Bafilomycin A1 activates respiration of neuronal cells via uncoupling associated with flickering depolarization of mitochondria

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Abstract Bafilomycin A1 (Baf) induces an elevation of cytosolic Ca\(^2+\) and acidification in neuronal cells via inhibition of the V-ATPase. Also, Baf uncouples mitochondria in differentiated PC12 (dPC12), SH-SY5Y cells and cerebellar granule neurons, and markedly elevates their respiration. This respiratory response in dPC12 is accompanied by morphological changes in the mitochondria and decreases the mitochondrial pH, Ca\(^2+\) and \(\Delta\Psi_m\). The response to Baf is regulated by cytosolic Ca\(^2+\) fluxes from the endoplasmic reticulum. Inhibition of permeability transition pore opening increases the depolarizing effect of Baf on the \(\Delta\Psi_m\). Baf induces stochastic flickering of the \(\Delta\Psi_m\) with a period of 20 ± 10 s. Under conditions of suppressed ATP production by glycolysis, oxidative phosphorylation impaired by Baf does not provide cells with sufficient ATP levels. Cells treated with Baf become more susceptible to excitation with KCl. Such mitochondrial uncoupling may play a role in a number of (patho)physiological conditions induced by Baf.

Keywords Bafilomycin A1 • Mitochondrial uncoupling • Oxygen sensing • Neuronal cells • Calcium • Bioenergetics • Respiration • V-ATPase

Introduction

Since being described as an inhibitor of vacuolar H\(^+\)-ATPase (V-ATPase) [1], plecomacrolide bafilomycin A1 (Baf) has become a powerful pharmacological tool in cell physiology research. V-ATPases comprise a family of structurally conserved proteins with multiple functions, which pump protons against the concentration gradient into acidic organelles, and create conditions for protein degradation, receptor–ligand dissociation, and turnover of both neurotransmitters and Ca\(^{2+}\) [2, 3]. In neuronal cells one of their main functions is to provide the driving force for filling vesicles with neurotransmitters (NT) by the vacuolar H\(^+\)/NT antiporters. Recent study has demonstrated that the V-ATPase is also involved in synaptic vesicle swelling which precedes neurotransmission [4]. As neuronal activity requires frequent NT release and vesicle recharge, V-ATPase consumes a considerable proportion of ATP produced in neurons.

V-ATPase activity is regulated by actin cytoskeleton remodelling, the activity of Cl\(^-\) channels, PI3K signalling, glucose supply, and the assembly of its subunits [2]. Along with Baf, V-ATPases can be inhibited by concanamycin A (CMA) and other pharmacological compounds [5]. Baf [6] interacts with the V0 subunit c [7, 8] and impairs translocation of protons into acidic compartments. Such inhibition has severe implications and leads to lysosome dysfunction, neurotransmission failure, cytosol acidification, impairment of polarized Ca\(^2+\) signalling and elevation of cytosolic Ca\(^{2+}\) [2, 9–13]. The decrease in pH and...
increase in Ca\(^{2+}\) in the cytosol, in turn, can induce opening of the permeability transition pores (PTP) [14] and cell death. The anticancer effect of Baf is well known and is attributed mainly to the inhibition of autophagy [15] by preventing the fusion of autophagosomes with dysfunctional lysosomes [16, 17], consequently triggering apoptosis [15]. Other mechanisms of cancer inhibition by Baf have also been proposed. Thus, by stabilizing the HIF-1\(z\), Baf has been shown to induce the p21\(^{WAF1/CIP1}\)-mediated growth arrest in a number of cancer cell lines and to stimulate direct interaction of the V0 subunit \(c\) with HIF-1\(z\) [18–20]. Also, both Baf and CMA induce mitochondrial depolarization and apoptosis in leukaemic monocytes by activating NO production [21]. On the other hand, Baf at subnanomolar concentrations has been shown to inhibit chloroquine-induced caspase-3 activity and apoptosis of the noncancerous cerebellar granule neurons (CGN) [22].

So far, most of the effects of Baf have been attributed to its V-ATPase inhibitory function. Little attention has been paid to its uncoupling effect demonstrated on isolated rat liver mitochondria, which was attributed to its K\(^+\) ionophore activity [23]. This, however, may be linked to some of the effects of Baf observed in vitro and in vivo, since mitochondrial uncoupling is implicated in cell and organ-specific toxicity of many drugs [24].

Considering the multiple targets and signalling pathways described for Baf, we undertook a detailed investigation of its effects on the mitochondrial function and bioenergetic parameters of neuronal cells using differentiated neurosecretory PC12 cells (\(\alpha\)PC12) as a model. Derived from rat adrenal phaeochromocytoma, \(\alpha\)PC12 cells demonstrate gene expression profiles, NT release and other features typical of neuronal cells [25, 26], while both oxidative phosphorylation (OxPhos) and glycolysis serve as effective suppliers of cellular ATP [27, 28]. An intracellular oxygen (O\(_2\)) sensing technique, which allows real-time monitoring of O\(_2\) levels in the monolayer of cells, both at rest and upon stimulation [28, 29], was used to study the metabolic effects of Baf on PC12 cells. The results were correlated with the other key metabolic markers and parameters of cellular function. The effects found in \(\alpha\)PC12 cells were subsequently confirmed in differentiated neuroblastoma SH-SY5Y cells and CGN from rat brain.

Materials and methods

Materials

PC12 and SH-SY5Y cells were obtained from the European and American collections of cell cultures. The oxygen-sensitive probe MitoXpress and extracellular acidification probe pH-Xtra were from Luxcel Biosciences (Cork, Republic of Ireland). Bafilomycin A, CMA, nerve growth factor (NGF), collagen IV, 2',7'-bis(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), valinomycin, nigericin and other reagents were from Sigma-Aldrich. Endo-Porter was from Gene Tools, LLC (Philomath, OR). Lipofectamine 2000, OptiMEM I, Fluo-4 AM, bis-(1,3-diethylthio Barbicarb acid) trimethine oxonol (DiSBAC\(_2\)(3)), tetramethyl rhodamine methyl ester (TMRM), 5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), Hoechst 33342, 5-carboxy-2',7'-dichloro-dihydrofluorescein diacetate, diAM (carboxy-H\(_2\)DCFDA) and Image-iT LIVE reactive oxygen species (ROS) detection kit were from Invitrogen Life Technologies (Carlsbad, CA and Dun Laoghaire, Republic of Ireland). Camptothecin, 2,5-di-t-butyl-1,4-benzoquinone (BHQ) and 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzotiazepin-2(3H)-one (CGP37157) were from Tocris (Bristol, UK). Plasticware was from Sarstedt (Wexford, Republic of Ireland), MatTek (Ashland, MA) and Greiner Bio One (Frickenhausen, Germany). The peptide caloxin 1C2 (TAWSEVLDDLRRGGSK-amide) was from GenScript (Piscataway, NJ). The mitochondria-targeted version of GFP-tagged Ca\(^{2+}\) sensor Case12, \(\text{minCase12} [30–32]\) was from Evrogen JSC (Moscow, Russia). The GFP-based plasmid encoding the mitochondrial pH sensor mtAlpHi [33] was kindly provided by Prof. Tullio Pozzan (University of Padua, Italy). Antibodies were: polyclonal anti-LC3A/B from Cell Signaling Technology (Danvers, MA), rabbit monoclonal anti-Smac/DIABLO from Novus Biologicals (Littleton, CO), mouse monoclonal anti-tubulin from Sigma, goat polyclonal anti-rabbit and anti-mouse IRDye 800CW from LI-COR Biosciences (Lincoln, NE), donkey anti-rabbit Cy3-conjugated from Jackson ImmunoResearch Laboratory (West Grove, PA).

Cell culture

PC12 cells were cultured in RPMI 1640 medium supplemented with 2 mM l-glutamine, 10% horse serum (HS), 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin (P/S) in an atmosphere containing 5% CO\(_2\). For the O\(_2\) and ATP measurements, the cells were seeded at \(5 \times 10^4\) cells/well into 96-well plates (Sarstedt) coated with 0.01% collagen IV, and differentiated for 3–5 days in RPMI supplemented with 1% HS, P/S, 100 ng/ml NGF. For live cell imaging, PC12 cells were differentiated on glass bottom mini-dishes (MatTek) coated with a mixture of collagen IV (0.007%) and poly-\(\alpha\)-lysine (0.003%).

SH-SY5Y cells were cultured in DMEM supplemented with 10% FBS, 2 mM l-glutamine and P/S and differentiated, as described previously [34]. Briefly, the cells were seeded at \(6 \times 10^4\) cells/well in a 96-well plate coated with
collagen IV, and at $3 \times 10^4$ cells/well in collagen IV/poly-

-lysine-coated glass-bottom MatTek mini-dishes [30].

Then the medium was changed to DMEM supplemented

with 10% FBS and 10 μM retinoic acid, and the cells were

further incubated for 5 days. After that, the medium was

changed to DMEM containing 50 ng/mL brain-derived

neurotropic factor (BDNF), and differentiation was con-

tinued for 7 days before the experiments.

CGN from 7-day-old rats were prepared, as described

previously [35], seeded at $2 \times 10^5$ cells/well and main-

tained in culture in the presence of 10 μM cytosine

arabinoside for 7 days prior to use.

Preparation of the cells and loading with probes

For the O$_2$ assay, dPC12 cells and CGN prepared in

96-well plates were loaded by incubating them for 28 h in

regular medium containing 1.2 μM MitoXpress probe and

6 μM Endo-Porter transfection reagent, as described pre-

viously [28]. dSH-SY5Y cells, which are poorly loaded

with the MitoXpress probe, were loaded with the cell-

penetrating probe PtCPTE-CFR9 [36] by incubating them

with 10 μM probe in DMEM/BDNF for 16 h. After load-
ing, the cells were washed with fresh medium three times

and used for O$_2$ measurements.

dPC12 and dSH-SY5Y cells were transfected with

mitoCase12 and mtAlpHi plasmids using lipofectamine and

OptiMEM I, as per the manufacturer’s procedure. The cells

were loaded with the following fluorescent indicators in

OptiMEM I: 20 nM TMRM (mitochondrial membrane

potential, ΔΨm), 2.5 μM Fluo-4 AM (cytosolic Ca$^{2+}$),

2.5 μM JC-1 (ΔΨm), 1 μM DiSBAC$_2$(3) (plasma mem-

brane potential, ΔΨp) and 2.5 μM BCECF AM (cytosolic

pH) for 30 min, 1 μM Hoechst 33342 (nuclear counter-

stain) for 5 min, and 10 μM carboxy-H$_2$DCFDA (ROS

probe) for 45 min. All AM probes were de-esterified for

15 min prior to each experiment. During the measure-

ments, TMRM and DiSBAC$_2$(3) probes were maintained in

solution at 20 nM and 0.25 μM, respectively. In the major-

ity of the experiments the probes were used individu-

ally. Probe combinations were used only when their

spectral, spatial and functional characteristics allowed

simultaneous loading and measurement. Each spectral

signal was acquired in sequential laser mode with emission
gates adjusted to avoid overlaps.

Experimental conditions

The experiments with dPC12 cells were carried out in the

presence of NGF in serum-free DMEM buffered with

25 mM HEPES, pH 7.4 (O$_2$ assays) and in OPTIMEM I

with additional Ca$^{2+}$ at 1 mM (imaging experiments). The

experiments with dSH-SY5Y cells were performed in

DMEM buffered with 25 mM HEPES, pH 7.4, and sup-

plemented with BDNF. For both cell lines, most of the

experiments were carried out in medium supplemented

with 10 mM glucose [glucose(+)] condition. To inhibit

the glycolytic pathway of ATP generation [37], the cells were

incubated in serum and glucose-free medium, containing

10 mM galactose and 1 mM pyruvate [galactose(+)] con-

dition] for 3 h prior to the measurements. The measure-

ments on CGN were carried out in buffer con-

taining 120 mM NaCl, 3.5 mM KCl, 0.4 mM KH$_2$PO$_4$

20 mM HEPES, 5 mM NaHCO$_3$, 1.2 mM Na$_2$SO$_4$

1.2 mM CaCl$_2$, 1.2 mM MgCl$_2$, and 15 mM glucose, pH

7.4, as described previously [38]. Conditions of treatment

with various effectors (dose, timing and media) are described in the “Results”.

Intracellular O$_2$ sensing assay

Typically, a standard 96-well plate containing 24 samples of
dPC12 and dSH-SY5Y cells preloaded with the O$_2$ probe

in 90 μl of air-saturated medium was measured on a time-

resolved fluorescence (TR-F) plate reader (Victor 2;

PerkinElmer Life Science) at 37°C using 340 nm excita-

tion and 642 nm emission filters. Each sample well was

measured repeatedly every 1.3–6 min by taking two

intensity readings at delay times of 30 and 70 μs and a gate
time of 100 μs [29]. The plate was initially monitored for

10–20 min to allow O$_2$ and temperature equilibrium to be

reached and to obtain baseline signals. The plate was then

quickly withdrawn from the reader, compounds were added
to the cells (10 μl of 10× stock solution) and monitoring

was resumed. Measured TR-F intensity signals for each

sample well were converted into phosphorescence life-time

(τ) values and plotted as time profiles. These profiles

were related to O$_2$ concentration (μM) according to the

following equation: [O$_2$] = −0.0027τ$^3$ + 0.5649τ$^2$ −

40.104τ + 972.23. This equation was obtained from the

calibration of the O$_2$ probe [39], performed in a hypoxia

chamber (Coy Scientific) equilibrated at given O$_2$ satu-

ration levels (0–20.9% of air saturation). Normoxic 20.91%

O$_2$ in the air roughly corresponded to 200

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O$_2$ in the air roughly corresponded to 200 μM O$_2$ in

solution at +37°C [40]. Control samples were incorporated

as appropriate.

Measurement of ΔΨ$_m$, ΔΨ$_p$, intracellular Ca$^{2+}$, pH,

ATP and ROS

Intracellular pH, cytosolic and mitochondrial Ca$^{2+}$, ΔΨ$_m$

ΔΨ$_p$ and ROS production were measured using an Olympus

FV1000 confocal laser scanning microscope under con-

trolled conditions of CO$_2$, humidity and temperature. The

BCECF, Fluo-4, carboxy-H$_2$DCFDA (Image-iT LIVE ROS

detection kit), minoCase12 and mtAlpHi probes were excited
at 488 nm (0.5, 5, 15, 1.5 and 10% of laser power, respectively) with emission collected at 500–550 nm. TMRRM and DiSBAC$_2$(3) were excited at 543 nm (1.5 and 1% laser power) collecting the emission with a 555–600 nm filter. JC-1 probe was excited at 488 and 543 nm (1 and 5% laser power) with emission collected at 500–540 and 555–600 nm. Hoechst 33342 was excited at 405 nm (3% laser power) with emission collected at 440–480 nm. In all the experiments fluorescent and differential interference contrast (DIC) images were acquired with a 60× oil immersion objective in two planes using a 0.5 μM step and 5–120 s intervals. The resulting z-stacked images were analysed using a FV1000 viewer (Olympus), Adobe Photoshop and Illustrator software. Mitochondria within neurites (rather than cell bodies) were used in the analyses of the TMRRM fluctuations, because in this case movements of the mitochondria are constrained and they are readily observable. Cellular ATP was measured using a CellTiter-Glo assay kit (Promega, Madison) on a white 96-well plate (Greiner Bio One). ROS levels were measured using a carboxy-H$_2$DCFDA probe and fluorescence activated cell sorter (FACS) analysis (Becton–Dickinson, NJ).

Extracellular acidification assay

The rate of extracellular acidification was monitored as described previously [41]. PC12 cells were differentiated for 4–5 days under standard CO$_2$ conditions, then the medium was replaced with unbuffered DMEM, supplemented with 1 mM sodium pyruvate and either 10 mM glucose or 10 mM galactose, pH 7.4 (pH buffer). Cells were maintained in a CO$_2$-free incubator at 37°C for 3 h prior to measurement and then the medium was replaced with 100 μl of fresh medium containing 1 μM of pH-Xtra probe and the stimulants. The plate was then measured kinetically on the Victor 2 plate reader at 37°C for a minimum of 30 min in the TR-F mode using a europium filter set (340 ± 35 nm excitation and 615 ± 8.5 nm emission). Two TR-F intensity signals were measured at delay times of 100 and 300 μs and a measurement window of 30 μs. The readings were converted into life-time values $	au = (t_1 – t_2)/ln(F_1/F_2)$, where $	au$ is the probe fluorescence life-time, $t_1$, and $t_2$ are the two delay times, and $F_1$ and $F_2$ are the corresponding TR-F intensity signals; $\tau$ was converted into pH and H$^+$ values [41].

Detection of autophagic flux and apoptosis

The level of autophagy was assessed by LC3 degradation using Western blot analysis [42]. Briefly, dPC12 cells were incubated under normal or starving (HBSS supplemented with 100 ng/ml NGF) conditions for 2 h and then treated with 0.25 μM Baf or CMA under starving conditions for 4 h. Whole-cell lysate proteins were separated with gradient gel electrophoresis, transferred onto a PVDF membrane and probed with anti-LC3A/B and IRDye 800CW antibodies. Immunoblotting results were analysed using the Odyssey infrared imaging system (LI-COR Biosciences).

The level of apoptosis was measured by Smac/DIABLO translocation (immunofluorescence), and caspase-3 activation (fluorescent plate reader). Immunofluorescence analysis was performed as described previously [43]. Briefly, cells treated for 2–4 h with Baf, CMA or 5 μM camptothecin were fixed with 3.7% PFA, permeabilized with 0.25% TX100, incubated with anti-Smac and stained with Cy3-conjugated secondary antibodies. Results were analysed by confocal microscopy.

Caspase-3 activation was determined using a kit from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer’s protocol. Briefly, dPC12 cells were incubated with drugs as described in the “Results”, washed in assay buffer and lysed. After addition of the enzyme substrate, caspase-3 activity was measured in a 96-well plate using the Victor 2 reader at 485 nm/535 nm (fluorescence excitation/emission).

Statistics

The data were evaluated for the significance of differences using the two-tailed Student t-test. The 0.01 level of confidence was accepted as statistically significant. Plate reader data are presented as average values ± standard deviation for six to eight repeated wells (error bars on the plots). Imaging data are presented as the averages from five to ten individual cells. All the experiments were repeated three to five times to ensure consistency of the results.

Results

Bafilomycin A1 activates the respiration and induces sustained deoxygenation of neural cells

Although uncoupling of isolated mitochondria by Baf has been described [23], its effects on cell respiration have not been investigated. Using the intracellular O$_2$ sensing technique [29], we found that exposure of dPC12 cells to 0.25 μM Baf gradually decreased the oxygenation of the cell monolayer from 140–160 μM down to 80±5 μM O$_2$ (Fig. 1a) due to increased respiration. Maximal respiration was achieved in 50–100 min, and then it gradually decreased but still remained elevated after >5 h. The effect was dose-dependent, becoming significant at 50 nM and reaching a maximum at 0.5–0.8 μM Baf after 45 min of treatment (Fig. 1b). In the presence of mitochondrial complex III inhibitor antimycin A the effect was abolished.
If Baf was subsequently removed from the medium, respiration returned to the basal level. When the cells were treated with 0.25 \( \mu \)M CMA (another V-ATPase inhibitor, which does not uncouple the mitochondria), no changes in cell oxygenation and respiration were seen (Fig. 1c), while the dissipation of acidic lysosomal compartments was similar to that seen with Baf (Fig. 1d, shown for Baf). This indicates that the respiratory response to Baf is not a consequence of V-ATPase inhibition. To confirm this, we inhibited V-ATPase via the dissociation of V0 and V1 domains prior to Baf treatment by replacing glucose in the medium with galactose [44]. Galactose also dramatically reduces the glycolytic ATP production and increases OxPhos [20], thus causing a significant decrease in cellular O2. We found that in the cells preconditioned for 3–4 h on galactose, Baf induced even higher deoxygenation (Fig. 1c) signifying that the activation of O2 consumption is not related to V-ATPase integrity and activity.

Uncoupling by the classical uncoupler FCCP or the K+ ionophore valinomycin is normally associated with complete dissipation of the mitochondrial membrane potential (\( \Delta \Psi \)m). However, treatment of dPC12 cells with 0.25 \( \mu \)M Baf for 30–60 min decreased the TMRM signal only by 40±10% (Fig. 1e). Under these conditions a large proportion of the mitochondria underwent substantial transformation from an elongated (5±3 to 0.2±0.1 \( \mu \)m) to a more round (3±2 to 0.4±0.2 \( \mu \)m) shape. The decrease in TMRM signal reflected only changes in \( \Delta \Psi \)m polarization, since plasma membrane potential (\( \Delta \Psi \)p) assessed using the DiSBAC2(3) probe remained unaffected (not shown). A substantial drop in JC-1 probe fluorescence confirmed significant depolarization of \( \Delta \Psi \)m (Supplementary Fig. S1).

Dissipation of lysosomes by Baf and CMA was coupled with significant inhibition of autophagic flux (seen as an increase in cellular LC3A/B II levels, Fig. 1f), and an increase in ROS production (Fig. 2a, b; Supplementary Fig. S2). Both inhibition of autophagy and elevation of ROS are known to activate apoptosis. We found that caspase-3 activity (marker of apoptosis) was increased by 150–200% and about 100% after 4 h of treatment with Baf.
and CMA, respectively (Fig. 2c). Under the same conditions, only minor changes in Smac/DIABLO location were observed (Supplementary Fig. S3).

The respiratory responses of dSH-SY5Y and primary CGN to Baf treatment were similar to those observed for dPC12 (Supplementary Fig. S4). We therefore concluded that Baf induces mitochondrial uncoupling and increases respiration in neuronal cells which may lead to a sustained reduction in cellular O\(_2\) levels and induction of apoptosis.

Bafilomycin A1 perturbs energy balance in neural cells

To maintain their energy status, uncoupled cells normally increase ATP production through glycolysis, thus elevating lactate production and increasing the rates of cytosolic and extracellular acidification. Inhibition of the V-ATPase activity can also contribute to both intra- and extracellular acidification due to impaired proton transport. In dPC12 cells treated with Baf for >30 min distinct acidification of the cytosol was observed, detected with the pH probe BCECF (Fig. 3a), with the distribution of BCECF showing a punctate pattern. Cells treated with CMA revealed similar changes in the intensity and distribution of BCECF. We therefore concluded that cytosol acidification by Baf is mainly due to the inhibition of V-ATPase activity.

Using the fluorescent extracellular acidification probe and assay [41], we found that Baf significantly increased the rate of extracellular acidification, similar to valinomycin and FCCP (Fig. 3b), whereas CMA caused no such effect. These results indicate that the activation of glycolysis in dPC12 cells is due to uncoupling rather than V-ATPase inhibition by Baf. Further analysis of bioenergetic parameters showed that in dPC12 cells grown on galactose, ATP levels were reduced by Baf in a time- and concentration-dependent manner, while under glucose(+) conditions they remained unchanged (Fig. 3c, d). In contrast, CMA did not influence ATP levels even in the absence of glycolytic ATP supply (not shown). A similar decrease in ATP upon Baf treatment in galactose(+)/

medium was seen in dSH-SY5Y cells (Supplementary Fig. S5). Interestingly, an additional decrease in ATP of about 20% was observed in Baf(+) dPC12 cells upon inhibition of F0F1 ATP synthase (mitochondrial complex V) with 10 \(\mu\)M oligomycin (Fig. 3d). This suggests that although Baf uncoupled \(\text{O}_2\) consumption from ATP production, the cells were still able to produce ATP through OxPhos, at least at <0.25 \(\mu\)M Baf.

To confirm this, we analysed the changes in respiration, \(\Delta\text{pHm}\) and \(\Delta\Psi_m\), induced in Baf(+) dPC12 cells by oligomycin. Normally, inhibition of ATP synthase (the main consumer of the H\(^+\) gradient across the mitochondrial membrane) reduces \(\text{O}_2\) consumption and leads to a rapid and sustained increase in \(\Delta\text{pHm}\) [45]. We observed a 10–20% decrease in the respiratory response to Baf in the cells pretreated with oligomycin (Fig. 4a). Using the mitochondrial pH probe mtAlpHi [33], we found that oligomycin transiently elevated mitochondrial pH in Baf(+) cells (Fig. 4b). Being significantly smaller than in the control, this increase suggests that complexes I–IV of the electron transport chain (ETC) generate notable H\(^+\) gradients it the mitochondria uncoupled by Baf. This result also shows that in the presence of Baf, ATP synthase still works in direct mode utilising H\(^+\) gradients for ATP production. On the other hand, after oligomycin addition, the intensity of TMRM in Baf(+) cells gradually decreased (Fig. 4c), indicating that the complex V also participates in the maintenance of \(\Delta\Psi_m\) acting as an ATPase.

Finally, we found that upon addition of FCCP to Baf(+) cells, the relative drop in mtAlpHi fluorescence was similar to that of the control (Fig. 4d), thus confirming that a large proportion of the \(\Delta\text{pH}\) is retained by the mitochondria. The relative decrease in TMRM signal induced by FCCP in...
Baf(+) cells was significantly smaller than in the control, confirming partial depolarization of ΔΨm in Baf(+) cells (see Fig. 1e).

Taken together, these data indicate that mitochondrial uncoupling induced by Baf is associated with large perturbations in cellular metabolism and energy balance.

Bafilomycin A1 induces flickering depolarization of the mitochondria in dPC12 cells

The marked decrease in cellular TMRM signal upon Baf treatment (Fig. 1e) can be interpreted as partial depolarization of the whole mitochondrial pool of the cell. However, when analysing confocal images for spatial distribution of TMRM (ΔΨm) and mitoCase12 (mitochondrial Ca²⁺) within individual cells, we found a significant (25 ± 10%) decrease in their colocalization 45 min after Baf addition (Fig. 5a). Since the mitoCase12 probe has no tendency to leak (being a mitochondria-targeted protein), this result pointed to a decrease in the proportion of polarized mitochondria, rather than a more general partial depolarization. Indeed, further analysis of the TMRM signal in individual mitochondria in dPC12 cell neurites visualized by mitoCase12, revealed random, flickering depolarization, which can be seen 10 min after Baf application and lasts for several hours (Fig. 5b, c; Supplementary movie). In galactose(+) medium the flickering developed even more rapidly (within 5 min in individual cells). Although such flickering had rather high interexperiment variability complicating its detailed statistical analysis, it was clearly visible after 45-min incubation with Baf in about 15–55% of cells, and progressed to about 100% of the cell population in 1–3 h. Detailed time-lapse image analysis of cells after 1 h of Baf treatment (images taken every 0.5 s) revealed that ΔΨm flickering between the polarized and depolarized states occurred with an average period of 20 ± 10 s (Supplementary Fig. S6). Marked fluctuations in TMRM fluorescence (90 ± 5%) showed that most individual mitochondria underwent full depolarization and restoration of the ΔΨm. In control cells, transient fluctuations in TMRM signal without prominent flickering were observed in <3% of cells. Confocal images recorded at different sampling frequencies (every 5–20 s) showed that ΔΨm fluctuations were independent of illumination of the
We also observed fluctuations in mitoCase12 fluorescence of around 10–25% (Fig. 5d, e), mostly in-phase with the changes in $\Delta \Psi_m$. This synchronism was to be expected, since mitochondrial Ca$^{2+}$ uptake can depolarize $\Delta \Psi_m$ [47, 48]. In turn, $\Delta \Psi_m$ depolarization inhibits Ca$^{2+}$ influx through the electrogenic mitochondrial Ca$^{2+}$ uniporter [49] and shifts the balance of the mitochondrial Ca$^{2+}$ fluxes towards Ca$^{2+}$ extrusion. Since the endoplasmic reticulum (ER) is considered to be the main contributor to mitochondrial Ca$^{2+}$ fluxes [50, 51], we studied the role of this compartment in the Baf-specific flickering. Figure 5f shows that inhibition of sarco/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) with thapsigargin noticeably changed the morphology of mitochondria in $\alpha$PC12 cells treated with Baf. After 1 h of thapsigargin/30 min of Baf treatment, the mitochondria were transformed into large round structures (1.5–3.5 μm in diameter), which could be seen on the DIC images. TMRM staining remained intense (Fig. 5f, g) and became more diffuse, whereas the amplitude and frequency of flickering decreased. When Baf was applied to cells pretreated with thapsigargin for 30 min, this effect started to develop within 7–10 min (Supplementary Fig. S7). Thapsigargin alone did not affect the morphology and polarization of the mitochondria. These results demonstrate the importance of the ER in the development of stochastic $\Delta \Psi_m$ fluctuations and in the maintenance of mitochondrial function.

One of the mechanisms proposed for Ca$^{2+}$-mediated fluctuations in $\Delta \Psi_m$ is transient opening of the PTP [52]. Similarly, PTP opening followed by $\Delta \Psi_m$ depolarization has been described for mitochondria overloaded with K$^+$ [53]. To investigate the contribution of PTP to the $\Delta \Psi_m$...
flickering, we applied Baf to dPC12 cells pretreated with cyclosporine A (CsA, 2 μM), which binds cyclophilin D and prevents the formation of PTP. CsA itself did not affect the polarization of mitochondria, but dramatically increased the effect of Baf on the $\Delta$$\Psi$m (Fig. 5f, g). Monitored after 1 h of the double treatment, the majority of cells lost TMRM staining, and only a minor population of the mitochondria revealed the characteristic flickering of $\Delta$$\Psi$m with low TMRM intensity. When Baf was applied to cells pretreated with CsA for 30 min, it triggered depolarization and flickering of the $\Delta$$\Psi$m within minutes, whereas after 15 min TMRM staining decreased by 70 ± 20% (Fig. 5g). The effect of CsA on $\Delta$$\Psi$m depolarization by Baf was dose-dependent and barely visible at CsA concentrations <0.75 μM. Finally, CsA did not inhibit activation of caspase-3 by Baf (data not shown).

In contrast, thapsigargin (10 μM, Thaps) does not change the effect of Baf on the TMRM signal. However, the morphology of the mitochondria changes dramatically: they become large round compartments with an average diameter of 1–2 μm. g 15 min after the addition of Baf to the cells preincubated with CsA, TMRM intensity decreases to 15–50%, which is significantly different to the cells treated with Baf alone. Inhibition of F0F1 ATPase with oligomycin (10 μM, Olig) has a similar effect. Antimycin A completely depolarizes the mitochondria. Thapsigargin strongly increases variability of the response to Baf. All preincubations were done for 30 min. DMSO was used as a negative control. Bar 20 μm; asterisks significant differences.

Fig. 5 Baf induces flickering depolarization of the $\Delta$$\Psi$m in dPC12 cells. a Colocalization of the TMRM and mitoCase12 probes in cells treated with Baf for 45 min decreases from 90–95% to 50–80%. b, c Large fluctuations of TMRM signals in individual mitochondria located in cell neurites and costained with mitoCase12 probe (b inset) reveal a flickering pattern of $\Delta$$\Psi$m depolarization. The dashed line (c) shows a cross-section of representative mitochondria selected for analysis. d, e In individual mitochondria localized in neurites (d arrows), TMRM intensity periodically drops by 90 ± 5% and then returns to the basal level, while the mitoCase12 fluorescence shows 15–25% fluctuations. f Inhibition of PTP opening by CsA (2 μM) strongly increases the effect of Baf on $\Delta$$\Psi$m depolarization.

Since $\Delta$$\Psi$m and F0F1 ATP synthase activity are reciprocally regulated, the latter can modulate $\Delta$$\Psi$m flickering. We inhibited ATP synthase with oligomycin and observed a fast progressive decrease in TMRM fluorescence after Baf addition, with only minor reductions in flickering. When the mitochondrial complex III was inhibited by antimycin A, mitochondria lost $\Delta$$\Psi$m within 5–7 min after Baf addition without any flickering (not shown).

Overall, we concluded that the characteristic random $\Delta$$\Psi$m flickering of individual mitochondria in PC12 cells is associated with Baf uncoupling.
The interplay between bafilomycin A1 and Ca$^{2+}$
in the regulation of respiration

In $\mu$PC12 cells treated with 0.25 μM Baf for 30 min or longer, we observed a dramatic elevation of cytosolic Ca$^{2+}$, seen as an increase of 140 ± 40% in Fluo-4 fluorescence (Fig. 6a). In approximately 30% of the cells, Fluo-4 fluorescence was distributed diffusely throughout the cytoplasm, including neurites. More than 50% of the cells had bright punctate Fluo-4 staining with poorly identifiable localization. Over time, the distribution of cytosolic Ca$^{2+}$ became more uneven and punctate. A similar effect was observed in $\mu$SH-SY5Y and CGN cells (not shown).

Since the $\Delta\Psi_{m}$ remained relatively unchanged upon Baf treatment, Ca$^{2+}$ influx through voltage-gated calcium channels was ruled out. Therefore, the increase in cytosolic Ca$^{2+}$ was provided by intracellular stores. The two candidate organelles affected by Baf directly are the acidic compartments and the mitochondria. Indeed, the impairment of V-ATPase activity dissipates the H$^{+}$ gradients across the membrane and abolishes the deposition of Ca$^{2+}$ into acidic stores. On the other hand, it is known that partial depolarization of $\Delta\Psi_{m}$ and decrease in matrix pH can inhibit Ca$^{2+}$ influx into the mitochondria and activate Ca$^{2+}$ release through the mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger (mNCX) [49, 54]. In assessing this, we found that the pool of acidic compartments is the main contributor to the elevation of cytosolic Ca$^{2+}$, because the inhibition of V-ATPase with CMA caused a similar increase in Fluo-4 fluorescence (not shown).

To estimate the contribution of mitochondria to sustained elevation of cytosolic Ca$^{2+}$ and the long-term effect on mitochondrial Ca$^{2+}$ levels, we preincubated $\mu$PC12 cells with Baf for 1–2 h and then treated with FCCP (0.5 μM), which is known to induce a drop in the mitochondrial Ca$^{2+}$ [55]. A large and rapid decrease in mitoCase12 fluorescence was observed irrespective of Baf pretreatment (Fig. 6b), suggesting that mitochondria retained a large proportion of Ca$^{2+}$ after the prolonged exposure of cells to Baf.

High cytosolic Ca$^{2+}$ increases Ca$^{2+}$ fluxes and inter-compartmental Ca$^{2+}$ exchange, thus regulating cell metabolism and particularly OxPhos [56]. Considering the persistent fluctuations in mitochondrial Ca$^{2+}$ (Fig. 5d, e), we examined the role of Ca$^{2+}$ fluxes in the respiratory response to Baf. The increase in respiration was completely abolished by blocking cytosolic Ca$^{2+}$ fluctuations with the intracellular chelator BAPTA (Fig. 6c) or by inhibiting the SERCA with thapsigargin (10–15 μM). A similar effect was observed when the SERCA was inhibited by BHQ (50 μM). The effect of Baf was partially decreased by CGP37157 (inhibitor of the mNCX, 10–15 μM). In contrast, neither the removal of extracellular Ca$^{2+}$ with EGTA nor the inhibition of plasma membrane Ca$^{2+}$-ATPase with 100 μM caloxin 1C2 [57] affected the respiratory response to Baf. This reveals that respiration is increased by a mechanism independent of Ca$^{2+}$ fluxes across the plasma membrane.

Taken together, the respiratory response to Baf, although attributed to mitochondrial uncoupling, is strongly regulated by the cytosolic Ca$^{2+}$ fluxes and the Ca$^{2+}$ buffering capacity of cellular compartments, particularly the ER which is the main partner for Ca$^{2+}$ exchange with mitochondria.

Bafilomycin A1 increases cell susceptibility to excitatory stimulation

Mitochondrial uncoupling increases the risk of energy crisis upon cell excitation, while for the cells lacking acidic

![Fig. 6](image-url) The interplay between Baf and Ca$^{2+}$ in $\mu$PC12 cell. a Cytosolic Ca$^{2+}$ levels increase dramatically after 45–60 min of treatment with 0.25 μM Baf, as detected with the Fluo-4 probe. b Uncoupling with 0.5 μM FCCP causes a dramatic drop in mitoCase12 fluorescence in Baf(+) cells, indicating a deep decrease in mitochondrial Ca$^{2+}$, similar to that seen in the control. c The effect of Baf on cell respiration is abolished by increasing the Ca$^{2+}$ buffering capacity of the cytosol with 25 μM BAPTA-AM or by inhibiting the SERCA with 10 μM thapsigargin. The respiratory response is not changed under extracellular Ca$^{2+}$-free conditions, or upon inhibition of plasma membrane Ca$^{2+}$-ATPase with caloxin 1C2 (100 μM). It is not significantly downregulated by the inhibition of mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger with CGP37157 (10 μM). DMSO was used as a negative control. Bars 20 μm; asterisks significant differences.
segments and overloaded with Ca\(^{2+}\) the risk increases further. We investigated how Baf affects respiratory responses to excitatory stimuli.

Depolarization of the ΔΨp by high K\(^+\) triggers a rapid transient decrease in the O\(_2\) level and substantial elevation of ATP level in vPC12 cells, which reaches a maximum in 2–5 min after K\(^+\) addition [28]. Preincubation with Baf significantly increased the respiratory response to K\(^+\) (Fig. 7a, b). This increase was further augmented by thapsigargin. Under extracellular Ca\(^{2+}\)-free conditions the response of Baf-treated cells to K\(^+\) was abolished (Fig. 7b).

While measuring the changes in ATP levels induced by high K\(^+\), we found that in Baf-treated cells grown on glucose the characteristic increase in ATP was partially inhibited (Fig. 7c), whereas under galactose(+) conditions the addition of K\(^+\) caused a significant drop in cellular ATP. Pretreatment with CMA did not change the respiratory response and slightly reduced the ATP response to K\(^+\). For comparison, valinomycin (0.25 μM) did not affect the ATP response to K\(^+\) in glucose(+) medium, while under galactose(+) conditions it dramatically reduced ATP irrespective of K\(^+\) addition.

Glutamate was shown to induce cytotoxicity in PC12 cells [58]. We applied 5, 10 and 20 mM glutamate to Baf(+) and Baf(−) vPC12 cells and found that Baf significantly increased the respiratory response to glutamate (Fig. 7d). Moreover, Baf markedly reduced the viability of the cells, treated with glutamate (Fig. 7e); determined by measuring total cellular ATP levels 20 h after glutamate application, cell viability decreased with the elevation of glutamate. These effects were not observed in the cells treated with CMA.

These results demonstrate that susceptibility of Baf(+) vPC12 cells to excitatory stimulation increases significantly, while their energy demand upon excitation is not met by the ATP production.

Fig. 7 Baf increases susceptibility of vPC12 to excitatory stimulation. a Pretreatment with 0.25 μM Baf dramatically increases the respiratory response to ΔΨp depolarization by high K\(^+\). The response is not reduced by the SERCA inhibitor thapsigargin. The dashed line shows the level of deoxygenation induced by K\(^+\) in the Baf(−) cells treated with thapsigargin. b In Ca\(^{2+}\)-free medium Baf(+) cells do not respond to high K\(^+\) and rapidly lose their respiratory activity. c Pretreatment with Baf (25 μM, 30 min) in glucose(+) medium reduces elevation of ATP in response to ΔΨp depolarization by high K\(^+\), whereas in galactose(+) medium K\(^+\) stimulation strongly decreases cellular ATP. The effect of CMA (0.25 μM) on the ATP response to K\(^+\) is less pronounced. Valinomycin (Val, 0.25 μM) in galactose(+) conditions reduces the ATP by >90%, irrespective of K\(^+\) treatment. d Pretreatment of the cells with 25 μM Baf for 30 min amplifies the dose-dependent response to glutamate (5–20 mM), while CMA does not affect the response to glutamate. e Relative decrease in cellular ATP (measured 20 h after glutamate treatment) reveals a negative effect of Baf on the viability of vPC12 cells, exposed to glutamate. DMSO was used as a negative control. Asterisks significant differences
Discussion

Known for two decades as an effective inhibitor of V-ATPases [1], Baf has been recently recognized also as a K\(^+\) ionophore with uncoupling potency on isolated mitochondria [23]. The key finding of this work was the sustained activation of respiration of intact neural cells leading to decreased cellular O\(_2\). The respiratory response to Baf developed gradually over 50–100 min, and then persisted for many hours (Fig. 1). The response became detectable at \(\geq 50\) nM, which is in the range of Baf concentrations found in the literature (from subnanomolar to 10 \(\mu\)M [18, 22]). The ability of Baf(+) cells to sustain uncoupling and maintain elevated respiration for many hours without serious change in viability arises from resultant alterations in certain bioenergetic parameters. In particular, free cytosolic Ca\(^{2+}\) was strongly elevated as a consequence of V-ATPase inhibition. \(\Delta\Psi\)m was partially reduced, whereas mitochondrial Ca\(^{2+}\) and \(\Delta\rho\)Hm were moderately decreased, thus allowing ATP production by the OxPhos. However, in the absence of glycolytic ATP supply, the total energy level in Baf(+) cells gradually decreased (Fig. 3c) as a consequence of increased ATP expenditure and a transient reversal of F0F1 ATP synthase activity. The latter process correlated with large continuous fluctuations of the \(\Delta\Psi\)m in individual mitochondria upon Baf treatment (Fig. 5, Supplementary Fig. S6, Supplementary movie), which were not observed in the cells treated with the traditional uncoupler FCCP or the K\(^+\) ionophore valinomycin.

Orchestrated by Baf, gradual depolarization of the \(\Delta\Psi\)m developed due to progressive establishment of a new ion and energy state, which was not lethal to \(\mu\)PC12 cells (for at least 6 h). This process was accompanied by an increased proportion of mitochondria that exhibit flickering (i.e. transiently depolarized). Indeed, live imaging of the Baf-treated cells demonstrated that stochastically flickering TMRM signals were colocated with the mitochondria-targeted calcium indicator \(\text{minCase}_{12}\) only in 50–80% of mitochondria (Fig. 5a), in contrast to 90–95% in control cells. The degree of observed colocalization represents the probability of being in the polarized state for each individual mitochondrion at a given time point.

It is known that in response to \(\Delta\Psi\)m depolarization by FCCP, F0F1 ATP synthase reverts to an ATPase consuming ATP to restore \(\Delta\Psi\)m, especially when glycolytic ATP supply is inhibited [59, 60]. Our data suggest that in Baf-treated \(\mu\)PC12 cells F0F1 ATP synthase works in both direct and reverse mode, depending on the \(\Delta\Psi\)m of individual mitochondria and the whole mitochondrial network. ATP synthase activity was evident, since oligomycin partially inhibited the respiratory response to Baf and induced transient ETC-mediated alkalization of the mitochondrial matrix (Fig. 4b). When the mitochondrion was depolarized by Baf, F0F1 ATP synthase switched to the reverse mode and consumed ATP for \(\Delta\Psi\)m restoration.

In Baf(+) \(\mu\)PC12 cells, the rate of glycolysis was increased to meet energy expenditure (Fig. 3), including the energy spent on \(\Delta\Psi\)m restoration by F0F1 ATP synthase. As a result, total cellular ATP levels remained practically unchanged and \(\Delta\Psi\)m decreased only partially. However, when complex V was inhibited by oligomycin and could not utilize ATP, the \(\Delta\Psi\)m decreased faster (Fig. 4c). If the glycolytic ATP supply was cut off, total cellular ATP decreased progressively. A further decrease in ATP upon oligomycin treatment in Baf(+) cells grown on galactose was somewhat surprising (Fig. 3d); it suggests that Baf-treated cells still produced ATP by OxPhos. The ability of the cells to maintain \(\Delta\Psi\)m flickering in galactose(+) medium (data not shown) supports this conclusion. Interestingly, inhibition of the ETC complex III, but not complex V, abolished the flickering and rapidly depolarized the mitochondria (Fig. 5g). This result suggests that the activity of F0F1 ATP synthase is mediated by \(\Delta\Psi\)m flickering, rather than drives it.

The phenomenon of flickering mitochondrial depolarization is known. \(\Delta\Psi\)m may be modulated by the photostimulation of TMRM described for the tetramethylrhodamine probes [61]. It has been shown that spontaneous depolarization of individual mitochondria isolated from heart tissue and the decrease in \(\Delta\Psi\)m in the population of mitochondria are caused by the repeated opening of the PTP due to light-induced generation of free radicals by TMRE probe [62]. Similar results have been demonstrated for intact HeLa cells loaded with TMRM [46]. In contrast, in cultured forebrain neurons the low-amplitude spontaneous fluctuations in \(\Delta\Psi\)m of individual mitochondria were detected with TMRM and JC-1 probes, and were not related to light exposure [63]. The authors concluded that such \(\Delta\Psi\)m oscillations reflect the alternation between active and inactive states of OxPhos. In our study, we did not observe significant light-induced oscillations or a gradual decrease in TMRM signal in intact cells upon light exposure.

PTP opening has been suggested to be the mechanism involved in transient depolarizations of \(\Delta\Psi\)m [52, 53]. We showed that inhibition of PTP opening by CsA strongly increased the depolarization of mitochondria by Baf (Fig. 5, Supplementary Fig. S7), and did not prevent or decrease \(\Delta\Psi\)m flickering. Instead, the oscillations begin sooner than with Baf alone. This indicates that the initiation of flickering does not require PTP opening and that PTP opening is involved in the maintenance of mitochondrial polarization and function under conditions of impaired K\(^+\), H\(^+\) and Ca\(^{2+}\) balance. It is possible that PTP opening is involved in the release of excessive osmotic pressure from the mitochondrial matrix, which is generated through K\(^+\) ionophore activity of Baf. Regulation of PTP
opening by K\textsuperscript{+} influx into the matrix is discussed in [53]. Therefore, PTP opening may serve to restore the shape and function of the mitochondria. When this process is inhibited by CsA, the ΔΨ\textsubscript{m} depolarizes more quickly. On the other hand, the fact that ΔΨ\textsubscript{m} flickering occurs in the presence of CsA, suggests that a different mechanism may also be involved in the stochastic depolarization and repolarization of mitochondria.

Ca\textsuperscript{2+} is an important regulator of mitochondrial function [64, 65]. To transport Ca\textsuperscript{2+} into the matrix, the mitochondrial uniporter requires polarized ΔΨ\textsubscript{m} [66] and a high concentration of Ca\textsuperscript{2+} in the vicinity of the mitochondria [67]. In Baf(+) cells both conditions are provided: the ΔΨ\textsubscript{m} is retained by a considerable proportion of mitochondria, while cytosolic Ca\textsuperscript{2+} is strongly and steadily elevated due to the dissipation of H\textsuperscript{+} gradients in the V-ATPase-regulated acidic Ca\textsuperscript{2+} stores. However, our results suggest that the effect of Ca\textsuperscript{2+} on the Baf-dependent increase in respiration is mediated by the ER, which is one of the main players in the Ca\textsuperscript{2+} turnover and a partner of the mitochondria in Ca\textsuperscript{2+} exchange [50, 51]. We hypothesize that high cytosolic Ca\textsuperscript{2+} could randomly activate Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+} release from the ER network surrounding the mitochondria. In turn, local increases in Ca\textsuperscript{2+} could activate the mitochondrial uniporter and trigger Ca\textsuperscript{2+} entry to the mitochondria. An elevation in Ca\textsuperscript{2+} transiently depolarizes ΔΨ\textsubscript{m} [63] and causes release of the mitochondrial Ca\textsuperscript{2+}, which is then again buffered by the ER. This is supported by the observation that thapsigargin and BHQ, which inhibit SERCA and prevent Ca\textsuperscript{2+} crosstalk between the ER and mitochondria, abolish the respiratory effect of Baf (Fig. 6). Moreover, when SERCA is inhibited, Baf induces dramatic changes in the mitochondrial morphology and the mode of ΔΨ\textsubscript{m} flickering (Fig. 5, Supplementary Fig. S7). A similar effect on the respiratory response to Baf can be achieved with the Ca\textsuperscript{2+} chelator BAPTA, which buffers Ca\textsuperscript{2+} fluxes in the cytosol and prevents intercompartmental Ca\textsuperscript{2+} exchange. In contrast, extracellular Ca\textsuperscript{2+} does not seem to be involved in the regulation of the respiratory response to Baf.

K\textsuperscript{+} balance is also important for the maintenance of ΔΨ\textsubscript{m}, mitochondrial integrity and function. It is controlled by the Ca\textsuperscript{2+}-dependent (K\textsubscript{Ca}) and ATP-dependent (K\textsubscript{ATP}) K\textsuperscript{+} channels responsible for influx, and by the K\textsuperscript{+}/H\textsuperscript{+} exchanger which performs K\textsuperscript{+} efflux from the matrix [53]. Despite the difficulties in the estimation of K\textsuperscript{+} fluxes across the inner mitochondrial membrane [68, 69], it is considered that the K\textsuperscript{+} efflux pathway exceeds influx, and the K\textsuperscript{+} concentration in the matrix is lower than in the cytoplasm [53]. An increase in matrix K\textsuperscript{+} leads to depolarization and swelling of the mitochondria. This can be observed in cells treated with valinomycin, which transports K\textsuperscript{+} into the matrix mimicking the activation of mitochondrial K\textsubscript{Ca} and K\textsubscript{ATP} channels. Characteristic morphological changes of the mitochondria from an elongated to a more round shape suggest that K\textsuperscript{+} transport by Baf may directly affect mitochondrial function and contribute to ΔΨ\textsubscript{m} depolarization and flickering by increasing matrix volume, causing swelling and physical damage to the mitochondria [53]. The dissipation of lysosomes, which are normally responsible for mitochondrial turnover [70], may also contribute to the unusual bioenergetic status of Baf(+) cells.

Inhibition of autophagy resulting from lysosomal dysfunction [71] does not seem to be affected by Baf uncoupling, since CMA causes a similar accumulation of LC3 II (Fig. 1f). Our data suggest that increased ROS production is caused by inhibition of V-ATPase rather than mitochondrial uncoupling (Fig. 2, Supplementary Fig. S2). However, caspase-3, the key enzyme in the apoptotic pathway, is activated by Baf to a larger degree than by CMA, suggesting that an overload of the mitochondria with K\textsuperscript{+} and frequent ΔΨ\textsubscript{m} depolarization contribute to the effect of Baf on apoptosis. So far, apoptosis has been considered mostly to result from V-ATPase inhibition and lysosomal dysfunction [15, 16].

Our data also show that neuronal cells uncoupled by Baf, overloaded with cytosolic Ca\textsuperscript{2+} and lacking functional acidic compartments become more susceptible to excitation. Stimulation with glutamate and depolarization of ΔΨ\textsubscript{p} by high K\textsuperscript{+} induce significantly stronger respiratory responses in Baf-treated cells (Fig. 7), and such amplified responses occur in the cells devoid of neurotransmission [72]. Increased susceptibility to excitatory stimulation and the evidence that increased energy demand upon excitation is not met by ATP production (Fig. 7) may have important physiological implications. Another interesting area for further investigation is the increased hypoxia in the cells uncoupled by Baf. We show here that upon Baf treatment at ambient O\textsubscript{2} (200 μM in extracellular medium), the concentration of O\textsubscript{2} in dense cell monolayers drops from about 150 μM to 100 μM and remains at this level for hours (Fig. 1). Considering the high O\textsubscript{2} consumption by excitable tissues and limited O\textsubscript{2} supply in solid tumours, we expect that Baf treatment may cause deep cell/tissue deoxygenation in vivo. Therefore, regulatory mechanisms modulated by hypoxia may be involved in cellular responses to Baf including apoptosis and HIF-1α stabilization. So far these responses have been attributed mostly to V-ATPase inhibition by this multifunctional drug.

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