Journal of Neurochemistry, 2003, **85**, 1109–1116 doi:10.1046/j.1471-4159.2003.01785.x

Chronic hypoxia potentiates capacitative Ca^{2+} entry in type-I cortical astrocytes

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Abstract

Prolonged hypoxia exerts profound effects on cell function, and has been associated with increased production of amyloid β peptides (AβPs) of Alzheimer's disease. Here, we have investigated the effects of chronic hypoxia (2.5% O_2, 24 h) on capacitative Ca^{2+} entry (CCE) in primary cultures of rat type-I cortical astrocytes, and compared results with those obtained in astrocytes exposed to AβPs. Chronic hypoxia caused a marked enhancement of CCE that was observed after intracellular Ca^{2+} stores were depleted by bradykinin application or by exposure to thapsigargin (1 μM). Exposure of cells for 24 h to 1 μM $A\beta P_{(1-40)}$ did not alter CCE. Enhancement of CCE was not attributable to cell hyperpolarization, as chronically hypoxic cells were significantly depolarized as compared with controls. Mitochondrial inhibition [by FCCP (10 μM) and oligomycin (2.5 μg/mL)] suppressed CCE in all three cell groups, but more importantly there were no significant differences in the magnitude of CCE in the three astrocyte groups under these conditions. Similarly, the antioxidants melatonin and Trolox abolished the enhancement of CCE in hypoxic cells. Our results indicate that chronic hypoxia augments CCE in cortical type-I astrocytes, a finding which is not mimicked by $A\beta P_{(1-40)}$ and appears to be dependent on altered mitochondrial function.

Keywords: Alzheimer's, amyloid, astrocytes, calcium, capacitative calcium entry, hypoxia.

J. Neurochem. (2003) **85,** 1109–1116.

Numerous studies performed in recent years have demonstrated that astrocytes and other non-neuronal cell types contribute significantly to intercellular signalling in the CNS (Verkhratsky *et al*. 1998; Bezzi and Volterra 2001; Bezzi *et al*. 2001). Astrocytes (and other glia), form close contacts with neurones, extending projections into neuronal synapses (Ventura and Harris 1999) and can form chemical synapses and gap–junction connections with neurones (Alvarez-Maubecin *et al*. 2000; Bergles *et al*. 2000). Astrocytes also possess a wide variety of transmitter receptors (Verkhratsky *et al*. 1998) and much evidence indicates they play important, active roles in synaptic activity: activation of astrocytes by neuronally derived transmitters has been demonstrated at levels of transmitter concentrations found close to synaptic clefts (Parpura *et al*. 1994; Porter and McCarthy 1996; Pasti *et al*. 1997; Kang *et al*. 1998; Kulik *et al*. 1999; Matyash *et al*. 2001; Araque *et al*. 2002). Astrocyte activation commonly leads to a rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) due in part to release of Ca^{2+} from internal stores, which can in turn lead to Ca^{2+} uptake from the extracellular space. This latter Ca^{2+} influx is often termed capacitative Ca^{2+} entry (CCE; Putney 2001; Berridge 1993, 1995).

CCE is a distinct mode of Ca^{2+} entry into excitable and non-excitable cells, and plays fundamental physiological roles, permitting store refilling and shaping and maintaining Ca^{2+} transients arising from cell stimulation (see Putney 2001 for review). A rise of $[Ca^{2+}]_i$ in one astrocyte can initiate Ca^{2+} waves that propagate across adjacent astrocytes (Giaume and Venance 1998; Araque *et al*. 2001; Pasti *et al*. 2001), and this means of intercellular signalling in the brain parallels and modulates classical neuronal synaptic communication. As such, this form of communication is of fundamental importance to central neuronal activity. (Fam *et al*. 2000; Bezzi and Volterra 2001). Within an individual astrocyte, a rise of $[Ca^{2+}]_i$ can also initiate glutamate release which modulates neuronal activity via extrasynaptic metabotropic glutamate receptors (Araque *et al*. 1998a,b; Newman and Zahs 1998; Carmignoto 2000; Pasti *et al*. 2001). Astrocytes are also capable of releasing glutamate via

Received January 17, 2003; accepted January 21, 2003.

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Abbreviations used: AβP, amyloid beta peptide; BK, bradykinin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCE, capacitative Ca^{2+} entry; CH, chronic hypoxia; ROI, regions of interest; ROS, reactive oxygen species.

regulated, Ca^{2+}-dependent exocytosis, in addition to reverse-mode uptake systems (Araque *et al*. 2001).

Exposure to prolonged hypoxia arising (for example) from chronic cardiorespiratory disease can lead to disturbances of higher brain functions (Incalzi *et al*. 1993; Kogure and Kato 1993; Koistinaho *et al*. 1996). Indeed, numerous clinical studies have shown an increased incidence of dementias in patients who have previously suffered prolonged hypoxic or ischaemic episodes (Tatemichi *et al*. 1994; Kokmen *et al*. 1996; Moroney *et al*. 1996). The cellular basis of these clinical links is poorly understood. However, we have previously shown that prolonged hypoxia can lead to increased formation of amyloid β peptides (AβPs; believed to be the neurotoxic agents in the most prevalent form of dementia, Alzheimer's disease) and application of exogenous AβPs mimics such effects of hypoxia (Taylor *et al*. 1999; Green *et al*. 2002). Given the clear clinical and cellular links between prolonged hypoxia and dementia, together with the increased awareness that astrocytes play key roles in central nervous function and rely on Ca^{2+} signalling for many functions, the present study was conducted in order to determine specifically any possible effects of prolonged hypoxia on CCE in cortical type-I astrocytes. We have also compared any such effects with exposure to $A\beta P_{(1-40)}$, the most common form of AβP which mimics other effects of hypoxia in model cell lines (Taylor *et al*. 1999; Green and Peers 2001; Green *et al*. 2002).

Experimental procedures

Primary cultures of astrocytes

Cerebral cortices were removed from neonatal (6- to 8-day-old) rats and placed in ice-cold buffer solution (10 mM NaH_2PO_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, whole cortices minced with a mechanical tissue chopper (McIlwain, St Louis, MO, USA) and dispersed in the same buffer containing 0.25 μg/mL trypsin (37°C, 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 I-S; Sigma, St Louis, MO, USA), 0.5 μg/mL DNase I (EC 3.1.21.1 type II from bovine pancreas; 125 kU/mL; Sigma) and 1.5 mM $MgSO_4$. The tissue was then pelleted by centrifugation at 1000 *g* 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 $MgSO_4$, and triturated with a fire polished Pasteur pipette. After allowing larger pieces of tissue to settle for 5 min, the cell suspension was removed and centrifuged at 1000 *g* 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, Paisley, UK). This was then aliquoted into 2 × 25-cm^2 flasks and onto glass coverslips in 6- and 24-well tissue culture plates. Cells were then kept in a humidified incubator at 37°C (95% air, 5% CO_2). This was designated passage 1 and cells were used up to a passage of 2. Four to six hours following plating, cells were washed vigorously several times with fresh media to remove non-adhered cells. This resulted in a culture of primarily type-I cortical astrocytes (as confirmed by positive immunostaining with an anti-glial fibrillary acidic protein 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 and 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% O_2, 5% CO_2 balanced with N_2 [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. Corresponding control cells were maintained in a 95% air, 5% CO_2 incubator for the same period, and those exposed to $A\beta P_{(1-40)}$ were maintained identically, except that they were also exposed to 1 μM of the peptide for 24 h prior to experimentation. Exposure of cells to hypoxia or to $A\beta P_{(1-40)}$ was without effect on cell viability (Ramsden *et al*. 2001; Smith *et al*. 2003).

Microfluorimetric measurement of $[Ca^{2+}]_i$

To measure cytosolic $[Ca^{2+}]$, glass coverslips onto which cells had grown were incubated in 2 mL of control solution containing 4 μM Fura-2AM for 1 h at 21°C–24°C in the dark, as previously described. Control solution was composed of: NaCl 135 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 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 gravity at a rate of 1–2 mL/min. $[Ca^{2+}]_i$ was determined using an Openlab System (Image Processing & Vision Company Ltd, Coventry, UK). Excitation was provided using a Xenon arc lamp (75 W) and excitation wavelengths (340 nm and 380 nm) were selected by a monochromator (Till Photonics, Planegg, Germany). A quartz fibreoptic guide transmitted light to the microscope and was reflected by a dichroic mirror (Omega Optical, Glen Spectra Ltd, Stanmore, UK) into the objective. Emission was collected through the objective and a 510-nm filter (40 nm band width). Digital images were sampled at 14-bit resolution by an intensified charge-coupled device camera (Hamamatsu Photonics, Hertfordshire, UK). Fura-2 was excited alternately at 340 and 380 nm and ratios of the resulting images were produced every 4 s. Regions of interest (ROI) were used to restrict data collection to individual cells. All the imaging was controlled by Improvision software that included Openlab 2.2.5 (Image Processing & Vision Company Ltd) and operated on a Macintosh PowerPC. Drugs and agonists were applied to cells as indicated in the Results by switching the inflow with a 6-way Hamilton tap to one supplied by a reservoir of the relevant composition. All experiments were conducted at room temperature (21–24°C). Because calibration of fluorescence into absolute $[Ca^{2+}]_i$-values can be subject to artefactual inaccuracies (Duchen 1992), data are presented as ratio signals. Mean time courses of CCE were measured every 4 s for averaging (e.g. Fig. 1b). Results are expressed as means ± SEM, together with example traces, and statistical comparisons were made using unpaired *t*-tests or ANOVA, as indicated.

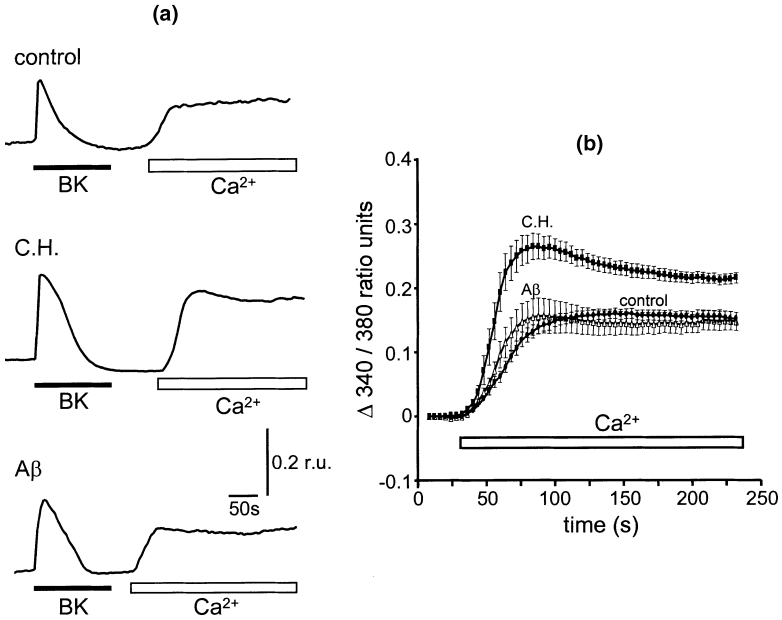

Fig. 1 Chronic hypoxia augments CCE in astrocytes. (a) Example recordings of $[Ca^{2+}]_i$ obtained from control (upper trace), chronically hypoxic (middle trace) and $A\beta P_{(1-40)}$-treated (lower trace) astrocytes. In each case, 100 nM bradykinin (BK) was applied for the period indicated by the solid horizontal bar in Ca^{2+}-free perfusate. Following removal of BK, Ca^{2+} was re-introduced into the perfusate, as indicated by the open bars. The subsequent rises of $[Ca^{2+}]_i$ are attributable to Ca^{2+} influx via CCE. Scale bars apply to all traces. (b) Mean (± SEM bars) rises of $[Ca^{2+}]_i$ obtained in the three cell groups as indicated ($n = 4$ recordings from each of which at least four cells for each group were analysed), following re-application of Ca^{2+} to the perfusate. Intracellular Ca^{2+} stores were previously discharged with 100 nM BK [as in (a)]. Chronic hypoxic (CH) conditions significantly enhanced responses ($p < 0.001$, ANOVA) as compared with controls.

Membrane potential measurements

To obtain measurements of resting membrane potential, whole-cell patch-clamp recordings were used. Cells were perfused with the standard control extracellular solution, and pipettes contained: KCl 140 mM, $MgCl_2$ 1 mM, $CaCl_2$ 0.5 mM, EGTA 5 mM, HEPES 10 mM, K_2ATP 2 mM (pH 7.2). After achieving the whole-cell configuration, the amplifier was immediately switched to current-clamp (I = 0) mode, and membrane potential recorded for up to 1 min, during which time a stable resting value could be determined. Data were acquired using an Axopatch 200D amplifier (Axon Instruments, Union City, CA, USA), filtered at 1 kHz, digitized at 5 kHz and stored using the Patch v6.0 program by Cambridge Electronic Design. Data were averaged for each cell group and plotted as mean (± SEM) values.

Results

In numerous cell types, receptor activation by agonist application leads to generation of inositol 1,4,5-trisphosphate, which liberates Ca^{2+} from endoplasmic reticulum stores. This store depletion then triggers Ca^{2+} influx via CCE (Berridge 1993; Putney 2001). In order to separate temporally the release of Ca^{2+} from stores and Ca^{2+} influx via CCE, we employed the protocol indicated in Fig. 1(a). During perfusion of astrocytes with a Ca^{2+}-free perfusate (replaced with 1 mM EGTA), 100 nM bradykinin (BK) was applied in order to discharge intracellular stores. This agonist evoked a transient rise of $[Ca^{2+}]_i$ due to liberation of Ca^{2+} into the cytosol (Fig. 1a). BK was washed from cells once $[Ca^{2+}]_i$ had returned to basal levels, then Ca^{2+} (2.5 mM) was re-admitted to the perfusate. The subsequent, second rise of $[Ca^{2+}]_i$ arises due to CCE. As indicated by the example traces of Fig. 1(a), and more clearly from the averaged data of Fig. 1(b), CH caused a marked, significant ($p < 0.001$, ANOVA) potentiation of CCE as compared to normoxically cultured (control) astrocytes. By contrast, treatment of cells under normoxic conditions with 1 μM $A\beta P_{(1-40)}$ was without significant effect on CCE.

Because $A\beta P_{(1-40)}$ is known to form Ca^{2+}-permeable pores in membranes (Arispe *et al.* 1993; Rhee *et al.* 1998), and periods of CH can induce increased formation of AβPs in some cell types (Taylor *et al.* 1999; Green and Peers 2001), it was important to verify the claim that rises of $[Ca^{2+}]_i$ seen on re-admission of Ca^{2+} to the perfusate were due to CCE, rather than other pathways. Thus, we repeated the experimental protocol of Fig. 1, with the important exception that stores were not firstly depleted by application of BK in Ca^{2+}-free perfusate. In contrast to astrocytes whose stores were initially depleted (Fig. 2a), those whose stores were left intact (i.e. by not applying BK to cells) displayed no rise of $[Ca^{2+}]_i$ when Ca^{2+} was re-admitted to the perfusate (Fig. 2b). Thus, store depletion was required

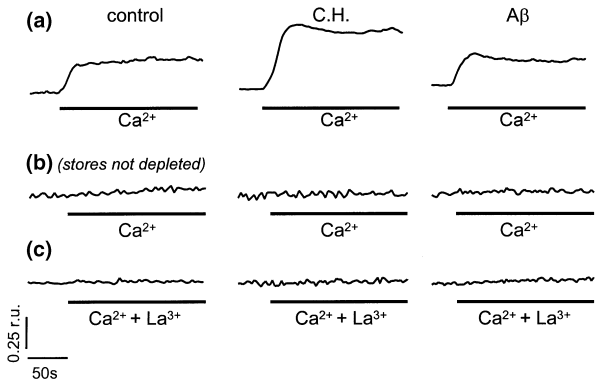

Fig. 2 Hypoxia does not alter the requirement for CCE of store depletion. Example recordings of $[Ca^{2+}]_i$ obtained from control (left-hand traces), chronically hypoxic (middle traces) and $A\beta P_{(1-40)}$-treated (right-hand traces) astrocytes. In each case, recordings were made during perfusion with a Ca^{2+}-free solution, then Ca^{2+} was re-introduced into the perfusate for the periods indicated by the solid bars. In (a), intracellular Ca^{2+} stores had previously been discharged with 100 nM BK (as in Fig. 1a). In (b), stores were not previously discharged. In (c), stores were initially discharged with 100 nM BK, but 1 mM La^{3+} was applied together with Ca^{2+}, as indicated. Scale bars apply to all traces, each of which is representative of at least four recordings.

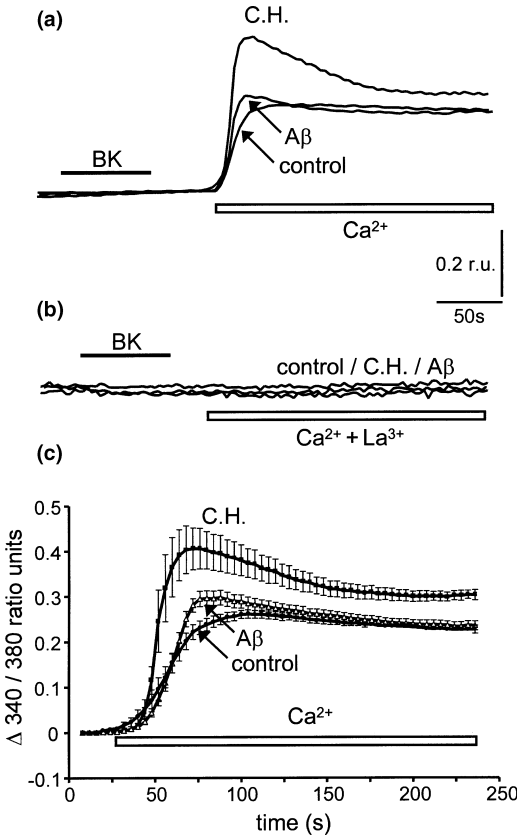

Fig. 3 Hypoxia augments CCE caused by complete store depletion. (a) Example recordings of $[Ca^{2+}]_i$ obtained from control, chronically hypoxic (CH) and $A\beta P_{1-40}$-treated astrocytes, as indicated. In each case, cells were initially incubated with 1 μM thapsigargin for 20 min before recording. 100 nM bradykinin (BK) was applied for the period indicated by the solid horizontal bar in Ca^{2+}-free perfusate. Following removal of BK, Ca^{2+} was re-introduced into the perfusate as indicated by the open bar. The subsequent rises of $[Ca^{2+}]_i$ are attributable to Ca^{2+} influx via CCE, activated by thapsigargin-induced store depletion. (b) as (a), except that La^{3+} (1 mM) was co-applied with Ca^{2+} for the period indicated by the open bar. (c) Mean (± SEM bars) rises of $[Ca^{2+}]_i$ obtained in the three cell groups as indicated (n = at least four recordings from each of which at least three cells for each group were analysed), following re-application of Ca^{2+} to the perfusate. Intracellular Ca^{2+} stores were previously discharged with 1 μM thapsigargin [as in (a)]. Chronic hypoxic (CH) conditions significantly enhanced responses ($p < 0.001$, ANOVA) as compared with controls.

to see rises of $[Ca^{2+}]_i$. Furthermore, when stores were depleted, but Ca^{2+} was re-admitted together with 1 mM La^{3+} (a known blocker of CCE; Putney 2001), no rise of $[Ca^{2+}]_i$ was observed (Fig. 2c). These observations were consistently seen in at least five cells from each group studied.

Previous studies have indicated that whilst CCE may be activated following store depletion caused by agonist application, the magnitude of the CCE response can be influenced by the degree to which stores are depleted (Leissring *et al.* 2000). In order to ensure stores were fully depleted, we treated astrocytes for 20 min with thapsigargin before repeating the experimental protocols indicated in Fig. 1. As shown in Fig. 3(a), subsequent application of BK (in Ca^{2+}-free perfusate) failed to elicit any rise of $[Ca^{2+}]_i$, indicating, as expected, that stores were indeed fully depleted. Following washout of BK, re-admission of Ca^{2+} to the perfusate evoked a rise of $[Ca^{2+}]_i$ due to CCE, as it was fully blocked by La^{3+} (Fig. 3b). As with CCE observed after store depletion evoked by BK application (Fig. 1), CCE observed in thapsigargin-treated cells was significantly greater in CH astrocytes than in controls or $A\beta P_{(1-40)}$-treated cells, and there were no significant differences between these latter two cell groups.

Following activation of CCE, the magnitude of the rise of $[Ca^{2+}]_i$ is dependent on the electrochemical driving force for Ca^{2+} entry. This in turn is determined by the Ca^{2+} concentration gradient between the external solution and cytosolic $[Ca^{2+}]$, and also by membrane potential. Thus, it was important to determine whether or not the increased CCE observed in CH astrocytes was simply due to hyperpolarization. To address this issue, we measured membrane potential in the three cell groups using whole-cell patch-clamp recordings. As shown in Fig. 4, membrane potential was actually significantly depolarized ($p < 0.001$) in CH cells (as well as $A\beta P_{(1-40)}$-treated cells) as compared with control cells. Thus the increased CCE seen following CH occurs despite, rather than because of, a change in membrane potential.

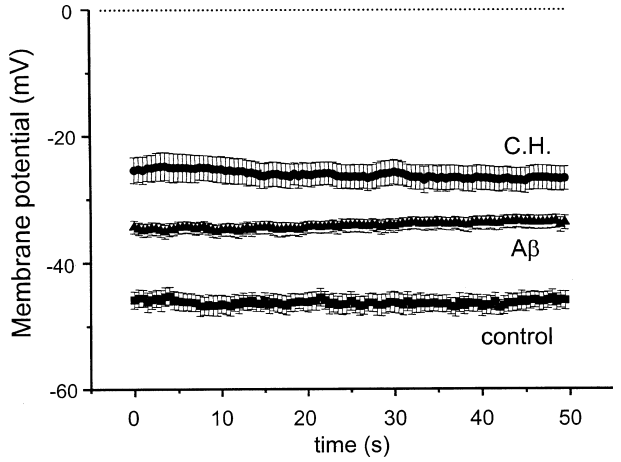

Fig. 4 Chronic hypoxia and amyloid peptide depolarize astrocytes. Each data set represents the mean (± SEM) membrane potential recorded from the three cell groups indicated ($n = 7$ cells for each group) immediately after achieving the whole-cell configuration. Values observed in chronic hypoxic (CH) cells and $A\beta P_{(1-40)}$-treated cells (Aβ) were significantly more depolarized ($p < 0.001$, unpaired *t*-test) than those obtained from control cells.

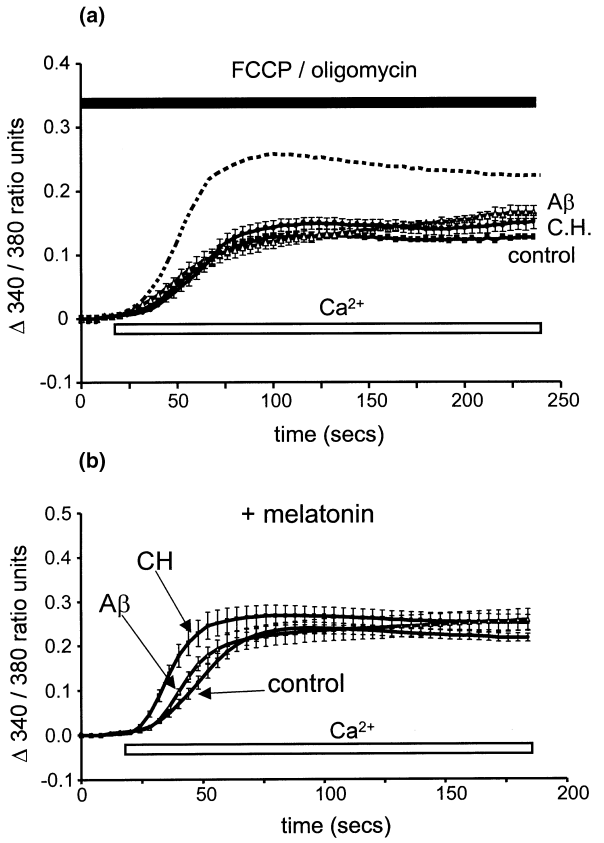

Fig. 5 Mitochondrial inhibition and antioxidants suppress CCE. (a) Mean (± SEM bars) rises of $[Ca^{2+}]_i$ obtained in the three cell groups as indicated ($n = 3$ recordings from each of which at least four cells for each group were analysed), following re-application of Ca^{2+} to the perfusate. Intracellular Ca^{2+} stores were previously discharged with 1 μM thapsigargin [as in (a)], and cells in each group were also exposed to 10 μM FCCP and 2.5 μg/mL oligomycin for 1 min before, and during the recording period illustrated. Dashed line is taken from control responses of Fig. 3, for ease of comparison. (b) Mean (± SEM bars) rises of $[Ca^{2+}]_i$ obtained in the three cell groups as indicated ($n = 3$ recordings from each of which at least four cells for each group were analysed), following re-application of Ca^{2+} to the perfusate. Intracellular Ca^{2+} stores were previously discharged with 1 μM thapsigargin, and cells were previously incubated for 24-h treatments in the additional presence of 150 μM melatonin.

Various studies have shown an intimate functional association between mitochondria and CCE in various cell types (Hoth *et al*. 1997; Gilabert *et al*. 2001). Becasue we have previously shown that mitochondrial Ca^{2+} buffering capacity is altered following a period of HC (Smith *et al*. 2003), we investigated the effects of mitochondrial inhibition on CCE in control, CH and AβP-treated astrocytes under investigation. To do this, stores were fully depleted by exposure to 1 μM thapsigargin (as in Fig. 3) in Ca^{2+}-free perfusate, then exposed to 10 μM FCCP and 2.5 μg/mL oligomycin for 1 min prior to, and during, re-application of Ca^{2+} to the extracellular solution. As compared with responses observed in the absence of mitochondrial inhibition (Fig. 3c; control response reproduced as dashed line in Fig. 5a), the rises of $[Ca^{2+}]_i$ observed were significantly smaller ($p < 0.002$, ANOVA) in each cell group (Fig. 5a). More importantly, perhaps, was the observation that during mitochondrial inhibition, CCE was similar in magnitude in all three of the cell groups, suggesting the involvement of mitochondria in the augmented responses normally seen in CH astrocytes.

A series of recent studies have indicated that mitochondria can be an important source of reactive oxygen species (ROS) during sustained hypoxia in various tissues (Chandel and Schumacker 2000; Chandel *et al*. 2000; Waypa *et al*. 2001). To investigate ROS as the messengers linking mitochondria to CCE, we examined the ability of two distinct antioxidants to modulate CCE in control, hypoxic and Aβ-treated astrocytes. As indicated in Fig. 5(b), melatonin (150 μM), like mitochondrial inhibition (Fig. 5a), suppressed the enhanced CCE of CH astrocytes. Exactly the same pattern of responses were observed when cells were treated with the structurally unrelated antioxidant, Trolox (data not shown). Together, the data of Fig. 5 suggest that hypoxic enhancement of CCE arises due to production of mitochondrial ROS.

Discussion

CCE is of fundamental physiological importance for numerous reasons, providing a route for Ca^{2+} entry which permits store re-filling following agonist-induced depletion, as well as a means of permitting prolonged rises of $[Ca^{2+}]_i$ and fine-tuning of transient Ca^{2+} spiking activity (reviewed by

Putney 2001), an important form of signalling in astrocytes. Given the central importance of Ca^{2+} signalling to astrocyte physiology, and the established disruption of higher brain functions as a consequence of prolonged hypoxia (see Introduction), the present study aimed to investigate the specific effects of CH on CCE in primary cultures of astrocytes. Our previous studies have also shown that some of the cellular effects of CH can be mimicked by (and indeed appear to be attributable to increased production of) AβPs (Taylor *et al*. 1999; Green and Peers 2001). Of these, the most common isoform, $A\beta P_{(1-40)}$, is capable of mimicking actions of CH, and so we compared effects of hypoxia with those of exogenous application of this peptide.

The major finding of the present study was that CH dramatically increased the magnitude of CCE (Figs 1–3). Thus, following discharge of intracellular stores by exposure of cells to BK, re-admission of Ca^{2+} to the perfusate permitted CCE, which was significantly greater following a period of hypoxia. Previous studies have indicated that the magnitude of CCE is dependent on the extent to which stores are depleted (Leissring *et al*. 2000), and so we also examined CCE following more complete store depletion which was achieved (as evidenced by the complete lack of response to BK; Fig. 3a) by exposure of cells to thapsigargin. Again, CCE was significantly greater in astrocytes previously exposed to CH. Thus, the augmentation of CCE by hypoxia could not be attributed to incomplete store depletion.

Once intracellular Ca^{2+} stores are depleted and the (presently unknown) channels which permit CCE are activated, the magnitude of Ca^{2+} entry is dependent solely on electrochemical driving forces, which strongly favour Ca^{2+} influx due to the steep Ca^{2+} concentration gradient and negative cell membrane potential. When present, extracellular $[Ca^{2+}]$ was maintained at 2.5 mM in the present study, and there was no discernible difference in resting Ca^{2+} levels in the three cell groups, suggesting that changes in Ca^{2+} concentration gradient could not account for enhancement of CCE by CH. However, it was conceivable that CCE was augmented because CH led to cell hyperpolarization. To address this, we measured resting membrane potential in the three cell groups (Fig. 4). Perhaps surprisingly, CH (and, to a lesser degree, exposure to $A\beta P_{(1-40)}$) caused significant depolarization of cells, an effect for which we cannot at present account, although it is noteworthy that amyloid peptides have been shown to inhibit neuronal Na^+ K^+ ATPase (Mark *et al*. 1995). However, this depolarization would act to suppress CCE, and so the observed hypoxic augmentation of CCE reported here is likely to be underestimated, as the electrochemical driving force for Ca^{2+} entry is less in CH cells.

Our previous studies, primarily employing the pheochromocytoma cell line, PC12, indicated that prolonged hypoxia led to an increased production of AβPs (primarily the most common form, $A\beta P_{(1-40)}$) which in turn caused disruption of Ca^{2+} homeostasis, through formation of a voltage-gated Ca^{2+} influx pathway and a selective upregulation of L-type Ca^{2+} channels (Taylor *et al*. 1999; Green and Peers 2001, 2002; Green *et al*. 2002). For this reason, we sought to examine whether any effects of CH on CCE in astrocytes could be mimicked by $A\beta P_{(1-40)}$. Our results indicate that exogenous application of $A\beta P_{(1-40)}$ failed to mimic the augmentation of CCE by CH, a finding which suggests that the actions of CH do not appear to involve amyloidogenesis in cortical astrocytes. It is noteworthy, however, that $A\beta P_{(1-40)}$ caused significant depolarization of astrocytes (Fig. 4). This would reduce the electrochemical driving force for CCE, yet the magnitude of CCE in $A\beta P_{(1-40)}$-treated astrocytes was similar to controls. It is conceivable therefore that if changes in membrane potential could be experimentally normalized, an augmentation of CCE caused by $A\beta P_{(1-40)}$ might be revealed. However, because the actions of CH were so clearly different from those of exogenous $A\beta P_{(1-40)}$, this possibility was not pursued further.

Several recent studies have indicated that mitochondria play an active role in regulating CCE: Gilabert *et al*. (2001) have shown in RBL-1 cells and lymphocytes that CCE is augmented when mitochondria are 'energized' (i.e. provided experimentally with a cocktail of substrates permitting increased respiration during whole-cell patch clamp dialysis), thereby increasing the dynamic range over which inositol 1,4,5-trisphosphate can modulate CCE. Hoth *et al*. (1997) have also shown regulation of CCE through mitochondrial buffering of Ca^{2+} entering T lymphocytes. These studies prompted us to investigate a potential role of mitochondria in mediating the actions of CH on CCE in astrocytes. Figure 5(a) indicates that when mitochondria were inhibited, CCE in all three groups of astrocytes was markedly suppressed (compare Fig. 5a with Fig. 3c). Perhaps most importantly, mitochondrial inhibition ablated the augmentation of CCE caused by CH. One possible interpretation of this finding is that CCE triggered Ca^{2+}-induced Ca^{2+} release (CICR) from mitochondria, causing the augmentation of the CCE signal in hypoxic traces. However, absolute levels of Ca^{2+} in mitochondria (even those from hypoxic astrocytes) are minor compared to Ca^{2+} elevations caused by CCE (see Smith *et al*. 2003), and CICR would also be transient. Thus CICR would be unlikely to be a major contributory factor in the elevated CCE sen in hypoxic astrocytes. It is also unlikely that this effect is due to inhibition of Ca^{2+} buffering: we have recently shown that hypoxia causes marked increases in mitochondrial Ca^{2+} loading, which would inhibit their ability to buffer Ca^{2+} influx via CCE (Smith *et al*. 2003). Instead, we suggest that augmentation of CCE in CH arises from increased mitochondrial production of ROS. Schumacker and colleagues (Duranteau *et al*. 1998; Chandel and Schumacker 2000) have shown in a number of different cell types that prolonged hypoxia causes increased produc-

tion of ROS, and that their source is mitochondrial and can be prevented by mitochondrial inhibitors. The ability of mitochondria to generate ROS is enhanced by mitochondrial hyperpolarization (Lee *et al*. 2002), which is also accompanied by increased Ca^{2+} loading (Lee *et al*. 2001, 2002), and we have recently demonstrated both mitochondrial hyperpolarization and Ca^{2+} loading to occur following prolonged hypoxia in astrocytes (Smith *et al*. 2003). Furthermore, CCE has been shown to be augmented in vascular endothelial cells by ROS (Graier *et al*. 1998). Taken together, these data suggest that chronic hypoxic augmentation of CCE in astrocytes, as shown here, arises from increased ROS production by mitochondria. In support of this notion, we found that the antioxidants melatonin (Fig. 5b) and Trolox acted in a manner comparable with that of mitochondrial inhibition to suppress CCE and abolish the augmentation caused by CH.

In summary, we have shown that CCE in type-I cortical astrocytes is markedly enhanced by a period of CH. This effect is observed despite a significant depolarization of astrocytes, and appears to involve an increased ability of mitochondria to generate ROS. Our findings have important potential implications for the cellular basis of disruption of higher brain functions caused by prolonged hypoxia.

Acknowledgements

This work was supported by The Wellcome Trust and the Medical Research Council. IFS holds a CASE studentship with Pfizer Central Research.

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