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Hypoxia Evokes Catecholamine Secretion from Rat Pheochromocytoma PC-12 Cells

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We have monitored exocytosis of catecholamines from individual PC-12 cells by amperometry using carbon fiber microelectrodes in order to investigate possible secretory responses to acute hypoxia. In normoxia, no secretion was detected from cells perfused with a solution containing 5mM K^+. However, when [K^+] was raised (10-100mM), exocytotic events were observed. Hypoxia (Po_2 11mmHg) stimulated secretion from PC-12 cells, and in hypoxic conditions exocytosis was greater at each [K^+] studied as compared with normoxia. Hypoxia-evoked secretion was abolished in Ca^{2+} free solutions containing 1mM EGTA and by the non-specific Ca^{2+} channel blocker, Cd^{2+} (200μM). Secretion was also largely inhibited by ω-conotoxin GVIA (1μM). Exocytosis was also observed in normoxia when cells were exposed to tetraethylammonium (1-10mM), but not 4-aminopyridine (3mM). Our findings indicate that hypoxia evokes exocytosis via depolarization arising from inhibition of a TEA-sensitive K^+ conductance, leading to Ca^{2+} influx primarily via N-type Ca^{2+} channels.

Recent studies have indicated that plasma membrane ion channels can be regulated by local Po_2 levels in a rapid, membrane-limited manner (1-3). This work originated with studies of isolated glomus cells from the carotid body arterial chemoreceptors (4, 5) and shortly after this, O_2 sensitive K^+ channels were reported in a diverse range of tissues (3, 6-9). In each case, the rapid inhibitory actions of hypoxia have been proposed or demonstrated to lead to membrane depolarization and subsequent secretion of transmitters or contraction as a result of increased activity of voltage-gated Ca^{2+} channels and hence increased Ca^{2+} influx. Although the mechanisms underlying O_2 sensing by ion channels have yet to be fully determined, the phenomenon is clearly of great physiological importance.

Rat pheochromocytoma (PC-12) cells have been utilized as a model system for studying different aspects of O_2 sensing. Millhorn and colleagues have demonstrated that gene expression and signal transduction pathways involved in the regulation of tyrosine hydroxylase production are strongly influenced by hypoxia (10). Tyrosine hydroxylase is the rate limiting step in catecholamine (CA) synthesis, and CAs are the predominant transmitters released from both PC-12 cells and carotid body glomus cells (11, 12). These observations suggest that PC-12 cells may represent a model system for examining the cellular responses to both acute and chronic hypoxia. Indeed, the effects of acute hypoxia on ion channels in PC-12 cells has recently been studied and, as is the case in carotid body glomus cells, hypoxia has been shown to inhibit whole cell K^+ currents (11, 13). Further studies indicated that the specific K^+ channel involved was likely to be the tetraethylammonium (TEA)-sensitive Kv1.2 (14). These findings imply that PC-12 cells might secrete CAs in response to acute hypoxia, but no such study has been described to date. Here, we have investigated the effects of hypoxia on CA release from PC-12 cells using amperometric techniques with carbon fiber microelectrodes which can resolve CAs released from individual vesicles in isolated cells (15-17). Since hypoxia-evoked transmitter release may involve membrane depolarization, we have compared the effects of hypoxia with release elicited by raised extracellular [K^+] to depolarize cells.

METHODS

PC-12 cells were originally obtained from the American Tissue Type Cell Collection (Rockville, MD, USA) and stored in liquid N_2. To grow cells in culture, an aliquot was thawed rapidly at 37°C, diluted 1:5 with RPMI 1640 culture medium (containing L-glutamine) supplemented with 20% fetal calf serum and 1% penicillin/streptomycin (from Gibco, Paisley, Strathclyde, UK) and incubated at 37°C for 24hr in a humidified atmosphere of 5% CO_2 / 95% air. Following this period, cells were centrifuged at 70 × g for 10 min, resuspended in fresh medium and re-seeded in flasks at low density. This preparation was designated passage 1, and cells were used for experiments for up to 20 passages. Each passage was conducted after 7 days when cells were resuspended in fresh medium and diluted 1:2. The prolonged period without medium change was believed to

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enhance evoked CA release (18). Cells used for experiments were transferred to smaller flasks in 10ml of medium and 1μM dexamethasone (from a stock solution of 1mM in Ultrapure water) was applied for 72-96 h to enhance CA secretion further (19).

Aliquots of cells were plated onto poly-L-lysine coated coverslips and allowed to adhere for approximately 1hr. Fragments of coverslip were then transferred to a recording chamber (volume *ca.* 80μl) which was continually perfused under gravity (flow rate 1-2ml / min) 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 *ca.* 300mOsm with sucrose, 21-24°C). For experiments using solutions of raised $[K^+]$, the $[Na^+]$ was reduced accordingly to maintain iso-osmolarity. Ca^{2+} free solutions contained 1mM EGTA and no added Ca^{2+}.

Drugs were applied in the perfusate except in the cases of ω-conotoxin GVIA toxin (ω-CgTx) and ω-agatoxin GIVA (ω-AgaTx). The effects of these agents were investigated by pre-incubation of cells in extracellular solution containing these agents for at least 10 min. Experiments were conducted within 3 min of transfer of these cells to the perfused recording chamber. Investigations of the effects of nifedipine were conducted at low light intensity, and nifedipine was added to the perfusate from a stock solution of 20mM in ethanol, made fresh each day. Hypoxic solutions were obtained by continually bubbling one or more of the reservoirs supplying the recording chamber with N_2, as required. Reservoirs were pre-equilibrated with N_2 for at least 30min before being applied to cells.

Carbon fiber microelectrodes (proCFE, Axon Instruments) with a diameter of 5μm were positioned adjacent to individual PC-12 cells and were polarized to +800mV to allow oxidation of released CA. Resulting currents were recorded using an Axopatch 200A amplifier (with extended voltage range), filtered at 1kHz and digitised at 2kHz 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). The same equipment was also used to monitor Po_2 levels in the recording chamber, except that the polarity of the microelectrode was reversed (20). Bath Po_2 was 11.0 ± 1.8 mmHg.

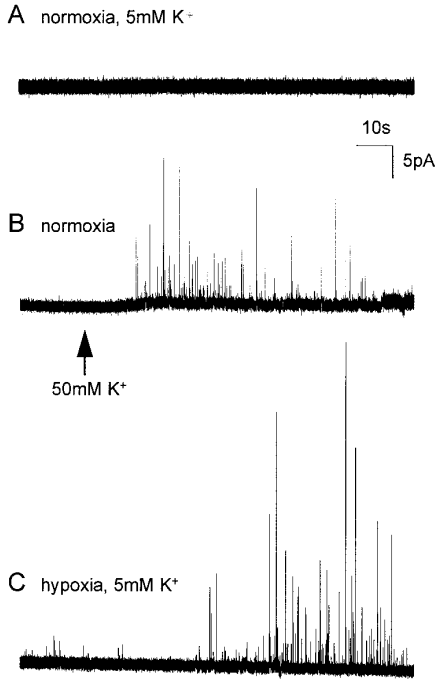

FIG. 1. Amperometric recordings from individual PC-12 cells. Each trace is the current recorded at a carbon fiber microelectrode tip, placed adjacent to a PC-12 cell and polarized to +800mV. (A) Lack of exocytotic events when cell was perfused with normoxic solution containing 5mM K^+. (B) When the perfusate was exchanged for one containing 50mM K^+ (at arrow), upward deflections were detected, each corresponding to oxidation of catecholamine released from a single vesicle. (C) Example of exocytosis evoked from a PC-12 cell by perfusion with a hypoxic solution (Po_2 11mmHg). Hypoxic solution was applied immediately before recording starts. Perfusate $[K^+]$ maintained at 5mM throughout. Scale bars apply to all traces.

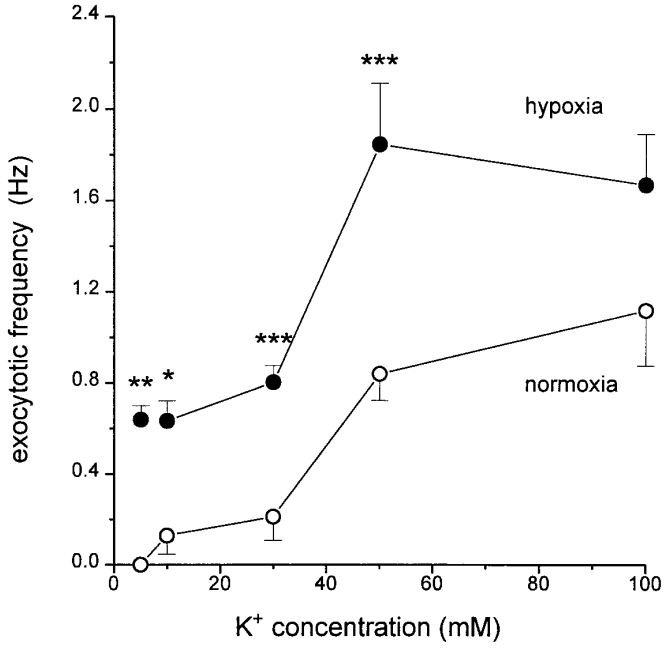

FIG. 2. Plot of mean (± SEM) frequency of exocytotic events detected in cells perfused with varying levels of $[K^+]$ under normoxic conditions (open circles) or under hypoxic conditions (solid circles). Each point was determined from between 7 and 26 cells (except 5mM $[K^+]$ in normoxia; n=58). Frequency was determined over a 55s period in normoxia, or over the same period following 90s exposure to hypoxia. Significant enhancement of release in hypoxia: *$P<0.002$; **$P<0.001$, ***$P<0.0005$.

Unless otherwise stated, each experiment consisted of current recordings of a brief control period during which cells were only perfused with standard external medium ($[K^+]$ 5mM). This was then exchanged for a test solution and amperometric signals were recorded for a further period of 1-4 min. CA secretion was apparent as discrete spike-like events, each corresponding to the released contents of a single vesicle of CA (15, 17). Quantification of release was achieved by counting spikes using Minian 16 software (Jaejin Software, Columbia, NY). This allowed visual inspection of each event so that artefacts (due, for example, to solution switches) could be rejected from analysis. Results are presented as individual examples or means ± standard error of the mean (SEM) and statistical comparisons were made using an unpaired Student's t-test.

RESULTS

Under normoxic conditions when cells were perfused with solution containing 5mM K^+, no secretory events were detected from PC-12 cells (n=58 cells; e.g. Fig. 1A). However, when the perfusate K^+ concentration was raised, for example to 50mM as illustrated in

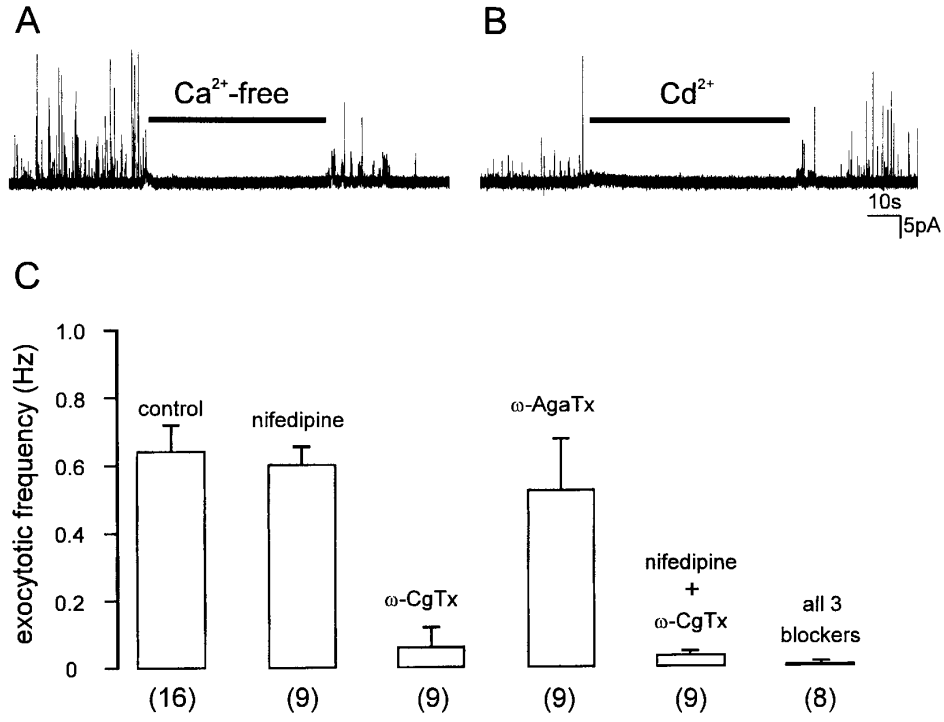

FIG. 3. (A) Example recording of exocytosis evoked from a PC-12 cell following exposure to hypoxia. Exocytosis was abolished by switching solution to one of the same Po_2 but containing no added Ca^{2+} and 1mM EGTA for a period indicated by horizontal bar. (B) Example recording of exocytosis evoked from a PC-12 cell following exposure to hypoxia. Exocytosis was abolished by switching solution to one of the same Po_2 but also containing 200μM Cd^{2+}. Scale bars apply to both (A) and (B). (C) Bar graph showing mean frequency of exocytosis evoked by hypoxia in the absence or presence of different selective blockers of voltage-gated Ca^{2+} channels. Cells were either exposed to nifedipine (2μM), or pretreated with ω-conotoxin GVIA (1μM; ω-CgTx) or ω-Agatoxin IVA (200nM; ω-AgaTx), or a combination of these, as indicated below each bar. Each bar represents mean ± SEM determined from the number of cells indicated in parentheses.

Fig.1B, discrete spike-like events were always observed, each corresponding to the released contents of a single vesicle of CA. When exposed to a hypoxic solution containing 5mM K^+, secretory events were observed in all PC-12 cells studied (e.g. Fig.1C). Fig. 2 indicates that in both normoxia and hypoxia, the frequency of exocytosis increased with increasing [K^+], but at each [K^+] studied except the highest (100mM) release under hypoxic conditions was much greater than in normoxia.

As described in the Introduction, hypoxia causes membrane depolarization and a rise of $[Ca^{2+}]_i$ in PC-12 cells. This would imply that hypoxia-evoked secretion as described in Fig.1 arises due to Ca^{2+} influx through voltage-gated Ca^{2+} channels in PC-12 cells. To test this, we exposed cells to a Ca^{2+}-free medium (containing 1mM EGTA) during exposure to hypoxia (Fig. 3A, representative of 8 cells) or to the non-selective Ca^{2+} channel blocker Cd^{2+} (200μM) in the continued presence of 2.5mM Ca^{2+} (Fig. 3B, representative of 5 cells). In both cases, exocytosis was completely abolished, suggesting an absolute dependence on Ca^{2+} influx for hypoxia evoked transmitter release. Release in response to 50mM K^+ was similarly abolished by Ca^{2+} free or Cd^{2+} containing solutions (data not shown).

In order to investigate which Ca^{2+} channels might be involved in exocytosis evoked by hypoxia, we determined the exocytotic frequency in hypoxia in the presence of 2μM nifedipine, or following pretreatment with either 1μM ω-CgTx or 200nM ω-Aga-VIA, or combinations of these blockers. Fig. 3C plots the results: the most important observation was that pretretment of cells with ω-CgTx resulted in approximately 90% inhibition of release ($P<0.005$), indicating that N-type Ca^{2+} channels were the primary route of Ca^{2+} entry under hypoxic conditions. The lack of statistically significant effects of nifedipine and ω-Aga IVA indicated that Ca^{2+} influx through L-type or P/Q type Ca^{2+} channels was not of physiological importance, although it is noteworthy that release in the presence of all 3 blockers was significantly ($P<0.05$) less that that observed following ω-CgTx treatment alone, suggesting a minor role for non N-type Ca^{2+} channels.

A previous study has reported that the O_2 sensitive K^+ current in PC-12 cells is also blocked by tetraethylammonium (TEA). This would suggest that TEA should, like hypoxia, evoke transmitter release from PC-12 cells. To investigate this, we examined the effects of TEA to evoke release. As Illustrated in Fig.

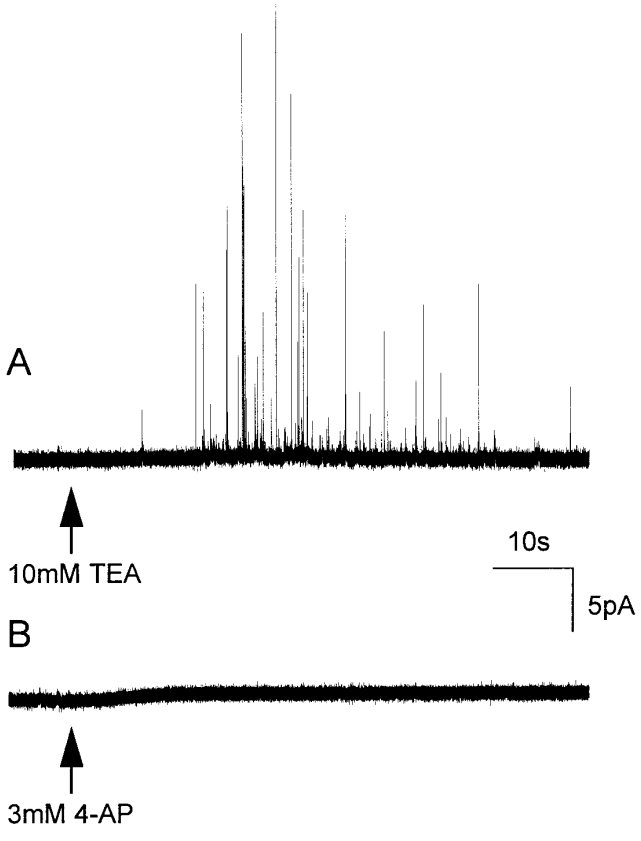

FIG. 4. (A) Example recording showing exocytosis evoked in normoxic solution (5mM [K^+]) containing 10mM tetraethylammonium (applied at arrow). (B) Example recording illustrating lack of effect of 4-aminopyridine (3mM, applied at arrow) on exocytosis from a cell perfused with normoxic solution containing 5mM [K^+]. Scale bars apply to both traces.

4A TEA (10mM) was able to evoke release, with mean release frequency of 0.32 ± 0.06 (n=13). Release (0.06 ± 0.02, n=10) was also evoked at a TEA concentration of 1mM. By contrast, another K^+ channel blocker, 4-aminopyridine (4-AP) was unable to evoke release at a concentration of 3mM (Fig. 4B, representative of 7 cells examined).

DISCUSSION

PC-12 cells have been previously utilized for studies of the cellular responses to prolonged hypoxia, such as modulation of transduction pathways regulating tyrosine hydroxylase gene expression (10). More recently, it has been proposed as a model system for investigating rapid responses to acute hypoxia similar to those reported in the carotid body type I chemoreceptor cell (11, 13, 14). Thus, PC-12 cells possess O_2-sensitive K^+ channels and acute hypoxia inhibits these channels, thereby causing depolarization. This is assumed to trigger CA secretion, but reports of hypoxia-evoked CA secretion are lacking. Indeed, although hypoxia-evoked CA release from the intact carotid body preparation has been demonstrated (12), only one group has successfully demonstrated hypoxia-evoked CA release from isolated type I carotid body cells (21, 22).

The present study is the first to demonstrate directly quantal release of CA from PC-12 cells in response to acute hypoxia (Fig.s 1 and 2). To do this, we employed the amperometric technique which utilizes carbon fiber microelectrodes polarized to allow oxidation of electroactive species at their surface. This is a high resolution technique which, whilst being limited in that it can only detect certain transmitter species (particularly amines), has advantages over membrane capacitance measurements of secretion from isolated cells: it is non-invasive (therefore loss of cell constituents through cell dialysis during patch-clamp recordings can be avoided), and secretion does not overlap with endocytotic events (15, 17, 23). Our results indicate that hypoxia-evoked release, like that evoked by depolarizing [K^+], was wholly dependent on $[Ca^{2+}]_o$ and could also be fully blocked by 200μM Cd^{2+}, indicating that Ca^{2+} influx through voltage-gated Ca^{2+} channels was a key step in this stimulus-secretion coupling.

PC-12 cells have long been known to possess both L- and N-type Ca^{2+} channels (24, 25) but a more recent study has also identified P/Q type Ca^{2+} channels in undifferentiated PC-12 cells (26). To determine the relative importance of these channels in permitting Ca^{2+} influx that is required to trigger exocytosis, we tested the actions of selective Ca^{2+} channel blockers to modulate evoked transmitter release (Fig. 3). N-type channels were clearly the predominant route of Ca^{2+} entry into cells in hypoxia, since approximately 90% of release was blocked by ω-CgTx. Other channel types (L-type and P/Q-type) play a minor, if any, role in mediating hypoxia evoked secretion.

The O_2 sensitive K^+ channel of PC-12 cells is sensitive to blockade by TEA (11), and recent molecular biological studies have suggested it belongs to the *Shaker* family of K^+ channels (14). Our demonstration that TEA applied under normoxic conditions can evoke quantal transmitter release (Fig. 4A) further supports the important role for a TEA-sensitive channel in hypoxia-evoked secretion from these cells. Furthermore, the lack of secretory response to another broad-spectrum K^+ channel blocker, 4-AP (Fig. 4B), provides further pharmacological characterization of the K^+ channel type underlying this response. It is important to note, however, that this secretory response of PC-12 cells to TEA may represent the limit to which comparisons between PC-12 cells and carotid body type I cells can be made. The O_2 sensitive K^+ current in type I cells is species dependent (3, 27), and even within the same species (rat), two different K^+ currents have been described as O_2 sensitive, although the relative impor-

tance of these currents to hypoxia-induced membrane depolarization has yet to be resolved (28, 29).

In summary, the present study demonstrates directly that acute hypoxia can evoke quantal CA release via a mechanism involving Ca^{2+} influx primarily through voltage-gated N-type Ca^{2+} channels. This fundamental stimulus-secretion response compares well with that described in the carotid body arterial chemoreceptor, and indeed may also compare well with the effects of acute hypoxia on airway chemoreceptor neuroepithelial cell bodies which also possess O_2 sensitive K^+ currents (7). Studies on both of these types of chemoreceptors are severely hampered by the lack of available tissue from which to obtain isolated cells. The present findings indicate that PC-12 cells represent a suitable model system for further investigating the mechanisms by which hypoxia can evoke transmitter release in chemosensory cells.

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