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Mitochondrial Inhibitors Evoke Catecholamine Release from Pheochromocytoma Cells

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Quantal catecholamine secretion evoked from individual pheochromocytoma (PC12) cells by exposure to mitochondrial inhibitors and uncouplers was monitored in real time using amperometry. Cyanide (0.05–5 mM) caused a concentration-dependent increase in the frequency of amperometric events. This secretory response was abolished by removal of extracellular Ca^{2+} and by the application of Cd^{2+} (200 μM), a nonselective blocker of voltage-gated Ca^{2+} channels. Secretion was also inhibited by *ca.* 75% following pretreatment of cells with ω-conotoxin GVIA to inhibit N-type Ca^{2+} channels selectively. Secretion was also detected when cells were exposed to rotenone (10 μM), dinitrophenol (250 μM) and *p*-trifluoromethoxyphenyl hydrazone (1 μM) and, as for cyanide, these secretory responses were abolished by removal of extracellular Ca^{2+} or application of 200 μM Cd^{2+}. These results indicate that, like hypoxia, mitochondrial inhibitors and uncouplers evoke catecholamine secretion from PC12 cells which is wholly dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels.

Key Words: **exocytosis; catecholamine; mitochondria; amperometry; pheochromocytoma; calcium channels.**

Numerous cell types sense and respond to acute hypoxia in a rapid and reversible manner (1). In the carotid body chemoreceptor hypoxic chemotransduction involves hypoxic inhibition of glomus cell K^+ channels, which causes membrane depolarization, opening of voltage-gated Ca^{2+} channels and exocytosis due to the resultant Ca^{2+} influx (2, 3). In recent years, the PC12 pheochromocytoma cell line, derived from rat chromaffin tissue, has been proposed as a model for studying chemoreception (4). These cells possess O_2-sensitive K^+ channels, and under hypoxic conditions they depolarize, thereby causing Ca^{2+} influx (5) and quantal catecholamine release (6). Thus, their secretory response to hypoxia can be abolished by blockers of voltage-gated Ca^{2+} channels (6). Also like carotid body glomus cells isolated from the rat (7), but not the rabbit (8), PC12 cells respond to acidic stimuli via a similar mechanism, and the effects of acidic and hypoxic stimuli are synergistic (9).

Mitochondrial inhibitors and uncouplers also excite the carotid body and stimulate catecholamine release from glomus cells (2), but the mechanisms by which these agents exert their effects are contested: previous studies have shown that such agents raise $[Ca^{2+}]_i$ in glomus cells (and so presumably trigger catecholamine release), yet hyperpolarize these cells by causing release of Ca^{2+} from intracellular stores, including mitochondria (10, 11). Others have suggested that mitochondrial uncouplers act as acidic stimuli and cause the plasma membrane Na-Ca exchanger to operate in reverse mode, due to Na^+ loading of cells via high Na-H exchanger activity (8). More recently, Buckler and Vaughan-Jones (12) have provided evidence that mitochondrial uncouplers, like hypoxia, depolarize glomus cells of the rat carotid body via inhibition of K^+ channels, causing Ca^{2+} entry via voltage-gated Ca^{2+} channels. In the present study, we have investigated the effects of two mitochondrial inhibitors (cyanide and rotenone) and two mitochondrial uncouplers (dinitrophenol (DNP) and carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP)) to stimulate catecholamine secretion from model chemoreceptor PC12 cells. Our results suggest that these agents share with hypoxia and acidity the ability to evoke catecholamine secretion that is dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels.

MATERIALS AND METHODS

PC12 cells were maintained in RPMI 1640 culture medium (containing L-glutamine) supplemented with 20% fetal calf serum and 1% penicillin/streptomycin (from Gibco, Paisley, Strathclyde, UK) at 37°C for 24 h in a humidified atmosphere of 5% CO_2/95% air. Cells were passaged every 7 days by resuspension in fresh medium, diluted 1:2. The prolonged period without medium change enhanced evoked catecholamine release (13). 72–96 h before experiments, cells were transferred to smaller flasks (10 ml of medium) and 1 μM dexamethasone was applied for to enhance catecholamine secretion

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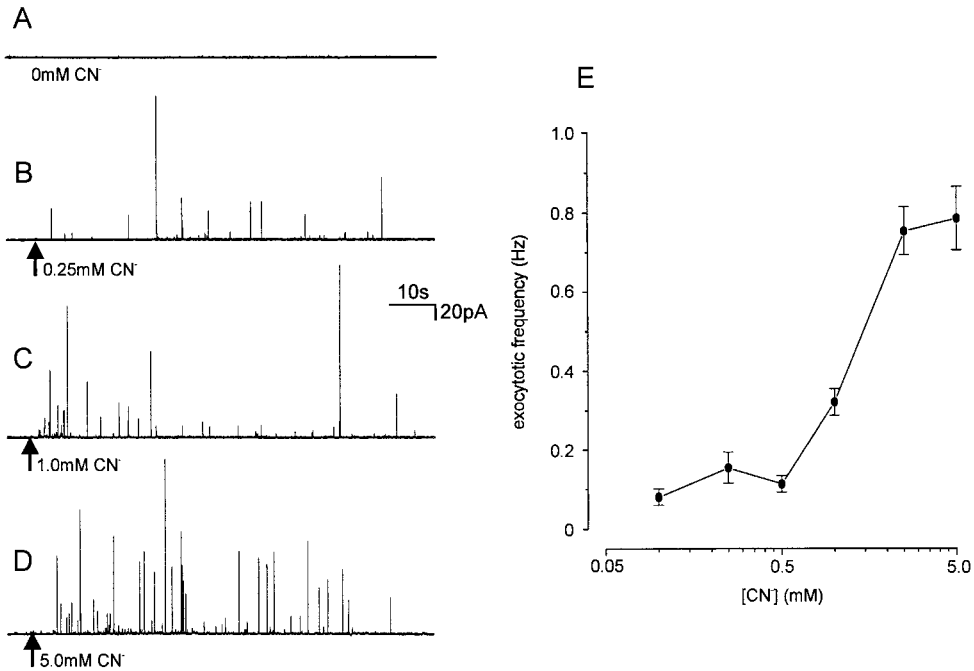

FIG. 1. (A–D) Amperometric recordings from representative example PC12 cells. Recordings were made either in the absence of cyanide (CN^-; A) or cyanide was applied to the cells (at the concentrations shown) at the point indicated by the upward arrow (B–D), and was present throughout the rest of the recording. Scale bars apply to all traces. (E) Concentration-response relationship for the effects of cyanide on the frequency of occurrence of quantal secretory events. Frequency was determined over a 55 s period, 30 s after switching to the cyanide-containing solution. Each plotted point is the mean ± s.e.m. taken from between 9 and 21 cells.

further (14). Each experimental day, PC12 cells were plated onto poly-D-lysine coated coverslips ($0.5–1.0 \times 10^5$ cells per coverslip) and allowed to adhere for *ca.* 1 h. Fragments of coverslip were then transferred to a recording chamber (volume *ca.* 80 μl) which was continually perfused under gravity (flow rate 1–2 ml/min) with a control 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. 300 mOsm with sucrose, 21–24°C). Ca^{2+}-free solutions contained 1mM EGTA and no added Ca^{2+}. All drugs were applied in the perfusate except in the cases of ω-conotoxin GVIA (ω-CgTx) and ω-agatoxin GIVA (ω-AgaTx). The effects of these agents were investigated by pre-incubation of cells in extracellular solutions containing these agents for at least 10 min. Experiments were conducted within 3 min of transfer of these cells to the perfused recording chamber. Experiments investigating the effects of nifedipine (applied from a stock solution of 20 mM in ethanol) were conducted at low light intensity.

Carbon fiber microelectrodes (proCFE, Dagan Corporation, diameter 5 μm) were positioned adjacent to individual PC12 cells and were polarized to +800 mV to allow oxidation of released catecholamine. Quantal secretion was detected as transient upward current deflections, each arising from the oxidation of the released contents of a single vesicle of catecholamine (15, 16). Currents were recorded using an Axopatch 200A amplifier (with extended voltage range), filtered at 1 kHz and digitised 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, Foster City, CA). Exocytosis is expressed as the frequency of quantal events, determined by counting the number of events over a 55 sec period, 30 sec after switching to test solutions, using Mini Analysis Program (Synaptosoft Inc., Leonia, NJ). All data are expressed as means ± s.e.m., and statistical comparisons were made using unpaired *t*-tests, with $P < 0.05$ being considered significant.

RESULTS AND DISCUSSION

The ability of cyanide to evoke catecholamine secretion from PC12 cells was investigated by bath application of cyanide (0.1–5.0 mM) during continuous amperometric recordings from individual cells. In the absence of cyanide, no electrochemical events were observed (e.g., Fig. 1A, representative of >80 cells), indicating that under control conditions, PC12 cells do not undergo detectable exocytosis, as we have previously reported (9, 17). Bath application of cyanide (0.05–5 mM) caused the appearance of spike-like amperometric events (e.g., Figs. 1B–1D), each arising from the oxidation of a single quantum (vesicle) of catecholamine. The concentration-response relationship for this effect of cyanide is plotted in Fig. 1E, which shows that the EC_{50} for cyanide was *ca.* 0.6 mM, and maximal effects were seen at *ca.* 1–2 mM.

We next investigated the Ca^{2+} dependence of the secretory effect of cyanide. As illustrated in Fig. 2A representative of 6 cells), ongoing secretion from a cell continuously exposed to 2.5 mM cyanide could be completely abolished when extracellular Ca^{2+} was replaced with 1 mM EGTA, indicating an absolute dependency on extracellular Ca^{2+}. Furthermore, secretory responses to the same concentration of cyanide could be fully abolished when Cd^{2+} (200 μM) was applied to cells in the continued presence of Ca^{2+} in the perfusate (e.g.,

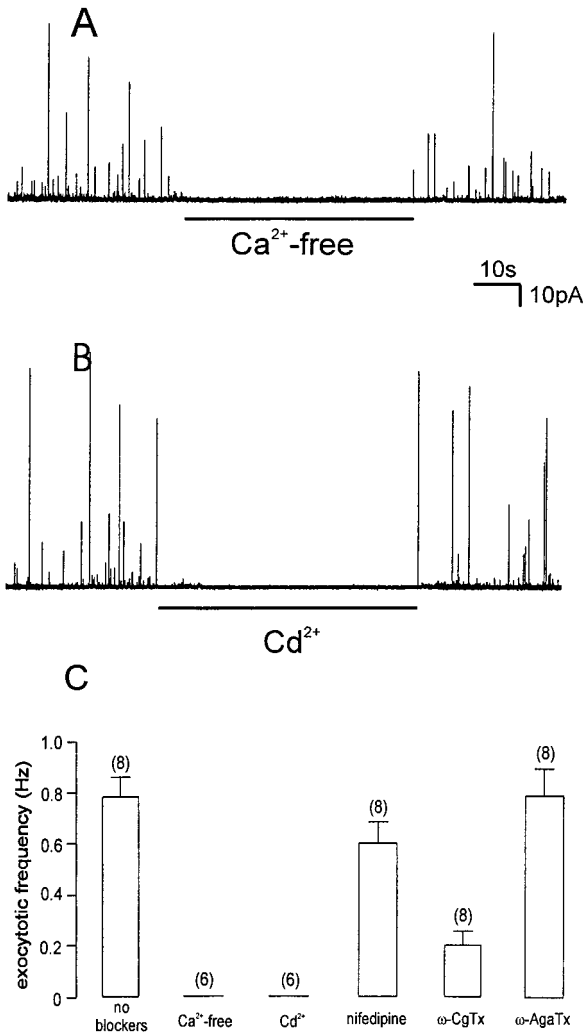

FIG. 2. Ongoing secretion evoked from 2 different PC12 cells by exposure to solution containing 2.5 mM cyanide. For the periods indicated by the horizontal bars, Ca^{2+} was removed from the perfusate and replaced with 1 mM EGTA (A) or Cd^{2+} (200 μM) was applied in the continued presence of Ca^{2+} (B). Scale bars apply to both traces. (C) Summary of the effects of cyanide (2.5 mM) on exocytotic frequency either in the absence of Ca^{2+} channel blockers, under Ca^{2+}-free conditions or in the presence of Cd^{2+} or the selective Ca^{2+} channel blockers nifedipine, ω-CgTxGVIA or ω-AgTxGIVA, as indicated. Each bar represents the mean (with vertical s.e.m. bar) exocytotic frequency obtained from the number of cells indicated in parentheses.

Fig. 2B, representative of 6 cells tested). These findings indicate that cyanide, like hypoxia (6) evokes catecholamine secretion from PC12 cells by stimulating Ca^{2+} influx through voltage-gated Ca^{2+} channels. We have previously shown that hypoxia evokes secretion from PC12 cells primarily by promoting Ca^{2+} influx through ω-CgTx-sensitive N-type Ca^{2+} channels (6). To investigate which Ca^{2+} channel subtype mediated Ca^{2+} influx coupled to secretion in response to cyanide, we tested the ability of selective Ca^{2+} channel blockers to interfere with cyanide-evoked secretion. Figure 2C summarizes the secretory responses of PC12 cells (in terms of exocytotic frequency) to 2.5 mM cyanide in the absence of Ca^{2+} channel blockers, in the absence of extracellular Ca^{2+}, or in the presence of Cd^{2+} (200 μM to block all voltage-gated Ca^{2+} entry), and also in the presence of nifedipine (2 μM) to block L-type Ca^{2+} channels. Responses of cells pre-treated with ω-CgTx (to block N-type channels) or ω-AgaTx (to block P/Q-type channels) are also shown. Of the selective agents tested, ω-CgTx clearly exerted the greatest effect, reducing secretion by *ca.* 75% ($P < 0.001$, unpaired *t*-test). No effect of ω-AgaTx was observed whilst a slight suppression of secretion was found in the presence of nifedipine, although this did not achieve statistical significance. Thus, like hypoxia (6), cyanide promotes Ca^{2+} entry to trigger exocytosis primarily though N-type Ca^{2+} channels.

In order to investigate whether cyanide exerted its effect to promote secretion from PC12 cells via its ability to inhibit mitochondrial function (rather than through any other, unknown action), we investigated the effects of another mitochondrial inhibitor, rotenone (10 μM), and also two mitochondrial uncouplers, DNP (250 μM) and FCCP (1 μM). As illustrated in Fig. 3 (A–C), all three agents were capable of promoting exocytosis. Furthermore, exocytosis elicited by all three agents could be fully inhibited either by removal of extracellular Ca^{2+} (replaced with 1 mM EGTA) or bath application of 200 μM Cd^{2+}. These findings indicate that mitochondrial inhibitors and mitochondrial uncouplers stimulate catecholamine release that is dependent on Ca^{2+} entry via voltage-gated Ca^{2+} channels.

This study indicates that four distinct agents, all of which interfere with mitochondrial function, are capable of stimulating quantal catecholamine release from PC12 cells. This supports the idea that this cell line serves as a useful model chemoreceptor cell (4). Mitochondrial inhibitors and uncouplers are, like hypoxia and acidosis, potent stimuli of the carotid body and cause catecholamine release from glomus cells (2). Hypoxia and acidosis similarly evoke catecholamine release from PC12 cells (6, 9) and, as we show here, secretory responses to mitochondrial inhibitors and uncouplers are apparent.

The mechanisms underlying the excitation of glomus cells by cyanide and other mitochondrial inhibitors/uncouplers has been disputed. Using rabbit glomus cells, Biscoe and Duchen (10) showed that cyanide hyperpolarized cells, due to activation of a Ca^{2+}-dependent K^+ current via release of Ca^{2+} from intracellular stores, including mitochondria (11). Indeed, cyanide (and also rotenone and FCCP) depolarized the mitochondrial membrane potential which is required for these organelles to sequester Ca^{2+} (10, 11). In contrast, Rocher *et al.* (8), also employing rabbit carotid bodies, indicated that the uncoupler DNP evoked catecholamine release by acting as an acidic stimulus

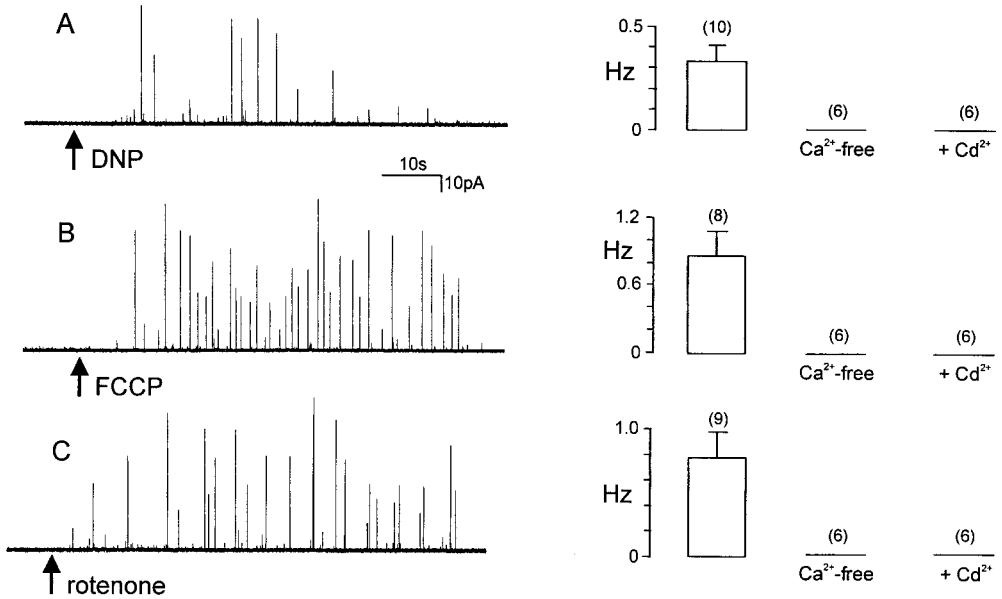

FIG. 3. Effects of mitochondrial inhibitors on secretory activity of PC12 cells. (A) left, example amperometric recording from a PC12 cell exposed to 250 μM 2,4-dinitrophenol (DNP), applied at the point indicated by the upward arrow and present throughout the rest of the recording. Right, bar graph indicating mean (with vertical s.e.m. bar) exocytotic frequency evoked by DNP applied in control solution, or in Ca^{2+}-free solution (containing 1 mM EGTA) or in the presence of 200 μM Cd^{2+}, as indicated. (B, C) as (A) except that cells were exposed to 1 μM FCCP (B) or 10 μM rotenone (C). Scale bars apply to all left-hand traces.

(DNP is a protonophore). Thus, DNP caused intracellular acidosis which stimulated plasma membrane Na-H exchange which in turn caused a sufficiently large rise of $[Na^+]_i$ to reverse the Na–Ca exchanger, so permitting Ca^{2+} entry via a transporter. Most recently, Buckler and Vaughan-Jones (12) studied the effects of two uncouplers, DNP and FCCP, rat carotid body cells. These agents only caused slight intracellular acidification, but evoked substantial rises of $[Ca^{2+}]_i$ due to cell depolarization and Ca^{2+} influx via voltage-gated Ca^{2+} channels. The depolarizing response was primarily due to inhibition of a K^+ conductance which appears similar to the one sensitive to hypoxia and hypercapnia (7, 18). Finally, cell depolarization was secondary to a depolarization of the mitochondrial membrane potential, and a small inward current was also observed. Interestingly, Inoue *et al.* (19) recently reported in chromaffin cells that cyanide and anoxia also stimulated a non-selective inward current, providing further evidence that mitochondrial inhibition is an important element of O_2-sensing. However, it would appear from the present study that this influx pathway, if present in PC12 cells, does not itself contribute significant Ca^{2+} influx, since Ca^{2+} channel blockers (rather than non-selective cation channel blockers) were able to reduce cyanide-evoked exocytosis dramatically (Fig. 2). Our results, using real-time measurements of catecholamine secretion from PC12 cells, are in good agreement with the work of Buckler and Vaughan-Jones (12). Both mitochondrial inhibitors (cyanide and rotenone) and uncouplers (DNP and FCCP) evoked catecholamine release via Ca^{2+} influx through voltage-gated Ca^{2+} channels (primarily N-type, at least in the case of cyanide). Thus, these agents are likely to cause membrane depolarization, presumably via inhibition of K^+ channels, as proposed for the effects of hypoxia and acidosis in these cells (5, 6, 9).

The major remaining question is what links the inhibition of mitochondrial respiration to inhibition of K^+ channels. One possibility is that reactive oxygen species (ROS) are involved, since studies in erythropoeitin-secreting Hep3B cells have shown that hypoxia causes an *increase* in ROS production which may mediate hypoxia-induced transcriptional events (20). However, the time-course of increased production of ROS was much slower than the effects of the agents used in the present study. Furthermore, a most recent report has shown that, whilst ROS production also increases in PC12 cells during hypoxia or in the presence of rotenone, the effects do not appear rapid, and do not account for hypoxia-induced elevation of tyrosine hydroxylase mRNA production (21). Despite these findings, the present evidence, together with the study of Buckler and Vaughan-Jones (12) suggests that there is a clear but undefined link between inhibition of mitochondrial respiration and inhibition of plasma membrane K^+ channels which leads to the observed secretory events. Further studies are required to understand this link, and such studies are also likely to shed important new light on the mechanisms underlying hypoxia-evoked exocytosis in PC12 and also carotid body glomus cells.

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