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Hypoxia potentiates exocytosis and Ca^{2+} channels in PC12 cells via increased amyloid β peptide formation and reactive oxygen species generation

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Exposure of PC12 cells to chronic hypoxia (CH; 10 % O_2, 24 h) augments catecholamine secretion via formation of a Cd^{2+}-resistant Ca^{2+} influx pathway, and up-regulates native L-type Ca^{2+} channels. These effects are mimicked by exposure of cells to Alzheimer's disease-associated amyloid β peptides (AβPs). Since pathological effects of AβPs have been associated with increased levels of reactive oxygen species (ROS), the involvement of ROS in hypoxia-mediated up-regulation of exocytosis and Ca^{2+} channel activity was examined. Both melatonin and ascorbic acid (two structurally unrelated antioxidants) fully blocked the enhancement of catecholamine secretion caused by CH (as determined amperometrically). Enhanced immunofluorescence, observed in chronically hypoxic cells using a primary monoclonal antibody raised against the N-terminus of AβP, was also suppressed by melatonin. Ascorbic acid, melatonin and ebselen (an additional antioxidant) also fully prevented augmentation of whole-cell Ca^{2+} currents caused by CH (as monitored using whole-cell patch-clamp recordings). Exposure of normoxic cells to H_2O_2 (40 μM, 24 h), like hypoxia, caused Ca^{2+} channel up-regulation. Importantly, AβP formation appeared to be an absolute requirement for the effects of hypoxia, since the ability of CH to augment exocytosis and Ca^{2+} channel activity was blocked by two novel inhibitors of γ secretase, an enzyme complex required for AβP formation. Our results indicate that the effects of hypoxia require ROS generation from AβPs, and suggest that elevated levels of ROS mediate hypoxic and AβP-mediated pathological remodelling of Ca^{2+} homeostasis.

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The incidence of dementias such as Alzheimer's disease (AD) is significantly increased in patients who have previously suffered prolonged hypoxic or ischaemic episodes arising, for example, as a consequence of cardiovascular dysfunction such as stroke or arrhythmia (Tatemichi *et al.* 1994; Kokmen *et al.* 1996; Moroney *et al.* 1996). Ischaemia involves alteration of a number of parameters, including lack of substrates, accumulation of metabolic products, acidosis and reduction of ATP and O_2 levels, each of which is essential to cellular homeostasis, yet our understanding of the influences of each of these parameters to cell injury or destruction is far from complete. However, the clear link between hypoxic/ischaemic episodes and increased incidence of AD strongly suggests that one or more of these parameters are capable of precipitating this increasingly widespread disease.

A defining feature of AD is the appearance of fibrillar deposits consisting of amyloid β peptides (AβPs; reviewed by Mattson, 1997; Selkoe, 2001). AβPs are 39–43 amino acid peptide cleavage products derived from amyloid precursor protein (APP; Glenner & Wong, 1984; Masters *et al.* 1985). APP is one of only a few gene products whose expression is increased following a period of cerebral ischaemia (Kogure & Kato, 1993; Koistinaho *et al.* 1996). The major (non-amyloidogenic) cleavage product of APP, sAPPα, is neuroprotective (Mattson, 1997; Selkoe, 2001), and so increased expression of APP may be considered a defence mechanism against ischaemia. However, increased APP levels would also provide increased substrate for formation of toxic AβPs (Mattson, 1997; Selkoe, 2001) and, indeed, AβP production is increased following ischaemia (Yokoto *et al.* 1996; Jendroska *et al.* 1997).

The mechanisms underlying the neuronal toxicity of AβPs appear complex and remain to be fully resolved. Toxicity involves disruption of Ca_i^{2+} homeostasis (Fraser *et al.* 1997; Mattson, 1997) which may be oxidative and involve free radical damage (Behl *et al.* 1994; Schubert *et al.* 1995). In addition, other studies have shown that AβPs disrupt Ca_i^{2+} homeostasis by forming Ca^{2+}-permeable pores or channels (Arispe *et al.* 1996; Kawahara *et al.* 1997; Rhee *et al.* 1998) which may account for increased central synaptic activity. Indeed, increased activity such as enhancement of long-

term potentiation and elevated glutamate release has been demonstrated in hippocampal neurones exposed to AβPs *in vitro* (Arias *et al.* 1995; Wu *et al.* 1995).

Our recent studies have provided clear evidence that Ca^{2+} homeostasis and neurosecretion are altered when cells are exposed to hypoxic conditions, and that these effects are mimicked by AβPs. Using the catecholamine secreting cell line PC12, we have shown that chronic hypoxia (10 % O_2, 24 h) leads to excessive stimulus-evoked neurosecretion due to the emergence of a Cd^{2+}-resistant Ca^{2+} influx pathway tightly coupled to the exocytotic machinery (Taylor *et al.* 1999). In addition, a selective up-regulation of L-type Ca^{2+} channels was observed (Green & Peers, 2001). These effects were mimicked by direct application of AβPs to the cells (Taylor *et al.* 1999). In the present study, we have examined whether AβP formation is a necessary step in these effects of hypoxia, and also report the involvement of reactive oxygen species (ROS) in mediating these effects of chronic hypoxia. Such studies were prompted not only by the well-recognized involvement of oxidative stress associated with neurotoxic effects of AβPs (Mattson, 1997; Miranda *et al.* 2000; Varadarajan *et al.* 2000; Selkoe, 2001), but also by recent, contested reports which suggest that ROS levels increase during prolonged hypoxia (Chandel *et al.* 1998; Hohler *et al.* 1999; Chandel & Schumacker, 2000). In addition to this it has recently been shown that oxidative stress increases AβPs, either by direct addition of H_2O_2 (Misonou *et al.* 2000), or indirectly through addition of mercury (Olivieri *et al.* 2000). Our results indicate that ROS are likely important mediators in the effects of both chronic hypoxia and AβPs and, importantly, that these effects of hypoxia require AβP production.

METHODS

Cell culture

PC12 cells were cultured in RPMI 1640 culture medium (containing L-glutamine) supplemented with 20 % fetal calf serum and 1 % penicillin–streptomycin (Gibco, Paisley, Strathclyde, UK) as previously described (Taylor *et al.* 1999; Taylor & Peers, 1999). Cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2–95 % air, passaged every 7 days and used for up to 20 passages. Cells used for experiments were transferred to smaller flasks in 10 ml of medium, to which was added 1 μM dexamethasone (Sigma, Poole, UK, from a stock solution of 1 mM in Ultrapure water), and were cultured for a further 72–96 h to enrich catecholamine stores (Tischler *et al.* 1983). Cells exposed to chronic hypoxia were treated identically, except that for 24 h prior to experiments they were transferred to a humidified incubator equilibrated with 10 % O_2, 5 % CO_2 and 85 % N_2. Following this period in chronic hypoxia, cells were exposed to room air for no longer than 1 h before experimentation. Peptides used in this study were dissolved in Ultrapure water and stored frozen in aliquots until required, so that they only underwent one freeze–thaw cycle before being applied directly to the cells. Gel electrophoresis of peptide samples revealed that they were applied to cells in the unaggregated form. When used, antioxidants were included for the culture period in normoxia, hypoxia or during exposure to $A\beta P_{(1–40)}$, as were the dipeptide-aldehyde γ secretase inhibitors, 2-napthyl-Val-Phe-CHO (NVP) and Boc-Gly-Val-valinal (GVV).

Amperometry

Each experimental day, PC12 cells were plated onto poly-L-lysine-coated coverslips and allowed to adhere for 1 h under either normoxic or hypoxic (10 % O_2) conditions, as required. Fragments of coverslip were then transferred to a recording chamber (volume 80 μl) which was continually perfused under gravity (flow rate 1–2 ml min^{-1}) with a solution of composition (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 mosmol l^{-1} with sucrose, 21–24 °C). High $[K^+]$ solutions contained 50 mM K^+ and the $[Na^+]$ was reduced accordingly to maintain iso-osmolarity.

Carbon fibre microelectrodes (proCFE, Dagan Instruments, MN, USA) with a diameter of 5 μm were positioned adjacent to individual PC12 cells using a micromanipulator and were polarized to +800 mV to allow the oxidation of released catecholamine. Resulting currents were recorded using an Axopatch 200A amplifier (with extended voltage range), filtered at 1 kHz and digitized at 2 kHz before storage on computer. All acquisition was performed using a Digidata 1200 interface and Fetchex software from the pCLAMP 6.0.3 suite (Axon Instruments). Unless otherwise stated, each experiment consisted of current recordings of a control period during which cells were perfused only with normoxic external medium. This was then exchanged for a depolarizing test solution (containing 50 mM K^+) and amperometric signals were recorded for a further period of 1–4 min. Catecholamine secretion was apparent as discrete spike-like events, each corresponding to the released contents of a single vesicle of catecholamine (Wightman *et al.* 1991; Chow & Von Ruden, 1995). The perfusate was then exchanged for one containing 50 mM K^+ but also 200 μM Cd^{2+} to block native, voltage-gated Ca^{2+} channels (see Taylor *et al.* 1999). Secretory events were never seen unless the electrode was polarized and adjacent to a cell. Quantification of release was achieved by determining spike frequency using Mini Analysis Program (Synaptosoft Inc., Leonia, NJ, USA). This allowed visual inspection of each event so that artefacts (due, for example, to solution switches) could be rejected from the analysis. Results are presented as individual examples or means ± standard error of the mean and statistical comparisons were made using Student's unpaired *t* test.

Electrophysiology

Cells were plated onto coverslips, fragments of which were subsequently placed in a perfusion chamber exactly as for amperometric recordings, except that perfusing solution was of composition (in mM): NaCl 110, CsCl 5, $MgCl_2$ 0.6, $BaCl_2$ 20, Hepes 5, glucose 10 and tetraethylammonium chloride 20 (pH 7.4). The osmolarity of the perfusate was adjusted to 300 mosmol l^{-1} by addition of sucrose. Patch pipettes (5–7 MΩ resistance) were filled with a solution of (in mM): CsCl 130, EGTA 1.1, $MgCl_2$ 2, $CaCl_2$ 0.1, NaCl 10, Hepes 10 and Na_2ATP 2 (pH 7.2). After establishing the whole-cell configuration, cells were voltage-clamped at −80 mV and whole cell capacitance determined from analog compensation. To evoke whole-cell Ca^{2+} channel currents, 200 ms voltage ramps were applied from −100 to +100 mV at a frequency of 0.2 Hz (Green & Peers, 2001). Evoked currents were filtered at 1 kHz, digitized at 2 kHz and stored on computer for off-line analysis. All results are presented as mean ± S.E.M. current densities spanning the voltage range −60 to +60 mV, which covers their full activation range, and statistical analysis performed using

Student's unpaired *t* tests. All data tested were taken from current densities measured at +20 mV (where *I–V* relationships were maximal).

Immunocytochemistry

Immunofluorescent labelling with a monoclonal antibody raised against the extracellular N-terminal five residues of AβP (3D6 antibody; Johnson-Wood *et al.* 1997) was performed as previously described (Taylor *et al.* 1999) with cells plated onto coverslips and subjected to normoxic or other conditions as described above. Cells were fixed by immersion in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min, and then rinsed thoroughly in several changes of 0.1 M phosphate-buffered saline (PBS). The cells were then incubated for 20 min at room temperature in PBS containing 10 % normal goat serum (NGS). At this time cells which were to be permeabilized (insets of Fig. 7) also had Triton X-100 (0.2 %) added. Cells were then thoroughly washed again with PBS for several changes. The coverslips were then incubated with the 3D6 antibody (diluted to 0.5 μg ml^{-1} in PBS) in 24-well microtitration plates on a shaker for 18 h at 4 °C. After two 10 min rinses in PBS, the cells were incubated for 2 h in a 1/200 dilution of Cy2 conjugated anti-mouse IgG (Jackson ImmunoResearch, PA, USA). Control experiments (Fig. 2*E* and *F*) varied as detailed in the legend of Fig. 2. After two further 10 min rinses in PBS, the coverslips were mounted onto glass microscope slides with glycerol–PBS and the edges of the coverslips were sealed with clear nail polish. The cells were examined using a Zeiss Axioskop epifluorescence microscope using a No. 10 (fluorescein) filter set. Photographs were taken using a Kodak MDS120 digital camera system.

RESULTS

Exposure of control (normoxically cultured) PC12 cells to perfusate containing 50 mM K^+ evokes exocytotic release of catecholamine which is dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels, since it is almost completely abolished in the presence of 200 μM Cd^{2+} (Fig. 1*A* and *E*; see also Taylor & Peers, 1999). Following a 24 h period of chronic hypoxia (10 % O_2), secretory responses were significantly enhanced as compared with controls ($P < 0.03$), in agreement with our earlier studies (Taylor *et al.* 1999; see Fig. 1*B* and *E*), and approximately 35 % of the K^+-evoked secretion remained in the presence of Cd^{2+} (significantly greater secretion than seen in controls; $P < 0.001$), indicating that chronic hypoxia induces a Ca^{2+} influx pathway coupled to exocytosis which is resistant to Cd^{2+} blockade. However, when the antioxidants melatonin (150 μM; Fig. 1*C* and *E*) or ascorbic acid (200 μM; Fig. 1*D*

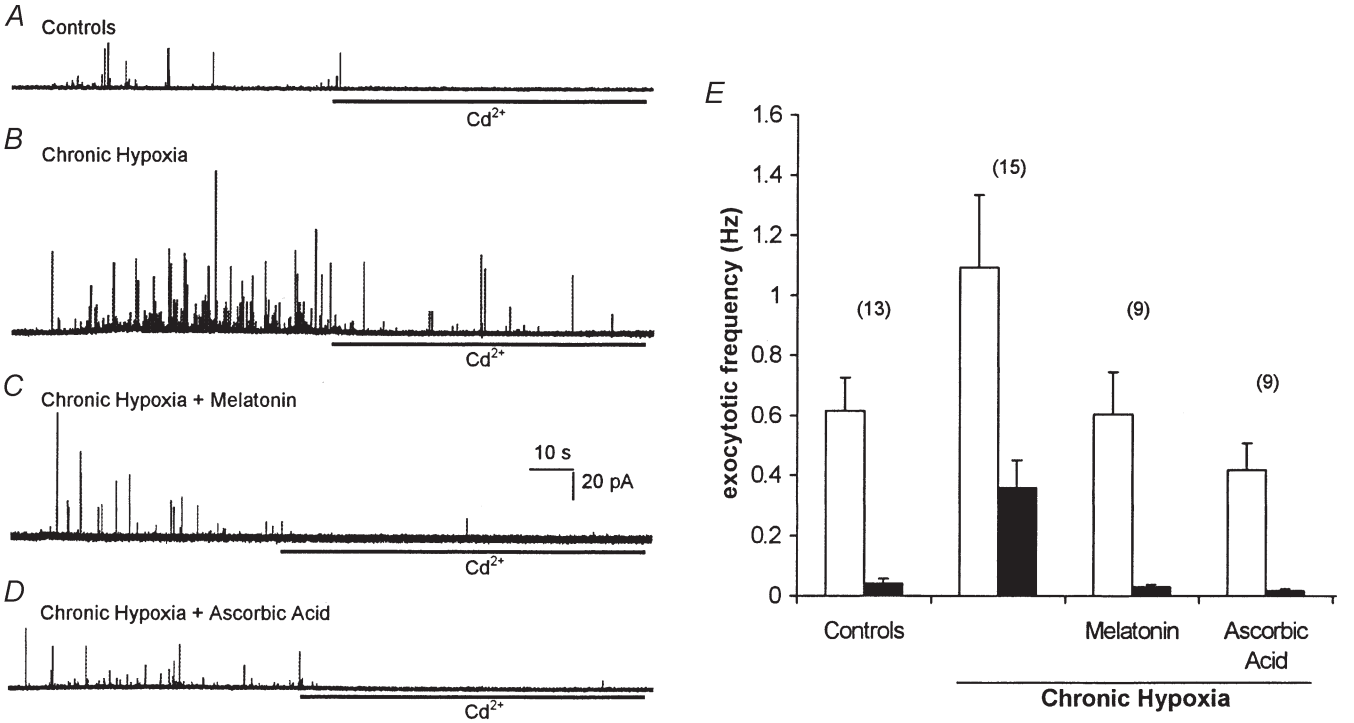

Figure 1. Antioxidants prevent Cd^{2+}-resistant exocytosis in chronically hypoxic PC12 cells

A, amperometric recording of exocytosis from a representative control PC12 cell. Secretion was evoked by exposure to a perfusate containing 50 mM K^+ (application period commencing at the beginning of the trace). In this trace and those of *B–D,* cells were exposed to 200 μM Cd^{2+} in the continued presence of 50 mM K^+ for the period indicated by the horizontal bars. *B*, as *A*, except that the recording was made from a cell previously cultured for 24 h in 10 % O_2. Note that Cd^{2+} does not prevent fully the secretory response. *C* and *D*, as *B*, except that during the exposure to hypoxia, the cells were also exposed to 150 μM melatonin (*C*) or 200 μM ascorbic acid (*D*). Scale bars apply to all traces. *E*, bar graph showing mean (with vertical S.E.M. bars, taken from the number of cells indicated above each bar) exocytotic frequency in the four cell groups indicated in *A–D* before (□) and during (■) exposure to Cd^{2+}.

and *E*) were added to the culture medium during the 24 h period of chronic hypoxia, no such increases in exocytosis were observed ($P > 0.5$ for each antioxidant *vs.* controls), and Cd^{2+} almost completely prevented secretion ($P < 0.01$ for each antioxidant *vs.* Cd^{2+}-resistant secretion observed in the absence of antioxidant).

We have previously shown that chronic hypoxia induces the appearance of AβPs in the plasma membrane of PC12 cells (Taylor *et al.* 1999). Figure 2 shows fluorescence images of intact (i.e. non-permeabilized) PC12 cells following exposure to a monoclonal antibody, 3D6 (Johnson-Wood *et al.* 1997), raised against the N-terminus of AβP. Clearly,

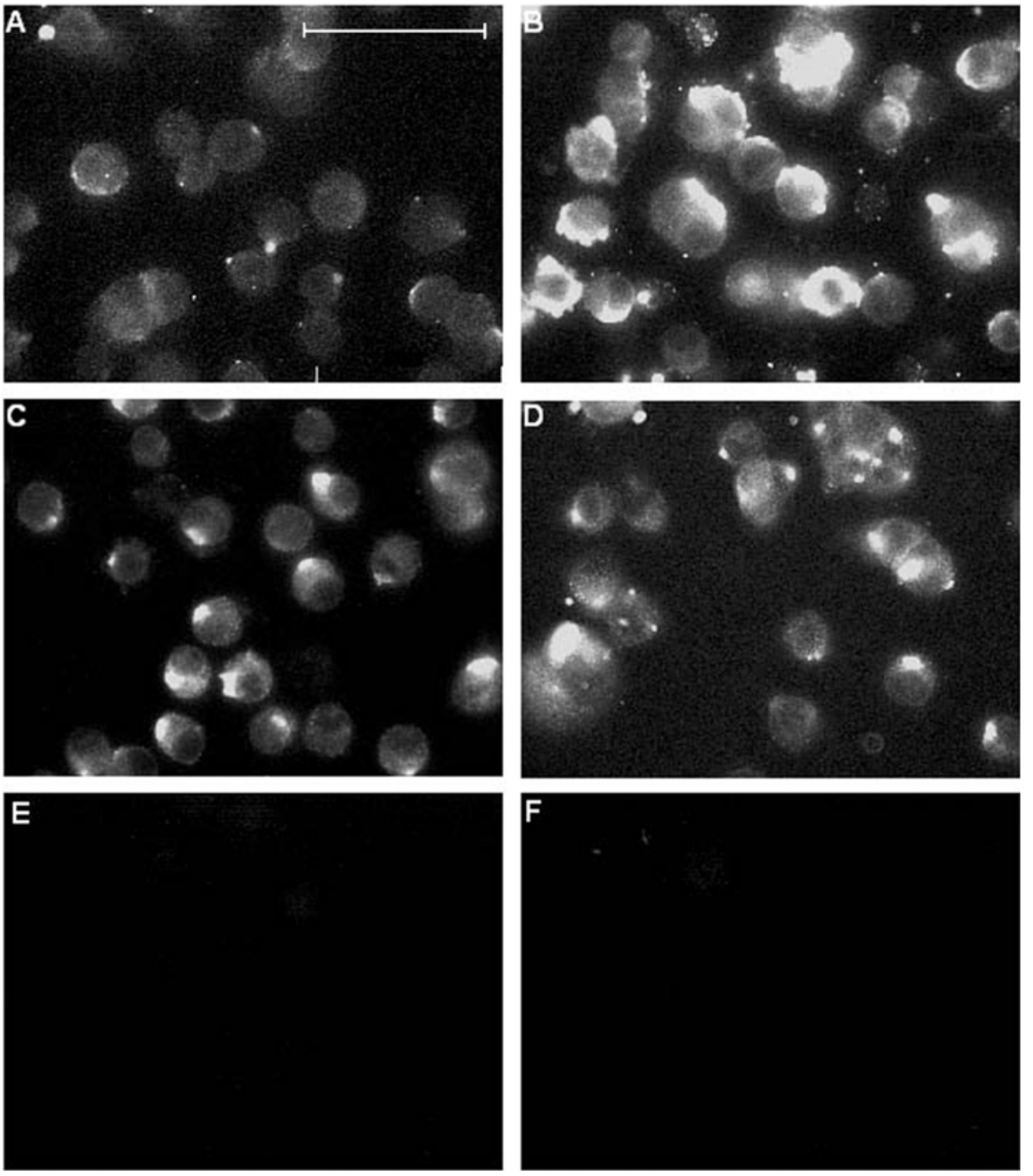

Figure 2. Immunofluorescent labelling of chronically hypoxic cells is suppressed by melatonin

Fluorescence images of PC12 cells cultured in the presence or absence of 150 μM melatonin either normoxically (*A* and *C*, respectively), or under chronically hypoxic conditions (*B* and *D*, respectively). Fluorescence was detected using the 3D6 monoclonal antibody raised against the extracellular N-terminus of AβP as the primary antibody. *E*, background fluorescence (obtained by repeating staining procedure but omitting application of primary (3D6) antibody). *F*, immunofluorescence from cells conducted as in *A* and *B*, except that the primary antibody (3D6) was pre-incubated in excess (3 μM) A$\beta P_{(1–40)}$ before being applied to cells. Scale bar in *A* represents 40 μm and is applicable to all panels.

immunofluorescence was enhanced in cells exposed to chronic hypoxia for 24 h (Fig. 2*B*) as compared with controls (Fig. 2*A*). The enhanced fluorescence was greatly attenuated, however, when cells were exposed to 150 μM melatonin (Fig. 2*D*), despite the fact that melatonin exposure caused a slight increase in autofluorescence itself (Fig. 2*C*).

We have recently reported that native L-type voltage-gated Ca^{2+} channels, whilst contributing approximately 40 % to the total whole-cell Ca^{2+} current, are not coupled to depolarization-evoked catecholamine secretion (Taylor & Peers, 1999; Green & Peers, 2001). However, exposure of cells to chronic hypoxia or to amyloid peptides $A\beta P_{(1–40)}$, $A\beta P_{(1–42)}$ or $A\beta P_{(25–35)}$ (but not the reverse sequence peptide $A\beta P_{(40–1)}$) causes a selective up-regulation of L-type Ca^{2+} channel activity or expression (Green & Peers, 2001), an effect which is clearly distinct from potentiation of exocytosis. Figure 3*A* illustrates this enhancement of whole-cell Ca^{2+} current in response to chronic hypoxia. Importantly, hypoxic augmentation of currents was fully reversed by 150 μM melatonin (Fig. 3*B*; a statistically significant effect; $P < 0.02$). This action of melatonin was most likely due to its antioxidant properties, since hypoxic augmentation of currents was also prevented by ascorbic acid (200 μM; Fig. 3*C*) and ebselen (10 μM; Fig. 3*D*). Interestingly, ascorbic acid treatment in itself caused a small but significant increase in Ca^{2+} channel current density ($P < 0.02$ *vs.* controls; Fig. 3*C*), an effect for which we cannot presently account, but no further augmentation was observed following CH treatment. Thus ROS production appears to mediate both augmentation of exocytosis and enhancement of Ca^{2+} current density.

To test this idea further, we attempted to mimic the effects of chronic hypoxia by exposing cells for 24 h to 40 μM H_2O_2. As illustrated in Fig. 4, Ca^{2+} current densities were significantly enhanced ($P < 0.005$) following exposure to H_2O_2. Furthermore, this enhancement was selectively due to up-regulation of L-type Ca^{2+} channels, since exposure of H_2O_2-treated cells to 2 μM nifedipine (a selective blocker of L-type channels) reduced current densities by *ca* 77 %.

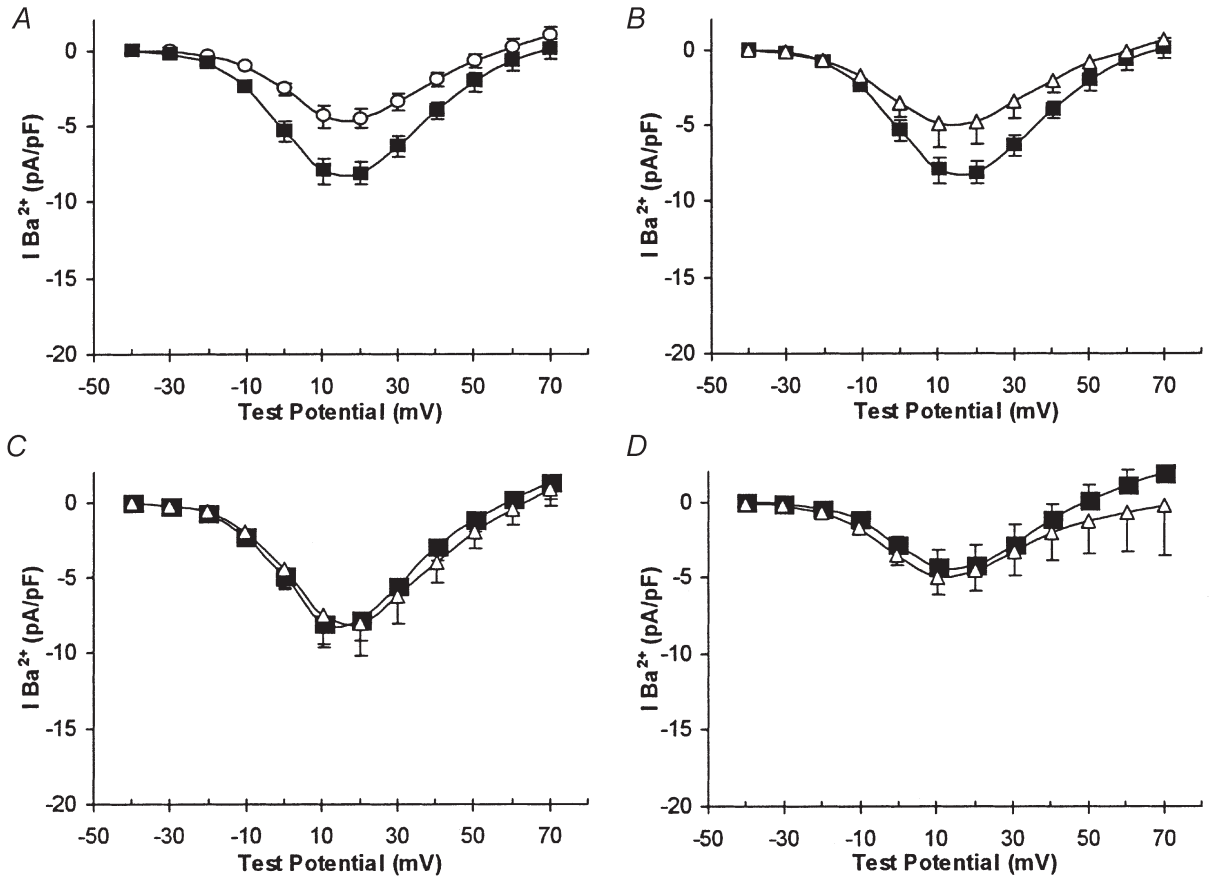

Figure 3. Antioxidants prevent augmentation of Ca^{2+} currents by chronic hypoxia

All plots are of mean current density *vs.* voltage plots (with vertical S.E.M. bars). *A*, recordings obtained from control cells (○, $n = 9$) and cells cultured under CH conditions (■, $n = 8$). *B*, recordings obtained from CH cells in the absence (■, $n = 8$) and presence (△, $n = 6$) of 150 μM melatonin. *C*, recordings obtained from control (△, $n = 11$) and CH (■, $n = 6$) which were also incubated in the presence of 200 μM ascorbic acid. *D*, control (△, $n = 8$) and CH (■, $n = 10$) currents recorded in cells exposed to 10 μM ebselen during the 24 h period of CH or normoxia.

Such a degree of inhibition is almost identical to the effects of nifedipine on chronically hypoxic and amyloid peptide treated cells (Green & Peers, 2001), and is significantly greater ($P < 0.01$) than the inhibitory effects of nifedipine on control cells (Green & Peers, 2001), indicating a selective enhancement of L-type channels caused by H_2O_2. In contrast to this effect on L-type Ca^{2+} channels, incubation of cells with 40 μM H_2O_2 failed to induce Cd^{2+}-resistant, K^+-evoked exocytosis. As exemplified in Fig. 4*B* (and the inset bar graph which shows mean data), application of 200 μM Cd^{2+} almost completely abolished ongoing secretory responses when cells previously incubated with 40 μM H_2O_2 were stimulated with 50 mM K^+. Thus, whilst H_2O_2 could mimic the effects of chronic hypoxia and exposure to AβP on up-regulation of L-type Ca^{2+} channels, it was unable to induce Cd^{2+}-resistant Ca^{2+} influx.

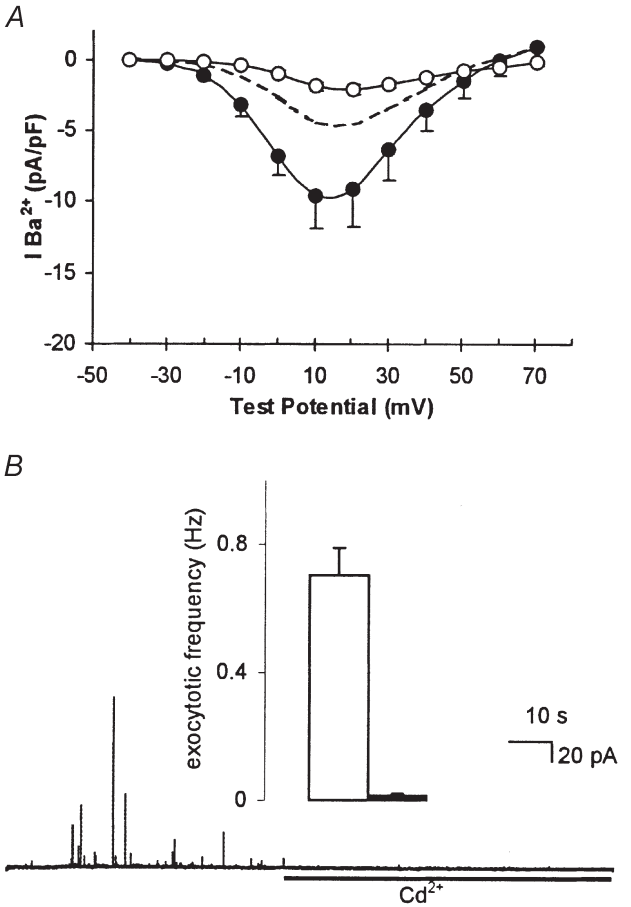

Figure 4. H_2O_2 mimics hypoxic and amyloid peptide augmentation of current density, but does not induce Cd^{2+}-resistant evoked exocytosis

A, mean current density *vs.* voltage plots (with vertical S.E.M. bars, where visible behind symbols) obtained from seven cells exposed to 40 μM H_2O_2 for 24 h. Recordings were made in the absence (●) or presence (○) of 2 μM nifedipine. Dashed line indicates, for ease of comparison, control current density (taken from Fig. 3*A*). *B*, amperometric recording of exocytosis from a representative PC12 cell exposed to 40 μM H_2O_2 for 24 h. Secretion was evoked by exposure to a perfusate containing 50 mM K^+ (application period commencing at the beginning of the trace), and 200 μM Cd^{2+} was applied for the period indicated by the horizontal bar. Inset shows mean ± S.E.M. exocytotic frequency in H_2O_2-treated cells before (□) and during (■) exposure to Cd^{2+}.

The findings reported thus far, together with our previous studies (Taylor *et al.* 1999; Green & Peers, 2000) indicated that the effects of CH and exposure to AβPs were qualitatively indistinguishable, raising the distinct possibility that the effects of chronic hypoxia were, in fact, mediated by AβP production. To investigate this possibility, we examined the actions of two novel γ secretase inhibitors to interfere with the actions of CH (γ secretase is an enzyme complex required to cleave AβP from amyloid precursor protein; see Discussion). These dipeptide-aldehyde inhibitors were 2-napthyl-Val-Phe-CHO (NVP) and Boc-Gly-Val-valinal (GVV), and when cells were incubated with either compound (each at a concentration of 10 μM, added for the 24 h hypoxic period), the effects of hypoxia were fully prevented. Thus, the Cd^{2+}-resistant component of catecholamine secretion, and indeed the augmentation of total secretion, evoked by exposure of cells to 50 mM K^+ was fully reversed, being not significantly different from control (normoxically cultured) cells ($P > 0.4$ for both compounds; Fig. 5*A–C*). In addition, whilst neither inhibitor exerted significant effects on control Ca^{2+} channel currents (Fig. 6*A*), hypoxic enhancement of whole cell Ca^{2+} channel currents was fully prevented (Fig. 6*B*), whilst currents recorded from AβP-treated cells remained significantly greater than controls in the presence of either inhibitor (e.g. $P < 0.01$ at +20 mV, Fig. 6*C*). The ability of these inhibitors to prevent hypoxic augmentation of secretion and Ca^{2+} current density were associated with prevention of increased immunofluorescence detected as described above using the monoclonal antibody 3D6 (Fig. 7*A–D*). The simultaneous addition of 10 μM NVP to cells treated with 40 μM H_2O_2 had no effect on the current enhancement caused by the H_2O_2 (Fig. 4); current densities still had a 2.4-fold increase compared to control cells ($n = 6$; data not shown). These results strongly suggest that AβP formation is an absolute requirement for hypoxia to exert its effects on exocytosis and Ca^{2+} current augmentation.

DISCUSSION

Prolonged periods of hypoxia induce a wide variety of cellular responses which, physiologically, form part of the adaptive response to environmental changes such as acclimatization to high altitude (Bunn & Poyton, 1997; Lopez-Barneo *et al.* 2001). However, such induced changes may also contribute to pathological remodelling of cellular processes, as part of the reaction to reduced availability of O_2 caused by disease or accident. Importantly, hypoxic/ischaemic episodes can precipitate the onset of dementias including AD (Tatemichi *et al.* 1994; Kokmen *et al.* 1996; Moroney *et al.* 1996), and so an understanding of

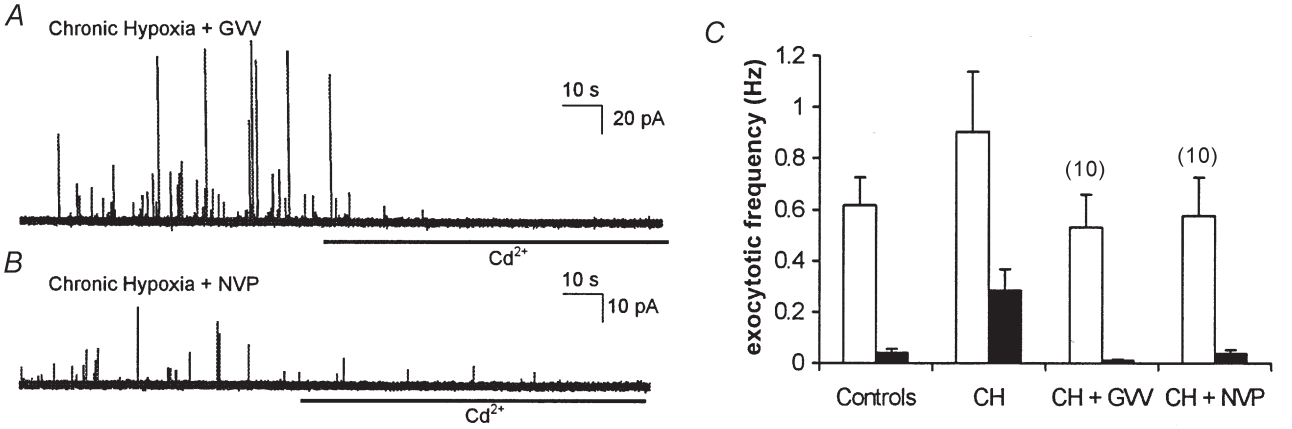

Figure 5. Inhibitors of γ secretase prevent hypoxic augmentation of exocytosis

A, amperometric recording of exocytosis from a representative PC12 cell which had been cultured under chronically hypoxic conditions but in the additional presence of the γ secretase inhibitor, GVV (10 μM). Secretion was evoked by exposure to a perfusate containing 50 mM K^+ (application period commencing at the beginning of the trace). *B*, as *A*, but the cell was incubated with another γ secretase inhibitor, NVP (10 μM). In both traces cells were exposed to 200 μM Cd^{2+} in the continued presence of 50 mM K^+ for the period indicated by the horizontal bars. *C*, bar graph showing mean (with vertical S.E.M. bars, taken from the number of cells indicated above each bar) exocytotic frequency in the two cell groups indicated in *A* and *B* before (□) and during (■) exposure to Cd^{2+}. Control and CH data taken from Fig. 1 for ease of comparison.

the mechanisms underlying pathological remodelling of cell function following prolonged hypoxia is of great potential importance in the future design of therapeutic strategies aimed at preventing the onset of AD following O_2 deprivation.

Our previous studies demonstrated two distinct alterations of cell function induced by hypoxia which are and mimicked by AβPs. Firstly, CH induced a Cd^{2+}-resistant Ca^{2+} influx pathway which, whilst difficult to detect electrophysiologically due to its small amplitude (Green & Peers, 2001), was tightly coupled to exocytosis (Taylor *et al.* 1999; see also Figs 1 and 2). Secondly, chronic hypoxia dramatically increased the component of whole-cell Ca^{2+} current attributable to L-type channels, whilst Ca^{2+} influx through other, non-L-type channel types (N-type and P-/Q-type; Liu *et al.* 1996; Taylor & Peers, 1999) was unaffected (Green & Peers, 2001). Disruption of Ca^{2+} homeostasis is an important factor leading to neurodegeneration in AD, and numerous studies have indicated that AβPs form Ca^{2+}-permeable channels in lipid bilayers and vesicles (Arispe *et al.* 1996; Kawahara *et al.* 1997; Rhee *et al.* 1998). Thus, the Ca^{2+} influx pathway coupled to secretion, together with the increase in L-type Ca^{2+} channel activity or expression (Taylor *et al.* 1999; Green &

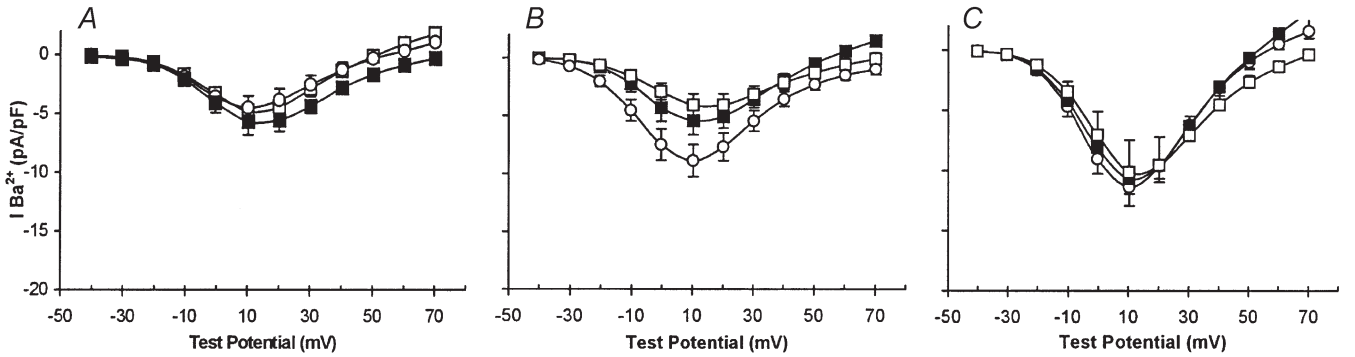

Figure 6. Inhibitors of γ secretase suppress enhancement of currents by hypoxia, but not by AβP

A–C, plots of mean current density *vs.* voltage (with vertical S.E.M. bars). *A*, data obtained from control cells in the absence (○, $n = 8$) and in the presence of the γ secretase inhibitors GVV and NVP (■, $n = 7$; □, $n = 8$). *B*, data obtained from cells cultured under CH conditions in the absence (○, $n = 9$) and presence of the γ secretase inhibitors GVV and NVP (■, $n = 10$; □, $n = 8$). *C*, recordings obtained from normoxically cultured cells exposed to 100 nM A$\beta P_{(1–40)}$ cells in the absence (○, $n = 8$) and presence of the γ secretase inhibitors GVV and NVP (■, $n = 8$; □, $n = 11$).

Peers, 2001) is likely to contribute to the excessive Ca^{2+} influx associated with amyloid neurodegeneration.

Reactive oxygen species (ROS) have long been known to be involved in cell damage and death induced by amyloid peptides, and the ability of these peptides to increase ROS levels is closely associated with disruption of Ca^{2+} homeostasis and toxicity (e.g. Behl *et al.* 1994; Harris *et al.* 1995; Schubert *et al.* 1995; Guo *et al.* 1999; Miranda *et al.* 2000; Pratico & Delanty, 2000; Varadarajan *et al.* 2000; Selkoe, 2001)). Indeed, antioxidant therapy has shown improvements in AD patients (reviewed by Pratico & Delanty, 2000).

Given (i) the strong association of ROS-mediated cell damage and disruption of Ca^{2+} homeostasis with amyloid peptides, (ii) the association of hypoxic/ischaemic episodes with increased subsequent development of dementia and (iii) our previous evidence suggesting that CH alters Ca^{2+} influx via amyloid peptide formation, the present study was directed firstly at investigating the potential involvement of ROS in mediating Cd^{2+}-resistant exocytosis and up-regulation of L-type Ca^{2+} channels caused by CH. To this end we examined the ability of various antioxidants to interfere with these effects of hypoxia, and particularly focused on the actions of melatonin, an endogenous antioxidant whose levels decline with age in humans, as late onset dementias become more prevalent (Reiter, 1995). Clearly, all antioxidants examined abolished the effects of CH, indicating that induction of a Cd^{2+}-resistant Ca^{2+} influx pathway coupled to exocytosis, and the up-regulation of native voltage-gated Ca^{2+} channels, was mediated by increased ROS levels. In further support of this, exposure of cells to H_2O_2 (Fig. 4) mimicked the actions of CH in enhancing L-type current density. Such a finding is in accordance with previous studies described above which indicate that many cellular effects of this and related peptides are mediated by ROS. Indeed, Thomas *et al.* (1998) have shown that H_2O_2 can specifically stimulate current through L-type Ca^{2+} channels. Furthermore, we have recently shown that L-type Ca^{2+} channel up-

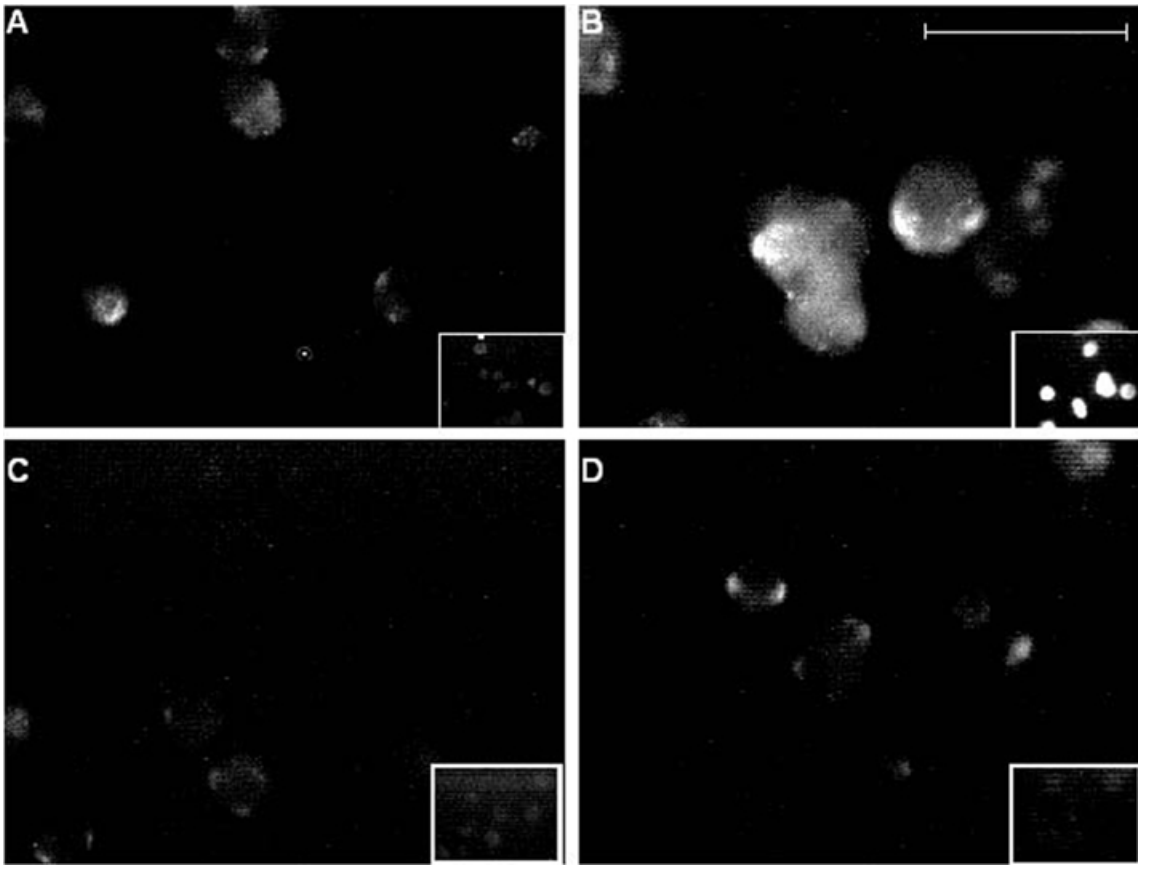

Figure 7. Immunofluorescent labelling of chronically hypoxic cells is suppressed by γ secretase inhibitors

Fluorescence images of PC12 cells cultured normoxically (*A*) or under chronically hypoxic conditions (*B–D*). Hypoxic cells shown in *C* were also exposed to 10 μM GVV during the hypoxic period, and in *D* were exposed to 10 μM NVP. Fluorescence was detected using the 3D6 monoclonal antibody raised against the extracellular N-terminus of AβP as the primary antibody, and was applied to intact (non-permeabilized; main images) and permeabilized cells (inset images) in each case. Scale bar represents 40 μm (200 μm for insets) and is applicable to all panels.

regulation by $A\beta P_{(1–40)}$ is fully prevented by a variety of antioxidants (Giles *et al.* 2001).

In addition to the augmentation of L-type Ca^{2+} channels, we also show that the Cd^{2+}-resistant Ca^{2+} influx pathway tightly coupled to secretion, apparent after a period of CH, is also inhibited by antioxidants (Fig. 1). It is possible that antioxidants merely prevent the coupling of this novel channel to exocytosis or, more likely, that they act by preventing peptide aggregation and insertion into the membrane. Presumably, channel formation requires soluble peptides to aggregate and be inserted into the membrane, as previously suggested (Arispe *et al.* 1996; Kawahara *et al.* 1997; Rhee *et al.* 1998). It has been established in PC12 cells that aggregation can be prevented by antioxidants (Tomiyama *et al.* 1996). Our immunohistochemical studies (Fig. 2) also suggest that this is the case, since hypoxia-induced 3D6 immunoreactivity was suppressed by melatonin, indicating a reduced presence of $A\beta$Ps in the membrane. Importantly, although others (e.g. Reeve *et al.* 2001) have suggested ROS decrease in chronic hypoxia, the findings presented here indicate that ROS levels increase during prolonged hypoxia, as demonstrated previously in these cells (Hohler *et al.* 1999), and that increased ROS levels mediate both the induction of the Cd^{2+}-resistant Ca^{2+} influx pathway and the up-regulation of native Ca^{2+} channels, two distinct effects. An important observation, however, was that H_2O_2 could mimic only the effect of hypoxia to up-regulate Ca^{2+} channels and not the ability of hypoxia to induce Cd^{2+}-resistant evoked exocytosis (Fig. 4*B*). This is because the latter effect (induction of a Cd^{2+}-resistant Ca^{2+} influx pathway coupled to evoked exocytosis) requires increased levels of amyloid peptides to aggregate and form channels, whereas the former (L-type channel up-regulation) requires ROS derived from amyloid peptides, and this effect could be mimicked by H_2O_2 application. It is noteworthy, however, that we cannot at present discount other ROS species in these effects.

Whether prolonged hypoxia was indeed increasing ROS levels independently of the $A\beta$P and that these ROS were leading to increased production of $A\beta$P as suggested (Misonou *et al.* 2000; Olivieri *et al.* 2000), or CH was inducing formation of the $A\beta$P through other pathways and that the produced $A\beta$P was then increasing ROS levels (Huang *et al.* 1999; Varadarajan *et al.* 2000) which were mediating the effects shown, needed to be determined. The antioxidants would prevent the effects shown in either case. To that end we then looked at these CH-induced ROS-mediated effects in the presence of $A\beta$P production inhibitors.

Both $A\beta P_{(1–40)}$ and $A\beta P_{(1–42)}$ are cleaved from amyloid precursor protein by the sequential actions of β and γ secretase (e.g. Mattson, 1997). Recent evidence suggests that γ secretase is an enzyme complex, consisting of presenilin 1 fragments coupled to additional proteins. Our understanding of the role of this complex in both physiological and pathophysiological cellular events is incomplete, yet is likely to benefit from recently developed γ secretase inhibitors. We examined the actions of two such compounds to interfere with the effects of chronic hypoxia (Figs 6, 7 and 8). Production of $A\beta P_{(1–40)}$ and $A\beta P_{(1–42)}$ by γ secretase are both prevented by NPV (Sinha & Lieberberg, 1999), whereas GVV preferentially inhibits production of $A\beta P_{(1–40)}$ (Murphy *et al.* 2000). We found that both agents were effective in inhibiting the actions of hypoxia, indicating that $A\beta$P production is a necessary step in hypoxic augmentation of currents and exocytosis in PC12 cells. Furthermore, given the preferential ability of GVV to block $A\beta P_{(1–40)}$ formation, our results suggest that $A\beta P_{(1–40)}$ may be the form of peptide required to mediate this action of hypoxia. Clearly ROS are therefore mediating the effects of $A\beta$P rather than its production during prolonged hypoxia; in the absence of $A\beta$Ps due to γ secretase inhibition, CH no longer caused current augmentation. If CH did increase ROS levels independently from $A\beta$P production then we would expect this ROS to cause current enhancement since addition of H_2O_2 augmented Ca^{2+} currents.

The question of whether or not ROS levels increase during hypoxia is not generally established and requires further study. Indeed, in our study, hypoxia seems to induce formation of $A\beta$Ps without increasing ROS levels and ROS production may only arise due to the increased presence of $A\beta$Ps. A generally accepted view is that ROS levels decline as available O_2 declines (e.g. Reeve *et al.* 2001). However, Schumacker and colleagues have provided evidence which points to mitochondria as a source of increased ROS production during hypoxia (Chandel *et al.* 1998; Chandel & Schumacker, 2000), and indeed Hohler *et al.* (1999) have demonstrated such an effect in PC12 cells. Such contrasting findings most likely arise from the difficulties associated with directly measuring ROS levels (Semenza, 2000). This is a crucial point to clarify in the context of the present study, especially since oxidative stress (i.e. increased ROS levels) can precipitate $A\beta$P accumulation (Misonou *et al.* 2000). Our data support the idea proposed earlier (Hohler *et al.* 1999) that ROS levels do indeed increase in these cells during prolonged hypoxia although through increased production of $A\beta$Ps.

In summary, our results indicate that chronic hypoxia can precipitate increased formation of $A\beta$P, and lead to the induction of Cd^{2+}-resistant exocytosis and up-regulation of native Ca^{2+} channels via increased ROS production. Importantly, these actions of hypoxia are absolutely dependent on $A\beta$P formation, and these peptides are likely to be the site of ROS generation. We suggest, therefore, that pathological remodelling of cell function in response to prolonged hypoxia – as manifested here by excessive neurosecretion and Ca^{2+} channel up-regulation – involves

elevated ROS levels generated by increased levels of AβPs. These findings are important as they demonstrate a clear biochemical link between O_2 deprivation and AβP formation which mirror clinical findings of a link between ischaemic episodes and Alzheimer's disease.

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