, M., Hauser, W. A., and Mayeux, R. (1994) Neurology 44, 1885–1891
5. Kokmen, E., Whisnant, J. P., O’Fallon, W. M., Chu, C. P., and Beard, C. M. (1996) Neurology 46, 154–159
6. Moroney, J. T., Bagiella, E., Desmond, D. W., Paik, M. C., Stern, Y., and Tatetmichi, T. K. (1996) Stroke 27, 1283–1289
7. Verkhratsky, A., Orkand, R. K., and Kettenmann, H. (1998) Physiol. Rev. 78, 99–141
8. Bezi, P., and Volterra, A. (2001) Curr. Opin. Neurobiol. 11, 387–394
9. Bezi, P., Domerco, M., Vesce, S., and Volterra, A. (2001) Prog. Brain Res. 132, 255–265
10. Ventura, R., and Harris, K. M. (1999) J. Neurosci. 19, 6897–6906
11. Bergles, D. E., Roberts, J. D., Somogyi, P., and Jahr, C. E. (2000) Nature 405, 187–191
12. Alvarez-Maulete, V., Garcia-Hernandez, F., Williams, J. T., and Van Bocktela, E. J. (2000) J. Neurosci. 20, 4091–4098
13. Parpura, V., Basarski, T. A., Liu, F., Kefinjina, K., Kefinjina, S., and Haydon, P. G. (1998) Nature 399, 744–747
14. Kang, J., Jiang, L., Goldman, S. A., and Nedergaard, M. (1998) Nat. Neurosci. 1, 683–692
15. Grosche, J., Matyash, V., Muller, T., Verkhratsky, A., Reichenbach, A., and Kettenmann, H. (1999) Nat. Neurosci. 2, 139–143
16. Diezner, J. W., Verkhratsky, A. J., and Lohr, C. (1998) Cell Calcium 24, 405–416
17. Aragone, A., Carmignato, G., and Haydon, P. G. (2001) Annu. Rev. Physiol. 63, 785–813
18. Giame, C., and Venance, L. (1998) Glia 24, 50–64
19. Pasti, L., Zonta, M., Pozzan, T., Vicini, S., and Carmignato, G. (2001) J. Neurosci. 21, 477–484
20. Fam, S. R., Gallagher, C. J., and Salter, M. W. (2000) J. Neurosci. 20, 2800–2808
21. Carmignato, G. (2000) Prog. Neurobiol. 62, 561–581
22. Beintner-Thomas, D., Shull, G. E., Dedman, J. R., and Millhorn, D. E. (1997) Respir. Physiol. 110, 87–97
23. Mosmann, T. (1983) J. Immunol. Methods 55, 55–63
24. Smith, I. F., Boyle, J. P., Vaughan, P. F., Pearson, H. A., and Peers, C. (2001) J. Neurochem. 79, 877–884
25. Tsen, R. Y., and Waggoner, A. (1994) in Handbook of Confocal Microscopy (Pawley, J. ed.), pp. 267–280, Plenum Press, New York
26. Osumi, K., and Reeves, J. P. (2000) J. Biol. Chem. 275, 21549–21554
27. Collins, T. J., Berridge, M. J., Lipp, F., and Bootman, M. D. (2002) EMBO J. 21, 1616–1627
28. Choi, D. W. (1999) J. Neurosci. 19, 2493–2501
29. Choi, D. W. (1999) Trends Neurosci. 18, 58–60
30. Stempp, G. J., Cholewinski, A. J., Wilkin, G. P., and Djamgoz, M. B. (1993) Glia 6, 269–279
31. Gimpel, G., Walz, W., Oehmeyer, C., and Kettenmann, H. (1992) Neurosci. Lett. 144, 139–142
32. Sergeeva, M., Uhl, J. J., and Reiser, G. (2000) Neuroscience 97, 765–769
33. Wang, Y. X., Dhulipala, P. K., and Kotlikof, M. I. (2000) PASEB J. 14, 1731–1749
34. Barrett, E. P. (2001) J. Gen. Physiol 118, 79–82
35. Landolfi, B., Ceci, S., Debelels, L., Pozzan, T., and Hofer, A. M. (1998) J. Cell Biol. 142, 1235–1243
36. Rutter, G. A., and Rizzuto, R. (2000) Trends Biochem. Sci. 25, 215–221
37. Lee, I., Bender, E., and Kadenbach, B. (2002) Mol. Cell. Biochem. 234–235, 63–70
38. Lee, I., Bender, E., Arnold, S., and Kadenbach, B. (2001) Biochem. J. 382, 1629–1636
39. Nicholls, D. G., and Ward, M. W. (2000) Trends Neurosci. 23, 166–174
40. Chandal, N. S., and Schumacker, P. T. (2000) J. Appl. Physiol. 88, 1880–1889
41. Waypa, G. B., Chandal, N. S., and Schumacker, P. T. (2001) Circ. Res. 88, 1259–1266
42. Chandal, N. S., Maltepe, E., Goldwater, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11715–11720
43. Holter, B., Lange, B., Holstef, B., Goldenberg, A., Hanez, J., Sell, A., Testan, H., Muller, W., and Kummer, W. (1999) FEBS Lett. 457, 53–56
44. Nicholls, D. G., and Bud, S. L. (2000) Physiol. Rev. 80, 315–369
45. Budd, S. L. (1998) Pharmacol. Ther. 80, 203–229