Hypoxic Enhancement of Quantal Catecholamine Secretion

EVIDENCE FOR THE INVOLVEMENT OF AMYLOID β-PEPTIDES

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Prolonged exposure to hypoxia (10% O₂) enhanced quantal catecholamine release evoked from O₂-sensing pheochromocytoma (PC12) cells, as monitored using single-cell amperometric recordings. The enhancement of exocytosis was apparent after 12 h of hypoxia and was maximal at 24 h. Elevated levels of secretion were due to the emergence of a Ca²⁺ influx pathway that persisted during complete blockade of known voltage-gated Ca²⁺ channels. Secretion triggered by this Ca²⁺ influx was severely reduced by known inhibitors of Alzheimer's amyloid β-peptides (AβPs), including an N terminus-directed monoclonal antibody. The enhancing effect on secretion of chronic hypoxia was mimicked closely by direct application of AβP to cells under normoxic conditions, although the effects of AβPs were more rapid at onset, being maximal after only 6 h. The present results suggest that prolonged hypoxia can induce formation of Ca²⁺-permeable AβP channels and that such induction can lead directly to excessive neurosecretion. This is a potential contributory factor to AβP pathophysiology following cerebral ischemia.

Dementias in general and Alzheimer's disease in particular are more prevalent following periods of cerebral ischemia caused by cardiovascular dysfunctions such as stroke (1–3). Ischemia is a complex condition, causing perturbations in several parameters that are essential to neuronal survival such as lack of substrates, accumulation of metabolic products, acidosis, and reduction of oxygen levels. However, the specific contributions of each of these factors to cellular dysfunction and death are presently unknown.

A fundamental feature of Alzheimer's disease is the accumulation of fibrillar deposits consisting of amyloid β-peptides (AβPs) (reviewed in Ref. 4). AβPs are 40–42-amino acid peptides and are cleavage products derived from amyloid precursor protein (APP) (5, 6). APP is one of only a few gene products whose expression is increased following a period of cerebral ischemia (7, 8). Since APP is generally perceived to be neuroprotective, this increased expression of APP can be considered a defense mechanism against ischemia. However, increased APP levels seen in ischemia would also permit increased formation of AβPs, which cause neuronal damage and death (4); and indeed, AβP production is increased following both mild and severe ischemia (9, 10). Thus, a clear link exists between ischemic insult and elevation of damaging AβP levels. However, it remains completely unknown how ischemia might increase AβP levels, and the mechanism(s) by which AβPs cause cellular dysfunction and death remain speculative.

Reduction of available O₂ (hypoxia) is a key feature of ischemia. Hypoxia is known to exert a diverse range of responses in cells, each of which serves a specific physiological purpose (11). Acute hypoxia can evoke extremely rapid responses such as selective, membrane-delimited inhibition of ion channels (12, 13). Prolonged (chronic) hypoxia can alter gene expression and so, for example, permit increased production of erythropoietin (14) or, in electrically excitable cells, tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis (15, 16). In this study, we have utilized the catecholamine-secreting pheochromocytoma (PC12) cell to investigate the effects of both acute and chronic hypoxia on exocytosis, as determined in real time using amperometry (17). PC12 cells represent a well-defined, model excitable cell system, which has been used extensively to study the effects of both acute and chronic hypoxia on various cellular processes, including ion channel activity and gene expression (16, 18–20). PC12 cells have also been extensively characterized as a model system for studying exocytosis (21).

Our recent work has demonstrated that acute hypoxia evokes catecholamine release from PC12 cells by causing membrane depolarization and subsequent Ca²⁺ influx, primarily through N-type voltage-gated Ca²⁺ channels (20). Furthermore, chronic mild hypoxia (10% O₂ for 24 h) enhances this secretory response of PC12 cells to acute hypoxia (22). This enhancement is in part due to increased expression of O₂-sensitive K⁺ channels that influence membrane potential, but is also due to the emergence of a Ca²⁺ influx pathway that is resistant to known blockers of voltage-gated Ca²⁺ channels (22). The present study was aimed at identifying this induced Ca²⁺ influx pathway, and our results indicate that chronic hypoxia increases evoked exocytosis by increasing the production of AβPs that form Ca²⁺-permeable membrane channels that are tightly coupled to exocytosis. Given the known increased incidence of Alzheimer's disease following ischemia as described above, these findings are likely to be of widespread importance in understanding causes and effects of increased dementias following ischemic insult.

EXPERIMENTAL PROCEDURES

PC12 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured as described previously (20, 22) in RPMI 1640 medium (containing l-glutamine) supplemented with 20% fetal calf serum and 1% penicillin/streptomycin (all from Gibco, Paisley, Strathclyde, United Kingdom). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air, passed every 7 days, and used for up to 20 passages. Cells used for experiments were transfected to smaller flasks in 10 ml of medium, to which was added 1 μM dexamethasone (Sigma, Poole, UK; from a stock solution of 1 mg/ml in ultrapure water), and were cultured for a further 72–96 h to enrich catecholamine stores (23). Cells exposed to chronic hypoxia were

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1 The abbreviations used are: AβPs, amyloid β-peptides; APP, amyloid precursor protein; PBS, phosphate-buffered saline.
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**RESULTS**

Exposure of PC12 cells to elevated extracellular \([K^+]_o\) (50 mM) evoked quantal catecholamine secretion, which was apparent as transient oxidative currents detected with a carbon fiber microelectrode (Fig. 1A). Release evoked by 50 mM \(K^+\) has previously been shown to be due to membrane depolarization and \(Ca^{2+}\) influx through voltage-gated \(Ca^{2+}\) channels (20, 27, 28) and so could be completely inhibited by removal of extracellular \(Ca^{2+}\) or by application of the nonselective inhibitor of voltage-gated \(Ca^{2+}\) channels, \(Cd^{2+}\) (Fig. 1A). When cells were cultured in an environment of reduced \(O_2\) (10% instead of the normal 21%) for 24 h, secretion evoked by the same stimulus (50 mM \(K^+\)) was significantly enhanced (Fig. 1B), from an average exocytotic frequency of 0.83 ± 0.12 Hz (n = 26 cells) observed in control cells to 1.93 ± 0.16 Hz (n = 19; \(p < 0.001\), unpaired t test). In both control cells (n = 70) and cells maintained in chronic hypoxia (n = 75), no secretion was detected until the cells were exposed to 50 mM \(K^+\) (data not shown). These findings for the effects of chronic hypoxia to enhance \(K^+\)-evoked secretion are extremely similar to our previously reported effects of chronic hypoxia on the secretory responses to acute hypoxia (22).

Enhancement of evoked secretion from cells cultured under chronically hypoxic conditions remained entirely dependent on the presence of extracellular \(Ca^{2+}\) since removal of \(Ca^{2+}\) from the perfusate (replaced with \(120 \text{mM} \text{NaCl \& 4 \text{mM} MgSO}_4\)) caused complete cessation of exocytosis (Fig. 1B, upper trace, representative of recordings from nine cells). However, \(Cd^{2+}\) (200 \(\mu\text{M}\)) was unable to prevent fully the secretory response to 50 mM \(K^+\) (Fig. 1B, lower trace, representative of 19 cells). In the presence of \(200 \mu\text{M} \text{Cd}^{2+}\), secretion was significantly reduced (\(p < 0.002\)) to 0.72 ± 0.11 Hz. Thus, a period of chronic hypoxia induced an enhanced secretory response from PC12 cells that was entirely \(Ca^{2+}\)-dependent, but ~37% of which was resistant to \(Cd^{2+}\) (i.e.
FIG. 2. Chronic hypoxia induces a Ca\(^{2+}\) influx pathway resistant to blockade by Cd\(^{2+}\). A and B, microfluorometric recordings of [Ca\(^{2+}\)]\(_i\) taken from representative control PC12 cells and PC12 cells previously kept chronically hypoxic (10% O\(_2\) for 24 h), respectively. In each trace, cells were exposed to a solution containing 50 mM K\(^+\) for the periods indicated by the horizontal bars. In the left-hand traces of A and B, no Cd\(^{2+}\) was present; but in the right-hand traces, Cd\(^{2+}\) (200 \(\mu\)M) was co-applied with the 50 mM K\(^+\) solution. C, mean (with S.E. bars) changes in [Ca\(^{2+}\)]\(_i\) determined from control PC12 cells (open bars) and PC12 cells maintained in 10% O\(_2\) for 24 h (closed bars) evoked by a perfusate containing 50 mM K\(^+\) either with or without Cd\(^{2+}\) (200 \(\mu\)M) as indicated. The *numbers in parentheses* indicate the number of recordings made under each condition.

was not mediated by Ca\(^{2+}\)-influx through known voltage-gated Ca\(^{2+}\) channels.

A Cd\(^{2+}\)-resistant Ca\(^{2+}\) entry pathway induced by 24 h of hypoxia was also indicated by microfluorometric recordings, as shown in Fig. 2. For control cells (Fig. 2A), both application of solution containing 50 mM K\(^+\) caused a rapid and reversible rise of [Ca\(^{2+}\)]\(_i\), that could be attributed to Ca\(^{2+}\)-influx through voltage-gated Ca\(^{2+}\) channels as it was completely abolished by 200 \(\mu\)M Cd\(^{2+}\) (co-applied as indicated by the horizontal bars in Fig. 2). By contrast, rises of [Ca\(^{2+}\)]\(_i\), measured in chronically hypoxic PC12 cells could only be reduced by \(\sim\)60% in the presence of 200 \(\mu\)M Cd\(^{2+}\) (Fig. 2B). Mean changes in [Ca\(^{2+}\)]\(_i\), evoked by 50 mM K\(^+\) in control (open bars) and chronically hypoxic (closed bars) PC12 cells are summarized in Fig. 2C.

To characterize further the Cd\(^{2+}\)-resistant Ca\(^{2+}\) influx pathway coupled to secretion that was apparent after chronic hypoxia, we tested the effects of other known blockers of Ca\(^{2+}\) influx pathways in cells previously maintained in hypoxia for 24 h. We have previously found that Cd\(^{2+}\)-resistant secretion evoked in these cells by acute hypoxia could be significantly reduced by inorganic cations (22). These cations (Zn\(^{2+}\) and La\(^{3+}\)) also reduced Cd\(^{2+}\)-resistant secretion evoked by 50 mM K\(^+\) in chronically hypoxic PC12 cells (Fig. 3). Furthermore, Cd\(^{2+}\)-resistant release evoked by 50 mM K\(^+\) was also inhibited by Congo red (Fig. 3). Both Congo red and Zn\(^{2+}\) have been shown previously to inhibit A\(\beta\)P-mediated Ca\(^{2+}\) fluxes (29, 30), suggesting the surprising possibility that a 24-h period of hypoxia-induced formation of Ca\(^{2+}\)-permeable A\(\beta\)P channels that coupled closely to exocytosis. In further support of this, we found that exposure of cells to a monoclonal antibody (3D6) raised against the extracellularly located N-terminus of A\(\beta\)P, anti-A\(\beta\)P(1–5) (30, 31), for 1 h at 37 °C also significantly suppressed the Cd\(^{2+}\)-resistant component of the secretory response (Fig. 3). Similar inhibition of Cd\(^{2+}\)-resistant secretion evoked in response to acute hypoxia (PO\(_2\) \(\sim\)20 mm Hg) was also found with Congo red and the 3D6 antibody (data not shown).

Immunocytochemical labeling of cells with the 3D6 antibody demonstrated specific binding of this antibody to cells previously exposed to chronic hypoxia for 24 h (Fig. 4A). This was seen as diffuse fluorescence over most of the surface area of each cell. This was a consistent pattern of labeling in all cells examined (>100) at both concentrations of the antibody tested (0.5 and 2.0 \(\mu\)g/ml), and this is exemplified by comparison of the phase-contrast image of the same cells (Fig. 4B). Fluorescent labeling was detectable only at very low levels in the normoxic cells processed under identical conditions (Fig. 4C; phase-contrast view of the same image shown in Fig. 4D).

If chronic hypoxia did indeed induce formation of A\(\beta\)P channels that coupled to exocytosis, we anticipated that direct application of A\(\beta\)Ps to normoxically cultured cells would mimic the effects of chronic hypoxia. Therefore, in a separate series of experiments, we exposed cells to 100 nM A\(\beta\)P(1–40) for 24 h under normoxic conditions (21% O\(_2\)). These A\(\beta\)P-treated cells secreted catecholamines in response to both acute hypoxia and raised K\(^+\) levels (Fig. 5A). Like both control and chronically hypoxic cells, they showed no spontaneous exocytosis under normoxic, non-depolarizing conditions (\(n = 47\) cells; data not shown). Secretion evoked by either 50 mM K\(^+\) or acute hypoxia was significantly greater (\(p < 0.001\)) than in control cells (Table 1) and was wholly dependent on the presence of extracellular Ca\(^{2+}\), and a substantial fraction was resistant to Cd\(^{2+}\) (Fig. 5A; see also Table 1); such effects of A\(\beta\)P treatment were extremely similar, both qualitatively and quantitatively, to the effects of chronic hypoxia (see above). Furthermore, the Cd\(^{2+}\)-resistant component of the secretory response displayed the same sensitivity to other blockers as was observed in cells kept chronically hypoxic for 24 h, including the 3D6 monoclonal antibody (Fig. 5B). These findings strongly support the idea that chronic hypoxia appeared to induce formation of A\(\beta\)Ps tightly coupled to exocytosis.

Fig. 6 illustrates the time course of emergence of Cd\(^{2+}\)-
resistant, K+-evoked secretion in cells either kept chronically hypoxic (open circles) or exposed to 100 nM AβP-(1–40) (closed circles). It is clear from this figure that exposure to hypoxia for up to 6 h was without effect on the secretory responses to 50 mM K+ (closed circles). Thereafter, the Cd2+-resistant component of secretion increased steeply for cells exposed to chronic hypoxia for between 12 and 24 h. This component of the secretory response then declined gradually following 30–48 h of exposure to hypoxia. These findings indicate that enhancement of secretion caused by the ability of hypoxia to induce Cd2+-resistant, Ca2+-dependent secretion was transient in nature and was maximal after 24 h of chronic hypoxia. By contrast, in cells treated with AβP-(1–40), Cd2+-resistant secretion was apparent even after 1 h of treatment, was maximal at 6 h, and remained approximately constant for up to 24 h, after which time point there was a gradual decline in this component of secretion. Thus, there was a clear lag in the emergence of Cd2+-resistant, K+-evoked secretion induced by hypoxia as compared with AβP-(1–40)-treated cells.

DISCUSSION

Prolonged periods of hypoxia are well known to alter gene expression and hence functional expression of numerous proteins (11). Several groups have documented altered levels of ion channel expression (particularly K+ channel expression) following periods of chronic hypoxia in tissues such as pulmonary vascular smooth muscle (32) and the carotid body (33). In this study, we investigated the effects of prolonged hypoxia on the secretory responses of PC12 cells to both acute hypoxia and raised extracellular [K+] in response to either acute hypoxia (upper traces; PO2 ~ 20 mm Hg, application began 1 min before beginning of traces) or 50 mM K+ (lower traces; stimulus application began 10 s before beginning of traces). For the periods indicated by the horizontal bars, cells were exposed either to Ca2+-free perfusate (left-hand traces) or 200 μM Cd2+ in the presence of 2.5 mM Ca2+ (right-hand traces) in the continued presence of acute hypoxia or 50 mM K+ as indicated. The scale bars apply to all traces. B, bar graph showing mean (with vertical S.E. bars, determined from the number of cells indicated in parentheses) exocytotic frequency recorded in cells previously exposed to 100 nM AβP-(1–40) in response to acute hypoxia (open bars) or 50 mM K+ (hatched bars) in the presence of 200 μM Cd2+ alone or together with other blocking agents as indicated (applied as described in the legend Fig. 2). All blockers produced significant inhibition (p < 0.02 to 0.001) of Cd2+-resistant release.

pathway is due at least in part to Ca2+-permeable channels formed by AβPs. Thus, in the presence of a supramaximal concentration of Cd2+ to block voltage-gated Ca2+ channels, residual secretion (constituting ~57% of total secretion in response to 50 mM K+), arising from Ca2+ influx as indicated by microfluorometric recordings (Fig. 2), was further reduced by known blockers of AβPs, including Zn2+, Congo red, and the 3D6 monoclonal antibody (Fig. 3).

AβPs are neurotoxic products associated with Alzheimer’s disease (4). The mechanisms underlying neuronal toxicity caused by AβPs are complex and remain to be fully resolved, although most evidence indicates that toxicity involves AβP disruption of intracellular Ca2+ homeostasis (4, 35). There is also strong evidence that toxicity is oxidative and involves free radical damage (36–38). An alternative (or perhaps additional) hypothesis indicates that AβPs disrupt intracellular Ca2+ homeostasis by forming Zn2+-sensitive, Ca2+-permeable channels (39, 40). Such an effect may account for increased central synaptic activity. Indeed, enhancement of long-term potentiation and elevated glutamate release have been demonstrated in hippocampal neurons exposed to AβPs in vitro (41, 42).

It is possible that hypoxic induction of AβPs enhanced secre-
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Effects of β-amyloid treatment on secretory responses and their modulation by Ca\(^{2+}\) removal or application of Cd\(^{2+}\)

Cells were exposed for 21–26 h to 100 nm AβP(1–40) under normoxic conditions. The mean ± S.E. exocytotic frequency, evoked by acute hypoxia (PO\(_2\) ~ 20 mm Hg) or 50 mm K\(^+\), was determined from the number of cells indicated. The incomplete inhibition of secretion caused by Ca\(^{2+}\) in AβP-treated cells was statistically significant (see Footnotes a and b).

| Stimulus | [Ca\(^{2+}\)]\(_i\) | [Cd\(^{2+}\)]\(_i\) | Exocytotic frequency | No. of cells tested |
|----------|-----------------|-----------------|-------------------|-------------------|
| For β-amyloid-treated cells |               |                 |                   |                   |
| Acute hypoxia | 2.5 bm      | 0 bm           | 1.05 ± 0.12       | 8                 |
| Acute hypoxia | 0 bm        | 0 bm           | 0                 | 7                 |
| Acute hypoxia | 2.5 bm      | 0.2 bm         | 0.46 ± 0.09\(^a\) | 6                 |
| 50 mm K\(^+\) | 2.5 bm      | 0 bm           | 2.92 ± 0.41       | 9                 |
| 50 mm K\(^+\) | 0 bm        | 0 bm           | 0                 | 9                 |
| 50 mm K\(^+\) | 2.5 bm      | 0.2 bm         | 0.82 ± 0.07\(^b\) | 9                 |

\(^a\) p < 0.003 versus respective controls.
\(^b\) p < 0.0001 versus respective controls.

FIG. 6. Time course of development of Cd\(^{2+}\)-resistant exocytosis induced by hypoxia and AβP(1–40). Shown is a plot of mean exocytotic frequency determined in cells pre-exposed to chronic hypoxia (open circles) or AβP(1–40) (100 nm; closed circles) for periods up to 48 h. Secretion was evoked always in the presence of 200 μM Cd\(^{2+}\) by exposure of cells to 50 mm K\(^+\). Each plotted point is the mean ± S.E. determined from 6–10 cells.

It is noteworthy that the known blockers of AβPs failed to inhibit completely the Cd\(^{2+}\)-resistant secretion in cells previously exposed to chronic hypoxia (Fig. 3). This suggests that formation of Ca\(^{2+}\)-permeable AβP channels might not account fully for the Cd\(^{2+}\)-resistant Ca\(^{2+}\) influx pathway. Interestingly, these blockers also failed to inhibit fully the Cd\(^{2+}\)-resistant secretion observed in cells pretreated with AβP(1–40) (Fig. 5). We cannot at present account for this incomplete blockade. Nevertheless, our present findings indicate that AβPs provide a Ca\(^{2+}\) influx pathway that can specifically trigger exocytosis. It should be noted that only Ca\(^{2+}\) influx specifically through N-type voltage-gated Ca\(^{2+}\) channels, but not L- or P/Q-type channels, influences depolarization-evoked exocytosis in control PC12 cells (20, 22). This suggests the possibility of an intimate association of AβPs with vesicle docking/fusion sites in a manner that may compare with the interaction of known voltage-gated Ca\(^{2+}\) channels and synaptic proteins (43). Most important, we have demonstrated that reduced O\(_2\) levels alone can induce formation of AβP-mediated Ca\(^{2+}\) influx. As described in the Introduction, AβPs are the cleavage products derived from amyloid precursor protein (5, 6), and levels of this precursor have been shown to increase following ischemia (9, 10). The enhanced Ca\(^{2+}\) influx reported here may therefore be an important contributory factor to the increased incidence of dementias and amyloid deposition following cerebral ischemia, leading to neurotoxicity through excessive transmitter release.

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