Dynamics of Intracellular Oxygen in PC12 Cells upon Stimulation of Neurotransmission

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Neurotransmission, synaptic plasticity, and maintenance of membrane excitability require high mitochondrial activity in neurosecretory cells. Using a fluorescence-based intracellular O2 sensing technique, we investigated the respiration of differentiated PC12 cells upon depolarization with 100 mM K+. Single cell confocal analysis identified a significant depolarization of the plasma membrane potential and a relatively minor depolarization of the mitochondrial membrane potential following K+ exposure. We observed a two-phase respiratory response: a first intense spike lasting ~10 min, during which average intracellular O2 was reduced from 85–90% of air saturation to 55–65%, followed by a second wave of smaller amplitude and longer duration. The fast rise in O2 consumption coincided with a transient increase in cellular ATP by ~60%, which was provided largely by oxidative phosphorylation and by glycolysis. The increase of respiration was orchestrated mainly by Ca2+ release from the endoplasmic reticulum, whereas the influx of extracellular Ca2+ contributed ~20%. Depletion of Ca2+ stores by ryanodine, thapsigargin, and 4-chloro-m-cresol reduced the amplitude of respiratory spike by 45, 63, and 71%, respectively, whereas chelation of intracellular Ca2+ abolished the response. Uncoupling of the mitochondria with the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone amplified the responses to K+; elevated respiration induced a profound deoxygenation without increasing the cellular ATP levels reduced by carbonyl cyanide p-trifluoromethoxyphenylhydrazone. Cleavage of synaptobrevin 2 by tetanus toxin, known to reduce neurotransmission, did not affect the respiratory response to K+, whereas the general excitability of PC12 cells increased.

Neurotransmission operates via a multistage synaptic vesicle cycle, which includes the following main events: active vesicle filling with neurotransmitters (NT);2 transportation and release of vesicle content via clustering, docking, priming, and Ca2+-dependent exocytosis, and finally recycling of consumed vesicle components (1, 2). Membrane fusion, a key event of exocytosis, is not energy-demanding, because the formation of the trans-SNARE complex is thermodynamically favorable (3, 4). Conversely, vesicle filling, transport, and recycling events consume 10–20% of energy resources, whereas re-establishment of resting membrane potential and maintenance of Ca2+ equilibrium by ion pumps consume 60–80% of ATP in neuronal cells (5). Despite being only 2% of body weight, the central nervous system consumes about 20% of O2 inspired at rest (6) to fuel sustained neuronal activity with ATP supply through oxidative phosphorylation (OxPhos). When overproduced, ATP down-regulates the respiration by allosteric inhibition of cytochrome c oxidase (7). However, the detailed dynamics and regulation of metabolism in neuronal cells upon activation with different stimuli are not very well studied. Ca2+ oscillations in the mitochondrial matrix are known to regulate mitochondrial membrane potential (ΔΨm), electron transport chain (ETC) activity, and ATP production. Transient Ca2+ influxes activate tricarboxylic acid cycle enzymes and modulate the activity of ETC complex IV (cytochrome c oxidase) and complex V (F0F1 ATP synthase) (8–11). To provide fast transport into matrix, the Ca2+ uniporter requires high cytosolic Ca2+ in the vicinity of mitochondria (12), and mitochondrial Ca2+ transients can reach submillimolar levels (13). Because of the low efficiency of Na+/Ca2+ and H+/Ca2+ exchangers and high buffering capacity, the mitochondria can transiently retain Ca2+ released from ER during excitation, thus contributing to the regulation of neurotransmission and modulating synaptic plasticity (14, 15). On the other hand, overload of the mitochondria with Ca2+ can trigger permeability transition pore formation and apoptosis (16–18).

Elevation of Ca2+ induces fast NT release via activation of the vesicular Ca2+ sensor synaptotagmin 1 (19). Generally, evoked NT exocytosis is triggered by Ca2+ influx through voltage-gated Ca2+ channels (VGCC) controlled by the plasma membrane potential (for review see Ref. 2). In turn, Ca2+ influx stimulates Ca2+ release from the ER, which modulates Ca2+-signaling pathways. However, recent studies demonstrate that even in the absence of Ca2+, neuronal cells can perform evoked oxidative phosphorylation; ETC, electron transport chain; VGCC, voltage-gated Ca2+ channel(s); DAPI, 4',6'-diamino-2-phenylindole; CmC, 4-chloro-m-cresol; TeNT, tetanus neurotoxin; TR-F, time-resolved fluorescence; TMRM, tetramethyl rhodamine methyl ester; BAPTA-AM, 1,2-bis (2-aminoethoxy) ethane-N,N,N’,N”-tetraacetic acid tetraacetoxymethyl ester.

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2 The abbreviations used are: NT, neurotransmitters(s); ER, endoplasmic reticulum; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; OxPhos,
neurotransmission driven by the ER (20, 21) and mitochondrial (22) Ca\(^{2+}\) stores. Action potential-driven activation of VGCC and Ca\(^{2+}\) release from intracellular stores can be mimicked by depolarizing plasma membrane with high extracellular K\(^+\) (K\(^+\)). It has been shown that upon prolonged exposure to high K\(^+\), PC12 cells undergo sustained membrane depolarization (23) and can execute frequent NT exocytosis for several minutes (24). Derived from rat adrenal pheochromocytoma, PC12 cells have rather heterogeneous pool of Ca\(^{2+}\) stores and express L, N, T, and P/Q types of VGCC (25, 26). Such architecture provides diverse mechanisms of regulation of Ca\(^{2+}\), neurotransmission, and mitochondrial activity. Differentiated PC12 (\(\rho\)PC12) cells demonstrate gene expression profiles, evoked NT release, and many other features typical for neuronal cells (25, 26) and rely on both OxPhos and glycolysis as energy sources (27).

Oxygen supply and consumption within the cell are informative markers of OxPhos, cell energetics, and signaling. Thus far these parameters were not amenable to routine analysis. The well established O\(_2\) respirometry technique (28) has limited applicability to suspension cell lines, low resolution power, and information content (end-point measurement), whereas the O\(_2\) imaging technique (29), although allowing detailed single-cell analyses, has a high level of complexity and low sample throughput. The new fluorescence-based methodology of sensing intracellular O\(_2\) (30) addresses these limitations and provides simple, high throughput analysis of O\(_2\) gradients in cell populations and effects of various metabolic effectors and stimuli. In this study, we applied this technique to examine the respiratory responses of \(\rho\)PC12 cells to sustained membrane depolarization by high K\(^+\) and to metabolic and Ca\(^{2+}\) effectors. Some other cell lines and parameters relevant to mitochondrial and neurosecretory functions were also analyzed to elaborate fine mechanisms of responses in \(\rho\)PC12 cells. We demonstrate that a marked increase in O\(_2\) consumption induced by high K\(^+\) proceeds in two distinct phases and that this response requires fast Ca\(^{2+}\) release from ryanodine-sensitive stores. During the first phase of response, the cells transiently elevate ATP by 60% over the resting level. Uncoupling of the ETC with FCCP enhances the respiratory response without elevating ATP. Finally, hampered NT exocytosis was shown not to alter significantly the respiratory response of \(\rho\)PC12 cells to membrane depolarization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat pheochromocytoma (PC12), human neuroblastoma (SH SY5Y), human cervix epithelioid carcinoma (HeLa), human lung carcinoma (A549), and mouse muscle myoblast (C2C12) cells were obtained from the European Collection of Cell Cultures. Phosphorescent oxygen-sensing probe MitoXpress® was from Luelxel Biosciences (Cork, Ireland), and Endo-Porter was from Gene Tools, LLC (Philomath, OR). The antibodies were: mouse anti-synaptobrevin 2 (SY5Y; Göttingen, Germany), mouse anti-\(\beta\)-tubulin (Santa Cruz Biotechnologies; Santa Cruz, CA), and horseradish peroxidase-conjugated rabbit anti-mouse (DAKO; Glostrup, Denmark). ECL Plus Western blotting detection reagents were from GE Healthcare (Waukesha, WI), and protease inhibitor mixture tablets were from Roche Applied Sciences. Ca\(^{2+}\) fluorescent indicator Fluo-4 AM, MitoTracker® Red, DAPI, DiSBAC\(_{3}(3)\), and TMRM were obtained from Invitrogen. Cellular ATP assay kit CellTiter-Glo® was from Promega (Madison, WI), and BCA™ protein assay was from Pierce. Collagen IV was from Fluka, and 4-chloro-m-cresol (CmC) was from Merck. Black body, clear-bottomed 96-well plates for fluorescent measurement, and white 96-well plate for luminescence analysis were from Greiner Bio One (Frickenhausen, Germany); WillCo dishes were from WillCo Wells BV (Amsterdam, The Netherlands). Cell culture plasticware was from Sarstedt (Wexford, Ireland), and all other reagents were from Sigma-Aldrich.

**Cell Culture and Treatments**—PC12 cells were cultured in RPMI 1640 supplemented with 2 mM l-glutamine, 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO\(_2\). For oxygen sensing experiments, the cells were washed with phosphate-buffered saline, incubated for 3 min at 37 °C with 0.25% trypsin/2 mM EDTA solution, resuspended in RPMI medium, passed 6–8 times through the 23-gauge needle to separate individual cells, and seeded at 5 × 10\(^4\) cells/well in 96-well plates precoated with collagen IV (31). The cells were grown for 8 h and then differentiated for 6–8 days in RPMI 1640 supplemented with 1% horse serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 100 ng/ml nerve growth factor. Transfection was performed by incubating \(\rho\)PC12 with 1.2 µM MitoXpress®/6 µM Endo-Porter for 28 h in differentiating conditions. The cells were washed twice with serum-rich RPMI; washed once with RPMI without serum, phenol red, and l-glutamine; and then analyzed on a fluorescent reader in 100 µl of RPMI without serum and phenol red. The viability of cells loaded with probe was tested (after trypsinization) on a PCA-96 flow cytometer using ViaCount™ kit and Cytosoft 2.5.5 software (all Guava Technologies), as per the manufacturer’s instructions. To assess the contribution of glycolysis to ATP production, \(\rho\)PC12 cells were preincubated for 3 h in serum-free RPMI containing 10 mM galactose and 1 mM pyruvate (32). To reduce neurotransmission through cleavage of synaptobrevin 2, \(\rho\)PC12 were treated with 20 nM tetanus neurotoxin (TeNT) for 6 h after transfection. Chelation of Ca\(^{2+}\) was achieved by incubation of cells with 50 µM BAPEA-AM for 30 min; Ca\(^{2+}\) was removed with 2.5 mM EGTA; Ca\(^{2+}\) stores were depleted by the addition of 50–500 µM CmC, 2 µM ryanodine, or 10 µM thapsigargin.

SH-SY5Y, HeLa, A549, and C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. For oxygen sensing experiments SH-SY5Y and C2C12 were washed with phosphate-buffered saline, trypsinized for 5 min at 37 °C, resuspended in the same medium, and seeded on 96-well plate at 2 × 10\(^4\) and 1 × 10\(^4\) cells/well, respectively. The cells were grown for 1 day, differentiated for 3 days in Dulbecco’s modified Eagle’s medium containing 1% horse serum and then loaded with MitoXpress probe in differentiating conditions, as described for PC12 cells. HeLa and A549 cells having low transfection efficiency were loaded at 75–85% confluence in 75-cm\(^2\) flasks, incubating with 1.2 µM MitoXpress®/6 µM Endo-Porter for 28 h. The cells were then washed, trypsinized, seeded on 96-well plate at 3–5 × 10\(^5\) cells/well, and allowed to adhere for 3 h prior to fluorescence measurements.
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Oxygen Sensing Assay—Typically, up to 24 samples, including loaded cells and appropriate controls, were analyzed in parallel on a time-resolved fluorescent (TR-F) plate reader Victor 2 (PerkinElmer Life Science) as described in Ref. 30. Briefly, TR-F measurements were carried out in air-saturated medium at 37 °C with standard 340-nm excitation and 642-nm emission filters. Each sample well was measured periodically, by taking two intensity readings at delay times of 30 and 70 μs and a gate time of 100 μs. The plate was monitored for 10–20 min to reach O_2 and temperature equilibrium and obtain basal signals. The plate was then quickly withdrawn from the reader, compounds were added to the wells in 10-μl aliquots, and monitoring was resumed for the next 20–60 min. Measured TR-F intensity experiments (see “Results”).

PC12 cells were differentiated on poly-D-lysine-coated glass slides. The procedures described in this paper are the two delay times and corresponding concentrations of the samples were monitored in kinetic mode on the Victor 2 reader at 37 °C with 355-nm excitation and 460-nm emission filters, with positive control (maximal fluorescent signal), and FCCP (4 μM) as negative control (minimal signal).

Measurement of Intracellular Ca^{2+}—PC12 cells were incubated with 5 μM Fluo-4 (AM) for 1 h, washed, and incubated for further 30 min in phenol red free RPMI medium to complete de-esterification. The fluorescence of samples treated with different compounds (see results) in 0.1 ml of medium in clear 96-well plates was monitored in kinetic mode on the GENios Pro reader (TECAN), using a 485 ± 20-nm excitation filter and a 535 ± 20-nm emission filter.

NAD(P)H Measurement—NAD(P)H auto-fluorescence was monitored following a modified method (35). PC12 cells were incubated in suspension for 3 h in serum-free RPMI containing 10 mM glucose and pipetted into the wells of clear 96-well plate (4 × 10^5 cells in 0.1 ml). The fluorescence of samples was monitored in kinetic mode on the Victor 2 reader at 37 °C with 355-nm excitation and 460-nm emission filters, with and without effector addition. Antimycin A (4 μM) was used as positive control (maximal fluorescent signal), and FCCP (4 μM) was used as negative control (minimal signal).

Monitoring of Mitochondrial and Plasma Membrane Potentials—PC12 cells were differentiated on WillCo dishes precoated with collagen IV and loaded with 20 nM TMRM or 1 μM DiSBAC_2(3) (36) for 30 min at 37 °C (in the dark) in experimental buffer (120 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM HEPES, 5 mM NaHCO_3, 1.2 mM Na_2SO_4, 1.2 mM CaCl_2, 1.2 mM MgCl_2, and 15 mM glucose, pH 7.4). The Willco dishes with cells were washed in fresh medium after loading before being mounted in a nonperfusion (37 °C) holder and placed on the stage of a LSM 510 Meta Zeiss (Carl Zeiss, Jena, Germany) confocal microscope. TMRM and DiSBAC_2(3) were excited at 543 nm with a helium-neon laser (3%), and the emission was collected through a 560-nm-long pass filter. Fluorescence and differential interference contrast images were collected at 30-s intervals throughout and every 15 s following KCl (100 mM) excitation. The resulting fluorescence images were processed using Metamorph Software version 7.1 release 3 (Molecular Devices, Berkshire, UK).

RESULTS

Assessment of O_2 in Resting PC12 Cells—Activation of mitochondrial machinery and OxPhos is linked to elevated respiration leading to reduced O_2. Using a new O_2 sensing technique (30), we examined respiratory responses of neurosecretory β-cell cells to excitatory stimuli, known to induce NT release. Optimization of the procedure for the passive loading of dense populations of PC12 cell (Fig. 1A) with the phosphorescent oxygen-sensing probe MitoXpress® resulted in high and reproducible signals from the cells when assessed by TR-F. Phosphorescence intensity signals measured on a standard TR-F plate reader Victor 2 were in the region of 40,000–100,000 cps, with...
a signal-to-blank ratio of >150. Cell viability remained unaffected (93–95%). When monitoring resting cells in air-saturated medium at 37 °C, phosphorescence intensity signal was seen to decrease significantly (by 25–40% after 1 h of monitoring), because of probe photobleaching. In contrast, the phosphorescence lifetime signals calculated from pairs of intensity readings (at delay times 30 and 70 μs) remained stable and reproducible (33–35 μs). This measurement mode provided the basis for reliable and accurate real time monitoring of average O$_2$ levels in pc12 and other cells and their responses to various stimuli and metabolic effectors. Confocal fluorescent images of transfected and co-stained cells demonstrate that the probe is distributed predominantly in the cytoplasm and surrounds the mitochondria visualized with MitoTracker<sup>®</sup> Red (Fig. 1B).

Detailed calibrations performed with pc12 cells loaded with MitoXpress<sup>TM</sup> probe revealed good linearity of Stern-Volmer plots (30). Assuming that the minimal phosphorescence lifetime of the probe in integral but nonrespiring cells corresponds to O$_2$ in the extracellular medium (i.e. 100% of air saturation, or 230 μM) and the maximal lifetime corresponds to deoxygenated medium, we performed a simple two-point calibration. For pc12 cells treated with antimycin A (i.e. nonrespiring), the measured lifetime was 30.5 μs, and upon the addition of glucose/glucose oxidase (deoxygenated cells) it was 66.3 μs, which gives (see "Experimental Procedures") a quenching constant K$_q$ of 0.0117%$^{-1}$ and an average O$_2$ of 85–90% of air saturation for resting pc12 cells.

**Rapid Spike in Oxygen Consumption by pc12 Cells in Response to Membrane Depolarization by K<sup>+</sup>**

Excitable cells respond to certain stimuli in a fast energetic manner, with the excretion of bioactive molecules or cell contraction. Thus, membrane depolarization by K<sup>+</sup> at concentrations exceeding the threshold of 56 mM induces Ca<sup>2+</sup>-dependent evoked NT release in pc12 cells (38). To examine how sustained membrane depolarization affects O$_2$ consumption, pc12 cells loaded with the probe were stimulated with different concentrations of KCl while monitoring them on the Victor 2 reader at 37 °C (Fig. 2A). At 25 mM K<sup>+</sup> had no measurable effect, and subdepolarizing 50 mM K<sup>+</sup> caused a short minor spike in lifetime, reflecting a decrease
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in O₂ by ~6%, whereas 100 mM K⁺ caused a marked rise in respiration reducing O₂ by 20~30%. The response of PC12 to high K⁺ showed two distinct phases: a short intense spike lasting ~10 min, followed by a second wave of lower amplitude reaching a maximum ~30 min after stimulation. At K⁺ below the membrane depolarization threshold, only the first phase of reduced duration and amplitude was observed. Cell pretreatment with 10 mM ouabain, which inhibits Na⁺/K⁺-ATPase and perturbs plasma membrane potential, decreased the response to high K⁺ by ~20 ± 5% (Fig. 2A). Measurements performed with the probe added to the extracellular medium revealed that there are no global O₂ gradients in samples containing the cells, and the levels of bulk O₂ remain at ~100% of air saturation.

**Transient Increase in Oxygen Consumption in PC12 Cells upon Membrane Depolarization Is Specific to Neurosecretory Cells**—To examine whether the respiratory response to K⁺ is specific to excitable cells, we exposed to 100 mM K⁺ nondifferentiated PC12 (nPC12), cervical epithelial HeLa cells, nondifferentiated PC12 cells, human lung carcinoma A549 cells, and differentiated human neuroblastoma SH-SY5Y cells (Fig. 2B). PC12 cells, which are also prone to excitation, NT synthesis, and release, responded similarly to nPC12, but with 25~30% lower amplitude. This observation is in agreement with the previously observed increased membrane depolarization and NT release in PC12 cells by nerve growth factor (39). Neuronal SH-SY5Y cells also demonstrated pronounced response to K⁺; the initial fast phase had similar amplitude (75 ± 10%), but longer duration (~20 min) than observed in nPC12 cells, whereas a second phase was not seen. All of the other cell lines tested produced significantly weaker responses: 26 ± 5% for HeLa and A549 cells, 20~25% for nPC12, and 20~25% for nPC12 (although nPC12 demonstrated a strong response to Ca²⁺-ionophore ionomycin: 50~55% of that of dPC12; data not shown). These results suggest that the observed fast, transient increase in respiration in response to sustained membrane depolarization by high K⁺ is characteristic to neurosecretory cells.

**ETC Uncoupling Does Not Inhibit Fast Respiratory Response of PC12 Cells to Membrane Depolarization**—To investigate how changes in respiration induced by K⁺ relate to mitochondrial function, PC12 cells were treated with 1~2 μM rotenone (inhibitor of ETC Complex I) or 4 μM antimycin A (inhibitor of ETC Complex III). Following a 10-min pretreatment, activation of O₂ consumption by K⁺ was effectively inhibited for both compounds (Fig. 2C). The F₂F₄ ATP synthase inhibitor oligomycin at 10 μM also reduced the effect of high K⁺ by ~50% (Fig. 2C).

Considering the strong effect of K⁺ on PC12 cell respiration, we investigated whether K⁺ ionophore valinomycin can mimic it (Fig. 2D). Valinomycin selectively increases K⁺ currents across plasma and mitochondrial membranes, resulting in membrane depolarization and ETC uncoupling. In comparison with the respiratory response to K⁺, valinomycin was seen to cause a slower and smaller decrease in O₂ (20~30% of the response to K⁺). At 10 mM K⁺, 1~2 μM valinomycin induced a sustained increase in O₂ consumption for about 1 h. At subthreshold 30 mM K⁺, valinomycin produced a transient increase of respiration over 30 min with higher amplitude (35~45% of K⁺). To check whether the effect of valinomycin interferes with the response to K⁺, we incubated PC12 cells for 10 min with 2 μM valinomycin and 10 mM K⁺ and then stimulated them with 100 mM K⁺. Compared with untreated cells, valinomycin accelerated the first rapid phase of respiratory response to K⁺; the maximum was reached 1.5 min earlier. However, valinomycin did not affect the amplitude and duration of the first phase, whereas the second phase was abolished, and respiration returned to the level characteristic of valinomycin alone.

To further examine the relationship between ETC uncoupling and membrane depolarization, PC12 cells were treated with the protonophore FCCP (4 μM) for 15 min and then stimulated with 100 mM K⁺ (Fig. 2E). We found that the respiratory response of PC12 to FCCP had a characteristic profile, with a fast strong initial spike followed by a slower second wave in O₂ consumption reaching maximum after ~20 min. When 100 mM K⁺ was applied to PC12 cells uncoupled with FCCP, the increase in respiration by K⁺ was significantly stronger and longer than by K⁺ alone, and the second phase of response (15~40 min) showed an additive effect of FCCP and K⁺. Maximal respiration was observed ~8 min after the addition of K⁺, which corresponded to only ~15% oxygenation of cell cytoplasm (compared with 85~90% for the resting cells). Interestingly, cell response to such double treatment did not merge and clearly retained the phases, specific to each compound, suggesting different mechanisms of action. Taken together, these results indicate that ETC uncoupling does not eliminate the respiratory response to membrane depolarization and that uncoupling of the mitochondria with FCCP amplifies the response.

**Ca²⁺ Orchestrates Changes in PC12 Cell Respiration upon Membrane Depolarization**—Membrane depolarization in neuronal cells leads to Ca²⁺-dependent activation of signaling cascades and neurotransmission. We induced elevation of Ca²⁺ with 1 μM ionomycin and compared its effect with K⁺ (Fig. 3A). The influx of Ca²⁺ (~0.5 mM in RPMI) increased respiration to a greater degree and faster than K⁺; however, this seems to damage the cells leading to an irredeemable drop in O₂ consumption 10 min after the addition of ionomycin. As expected, pretreatment of the cells with 50 μM BAPTA-AM, which neutralizes Ca²⁺ delivered from intracellular and extracellular sources, dramatically reduced the response to both K⁺ and ionomycin. Chelation of Ca²⁺ with 2.5 mM EGTA abolished the response to ionomycin (Fig. 3B), whereas the response to K⁺ was reduced by only 15~20%. Preincubation with both 2.5 mM EGTA and 50 μM BAPTA-AM eliminated the effect of high K⁺ on PC12 cells. Finally, inhibition of N-, T- and P/Q types of VGCCs with 100 μM Cd²⁺ had an effect similar to EGTA, whereas neither activation of L type VGCCs with 10 μM Bay K 8644 nor their inhibition with 1~10 μM nifedipine was seen to alter the response to K⁺ (data not shown).

PC12 cells express ryanodine receptors, which can be activated by CmC triggering the release of Ca²⁺ from the ER (40). We applied 50, 250, and 500 μM CmC to PC12 cells for 10~15 min and then stimulated them with 100 mM K⁺ (Fig. 3C). Neither 50 nor 250 μM CmC influenced the respiration, whereas 500 μM CmC decreased it considerably. In cells pretreated with 250 and 500 μM CmC, the response to high K⁺ was reduced by 57 ± 8 and 71 ± 5%, respectively. This effect is likely due to the
depletion of Ca^{2+} stores that normally provide Ca^{2+} required for the observed rapid metabolic response to membrane depolarization. To verify this, dPC12 were pretreated for 15 min with 2 μM ryanodine (ryanodine receptor agonist) or 10 μM thapsigargin (potent inhibitor of Ca^{2+} ATPase responsible for refilling Ca^{2+} stores) and then stimulated with 100 mM K\(^+\). As expected, both compounds decreased the response to K\(^+\) by 45 ± 10 and 63 ± 5%, respectively. Finally, we studied how the depletion of Ca^{2+} stores in dPC12 influences the dynamics of Ca^{2+} increase by K\(^+\). The cells were loaded with 5 μM Fluo-4 AM, and fluorescence was monitored on a GENios Pro reader (Fig. 3D). High K\(^+\) considerably increased Ca^{2+} to a high steady level. Similarly, 500 μM CmC rapidly elevated Ca^{2+}; however, if the cells were exposed to high K\(^+\) at this stage, we observed only a minor further elevation of Ca^{2+}, indicating the depletion of ER Ca^{2+} stores by CmC. These results show that the respiration spike in response to membrane depolarization by K\(^+\) is largely mediated by Ca^{2+} and that Ca^{2+} stores play a major role in this process. On the other hand, elevation of Ca^{2+} by CmC is not sufficient to enhance the respiration.

Ca^{2+} Regulates Respiratory Response to Hypertonic Sucrose in PC12 Cell—Are intracellular Ca^{2+} stores involved in respiratory responses to other inducers of neurotransmission? As demonstrated recently, Ca^{2+} regulates the ability of cerebrocortical synaptosomes to continuously release NT in response to hypertonic concentrations of sucrose (41). To examine
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whether Ca\(^{2+}\) modulates the respiratory response to such non-specific stimulation, we applied 0.5 M sucrose to \(\text{dPC12}\) cells preincubated with 2.5 mM EGTA, 50 mM BAPTA-AM, both EGTA and BAPTA-AM, 4 \(\mu\)M antimycin A, and regular RPMI medium (Fig. 4). The initial nonspecific spike in fluorescence, which can be attributed to osmotic cell shrinkage, independent of pretreatment with antimycin A, was followed by a steady increase of respiration over 30 min. Maximal response was achieved 10–20 min after treatment, bringing \(\text{O}_2\) to 75 ± 5% of air saturation, and it was inhibited by antimycin A. We found that Ca\(^{2+}\) mediates the response to sucrose in a time-dependent manner, because chelation of Ca\(^{2+}\) with BAPTA-AM reduced the response by 45 ± 5% at 5 min and by 90 ± 5% at 15 min after the addition of sucrose. During the first 10 min, the removal of Ca\(^{2+}\) with EGTA had little effect on the amplitude of the response; thereafter \(\text{O}_2\) consumption rapidly decreased, most likely because of the depletion of Ca\(^{2+}\) stores. Simultaneous chelation of Ca\(^{2+}\) and Ca\(^{2+}\) inhibited the response in an additive manner. These results demonstrate the key role of Ca\(^{2+}\) in the regulation of respiratory activity related to the excitation of PC12 cells.

High K\(^+\) Elevates Cellular ATP in a Manner Dependent on ETC Integrity, Ca\(^{2+}\), and Glycolysis—The level of ATP in the cell depends on the rate and efficiency of OxPhos, glycolysis, and ATP consumption and is regulated by many factors including Ca\(^{2+}\), Na\(^+\), K\(^+\), \(\Delta\Psi_m\), ATP/ADP ratio, and \(\text{O}_2\). To check whether respiratory activity correlates with ATP levels during sustained membrane depolarization, we applied 100 mM K\(^+\) to \(\text{dPC12}\) cells and measured cellular ATP at different time intervals (3-min steps). A maximal elevation in ATP of 58 ± 12% over the resting level was observed 3 min after stimulation (Fig. 5A), which coincides with the early phase of \(\text{O}_2\) decrease. After 6 min, i.e. when \(\text{O}_2\) reaches its minimum, ATP was 27 ± 8% above the resting level and then returned to the resting values after 10–12 min. Measurement of ATP over the first 6 min (in 1-min steps) revealed a maximum 3 ± 1 min after membrane depolarization. In the cells treated with antimycin A for 20 min, ATP levels decreased by 40–45% and remained unaffected by 100 mM K\(^+\) (Fig. 5C). Uncoupling by FCCP reduced ATP by 30–35% (Fig. 5B), which is in agreement with previous report (42). Subsequent increases in \(\text{O}_2\) consumption by K\(^+\) had no effect on the ATP level. Although cellular ATP was not restored, 12–15 min after K\(^+\) treatment the cells rapidly decreased \(\text{O}_2\) consumption to a level above the resting state. These results indicate that upon ETC uncoupling high K\(^+\) transiently accelerates \(\text{O}_2\) consumption in a manner independent of cellular ATP levels. The removal of Ca\(^{2+}\) by the addition of 2.5 mM EGTA had no significant effect on resting ATP level, and the rise in ATP upon subsequent K\(^+\) application was only 15 ± 4% above the resting level (Fig. 5C). We found that ATP, reduced to 75 ± 5% by 500 \(\mu\)M CmC, increased only to the resting level upon membrane depolarization by K\(^+\) (Fig. 5D), proving that depletion of Ca\(^{2+}\) stores reduced energy reserves in \(\text{dPC12}\).

Because Ca\(^{2+}\) transients activate dehydrogenases of the tricarboxylic acid cycle (9–11), thus increasing NADH supply to the ETC, we measured NAD(P)H auto-fluorescence upon K\(^+\) treatment (Fig. 5G). This data revealed a two-phase rise in cellular NAD(P)H, in which the first spike coincided with the onset of the respiratory response.

Only partial reduction in ATP by antimycin A (Fig. 5C) suggests a significant contribution of glycolysis to ATP supply in PC12 cells. To assess this contribution, we removed the ability of the cells to generate ATP via glycolysis. The cells were exposed for 3 h to RPMI containing 1 mM pyruvate, 10 mM galactose, and no glucose and then treated with K\(^+\). Compared with glucose (+) cells, in galactose (+) cells resting ATP levels were similar, but ATP increase in response to membrane depolarization became smaller (Fig. 5E). The contribution of glycolysis to this response, calculated as the difference in ATP level between glucose (+) and galactose (+) cells, was ~30% (Fig. 5E, inset). For galactose (+) cells, both resting \(\text{O}_2\) and the first peak of respiration (\(\text{O}_2 = 30 ± 5%\) of air saturation) were significantly higher than for glucose (+) cells (Fig. 5F).

Single Cell Characterization of \(\Delta\Psi_m\) and \(\Delta\Psi_p\) Relative to \(\text{O}_2\) Consumption Following K\(^+\) Stimulation—The potentiometric probe TMRM has been used extensively as a tool for the single cell characterization of \(\Delta\Psi_m\) (36, 37, 43–46) in vitro. Here we have utilized a nonquenching concentration of TMRM (20 nM) to monitor mitochondrial bioenergetics in PC12 cells relative to changes in \(\text{O}_2\). Upon the addition of 100 mM K\(^+\) there was a significant decrease of the TMRM signal (Fig. 6, A and B) that was associated with a rapid increase in DiSBAC\(_2\)(3) fluorescence (depolarization of \(\Delta\Psi_p\); Fig. 6C). Because TMRM is sensitive to changes in both \(\Delta\Psi_m\) and \(\Delta\Psi_p\), much of the loss in TMRM fluorescence can be accounted for by a decrease in \(\Delta\Psi_p\). In an effort to quantify the changes in \(\Delta\Psi_m\) and \(\Delta\Psi_p\) following stimulation with K\(^+\), we modeled the data using models provided by Ward et al. (36), and Nicholls (37). From this we calculated that a 30–40-mV \(\Delta\Psi_p\) depolarization is coupled to a 5–10-mV depolarization of \(\Delta\Psi_m\) (Fig. 6D and supplemental figure). The responses at a \(\Delta\Psi_p\) and \(\Delta\Psi_m\) level following K\(^+\) were further coupled to a significant decrease in \(\text{O}_2\) (Fig. 6B) within the cells during the 10–12-min period following stimulation. These results imply that mitochondrial respiration, fueled by increased NADH availability (Fig. 5G), is increased to meet the energy demands associated with the extensive depolarization of \(\Delta\Psi_p\) by K\(^+\).
Inhibition of Neurotransmission with Tetanus Toxin Does Not Change the Respiratory Response of dPC12 to High K⁺—The increase in O₂ consumption by dPC12 cells in response to membrane depolarization with K⁺ can be linked to NT exocytosis or other events in the synaptic vesicle cycle. To examine how blockage of neurotransmission influences respiratory responses of dPC12, cells were differentiated for 7 days, loaded with MitoXpress® probe, and incubated for 6 h with 20 nM TeNT known to inactivate synaptobrevin 2, prevent the formation of synaptobrevin/SNAP-25/syntaxin complexes between NT granules and synaptic membranes (47, 48), and reduce basal and evoked neurotransmission (49). First, we analyzed the effect of TeNT treatment on synaptobrevin 2 level. Western blot showed that the concentration of synaptobrevin 2 in TeNT (+) cells was reduced by 65 ± 6% (Fig. 7A). Next, the effect of NT blockage on the dPC12 cells treated with 100 mM K⁺ was recorded and compared with that of 1 µM ionomycin and 4 µM FCCP to distinguish the effect of TeNT specific for membrane depolarization (Fig. 7B). Although statistically different only for ionomycin, the responses were all higher in TeNT (+) cells,
suggesting higher excitability. We think that reduced exposure of TeNT (+) cells to NT can up-regulate their excitability, because dopamine was shown to suppress the responses of entorhinal cortex and striatal neurons to excitation (50, 51). Taken together, these results indicate that high $\kappa^+$ activates respiration regardless of the efficiency of NT exocytosis in PC12 cells.

**DISCUSSION**

A new fluorescence-based method of sensing $\Omega_2$ in cell populations was applied to study physiological responses of neurosecretory PC12 cells. This method uses the phosphorescent $\Omega_2$-sensing probe MitoXpress loaded into cell cytosol that monitors alterations in the respiratory activity of the cells via changes of phosphorescence lifetime. The method operates with relatively large populations of cells; therefore the measured lifetime signals reflect the $\Omega_2$ levels averaged throughout the cell and entire population. Although the measured $\Omega_2$ concentration has a complex relationship with the rate of $\Omega_2$ consumption by the cell, it allows dynamic monitoring of relative changes in respiration. It provides simplicity, convenience, high sample throughput, and real time, quantitative assessment of cell metabolic status under resting conditions and upon stimulation. Our results demonstrate that $\Omega_2$ is a sensitive marker of physiological responses, particularly those driven by $\text{Ca}^{2+}$-dependent pathways and affecting mitochondrial activity and neurosecretion.

Here we examined the dynamics of $\Omega_2$ consumption by dPC12 in response to cell stimulation with high $\kappa^+$. The effect of $\kappa^+$ has a threshold between 50 and 100 mM, which is close to the threshold of 56 mM $\kappa^+$ reported for plasma membrane depolarization in PC12 cells (38). Inhibition of the $\text{Na}^+/\text{K}^+$ ATPase with ouabain, which perturbs plasma membrane potential, decreases respiratory response to high $\kappa^+$ by 20 ± 5%. We therefore relate this response to sustained depolarization of the cell membrane, which induces NT release. Among the cell lines tested, only SH-SY5Y cells revealed a rise in respiration comparable with PC12 cells. Furthermore, upon differentiation with nerve growth factor, known to increase NT secretion and generate numerous synapses and cell contacts, PC12 cells responded more strongly to $\kappa^+$. The specificity of respiratory response to $\kappa^+$ for neurosecretory cells can be explained by the fact that they are enriched with mitochondria positioned in active growth cones and synapses (52, 53). Neuronal mitochondria are subjected to repeated ion fluxes when $\text{Na}^+$ and $\text{Ca}^{2+}$ levels increase dramatically in the synaptic terminals. Such terminals contain a high amount of various ATP-dependent ion pumps to rapidly re-establish ion equilibrium, $\text{Ca}^{2+}$ homeostasis and plasma membrane potential (54). Thus, in contrast to nonexcitable cells, neurons possess the mitochondria capable of producing large amounts of ATP shortly after plasma membrane depolarization.

Membrane depolarization activates VGCC, which allow $\text{Ca}^{2+}$ influx and $\text{Ca}^{2+}$ release from intracellular stores, thus triggering active
NT exocytosis and the synaptic vesicle cycle (1, 2). Our observations suggest that Ca\(^{2+}\) is a major driver of the K\(^{+}\)-dependent respiratory response, which can be abolished by the intracellular Ca\(^{2+}\) chelator BAPTA-AM (Fig. 3A). Examining the role of extra- and intracellular Ca\(^{2+}\) in respiratory response to K\(^{+}\), we concluded that only a minor component of it is attributable to Ca\(^{2+}\). First, chelation of Ca\(^{2+}\) by EGTA only partly reduces the characteristic transient increase in respiration (Fig. 3B). This partial inhibition is also evident for the blockade of N, T, and P/Q types VGCC with CmC, whereas activation of L type VGCC with Bay K 8644 or inhibition with nifedipine do not influence the response. Second, depletion of ryanodine receptor-activated stores by CmC (Fig. 3C) and ryanodine reduces the respiratory response of \(\text{dPC12}\) by 50–70%, with ATP remaining at the levels seen in intact cells (Figs. 3C and 5D). Third, respiratory response to K\(^{+}\) is partly inhibited by blocking Ca\(^{2+}\)-ATPases with thapsigargin, which refills Ca\(^{2+}\) stores (Fig. 3C). Because the ER and mitochondria tightly co-localize and communicate (55), the ER is thought to represent the main source of Ca\(^{2+}\) involved in the regulation of O\(_2\) consumption. Surprisingly, ryanodine receptor agonists do not increase respiration of \(\text{dPC12}\) cells (Fig. 3C), and considerable elevation of Ca\(^{2+}\) by 500 nm CmC was seen to inhibit respiration and ATP production (Fig. 5D). This suggests that CmC and ryanodine are not able to provide the high rates of Ca\(^{2+}\) release required to generate local Ca\(^{2+}\) gradients sufficient for the activation of OxPhos in mitochondria. We consider that fast release of Ca\(^{2+}\) from ER to the vicinity of mitochondria together with efficient uptake through the Ca\(^{2+}\) uniporter can produce such characteristic respiratory response to sustained plasma membrane depolarization, without fatally damaging the cell. Conversely, rapid nonspecific influx of Ca\(^{2+}\) by ionomycin causes an intense respiratory spike followed by a fast collapse of mitochondrial activity (Fig. 3, A and D). This does not happen in response to sucrose, a nonphysiological hypertonic treatment known to produce repeatable NT release in a Ca\(^{2+}\)-dependent manner (41). We found that sustained osmotic shock enhanced the respiration of \(\text{dPC12}\) cells in a two-phase mode: the first, which is regulated by Ca\(^{2+}\) and can be inhibited by BAPTA-AM, and the second, which starts 10 min after sucrose application and can be abolished by chelation of Ca\(^{2+}\) or Ca\(^{2+}\) (Fig. 4).

Analysis of cellular NAD(P)H levels revealed that a significant rise in reduced NAD(P) levels, providing the ETC with an increased electron supply (Fig. 5G), is one of the key drivers of the transient increase in respiration upon the addition of K\(^{+}\). On the other hand, the subsequent decrease in NAD(P)H coincides with the respiratory spike, pointing to the significant contribution of ATP turnover in this effect. Furthermore, markedly enhanced respiratory response to K\(^{+}\) upon inhibition of glycolytic ATP synthesis (Fig. 5F) cannot be explained by the increased NAD(P)H supply. Finally, inhibition of F,\(_{0}\)F\(_{1}\) ATP synthase by oligomycin also considerably reduces the effect of K\(^{+}\) (Fig. 2C). These experimental data suggest that both tricarboxylic acid cycle and OxPhos orchestrate the respiratory response to such a stressful event as sustained plasma membrane depolarization.

Along with Ca\(^{2+}\), K\(^{+}\) also regulates OxPhos in mitochondria. Mitochondrial K\(^{+}\) balance is governed by ATP-dependent and Ca\(^{2+}\)-dependent K\(^{+}\) channels (influx) and by K\(^{+}\)/H\(^{+}\) exchanger (efflux). Valinomycin enables transport of K\(^{+}\) outside the cell and into mitochondrial matrix, thus perturbing plasma membrane potential and uncoupling the ETC. We observed a sustained increase of O\(_2\) consumption in \(\text{dPC12}\) cells by valinomycin. The increase is dependent on K\(^{+}\), probably because valinomycin transports K\(^{+}\) into mitochondria at a higher rate when the K\(^{+}\) gradient across plasma membrane decreases. In this case, K\(^{+}\) in the extracellular space, cytoplasm, and mitochondria tend to reach K\(^{+}\) levels, and the higher the latter, the stronger the ETC uncoupling. However, uncoupling of the ETC by valinomycin has little effect on the response to K\(^{+}\), thus suggesting different mechanisms of action (Fig. 2D). This is supported by the observation that in the cells pretreated with FCCP, sustained membrane depolarization by K\(^{+}\) has a synergistic respiratory effect, which leads to a deep deoxygenation of cell cytoplasm. Several factors can contribute to this large decrease in O\(_2\). First, K\(^{+}\) increases NAD(P)H level, thus feeding the ETC leak with electrons more intensively (Fig. 5G). Second, FCCP was shown to reduce mitochondrial Ca\(^{2+}\) (22). Therefore subsequent K\(^{+}\)-induced influx of Ca\(^{2+}\) into mitochondria may result in a more profound decrease in \(\Delta \Psi_{m}\), generating a stronger respiratory response. Third, low efficiency of F,\(_{0}\)F\(_{1}\) ATP synthase during uncoupling does not allow the cells to regulate respiration by allosteric inhibition of cytochrome c oxidase with ATP. Indeed, in intact \(\text{dPC12}\) cells, a ~60% spike in ATP by K\(^{+}\) (maximum at 3 ± 1 min) resembles the shape but surpasses the speed of respiratory response (Fig. 5A). Elevated mitochondrial ATP can transiently inhibit OxPhos, binding directly to cytochrome c oxidase after the removal of excess Ca\(^{2+}\) from mitochondria by the Na\(^{+}\)/Ca\(^{2+}\) exchanger, which works more slowly than the Ca\(^{2+}\) uniporter (54). In FCCP-treated cells, this inhibitory mechanism may not work, because ATP remains at a relatively constant low level. The large increase in O\(_2\) consumption leads to a profound deoxygenation of cell cytoplasm without any increase in ATP. However, 12–15 min after exposure to high K\(^{+}\), the cells reduce respiration to another activated steady state. The nature of such ATP-independent inhibition of respiration is unclear, however, O\(_2\)-sensing K\(^{+}\) channels and mitochondrial K\(_{ATP}\) channels activated by hypoxia may be involved (56–58).

TMRM has been widely used to monitor changes in \(\Delta \Psi_{m}\) in models of injury (36, 37, 45, 46, 59). However, TMRM is also sensitive to alterations in \(\Delta \Psi_{p}\); therefore care has to be taken in the interpretation of TMRM fluorescent signals. Fig. 6 demonstrates a significant drop in TMRM fluorescence following K\(^{+}\) stimulation; however, the majority of this response can be accounted for the redistribution of TMRM following the extensive depolarization of \(\Delta \Psi_{p}\) (verified by the increase in DiSBAC2(3) fluorescence; Fig. 6C) with only minor changes at the \(\Psi_{m}\) level. The changes in TMRM fluorescence caused by the depolarization of \(\Delta \Psi_{p}\) with K\(^{+}\) make the minor shifts in \(\Delta \Psi_{m}\) caused by an increase in respiration (Fig. 6B) very difficult to resolve and interpret. However, the difficulties in defining absolute changes \(\Delta \Psi_{m}\) and \(\Delta \Psi_{p}\) can be partially resolved when utilizing mathematical models that interpret changes in TMRM fluorescence (36, 37). From this we could establish that a 30–40 mV depolarization of \(\Delta \Psi_{p}\) was coupled with a
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5–10-mV depolarization of $\Delta \Psi_m$ (Fig. 6D). Intracellular oxygen sensing probes may be an important addition to the various probes and tools developed for the characterization of cellular bioenergetics but may be subject to other potential artifacts or misinterpretations. As demonstrated in the present study, the most informative approach may represent the characterization of both oxygen consumption and $\Delta \Psi_m$ (when TMRM fluorescence is corrected for $\Delta \Psi_p$ contributions). Indeed the analysis of NAD(P)H levels provides further detail on the energetic status of the cell (Fig. 5G).

Our data indicate that during plasma membrane depolarization by $K^+$, OxyPhos is the main source of ATP elevation (Fig. 5, E and F). In cultured PC12 cells grown on galactose/pyruvate, where OxyPhos is the only source of ATP, $K^+$ induces $\sim$45% increase in ATP, along with a strong rise of respiration and deep deoxygenation of the cytosol. However, glycolysis also contributes significantly to the enhanced ATP production in PC12 cells upon the excitation and maintains ATP at a $\sim$60% level when OxyPhos is blocked by antimycin A (Fig. 5C).

The increase in cellular ATP by $\sim$60% provides a considerable resource to cover increased energy demand in excited cells. On the other hand, the observed elevation in ATP level in depolarized cells can be explained by reduced ATP consumption upon termination of NT release, because PC12 cells excited by repeatable membrane depolarization were shown to perform multiple NT exocytosis with gradually decreasing amplitude and frequency (24).

Because neurotransmission is a main neuronal function, its inhibition is expected to down-regulate cell respiration. According to our results, the increase in respiration by $K^+$ is not affected by treating the cells with TenNT despite a considerable synaptobrevin 2 cleavage (Fig. 5). Moreover, general excitability of the cells grows, presumably because of the inhibition of spontaneous neurotransmission. Spontaneous NT release, which occurs randomly in the cell population, activates individual cells, making them resistant to sustained population-wide stimulation with high, $K^+$. In contrast, TenNT (+) cells, which are exposed to lower levels of dopamine, become more excitable than intact cells (50, 51). This effect is rather nonspecific because TenNT (+) cells respond more actively to FCCP and ionomycin. We think that a fast increase of respiration occurs in excited PC12 cells irrespectively of the efficiency of NT exocytosis. In normal conditions respiration and NT release are tightly regulated, thus providing a balance between production and utilization of ATP required for neurotransmission.

Our results were obtained with confluent populations of PC12 cells, which represent a useful model of brain architecture, where neuronal cells are packed densely enough to generate local $O_2$ gradients upon excitation. Continuous or frequent excitation in the brain may lead to a profound tissue deoxygenation. A similar effect was observed in PC12 cells exposed to ETC uncoupling and sustained membrane depolarization, when $O_2$ content dropped down to 15% of air saturation. In turn, large and prolonged deoxygenation could be a major factor responsible for tissue damage. On the other hand, it is clear that respiratory responses of individual cells can be more diverse and complex than bulk responses of cell populations. These aspects are outside the scope of this study and are the subject of a separate investigation using an $O_2$ imaging method (29).

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