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Effects of chronic hypoxia on Ca^{2+} stores and capacitative Ca^{2+} entry in human neuroblastoma (SH-SY5Y) cells

I. F. Smith,* J. P. Boyle,* P. F. T. Vaughan,* H. A. Pearson† and C. Peers*

**Institute for Cardiovascular Research and †School of Biological Sciences, University of Leeds, Leeds, UK*

Abstract

Microfluorimetric measurements of intracellular calcium ion concentration $[Ca^{2+}]_i$ were employed to examine the effects of chronic hypoxia (2.5% O_2, 24 h) on Ca^{2+} stores and capacitative Ca^{2+} entry in human neuroblastoma (SH-SY5Y) cells. Activation of muscarinic receptors evoked rises in $[Ca^{2+}]_i$ which were enhanced in chronically hypoxic cells. Transient rises of $[Ca^{2+}]_i$ evoked in Ca^{2+}-free solutions were greater and decayed more slowly following exposure to chronic hypoxia. In control cells, these transient rises of $[Ca^{2+}]_i$ were also enhanced and slowed by removal of external Na^+, whereas the same manoeuvre did not affect responses in chronically hypoxic cells. Capacitative Ca^{2+} entry, observed when re-applying Ca^{2+} following depletion of intracellular stores, was suppressed in chronically hypoxic cells. Western blots revealed that presenilin-1 levels were unaffected by chronic hypoxia. Exposure of cells to amyloid β peptide (1–40) also increased transient $[Ca^{2+}]_i$ rises, but did not mimic any other effects of chronic hypoxia. Our results indicate that chronic hypoxia causes increased filling of intracellular Ca^{2+} stores, suppressed expression or activity of Na^+/Ca^{2+} exchange and reduced capacitative Ca^{2+} entry. These effects are not attributable to increased amyloid β peptide or presenilin-1 levels, but are likely to be important in adaptive cellular remodelling in response to prolonged hypoxic or ischemic episodes.

Keywords: amyloid peptide, Ca^{2+} stores, capacitative Ca^{2+} entry, hypoxia, intracellular Ca^{2+}, presenilin.

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Prolonged exposure to low O_2 levels (chronic hypoxia; CH), can have marked and diverse effects on a variety of tissues, including neurones, by mechanisms which involve altered gene expression (see recent reviews by Lopez-Barneo *et al*. 2001; Semenza 2000, 2001). Particularly important to cell excitability is the altered expression of ion channels by CH, which leads to altered contractility (in muscle) and altered neurosecretion (in neuronal and neuroendocrine tissue; Smirnov *et al*. 1994; Conforti and Millhorn 1997; Taylor *et al*. 1999; Green and Peers 2001). This cellular remodelling can be considered a protective adaptation to CH, but may also reflect initial stages of deleterious effects on cell function.

We have previously reported that CH potentiates stimulus-secretion coupling in excitable cells in a manner that can be mimicked by exposure of cells to amyloid β peptides (AβPs; Taylor *et al*. 1999), the accumulation of which are associated with Alzheimer's disease (AD; see, e.g. Mattson 1997; Selkoe 2001 for reviews). Indeed, using pheochromocytoma (PC12) cells we have provided evidence to indicate that augmentation of Ca^{2+}-dependent exocytosis by CH is mediated by increased AβP production (Taylor *et al*. 1999; Green and Peers 2001). This is a potentially important pathophysiological observation, since the incidence of AD is greatly increased in individuals following a period of hypoxia/ischemia, such as is encountered in stroke (Tatemichi *et al*. 1994; Kokmen *et al*. 1996; Moroney *et al*. 1996). This clinical observation is supported by several experimental models, all of which point to increased amyloidogenic processing following hypoxic/ischemic insult (Kogure and Kato 1993; Koistinaho *et al*. 1996; Yokota *et al*. 1996; Jendroska *et al*. 1997).

Disruption of Ca^{2+} homeostasis is an important, contributory factor in neurodegeneration associated with AD

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Address correspondence and reprint requests to Dr Chris Peers, Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, UK. E-mail: c.s.peers@leeds.ac.uk

Abbreviations used: AD, Alzheimer's disease; AβP, amyloid β peptide; BSA, bovine serum albumin; CH, chronic hypoxia; CCE, capacitative calcium entry; $[Ca^{2+}]_i$, intracellular calcium ion concentration; ECL, enhanced chemiluminescence; HBS, HEPES-based saline; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; PS1, presenilin 1; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

(reviewed by Hardy 1997; Mattson 1997; Selkoe 2001). The mechanisms underlying AD-related disturbances of Ca^{2+} homeostasis remain unclear, but new studies have pointed to presenilins, particularly presenilin 1 (PS1), as being key candidate proteins in this process (Esler *et al*. 2000; Li *et al*. 2000; Wolfe and Haass 2001). Recent evidence indicates that PS1 is the elusive γ secretase, one of the secretases which cleave AβPs from amyloid precursor protein (APP; Esler *et al*. 2000; Li *et al*. 2000; Wolfe and Haass 2001). These studies suggested that Ca^{2+} mobilization from intracellular stores and capacitative Ca^{2+} entry (CCE) induced by depletion of stores are modified by altered PS1 levels (see also Begley *et al*. 1999; Chan *et al*. 2000; Mattson *et al*. 2000). Such observations provide strong mechanistic links between AD and altered Ca^{2+} homeostasis.

Given the association of altered Ca^{2+} stores/CCE with mutant PS1 associated with familial AD (FAD) described above, and the concept that CH may promote amyloidogenic pathways associated with AD, the present study was conducted to investigate whether CH might disrupt Ca^{2+} homeostasis in the human neuroblastoma SH-SY5Y, since one candidate mechanism by which chronic hypoxia could lead to increased AβP formation is via enhanced activity and/or expression of PS1. We have investigated the effects of chronic hypoxia on Ca^{2+} mobilization from intracellular stores and subsequent CCE, which are well characterized in these cells (Murphy *et al*. 1991, 1992; McDonald *et al*. 1995, 1997; Purkiss *et al*. 1995; Vaughan *et al*. 1997, 1998). We compare the effects of CH with those of exposure to $A\beta P_{(1-40)}$, since our previous studies have indicated that effects of hypoxia are mimicked by this peptide (Taylor *et al*. 1999; Green and Peers 2001).

Materials and methods

Human neuroblastoma SH-SY5Y cells were cultured in a mixture of Ham's F12 medium and Eagle's minimal essential medium (1 : 1 mixture), supplemented with 10% (v/v) fetal calf serum, 1% non-essential amino acids and 0.1% gentamicin, as previously described (e.g. Murphy *et al*. 1992; McDonald *et al*. 1995). Cells were incubated at 37°C in a humidified incubator gassed with 95% air and 5% CO_2, passaged every 7 days and used for up to 20 passages. When required for fluorimetric studies, cells were harvested in phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} and subcultured on poly-D-lysine-coated glass coverslips at a seeding density of 3×10^4 cells/mL. Cells maintained under chronically hypoxic conditions were subcultured in the same way, but, for 24 h prior to experiments, were transferred to a humidified environment continuously gassed with 2.5% O_2, 5% CO_2 and 92.5% N_2. Corresponding control cells were maintained in a 95% air/5% CO_2 incubator for the same period. To examine the effects of $A\beta P_{(1-40)}$, the peptide was added directly to cells in culture for 24 h, as previously described (Taylor *et al*. 1999).

Changes in $[Ca^{2+}]_i$ were measured in SH-SY5Y cells attached to glass coverslips after 4 days in culture using the methods described previously (McDonald *et al*. 1997; Roberts *et al*. 2001). In brief, cell layers were incubated with HEPES-buffered saline (HBS) containing 4 μM fura-2AM at room temperature (21–24°C) for 1 h. HBS was composed of (in mM): NaCl 135, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2.5, HEPES 5, and glucose 10 (pH 7.4, osmolarity adjusted to 300 mOsm with sucrose, 21–24°C). Fragments of coverslip were then placed in a perfusion chamber and intracellular calcium concentration ($[Ca^{2+}]_i$) measured with a Joyce Loeble Phocal apparatus (Applied Imaging, Newcastle upon Tyne, UK). Cells were continually perfused with HBS and $[Ca^{2+}]_i$ was indicated by the ratio of fluorescence emitted at 510 nm due to alternating excitation at 340 nm and 380 nm using a rotating filter wheel. Ca^{2+}-free HBS contained 1 mM EGTA and no added $CaCl_2$. In Na^+-free HBS, Na^+ was replaced with *N*-methyl-D-glucamine. Since calibration of fluorescence into absolute $[Ca^{2+}]_i$-values can be subject to artefactual inaccuracies (Duchen 1992), data are presented as ratio signals.

Several parameters were determined from collected data. Changes in $[Ca^{2+}]_i$ were taken from measuring peak or plateau values and expressing them as the change in fluorescence ratio from basal levels, determined for each recording. Rise-times of responses were taken as the time for a response to progress from 20% to 80% of the final (peak) value. Decay times are expressed as $t_{1/2}$-values, i.e. the time taken for a response to decline to 50% of its peak value. The size of muscarine-evoked stores was taken from the integral of transient responses recorded in Ca^{2+}-free perfusate (see Fig. 2). Mean time courses of CCE were measured every 10 s for averaging (indicated in Figs 4 and 5b). All results are expressed as means ± SEM, together with example traces, and statistical comparisons were made using unpaired *t*-tests.

For western blotting, SH-SY5Y cells were grown to confluence as detailed above, in 75 cm^2 flasks, washed free of media, harvested into Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and centrifuged at 4°C, ~1000 *g* for 10 min. The pellet was then re-suspended and the cells lysed in 1 mL M-Per™ mammalian protein extraction reagent (Perbio Science, Tattenhall, Cheshire, UK) containing Complete Mini protease inhibitors (Roche Diagnostics UK Ltd, Lewes, East Sussex, UK). Protein levels in the cell lysates were measured using the method of Bradford (1976).

Cell proteins (typically 10 μg protein per lane) were separated on 15%, 0.75 mm thick polyacrylamide–sodium dodecyl sulphate (SDS) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (30 V overnight). Membranes were blocked with 5% non-fat milk protein/2% bovine serum albumin (BSA) in PBS-Tween (0.05%) for 1 h and immunostained with antibodies raised against C-terminal (344–358) or N-terminal (2–15) fragments of human presenilin-1 for 3 h. The blots were washed and incubated with anti-rabbit Ig conjugated to HRP (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 Scion Image analysis software.

Results

Activation of muscarinic receptors in SH-SY5Y cells (as in many other cell types) leads to generation of IP_3 and

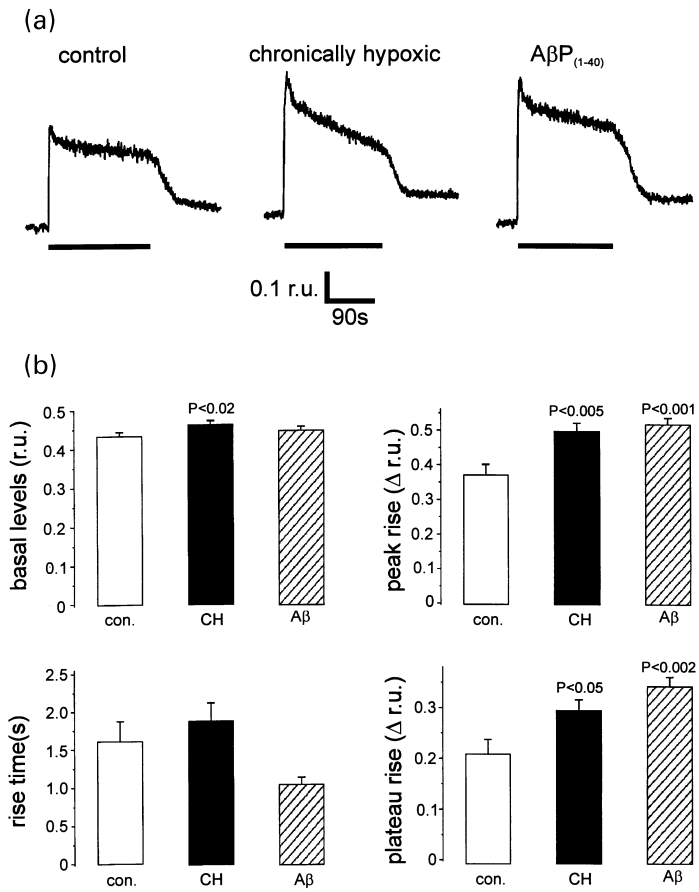

Fig. 1 (a) Example $[Ca^{2+}]_i$ changes evoked in response to 100 μM muscarine in control SH-SY5Y cells (left trace), cells cultured under chronically hypoxic conditions (2.5% O_2, 24 h; middle trace) and cells treated with 1 μM $A\beta P_{(1-40)}$ for 24 h (right trace). Muscarine was applied for the period indicated by the solid horizontal bar below each trace, and scale bars apply to all three traces (r.u.; ratio units). (b) Mean (with vertical SEM bars) measured parameters (basal Ca^{2+} levels, upper left panel; peak rise of Ca^{2+} above basal levels, upper right panel; rise time of peak response, lower left panel; and plateau level above basal, lower right panel) taken from experiments illustrated in (a) for control cells (con; open bars), chronically hypoxic cells (CH; solid bars) and cells exposed to $A\beta P_{(1-40)}$ (Aβ; hatched bars). *p*-values shown above bars indicate statistically significantly different values from control cells, determined using unpaired *t*-tests. Values were determined from 14 recordings for each group.

subsequent liberation of Ca^{2+} from intracellular stores. Store depletion in turn leads to activation of CCE (Purkiss *et al*. 1995; McDonald *et al*. 1997; Roberts *et al*. 2001). Figure 1(a) exemplifies responses to bath application of 100 μM muscarine in the three groups of cells studied: when cells were perfused with extracellular solution containing 2.5 mM Ca^{2+}, application of 100 μM muscarine evoked a rapid rise of $[Ca^{2+}]_i$ which subsequently declined to a plateau level, as previously described (e.g. McDonald *et al*. 1997). The initial peak rise of $[Ca^{2+}]_i$ is attributed to release of Ca^{2+} from intracellular stores, whilst plateau levels are indicative of Ca^{2+} entry from the extracellular environment. It is notable from the example traces that, compared with control cells (left-hand trace), peak responses were greater in cells exposed to chronic hypoxia (middle trace) and in cells exposed to 1 μM $A\beta P_{(1-40)}$ (right hand trace). Mean data taken from these recordings for each group of cells are shown in Fig. 1(b). Compared with control, normoxically cultured cells, those exposed to chronic hypoxia displayed slightly (yet significantly) higher baseline ratio values. More striking, however, was the significant increase in the peak rise of $[Ca^{2+}]_i$ in both CH and AβP-treated cells, whilst rise-times were not significantly different from those observed in control cells. $[Ca^{2+}]_i$ was also significantly raised in CH and AβP-treated cells at the same time point (180 s after muscarine application) at which plateau levels were determined in controls. Clearly, CH and exposure to $A\beta P_{(1-40)}$ augmented Ca^{2+} mobilization/influx in response to activation of muscarinic receptors in SH-SY5Y cells.

Release of Ca^{2+} from intracellular stores can be studied in the absence of CCE using Ca^{2+}-free solutions. Thus, application of 100 μM muscarine in Ca^{2+}-free perfusate (containing 1 mM EGTA) causes a transient rise of $[Ca^{2+}]_i$ arising from IP_3-mediated release from internal stores. In control cells (exemplified in Fig. 2a, left), $[Ca^{2+}]_i$ levels returned to basal values after approximately 130 s. In CH cells (e.g. Figure 2a, middle), the peak rise of $[Ca^{2+}]_i$ was significantly greater and there was also a marked prolongation of the time required for $[Ca^{2+}]_i$ to return to basal levels (see also Fig. 2b). In AβP-treated cells (Fig. 2a, right), the peak rise of $[Ca^{2+}]_i$ was again greater than in control cells, but the time taken for Ca^{2+} to return to its original level was similar to controls. A summary of the parameters measured in these studies is shown in Fig. 2(b). Thus, as compared with control cells, responses in CH cells were larger and decayed more slowly, suggesting that Ca^{2+} stores were far greater, as indicated by the integral of the transient signal. These effects of CH were not fully

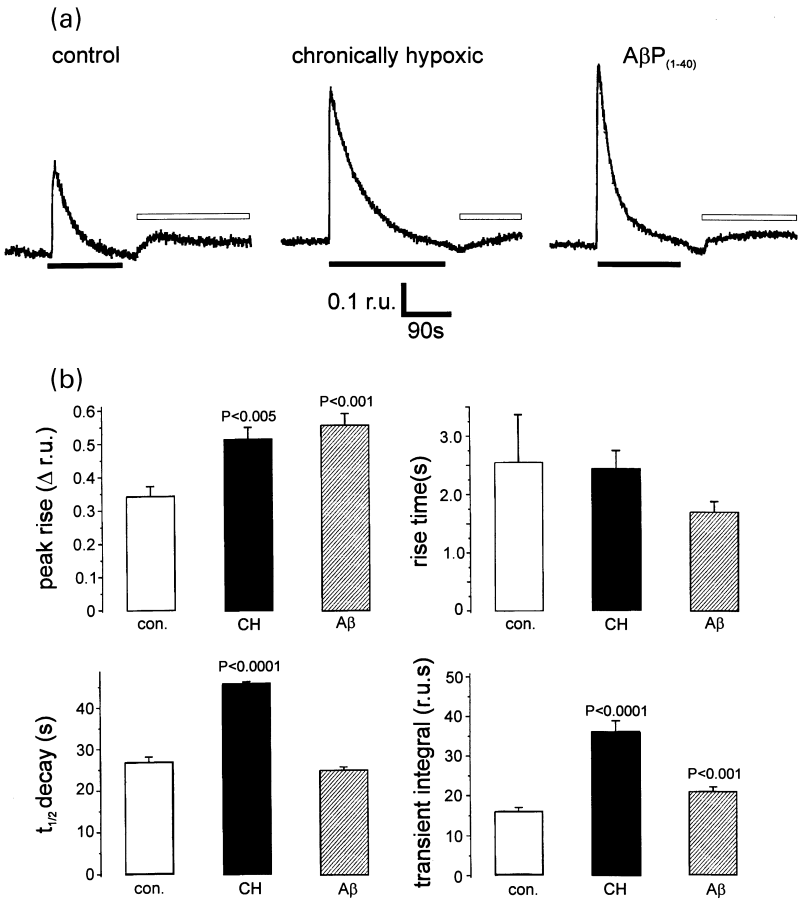

Fig. 2 (a) Example $[Ca^{2+}]_i$ changes evoked in response to 100 μM muscarine in control SH-SY5Y cells (left trace), cells cultured under chronically hypoxic conditions (2.5% O_2, 24 h; middle trace) and cells treated with 1 μM $A\beta P_{(1-40)}$ for 24 h (right trace). Muscarine was applied for the period indicated by the solid horizontal bar below each trace, in the absence of extracellular Ca^{2+} (replaced with 1 mM EGTA). For the periods indicated by the open bars, Ca^{2+} was re-admitted to the perfusate. Scale bars apply to all three traces (r.u.; ratio units). (b) Mean (with vertical SEM bars) measured parameters (peak Ca^{2+} rise, upper left panel; rise time of peak response, upper right panel; time for response to decay to 50% of peak value, lower left panel and the integral of the transient response, lower right panel) determined from experiments illustrated in (a) for control cells (con; open bars), chronically hypoxic cells (CH; solid bars) and cells exposed to $A\beta P_{(1-40)}$ (Aβ; hatched bars). *p*-values shown above bars indicate statistically significantly different values from control cells, determined using unpaired *t*-tests. Values were determined from 14 recordings for each group.

reproduced in AβP-treated cells, since although the peak responses were larger, the rate of decline to basal levels and the integral of the transient, although larger than control values, was markedly less that was seen in CH cells (Fig. 2b).

In Ca^{2+}-free solutions, the rise of $[Ca^{2+}]_i$ during continued agonist application is transient in part because Ca^{2+} is extruded from the cells. To investigate the role of plasmallemal Na^+/Ca^{2+} exchange in this process, and whether Na^+/Ca^{2+} exchanger function was altered in CH cells (which displayed a much longer decline in Ca^{2+}-free solution; Fig. 2), we investigated the effects of Na^+ removal from the perfusate. These studies were not conducted for AβP-treated cells since their decay rates were no different from controls (Fig. 2). As shown in Fig. 3(a), the peak response to 100 μM muscarine was far greater in control cells in the absence of Na^+, and the subsequent decline was significantly slowed (see also Fig. 3b). By contrast, the amplitude and time course of responses to muscarine seen in CH cells was unaffected by Na^+ removal (Figs 3a and b), suggesting that the activity or expression of the Na^+/Ca^{2+} exchanger was suppressed due to exposure to chronically hypoxic conditions.

After $[Ca^{2+}]_i$ levels had declined to basal levels during muscarine application in all three groups, re-addition of Ca^{2+} to the perfusate caused a secondary rise of $[Ca^{2+}]_i$, attributable to CCE. CCE was only observed on re-addition of Ca^{2+} if stores were firstly depleted by muscarine; in all cell groups, removal then re-application of Ca^{2+} without depletion of stores caused no rise of $[Ca^{2+}]_i$ ($n = 4$ for each group, data not shown). Examples are shown in Fig. 2 for all three cell groups examined, and Fig. 4 shows the averaged data (sampled at 10 s intervals) for all three groups. Clearly, compared with CCE observed in control cells, there was a marked slowing of CCE in both CH and AβP-treated cells. However, although this initial rate of rise of $[Ca^{2+}]_i$ was dramatically slowed over the first 50 s of readmission of Ca^{2+}, the maximum levels achieved were not significantly different between the three cell groups.

Leissring *et al.* (2000) have suggested that incomplete discharge of Ca^{2+} stores can lead to changes in the rate and magnitude of subsequent CCE. This would lead in turn to possible misinterpretation of CCE responses observed following muscarine application then removal [although it should be noted that second applications of muscarine in Ca^{2+}-free solutions always failed to evoke a response in any cell group; data not shown (see also McDonald *et al.* 1997)]. To investigate this possibility, cells were incubated in 1 μM thapsigargin for 20 min (in Ca^{2+}-free solution containing

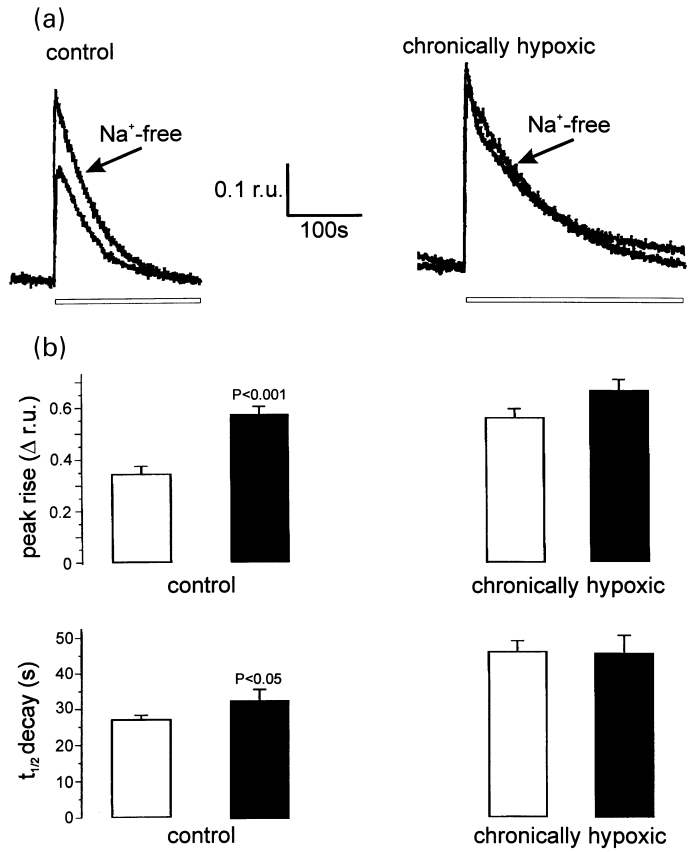

Fig. 3 (a) Example $[Ca^{2+}]_i$ changes evoked in response to 100 μM muscarine in control SH-SY5Y cells (left trace), and in cells cultured under chronically hypoxic conditions (2.5% O_2, 24 h; right trace). Muscarine was applied for the periods indicated by the open horizontal bar below each trace in the absence of extracellular Ca^{2+} (replaced with 1 mM EGTA). Superimposed on these example traces are responses recorded in the absence of extracellular Na^+ (which was replaced with equimolar *N*-methyl-D-glucamine). (b) Mean (± SEM) peak responses (upper bars) and $t_{1/2}$ decay times (lower bars) determined from experiments exemplified in (a) for control and chronically hypoxic cells, as indicated, in the presence (open bars, $n = 17$ for controls and 18 for chronically hypoxic cells) and absence (solid bars, $n = 6$ for both control and chronically hypoxic cells) of extracellular Na^+. *p*-values shown above bars indicate statistically significantly different values (indicative of the effect of Na^+ removal in control cells), determined using unpaired *t*-tests.

1 mM EGTA) to empty Ca^{2+} from internal stores completely, then Ca^{2+} was restored to the perfusate and CCE monitored. Representative example responses are shown in Fig. 5(a). Consistent with data presented in Fig. 4, CCE in thapsigargin-treated cells was significantly slowed and suppressed in CH cells as compared with controls (Fig. 5). Perhaps surprising, however, was the finding that CCE was slightly enhanced in AβP-treated cells, reaching a elevated plateau level (Fig. 5). This latter finding clearly distinguishes a further difference between exposure to chronic hypoxia and treatment with $AβP_{(1–40)}$.

The increase in Ca^{2+} stores and suppression of CCE seen in CH cells was consistent with the idea that CH might increase PS1 levels in SH-SY5Y cells, since over expression of PS1 has previously been shown to lead to similar effects in these and other neuronal cells (e.g. Yoo *et al*. 2000). We therefore employed western blots to compare the levels of PS1 protein in control and CH cells. Results obtained using antibodies raised against both C- and N-terminal epitopes are shown in Fig. 6. In agreement with other studies, PS1 was detected primarily at one point using SDS–polyacrylamide gel electrophoresis (PAGE), corresponding to fragmented PS1, although in both cell types a very small amount of intact PS1 was detected. Using either antibody, it was apparent that CH did not increase PS1 levels in SH-SY5Y cells; indeed, PS1 levels were slightly (although not statistically significantly) reduced in CH cells. Thus, importantly, the effects of CH on Ca^{2+} influx and release from stores cannot be accounted for by increased levels of PS1.

Discussion

In this study, we have characterized the effects of CH on Ca^{2+} mobilization from intracellular stores and subsequent CCE in the human neuroblastoma, SH-SY5Y. In addition, we compared the effects of CH with those of exposure to $AβP_{(1–40)}$, since our previous studies have shown this peptide [and also $AβP_{(1–42)}$ and $AβP_{(25–35)}$, but not $AβP_{(40–1)}$] mimics the effects of CH on up-regulation of L-type Ca^{2+} channels and induction of a Cd^{2+}-resistant Ca^{2+} influx pathway in another catecholaminergic cell line, PC12 (Green and Peers 2001). Our findings, which, to our knowledge constitute the first study on the effects of CH on cellular Ca^{2+} handling in neuronal tissue, reveal that CH has marked effects on Ca^{2+} stores and CCE and, importantly, that these are not mimicked by $AβP_{(1–40)}$ treatment.

Our results suggest that CH increases the levels of Ca^{2+} contained in intracellular stores. Thus, muscarine-evoked rises of $[Ca^{2+}]_i$ were greater in CH cells than in controls,

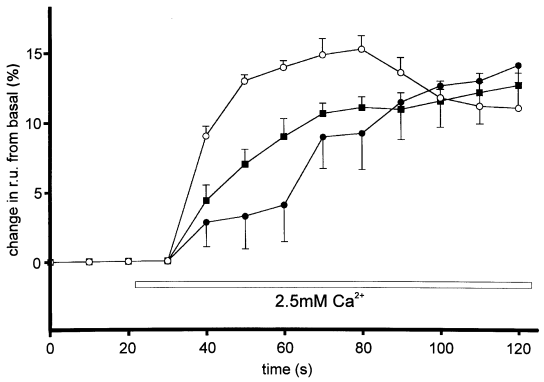

Fig. 4 Mean (± SEM) time courses of rises of $[Ca^{2+}]_i$ due to capacitative Ca^{2+} entry in control cells (○, $n = 6$), cells cultured under chronically hypoxic conditions (●, $n = 8$) and cells exposed to 1 μM $A\beta P_{(1-40)}$ for 24 h (■, $n = 6$). These data were averaged from experiments exemplified in Fig. 1, and show the rise of $[Ca^{2+}]_i$ observed when Ca^{2+} was readmitted to the perfusate after intracellular stores were depleted by exposure of cells to 100 μM muscarine in the absence of extracellular Ca^{2+}. For comparative purposes, data were sampled every 10 s and normalized to the average ratio value determined immediately before readmission of Ca^{2+} (indicated by open bar). Rises of $[Ca^{2+}]_i$ are expressed as percentage change from basal levels for each group.

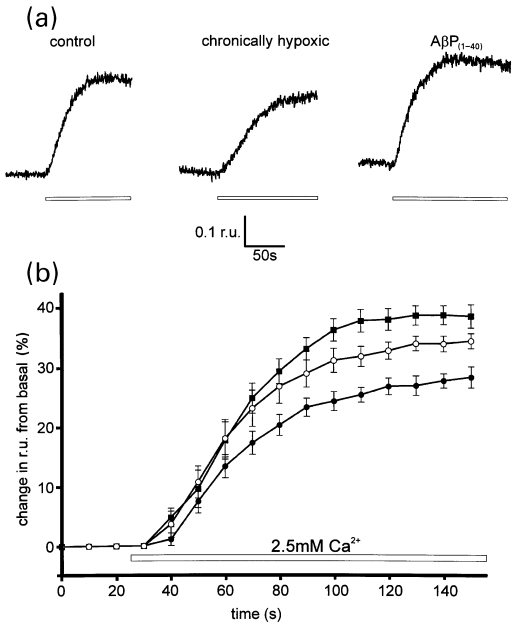

Fig. 5 (a) Example rises of $[Ca^{2+}]_i$ observed in response to re-addition of 2.5 mM Ca^{2+} to the perfusate in control SH-SY5Y cells (left trace), cells cultured under chronically hypoxic conditions (2.5% O_2, 24 h; middle trace) and cells treated with 1 μM $A\beta P_{(1-40)}$ for 24 h (right trace). In each case, cells were previously exposed to 1 μM thapsigargin in Ca^{2+}-free solution (containing 1 mM EGTA) for 20 min to deplete intracellular stores, before Ca^{2+} was re-applied for the periods indicated by the open bars. Scale bars apply to all three traces (r.u.; ratio units). (b) Mean (± SEM) time course of rise of $[Ca^{2+}]_i$ due to capacitative Ca^{2+} entry in control cells (○, $n = 8$), cells cultured under chronically hypoxic conditions (●, $n = 9$) and cells exposed to 1 μM $A\beta P_{(1-40)}$ for 24 h (■, $n = 9$). These data were averaged from experiments exemplified in (a). For comparative purposes, data were sampled every 10 s and normalized to the average ratio value determined immediately before readmission of Ca^{2+} (indicated by open bar). Rises of $[Ca^{2+}]_i$ are expressed as percentage change in ratio units from basal levels.

regardless of the presence (Fig. 1) or absence (Fig. 2) of extracellular Ca^{2+}. However, these results are complicated by the fact that the kinetics of Ca^{2+} transients rely not only on store size, but also Ca^{2+} buffering and extrusion mechanisms. We gained some insight into these factors by examining the effects of removing Na^+ from the extracellular solution in order to prevent Na^+/Ca^{2+} exchange. Interestingly, peak responses in control cells were significantly enhanced, and the $t_{1/2}$ of the evoked transients lengthened, indicating that Na^+/Ca^{2+} exchange is an important mechanism regulating Ca^{2+} homeostasis in these cells. These effects of Na^+ removal were not reproduced in CH cells (Fig. 3). The simplest interpretation of these data is that CH causes down-regulation or dysfunction of the Na^+/Ca^{2+} exchanger. Whilst further experiments are required to clarify this, it is noteworthy that acute hypoxia has recently been shown to suppress Na^+/Ca^{2+} exchange in pulmonary vascular smooth muscle cells (Wang *et al*. 2000) and, in other tissues, the expression of proteins which are modulated by acute hypoxia is altered following CH (e.g. Wyatt *et al*. 1995). It should also be noted, however, that the effects of CH to prolong the decline of $[Ca^{2+}]_i$ transients is unlikely to be due solely to suppression of Na^+/Ca^{2+} exchange, since the increase in $t_{1/2}$ caused by Na^+ removal in controls, whilst significant, was still less than was observed in CH cells (Fig. 3b). Thus, an additional effect of CH on other Ca^{2+} extrusion mechanisms (e.g. the plasmalemmal Ca^{2+}-ATPase) cannot be discounted.

Additional evidence to support the idea that Ca^{2+} stores are over-filled in CH cells [and, indeed, in $A\beta P_{(1-40)}$ treated cells] comes from recordings of CCE following previous muscarine application (Figs 2a and 4). CCE observed following muscarine-depletion of intracellular stores was significantly slowed in CH- and $A\beta P_{(1-40)}$-treated cells as compared with controls (Fig. 4), but this is likely attributable in part to incomplete emptying of stores by muscarine (despite the fact that second applications of muscarine always failed to alter $[Ca^{2+}]_i$ levels in all three cell groups; data not shown). This conclusion is drawn when comparing our study with recent reports which indicated that presenilins are intimately linked with Ca^{2+} homeostasis via modulation of CCE. Thus, Yoo *et al*. (2000) found that knockout of PS1 led to enhanced CCE following agonist-evoked store depletion. Furthermore, SH-SY5Y cells stably expressing an inactive form of PS1 also displayed enhanced

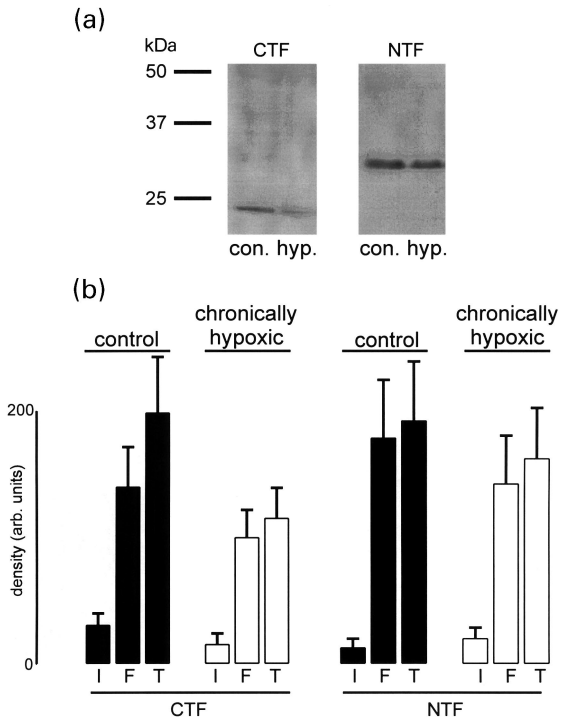

Fig. 6 (a) Example western blots illustrating presenilin-1 fragment levels in control (con.) and chronically hypoxic (hyp.) cells, as determined using a C-terminal fragment-directed antibody (CTF, left-hand blots) and a N-terminal fragment-directed antibody (NTF, right-hand blots). (b) Averaged densitometric values (arbitrary units, based on a 0–256 greyscale densitometric analysis, with vertical SEM bars) determined from experiments exemplified in (a). For both antibodies, tested on control cell homogenates (solid bars, $n = 9$) and homogenates of chronically hypoxic cells (open bars, $n = 6$), densitometric values are given for intact PS1 (I), PS1 fragment (F) and total protein level detected (T).

CCE. By contrast, cells expressing a FAD mutant form of PS1 displayed slowed CCE. The channels underlying CCE, termed CRAC channels (Ca^{2+} release-activated Ca^{2+} channels), were also suppressed in FAD PS1 mutants. Leissring *et al*. (2000) examined Ca^{2+} signalling in FAD PS1 mutant knock-in mice, and found that Ca^{2+} mobilization from intracellular stores was greatly increased, whilst CCE was, in agreement with Yoo *et al*. (2000), markedly decreased. However, when Ca^{2+} stores were depleted completely with thapsigargin in the absence of extracellular Ca^{2+}, subsequent re-exposure to Ca^{2+} produced rises of $[Ca^{2+}]_i$ which were similar in both the PS1 knock-in and control cells. These findings indicated that mutant PS1 caused over-filling of internal stores, such that application of agonist failed to deplete the store fully, leading to an apparent reduction of CCE. Thus, although the studies of Yoo *et al*. (2000) and Leissring *et al*. (2000) further supported the concept that FAD PS1 mutations lead to disturbances of Ca^{2+} homeostasis, the two studies reached strikingly different conclusions. Whether this was due to the fact that Ca^{2+} signalling was studied in neurones and recombinant expression systems (Yoo *et al*. 2000), as opposed to fibroblasts of transgenic mice (Leissring *et al*. 2000), remains to be determined. When applied to the present study, the conclusions of Leissring *et al*. (2000) suggest that both CH and $A\beta P_{(1-40)}$ treatment cause excessive filling of intracellular Ca^{2+} stores.

As detailed earlier, the effects of CH on Ca^{2+} channels are mimicked by, and indeed appear to be mediated by, increased levels of amyloid peptides (Green and Peers 2001). The reason why AβP levels are increased by CH are as yet undetermined, but one possibility was that hypoxia leads to increased levels of presenilin. Thus, if hypoxia caused an increased expression of PS1, this could lead to increased AβP formation. The present study shows directly that CH does not increase PS1 levels (Fig. 6), yet reveals a novel effect of CH on CCE. This was most clearly examined when Ca^{2+} stores were fully emptied by thapsigargin (Fig. 5). Following such treatment, CCE was suppressed as compared with controls, in contrast to the effects of $A\beta P_{(1-40)}$ treatment which caused slight enhancement of CCE. This occurred despite the likely suppression of Na^+/Ca^{2+} exchange (see earlier), an effect which would promote intracellular Ca^{2+} accumulation. Thus, since increased PS1 levels cannot account for this effect, the possibility that CRAC channel expression or function is altered is worthy of future investigation.

In conclusion, the present study reveals that CH exerts marked effects on Ca^{2+} homeostasis in the human neuroblastoma SH-SY5Y. Following exposure to CH, intracellular Ca^{2+} stores appeared to contain increased levels of Ca^{2+}, Na^+/Ca^{2+} exchanger expression or function was markedly diminished, and CCE was suppressed without significant changes in PS1 protein levels. We also found that $A\beta P_{(1-40)}$ treatment increased the size of mobilizable Ca^{2+} stores, but that this peptide could not mimic any other effects of CH. Such effects are likely to be of great importance in the remodelling of cell function as an adaptive response to chronic hypoxia, and may also contribute to neuronal dysfunction or death following prolonged hypoxic or ischemic episodes.

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