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Hypoxic remodelling of Ca^{2+} mobilization in type I cortical astrocytes: involvement of ROS and pro-amyloidogenic APP processing

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Abstract

Chronic hypoxia (CH) alters Ca^{2+} homeostasis in various cells and may contribute to disturbed Ca^{2+} homeostasis of Alzheimer's disease. Here, we have employed microfluorimetric measurements of $[Ca^{2+}]_i$ to investigate the mechanism underlying augmentation of Ca^{2+} signalling by chronic hypoxia in type I cortical astrocytes. Application of bradykinin evoked significantly larger rises of $[Ca^{2+}]_i$ in hypoxic cells as compared with control cells. This augmentation was prevented fully by either melatonin (150 μM) or ascorbic acid (200 μM), indicating the involvement of reactive oxygen species. Given the association between hypoxia and increased production of amyloid β peptides (AβPs) of Alzheimer's disease, we performed immunofluorescence studies to show that hypoxia caused a marked and consistent increased staining for AβPs and presenilin-1 (PS-1). Western blot experiments also confirmed that hypoxia increased PS-1 protein levels. Hypoxic increases of AβP production was prevented with inhibitors of either γ- or β-secretase. These inhibitors also partially prevented the augmentation of Ca^{2+} signalling in astrocytes. Our results indicate that chronic hypoxia enhances agonist-evoked rises of $[Ca^{2+}]_i$ in cortical astrocytes, and that this can be prevented by antioxidants and appears to be associated with increased AβP formation.

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Alzheimer's disease (AD), characterized by impairments in cognition and memory, is clearly associated with the slow accumulation of amyloid β peptides (AβPs) in the central nervous system (Selkoe 2001; Small *et al*. 2001). AβPs are generated via amyloidogenic processing of amyloid precursor protein (APP) by β- and γ-secretases, and recent evidence suggests that γ-secretase activity requires the formation of a complex between presenilin, nicastrin, APH-1 and pen-2 (Edbauer *et al*. 2003). Disruption of Ca^{2+} homeostasis has been strongly implicated in the neurodegeneration of AD; indeed, increased Ca^{2+}-dependent protease activity occurs in association with degenerating neurones in AD brain tissue (Nixon *et al*. 1994), and AβPs perturb Ca^{2+} homeostasis, rendering cells susceptible to excitotoxic damage (Mattson *et al*. 1992). Presenilin mutations are known to have effects on cellular Ca^{2+} homeostasis (Mattson *et al*. 2000a), and familial AD (FAD)-related mutations of presenilin-1 (PS-1) can alter inositol triphosphate-coupled intracellular Ca^{2+} stores as well as Ca^{2+} influx pathways (Leissring *et al*. 2000; Mattson *et al*. 2000a; Yoo *et al*. 2000). This may contribute to neurodegeneration, since disruption of Ca^{2+} homeostasis is an important mechanism underlying such loss of neurones (Chan *et al*. 2000; Mattson *et al*. 2000b; Yoo *et al*. 2000).

Periods of cerebral hypoxia or ischaemia can increase the incidence of AD (Tatemichi *et al*. 1994; Kokmen *et al*. 1996), and APP expression is elevated following mild and severe brain ischaemia (Kogure and Kato 1993). Since the non-amyloidogenic cleavage product of APP (sAPPα) is neuroprotective (Mattson 1997; Selkoe 2001), increased expression during hypoxia could be considered a protective

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Abbreviations used: AβPs, amyloid β peptides; AD, Alzheimer's disease; AICD, APP intracellular cleaved domain; APP, amyloid precursor protein; BK, bradykinin; β-SI, β-secretase inhibitor; CH, chronically hypoxic; ECL, enhanced chemiluminescence; γ-IV, 2-naphthoyl-VF-CHO; HIF, hypoxia inducible factor; NCX, Na^+/Ca^{2+} exchange; NGS, normal goat serum; PBS, phosphate-buffered saline; PS-1, presenilin-1; pVHL, von Hippel-Lindau tumour-suppressor protein; ROS, reactive oxygen species.

mechanism against ischaemia. However, increased APP levels would also provide an increased substrate for AβP formation of and, indeed, we have previously shown that AβP formation is increased following hypoxia in PC12 cells (Taylor *et al*. 1999; Green *et al*. 2002). Furthermore, we have shown in rat type I cortical astrocytes that bradykinin (BK)-induced Ca^{2+} release from intracellular stores is potentiated by prolonged hypoxia. This was due to dysfunction of mitochondria and plasmalemmal Na^{+}/Ca^{2+} exchanger (NCX; Smith *et al*. 2003). This observation is of particular importance, since astrocytes contribute significantly to intercellular signalling within the central nervous system (Verkhratsky *et al*. 1998; Bezzi and Volterra 2001; Bezzi *et al*. 2001). They express various receptors through which they can regulate both neuronal activity (Newman and Zahs 1997; Porter and McCarthy 1997) and local blood supply (Zonta *et al*. 2003). Astrocyte activation is usually manifest as a rise of $[Ca^{2+}]_i$ (Bezzi *et al*. 1998; Deitmer *et al*. 1998; Kang *et al*. 1998), and astrocytes communicate with neighbouring cells primarily via propagation of Ca^{2+} signals (Giaume and Venance 1998; Araque *et al*. 2001). These Ca^{2+} waves can spread via transmitter release (Fam *et al*. 2000) or via gap junctions (Charles *et al*. 1992).

Given the established link between periods of hypoxia and the development of AD, together with the increased awareness of the role for Ca^{2+} signalling in astrocytes in the central nervous system, we have sought the possible involvement of reactive oxygen species (ROS) in these effects of hypoxia, and examined whether or not these effects of hypoxia are associated with amyloidogenic processing of APP.

Methods

To obtain primary cultures of astrocytes, cerebral cortices were removed from 6- to 8-day-old Wistar rats (four per preparation) and placed immediately in ice-cold buffer solution consisting of 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 using fine forceps and cortices then minced with a mechanical tissue chopper (McIlwain, Missouri, USA) 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 (type I-S; Sigma, Poole, Dorset, UK), 0.5 μg/mL DNase I (EC 3.1.21.1 type II from bovine pancreas; 125 kU/mL; Sigma) and 1.5 mM $MgSO_4$. Tissue was pelleted by centrifugation at 1300 r.p.m. (1000 *g*) for 1 min. The supernatant was poured off and cell pellets resuspended in 2 mL of buffer solution containing 100 μg/mL soy bean trypsin inhibitor, 0.5 μg/mL DNase I and 1.5 mM $MgSO_4$. Tissue was subsequently triturated gently three times with a fire polished Pasteur pipette. After allowing larger pieces of tissue to settle for 5 min, the cloudy cell suspension was pipetted off and centrifuged at 1300 r.p.m. for 1.5 min before resuspension into 60 mL of media. The culture medium consisted of Eagle's minimal essential medium supplemented with 10% fetal calf serum (v/v) and 1% (v/v) penicillin-streptomycin (Gibco, UK, Paisley, Scotland, UK). The cell suspension 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 out the culture medium, cells were washed three times with fresh media to remove non-adhered cells. This resulted in a culture of primarily type I cortical astrocytes as confirmed by glial fibrillary acidic protein immunohistochemistry (data not shown). Culture medium was exchanged every 3–4 days and cells were grown in culture for up to 14 days. All recording 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% O_2, 5% CO_2 and 2.5% 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. Cells exposed to AβPs ($AβP_{1–40}$, $AβP_{1–42}$, $AβP_{25–35}$ and $AβP_{40–1}$; Sigma, UK) were treated in an identical manner to control cells but 24 h prior to experimentation amyloid β peptide was added to the culture medium to give a final concentration of 1 μM. AβPs were purchased from a number of different companies (Sigma; Biosource, Sunnyvale, CA, USA; Tocris, Bristol, UK), dissolved in sterile, distilled water (100 μM) and stored frozen in small aliquots. Aliquots were defrosted immediately before each experiment and were not subject to freeze–thaw cycles. Samples of AβPs stored in this manner were run on western blots to detect any aggregation of the peptides. No aggregation of the peptides stored in this manner was detected. Samples of the media to which AβPs had been added and which had been used to treat cells (see below) were also tested. In these samples extra bands were seen on the western blots indicating that aggregation had occurred but the vast majority of the peptide (as estimated from band density) was still in the monomeric form.

In order 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 21–24°C in the dark. Control solution was composed of 135 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM HEPES and 10 mM glucose. pH was adjusted to 7.4 using NaOH and osmolarity adjusted to 300 mOsm with sucrose. Following this incubation fragments of coverslips were transferred to the stage of an inverted microscope, where cells were continuously perfused under gravity at a rate of 1–2 mL^{-1}. Ca^{2+} measurements were made using an Openlab System [Image Processing & Vision Company Ltd (Improvision), Coventry, UK) using Fura-2 for Ca^{2+} detection. Excitation was provided using a Xenon arc lamp (75 W) and excitation wavelengths (340 and 380 nm) were selected by a monochromator (Till Photonics, Planegg, Germany). A quartz fibre-optic 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 captured at 14 bit resolution using an intensified charge-coupled device camera (Hamamatsu Photonics, Hertfordshire, UK). Fura-2 was excited

alternatively at 340 and 380 nm and ratios of the resulting images were produced every 4 s. Regions of interest were used to restrict data collection to individual cells. All the imaging was controlled by Improvision software that included Openlab 2.2.5 (Improvision, UK) and operated on a Macintosh PowerPC.

For cytosolic Ca^{2+} measurements, several parameters were determined from the collected data. Changes in $[Ca^{2+}]_i$ were taken from measuring peak values and expressing them as a change in fluorescence ratio from basal levels. The size of bradykinin-evoked stores was taken from the integral of transient responses recorded in Ca^{2+}-free perfusate. All results are expressed as means ± SEM, together with example traces, and statistical comparisons were made using unpaired Student's *t*-tests.

For immunofluoresence microscopy, cells were grown on 22 × 22-mm glass coverslips in 6-well plates. Cell layers were washed twice in phosphate-buffered saline plus Ca^{2+} (PBS + Ca^{2+}) to remove medium. Cells were then fixed in 4% (v/v) paraformaldehyde for 20 min at room temperature (22–24°C). The fixed cells were washed twice in PBS + Ca^{2+} and then permeabilized in PBS + Ca^{2+} containing 0.05% (v/v) TritonX 100 (Sigma, UK) and 10% (v/v) normal goat serum (NGS; Sigma, UK) for a further 20 min at room temperature. Permeabilized cells were then washed in PBS + Ca^{2+} with 1% (v/v) NGS (to block non-specific sites) and then incubated overnight at 4°C with the appropriate antibody (polyclonal antibody raised against N-terminal fragments of human presenilin-1 (PS-1, 1 : 2000 dilution in PBS + Ca^{2+}/1% NGS), or a monoclonal antibody raised against the N-terminal fragment of the AβP (1 : 1000 dilution in PBS + Ca^{2+}/1% NGS). Twenty-four hours later, each coverslip was washed three times with PBS + Ca^{2+}. After draining, coverslips were incubated in the dark with Cy-3 conjugated donkey anti-rabbit serum (Jackson Immunoresearch Ltd, Baltimore, MD, USA) for detection of N-terminal fragment of PS-1 or with Cy-3 conjugated donkey anti-mouse serum (Jackson Immunoresearch Ltd) for detection of N-terminus of AβP for 1 h at room temperature (1 : 1000 dilution in PBS + Ca^{2+}/1% NGS). Coverslips were then washed three times with PBS + Ca^{2+} and mounted on glass slides using Vectashield (Vector Laboratories Ltd, Peterborough, UK). Cells were then examined using a Zeiss Axioscop epifluorescence microscope (Axioskop, Carl Zeiss Ltd, Welwyn Garden City, UK).

For western blotting, astrocytes were grown to confluence as detailed above in 75-cm^2 flasks, washed free of media, lysed in 1 mL M-per TM mammalian protein extraction reagent (Perbio Science, Tattenhall, Cheshire, UK) containing Complete Mini protease inhibitors (Roche Diagnostics UK Ltd, Lewes, East Sussex, UK). Protein levels were determined using established methods (Bradford 1976).

Cell proteins were separated on 15%, 0.75 mm thick polyacrylamide-sodium dodecyl sulphate gels and transferred onto polyvinyl difluoride membranes (30 V overnight). Membranes were blocked with 5% non-fat milk protein/2% bovine serum albumin in PBS-Tween (0.05%) for 1 h and immunostained with antibodies raised against N-terminal fragment of human PS-1 for 3 h. Blots were then washed and incubated with anti-rabbit Ig conjugated to horseradish peroxidase (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK) and bands visualized using the enhanced chemiluminescence (ECL) detection system and hyperfilm ECL (Amersham, UK). Band intensities were measured using the Scion Image analysis software.

Results

When cells were cultured under CH conditions, application of 100 nM BK (Gimpl *et al*. 1992) in a Ca^{2+}-free perfusate (replaced with 1 mM EGTA) evoked transient rises of $[Ca^{2+}]_i$ that were significantly greater ($p < 0.01$) than those seen in control cells. Basal $[Ca^{2+}]_i$ was not affected by CH, being 0.52 ± 0.02 ratio units in control cells and 0.49 ± 0.02 ratio units in CH cells (taken from experiments of Fig. 1). Such responses are exemplified in Fig. 1(a), and quantified in terms of peak responses (Fig. 1b) and integral of transient responses (Fig. 1c). We have previously shown that prolonged exposure of astrocytes to CH enhances BK-evoked rises of $[Ca^{2+}]_i$ through a mechanism primarily involving decreased mitochondrial Ca^{2+} buffering (Smith *et al*. 2003). Since mitochondria are a source of ROS, and recent studies indicate that hypoxia leads to a rise of ROS (Chandel and Schumacker 2000; Chandel *et al*. 2000; Green *et al*. 2002), the involvement of ROS in hypoxic remodelling of astrocytes was investigated. To do this, cells were incubated with two structurally unrelated antioxidants, melatonin or ascorbic acid, during the 24-h period of exposure to CH. Exposure of control cells to melatonin (150 μM) or ascorbic acid (200 μM) for 24 h had no effect on BK-induced $[Ca^{2+}]_i$ rises (Figs 1b and c). However, the potentiation of BK-evoked rises of $[Ca^{2+}]_i$ seen under CH conditions was fully prevented by either of these antioxidants (Figs 1a–c). These antioxidants had no significant effect on basal $[Ca^{2+}]_i$ levels (in control cells, basal levels with ascorbate 0.46 ± 0.01 ratio units, and with melatonin 0.48 ± 0.02 ratio units; in CH cells basal levels with ascorbate 0.50 ± 0.02; with melatonin 0.53 ± 0.02 ratio units).

Our previous studies have shown in PC12 cells that specific effects of hypoxia are mimicked by – and, indeed, appear to require formation of – AβPs (Taylor *et al*. 1999; Green and Peers 2002; Green *et al*. 2002). Since many of the actions of AβPs are associated with increased levels of ROS (see, e.g. Hensley *et al*. 1994; Miranda *et al*. 2000), we next investigated the effects of exposure of astrocytes to $AβP_{(1–40)}$ (1 μM, 24 h). $AβP_{(1–40)}$ significantly ($p < 0.01$) enhanced BK-induced rises of $[Ca^{2+}]_i$ as compared to control cells (Figs 2a and b), although this enhancement was smaller than that caused by CH (Fig. 1). Surprisingly, however, neither melatonin nor ascorbic acid prevented the $AβP_{(1–40)}$-mediated enhancement of BK-induced Ca^{2+} rises (Figs 2a and b), suggesting that the effect of this peptide in rat type I cortical astrocytes is not dependent on the production of ROS. This potentiating effect was mimicked by exogenous application of $AβP_{(25–35)}$, but not by the reverse sequence peptide, $AβP_{(40–1)}$ (both at 1 μM; Fig. 2c). Interestingly, cells treated with $AβP_{(1–42)}$, reportedly the most toxic AβP fragment (Mattson 1997; Selkoe 2001), had no effect on BK-induced Ca^{2+} rises (Fig. 2c). All effects reported in Fig. 2 were

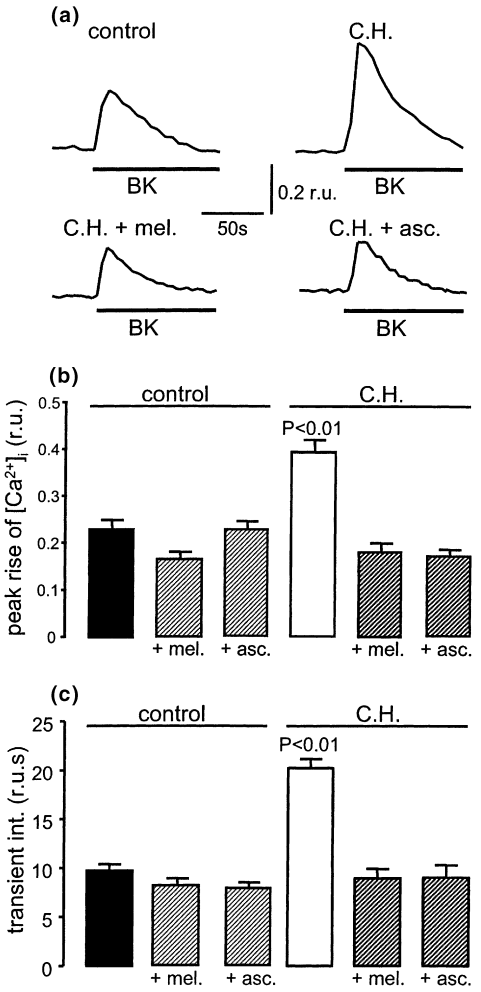

Fig. 1 Antioxidants prevent the augmentation of bradykinin-induced $[Ca^{2+}]_i$ rises caused by chronic hypoxia. (a) Example traces of $[Ca^{2+}]_i$ rises in response to 100 nM bradykinin (BK, applied for the period indicated by horizontal bars below each trace) in control cells (control), cells cultured under CH conditions (CH), and CH cells treated with 150 μM melatonin (+ mel.) or 200 μM ascorbic acid (+ asc.). Scale bars apply to all traces (r.u., ratio units). Experiments were conducted in a Ca^{2+}-free solution. (b) Bar graph indicating mean (± SEM bars) peak rises of $[Ca^{2+}]_i$ evoked by 100 nM BK under the conditions indicated ($n = 3$–6 experiments in each case). (c) Bar graph indicating mean (± SEM bars) transient integrals taken from the same experiments as in (b). *p*-values indicate significantly different from control values (unpaired *t*-test).

similar when peak rises of $[Ca^{2+}]_i$ were measured instead of transient integrals (data not shown). Thus, although $A\beta P_{(1-40)}$ appeared to mimic the augmenting effects of hypoxia, the underlying mechanisms appear to differ, and are not shared by the longer peptide form, $A\beta P_{(1-42)}$.

Despite the apparent lack of mechanistic similarities, it was clear that the effect of hypoxia to augment agonist-evoked rises of $[Ca^{2+}]_i$ in astrocytes was shared by $A\beta P_{(1-40)}$. Thus, as for PC12 cells (Taylor *et al*. 1999; Green *et al*. 2002), there was a possibility that the effects of hypoxia were somehow linked to amyloidogenic APP processing. To investigate this further, we used the monoclonal antibody 3D6 as a means to detect potential changes in AβP levels using immunohistochemistry. This antibody recognizes AβP species but, crucially, does not recognize APP or other fragments of APP (Johnson-Wood *et al*. 1997). As exemplified in Fig. 3, CH caused a marked increase in AβP immunofluorescence (Fig. 3b) as compared with control cells, where AβP immunofluorescence was barely detectable (Fig. 3a). One mechanism that may account for increased production of AβPs under CH conditions is altered processing of APP via secretase enzymes. The protein complex that comprises γ-secretase (Edbauer *et al*. 2003) is sensitive to the dipeptide-aldehyde, 2-naphthoyl-VF-CHO (γ-IV), which is a potent, cell-permeable agent known to prevent formation of either $A\beta_{1-40}$ and $A\beta_{1-42}$ (Sinha and Lieberburg 1999). Alternatively, amyloid production can be inhibited by blockade β-secretase activity, for example with H-KTEEI-SEVN-stat-VAEF-OH (β-secretase inhibitor, β-SI), which prevents the initial cleavage of APP and therefore subsequent Aβ production (Sinha *et al*. 1999). The enhanced fluorescence seen under CH conditions was attenuated when cells were incubated with either γ-IV (10 μM; Fig. 3c) or β-SI (100 nM; Fig. 3d). These findings were consistently seen in five separate experiments.

One candidate mechanism through which CH may be causing an increase in AβP production is via an increase in γ-secretase activity. Figure 4a shows fluorescence images of astrocytes that were stained using a polyclonal antibody raised against the N-terminal fragment of PS-1. Clearly, as compared with control cells, this immunofluorescence was increased in CH cells. This observation was supported and quantified using western blots (Fig. 4b): most of the PS-1 protein was detected in the cleaved, active form and since our antibody was directed at the N-terminal region of PS-1, we have termed this the N-terminal fragment. We were also able to detect low levels of intact PS-1 (Fig. 4b), but the major observation was that both intact and cleaved forms of PS-1 were significantly increased in cells exposed to CH.

Thus far, our data indicate that CH potentiates BK-evoked rises of $[Ca^{2+}]_i$ via a mechanism involving ROS production, and also that CH has pro-amyloidogenic effects, increasing both AβP and PS-1 levels in astrocytes. Since presenilins, particularly PS-1, are also known to have effects on cellular Ca^{2+} homeostasis (e.g. Leissring *et al*. 2000; Yoo *et al*. 2000), we investigated whether the pro-amyloidogenic effects of CH were in any way associated with effects on BK-evoked rises of $[Ca^{2+}]_i$. To do this, we examined the ability of γ-IV or β-SI to alter BK-evoked rises of $[Ca^{2+}]_i$, again in the absence of extracellular Ca^{2+}. As shown in Figs 5(a) and (b), 24-h incubation of cells with either γ-IV (10 μM) or β-SI (100 nM) failed to alter the

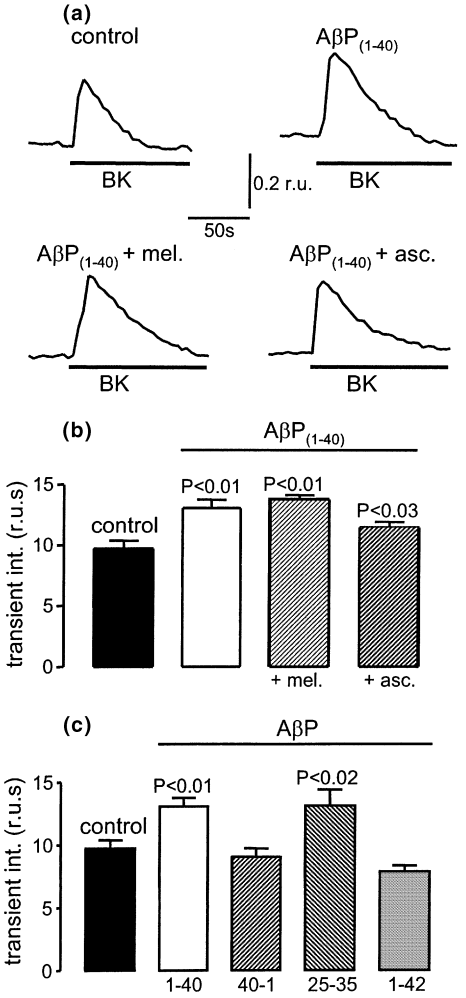

Fig. 2 Antioxidants do not influence augmentation of BK-induced $[Ca^{2+}]_i$ rises evoked by $A\beta P_{(1-40)}$. (a) Example traces of $[Ca^{2+}]_i$ rises in response to 100 nM BK (applied for the period indicated by the solid horizontal bar in a Ca^{2+}-free solution) in control cells and in cells treated with $A\beta P_{(1-40)}$ either alone, or together with 150 μM melatonin ($A\beta P_{1-40}$ + mel.) or 200 μM ascorbic acid ($A\beta P_{1-40}$ + asc.). Scale bars apply to all traces (r.u., ratio units). (b) Bar graph indicating mean (± SEM bars) transient integrals taken from the same experiments as exemplified in (a) ($n = 3$–6 experiments in each case). *p*-values indicate significantly different values from controls (unpaired *t*-tests). (c) Bar graph indicating mean (± SEM bars) transient integrals taken from rises of $[Ca^{2+}]_i$ evoked by BK in cells treated with various amyloid peptides, as indicated below each bar (all applied at 1 μM for 24 h). *p*-values indicate significantly different values from controls.

responses to BK of control cells. However, responses observed in CH cells were significantly attenuated ($p < 0.01$) using either blocker (Figs 5a and b). These data suggest that at least part of the enhancement of BK-evoked rises of $[Ca^{2+}]_i$ caused by CH is attributable to amyloidogenic APP processing.

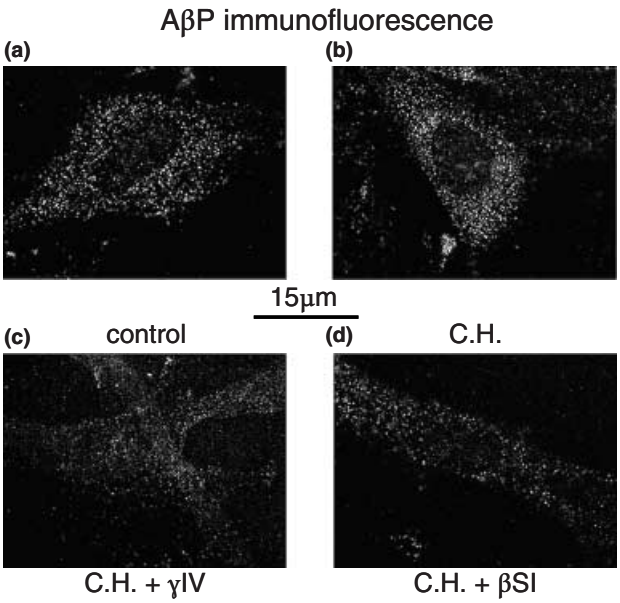

Fig. 3 Chronic hypoxia increases AβP accumulation in astrocytes. Fluorescent images of AβP detected in rat type I cortical astrocytes cultured under normoxic conditions (a) and following a period of CH (b). Also shown are immunofluorescent images from cells cultured hypoxically but in the additional presence of the γ-secretase inhibitor, γ-IV (10 μM; c) or the β-secretase inhibitor, β-SI (100 nM; d). Each image is representative of at least four separate experiments, and AβP fluorescence was detected using the 3D6 monoclonal antibody raised against the N-terminus of AβP as the primary antibody. Scale bar applies to all traces.

Discussion

The present study was conducted to investigate the mechanism(s) underlying the ability of CH to potentiate agonist-evoked rises of $[Ca^{2+}]_i$ observed in the absence of Ca^{2+} influx (i.e. liberation of Ca^{2+} from intracellular stores). We have previously shown that this action of CH arises not by an increase in the size of the ER Ca^{2+} store, but by a reduced buffering capacity of the cell, with the most functionally important site of reduced buffering capacity being the mitochondria (Smith *et al*. 2003). Mitochondria are less capable of buffering rises of cytoplasmic $[Ca^{2+}]$ following CH because they become hyperpolarized and so already contain excess Ca^{2+} levels (Smith *et al*. 2003). Importantly, such hyperpolarization has been shown to lead to increased production of ROS (Lee *et al*. 2002), and ROS have been implicated in the inhibition of the plasmalemmal NCX (Nicholls and Ward 2000), the second site of reduced Ca^{2+} buffering observed in CH astrocytes (Smith *et al*. 2003). The work in this present study was in part therefore conducted in order to determine the role for ROS in the CH-mediated disturbance of Ca^{2+} homeostasis. In addition, given the close association of CH-mediated alterations in cell function and those of AβPs, together with the observation that CH can

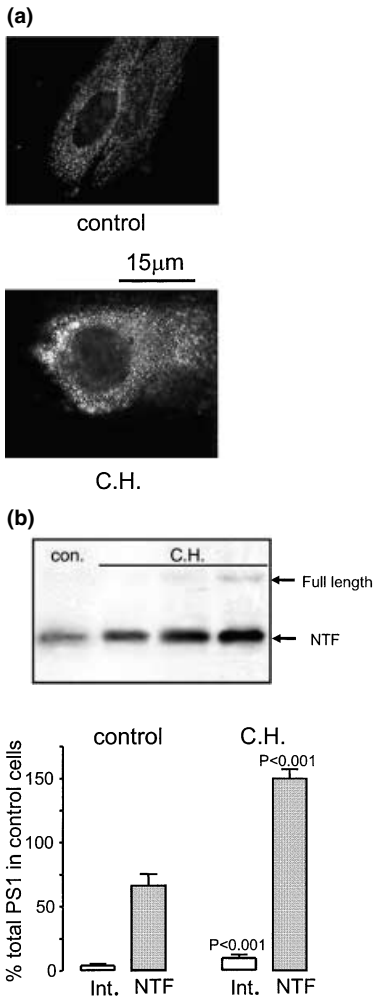

Fig. 4 Chronic hypoxia increases expression of PS-1 in astrocytes. (a) Fluorescent images of PS-1 in rat type I cortical astrocytes cultured under control (upper trace) or CH (lower trace) conditions. Fluorescence was detected using a polyclonal antibody raised against the N-terminal fragment of PS-1. Scale bar applies to both traces. (b) Western blots showing full length and N-terminal (NTF) fragment PS-1, as indicated. Bar graph (below) shows mean densitometric data expressed as percentage of the total PS-1 load in control cells ($n \geq 6$ for each condition). *p*-values shown above bars indicate statistically different values from relevant controls.

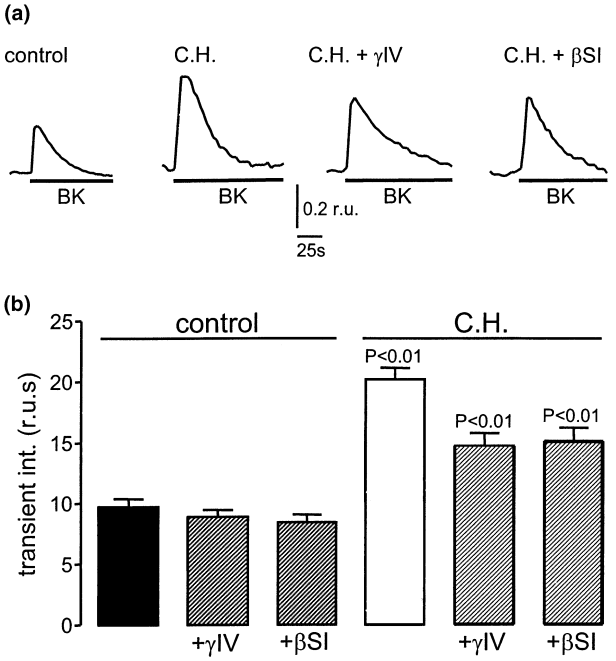

Fig. 5 Inhibitors of β and γ-secretases partially reverse the effects of chronic hypoxia. (a) Example traces of $[Ca^{2+}]_i$ rises evoked by 100 nM bradykinin (BK) in control cells (control) and in cells cultured under CH conditions alone (CH) or in the additional presence of 10 μM γ-IV (+ γ-IV) or 100 nM β-SI (+ β-SI). Scale bars apply to all traces (r.u., ratio units). BK was applied for the period indicated by the solid horizontal bars in Ca^{2+}-free solution. (b) Bar graph indicating mean values of transient integrals taken from from recordings exemplified in (a). Data are mean ± SEM, $n = 3$–6 experiments in each case, from each of which at least three cells were analysed. *p*-values shown above bars indicate statistically different values from control cells determined using unpaired *t*-tests.

increase AβP levels (Taylor *et al*. 1999; Green and Peers 2001, 2002; Green *et al*. 2002), led us to investigate a possible role for pro-amyloidogenic APP processing in these cells.

Our initial observation was that the effects of CH on BK-induced Ca^{2+} rises were fully reversed by the antioxidants melatonin or ascorbate (Fig. 1), implying that the effects of CH are mediated by ROS. This observation is in accordance with the recent report by Duchen and colleagues that mitochondrially generated ROS cause mitochondrial Ca^{2+} loading from ER stores in astrocytes (Jacobson and Duchen 2002). This Ca^{2+} loading into the mitochondria would in turn render the mitochondria less able to buffer cytosolic Ca^{2+} rises. The nature of the mechanism underlying NCX inhibition is not presently clear, but it has been suggested that mitochondrial Ca^{2+} accumulation specifically inhibits the NCX in COS cells transfected with a bovine NCX (Opuni and Reeves 2000), yet no mechanism for this was provided. Whether or not ROS derived from mitochondria interact directly with NCX to alter its function, or other mitochondrial factor(s) are responsible for NCX inhibition remains to be determined.

Several reports have found that AβP deposition is increased following a period of cerebral ischaemia (Yokota *et al*. 1996; Niwa *et al*. 2002). It has also been shown in PC12 cells that the actions of hypoxia on Ca^{2+} *influx* can be mimicked by the exogenous application of AβP, and also that the effects of this peptide were dependent on its ability to produce ROS (Green *et al*. 2002). Application of $A\beta P_{(1-40)}$ for 24 h significantly enhanced BK-induced Ca^{2+} rises in astrocytes, yet this rise was significantly smaller than that seen under CH conditions (Figs 2a and b). This effect was mimicked by $A\beta P_{(25-35)}$ but

not by the reverse sequence AβP fragment $A\beta P_{(40-1)}$. Surprisingly, $A\beta P_{(1-42)}$, generally considered to be the most toxic AβP fragment (Jarrett *et al*. 1993), also failed to influence Ca^{2+} homeostasis. It is important to note that we have previously demonstrated in PC12 cells that this peptide can indeed mimic the actions of $A\beta P_{(1-40)}$ (e.g. Green and Peers 2001), so the lack of effect shown here cannot be attributable to solubility status of this peptide.

One mechanism through which AβP is supposed to mediate its effects is via the production of ROS (Hensley *et al*. 1994). However, the antioxidants melatonin and ascorbic acid failed to influence $A\beta P_{(1-40)}$-enhanced, BK-induced $[Ca^{2+}]_i$ rises in astrocytes (Fig. 2). Defects in energy metabolism are a consistent feature of the AD-affected brain and AβPs have been shown to interact with the respiratory chain, thereby reducing cellular ATP levels (Casley *et al*. 2002). Given the importance of mitochondria in buffering cytosolic Ca^{2+} rises in astrocytes (Boitier *et al*. 1999; Smith *et al*. 2003), any interaction of the AβP with mitochondrial function might be expected to influence Ca^{2+} homeostasis. As has been reported, AβP itself can interact with NCX directly and interfere with plasma membrane Ca^{2+} transport. This has been shown not to be a ROS-mediated effect, as various antioxidants have failed to reverse the NCX inhibition (Wu *et al*. 1997). Thus, NCX inhibition by AβP itself seems a likely explanation for the small, yet significant enhancement of BK-induced Ca^{2+} rises seen in $A\beta P_{(1-40)}$ treated cells.

Recent evidence strongly suggests that $A\beta P_{(1-40)}$ and $A\beta P_{(1-42)}$ are cleaved from amyloid precursor protein (APP) by the sequential actions of the β- and γ-secretase (Selkoe and Podlisny 2002), with the γ-secretase being a heteromultimeric protein including PS-1 (Edbauer *et al*. 2003). Under hypoxic conditions, the degradation of the hypoxia inducible factor subunit, HIF-1α, is suppressed and the transcription of mRNAs encoding hypoxia responsive genes can occur (Safran and Kaelin 2003). Recently, HIF binding regions in the PS-1 gene promoter regions have been described (Bazan and Lukiw 2002) potentially representing a site at which O_2 levels can regulate PS-1 activity. Indeed following a period of chronic hypoxia, not only are PS-1 levels elevated (Fig. 4), but also the product of γ-secretase/PS-1 activity, AβPs (Fig. 3). Further evidence of increased PS-1 expression is provided by the ability of the β- and γ-secretase inhibitors to prevent hypoxic increases in AβP levels, as shown immunocytochemically (Figs 3c and d). Current lack of a viable ELISA assay for rat AβPs, coupled with the fact that even under hypoxic conditions AβP levels are too low to detect in western blots in these cells, has meant we currently have to rely on immunocytochemistry to detect AβPs. However, the monoclonal antibody used (3D6) is known not to recognize APP or derivatives other than AβPs (Johnson-Wood *et al*. 1997) and so results obtained may be considered reliable.

Recently, LaFerla and colleagues described a physiological signalling role for the γ-secretase-derived intracellular fragment of APP (Leissring *et al*. 2002). Cleavage of APP by γ-secretase/PS-1 liberates a fragment, the APP intracellular cleaved domain (AICD), which interacts with the nuclear adaptor protein, Fe65. Leissring *et al*. (2002) showed that blockade of AICD production impaired phosphoinositide-mediated Ca^{2+} signalling. In rat type I cortical astrocytes application of either the β- or γ-secretase inhibitors for a period of 24 h had no effect on control BK-induced $[Ca^{2+}]_i$ rises (Fig. 5), suggesting that AICD plays no role in controlling Ca^{2+} homeostasis under normoxic conditions. However, PS-1 expression is increased (Fig. 4), potentially increasing the production of AICD. When cells were cultured under a hypoxic environment for 24 h in the presence of either the β- or γ-secretase inhibitor, BK-induced Ca^{2+} rises were partially, but significantly, reduced as compared with $[Ca^{2+}]_i$ rises seen in CH cells (Fig. 5). Thus, part of the enhanced Ca^{2+} response seen upon application of BK may be via an amyloidogenic pathway.

Under normoxic conditions, proline residues in HIF-1α are hydroxylated. These hydroxylated prolines acts as recognition sites for the von Hippel-Lindau tumour-suppressor protein (pVHL), permitting ubiquitin-targeted degradation (Safran and Kaelin 2003). Under hypoxic conditions, proline hydroxylation does not occur, preventing pVHL binding and subsequent degradation (Hofer *et al*. 2002). However, recently, Chandel and colleagues have provided an alternative explanation for the stabilization of HIF-1α under hypoxic conditions that involves ROS (Schroedl *et al*. 2002). In this study, cells lacking mitochondrial DNA (so called ρ^0 cells) failed to generate ROS or stabilize HIF-1α in response to hypoxia. Therefore it is possible that under hypoxic

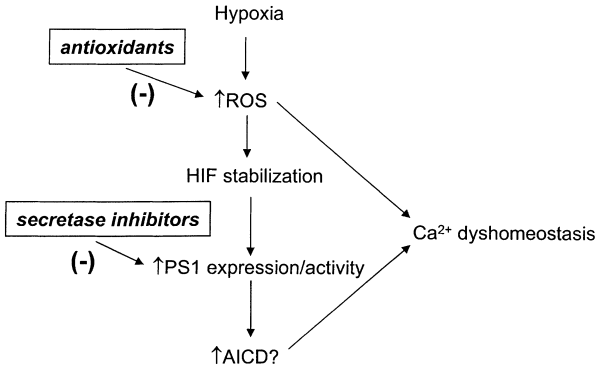

Fig. 6 Potential pathways through which chronic hypoxia may interact with amyloidogenesis. Under hypoxic conditions increased production of ROS (which in itself causes Ca^{2+} dyshomeostasis), stabilizes HIF-1α, which in turn interacts with the HIF binding domain of PS-1 thus increasing the production of the amyloid intracellular cleaved domain (AICD) complex, with a resultant disturbance in Ca^{2+} homeostasis. Antioxidants fully abolish the response to hypoxia by scavenging ROS, thereby preventing HIF-1α stabilization. Secretase inhibitors only partially reduce CH-induced enhancement of BK-induced rises of $[Ca^{2+}]$ rises as they only have an effect on AICD formation.

conditions, the increased production of ROS (which in itself causes Ca^{2+} dyshomeostasis), stabilizes HIF-1α and this in turn interacts with the HIF binding domain of PS-1, increasing the production of the AICD complex with further resultant disturbances in Ca^{2+} homeostasis (as illustrated in Fig. 6). In support of this hypothesis, when cells were incubated with the secretase inhibitors, there was only a partial reduction in enhancement of the BK-induced Ca^{2+} rise seen in CH levels (Fig. 5) presumably because these inhibitors have no effect on ROS-mediated disturbances of Ca^{2+} homeostasis. However, when cells were incubated with antioxidants, ROS are scavenged and therefore no ROS-mediated Ca^{2+} dyshomeostasis occurs, HIF-1α is not stabilized, PS-1 expression is not induced, thus AICD is not produced to mediate Ca^{2+} dyshomeostasis. Thus the model of O_2 sensing involving ROS production by mitochondria provided by Chandel and colleagues (Schroedl *et al*. 2002), provides a potential pathway in which O_2 sensing interacts with amyloidogenesis.

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