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ORIGINAL PAPER

Bafilomycin A1 is a potassium ionophore that impairs mitochondrial functions

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Abstract Novel activities of bafilomycin A1, a macrolide antibiotic known as an inhibitor of V-ATPases, were discovered. Bafilomycin A1 induced uptake of potassium ions by energized mitochondria and caused mitochondrial swelling, loss of membrane potential, uncoupling of oxidative phosphorylation, inhibition of the maximal respiration rates, and induced pyridine nucleotide oxidation. The mitochondrial effects provoked by nanomolar concentrations of bafilomycin A1 were connected to its activity as a potent, K^+-specific ionophore. The K^+ ionophoric activity of bafilomycin A1 was observed also in black lipid membranes, indicating that it was an inherent property of the bafilomycin A1 molecule. It was found that bafilomycin A1 is a K^+ carrier but not a channel former. Bafilomycin A1 is the first and currently unique macrolide antibiotic with K^+ ionophoric properties. The novel properties of bafilomycin A1 may explain some of the biological effects of this plecomacrolide antibiotic, independent of V-ATPase inhibition.

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Abbreviations

$\Delta\Psi$	membrane potential
$\Delta\Psi_m$	mitochondrial membrane potential
BLM	bilayer black lipid membranes
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
PN	pyridine nucleotides
RLM	rat liver mitochondria

Introduction

Bafilomycin A1 is an inhibitor of the vacuolar H^+-ATPase (V-ATPase) present in membranes of endoplasmic reticulum, Golgi apparatus and vacuoles, where the V-ATPase generates an electrochemical gradient for protons that is required for metabolite transport and pH regulation. V-ATPase is inhibited by nanomolar concentrations of bafilomycin A1, whereas P-ATPase is affected at micromolar concentrations and the ATP synthases of mitochondria and bacteria are insensitive to this antibiotic (Bowman et al. 1988; Bowman and Bowman 2002; Gagliardi et al. 1999). Inhibition of V-ATPase by bafilomycin A1 has been shown using purified enzymes (Bowman et al. 1988; Dröse and Altendorf 1997; Dröse et al. 2001; Bowman and Bowman 2002) and in vesicles containing this enzyme (Moriyama and Nelson 1989; Moriyama and Futai 1990).

At high concentrations bafilomycin A1 inhibits cell growth and induces apoptosis (Umata et al. 1990; Ohkuma

et al. 1993; Okahashi et al. 1997; Nishihara et al. 1995; Ohta et al. 1998; Xu et al. 2003). Inhibition of V-ATPase usually leads to acidification of the cytosol. In several cell lines cytosolic acidification preceded or was associated with apoptosis (Ohkuma et al. 1993; Hishita et al. 2001; Nakashima et al. 2003), but in other cells bafilomycin A1-induced apoptosis occurred independently of intracellular acidification (Kinoshita et al. 1996; Okahashi et al. 1997; Ohta et al. 1998; Xu et al. 2003). It also was demonstrated that an increase of lysosomal pH, provoked by ammonium chloride, did not induce apoptosis (Tanigaki et al. 2003) and that bafilomycin A1 induced M1 cells to differentiate into macrophage-like phagocyte cells independently from lysosomal pH changes (Kinoshita et al. 1994).

Of interest is also that bafilomycin A1 induced loss of mitochondrial membrane potential ($\Delta\Psi_m$) and release of cytochrome *c* depending on the cell type (Nakashima et al. 2003; Hong et al. 2006). In the mouse leukemia monocyte cell line RAW 264.7 bafilomycin A1 induced nitric oxide production, mitochondrial depolarization and decreased growth and survival (Hong et al. 2006). Exposure to 5–20 nM of bafilomycin A1 induced loss of motility and depolarization of mitochondria in boar spermatozoa (Hoornstra et al. 2004). These effects of bafilomycin A1 are unlikely explained by induction of increased lysosomal pH.

The aim of the present study was to explore direct effect of bafilomycin A1 on mitochondria. To this end, bafilomycin A1-induced changes of the $\Delta\Psi_m$, oxidative phosphorylation and volume regulation systems were studied in isolated rat liver mitochondria (RLM). The study revealed that the mitochondrial effects provoked by nanomolar concentrations of bafilomycin A1 were connected to its activity as a potent, K^+-specific ionophore.

Materials and methods

Chemicals

Bafilomycin A1, erythromycin, azithromycin, roxithromycin, cyclosporin A, valinomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA), and clarithromycin was from LKT laboratories, Inc. (Nuppulinna, Finland). Stock solutions were prepared in methanol. All reagents were of the highest purity commercially available.

Preparation of rat liver mitochondria

Rat liver mitochondria (RLM) from male Wistar rats were isolated, washed and purified by standard protocols and the effects on oxygen uptake, $\Delta\Psi_m$ (with the aid of a tetraphenylphosphonium (TPP^+)-selective electrode), the kinetics of potassium efflux and mitochondrial swelling measured as previously described (Teplova et al. 2006). $\Delta\Psi_m$ was also measured with rhodamine 123 as a fluorescent probe with excitation at the wavelength of 503 nm and emission at the wavelength of 527 nm (Emaus et al. 1986). The pyridine nucleotide (PN) redox state was determined by recording PN fluorescence by the Hitachi F4000 fluorometer with excitation at 340 nm and emission

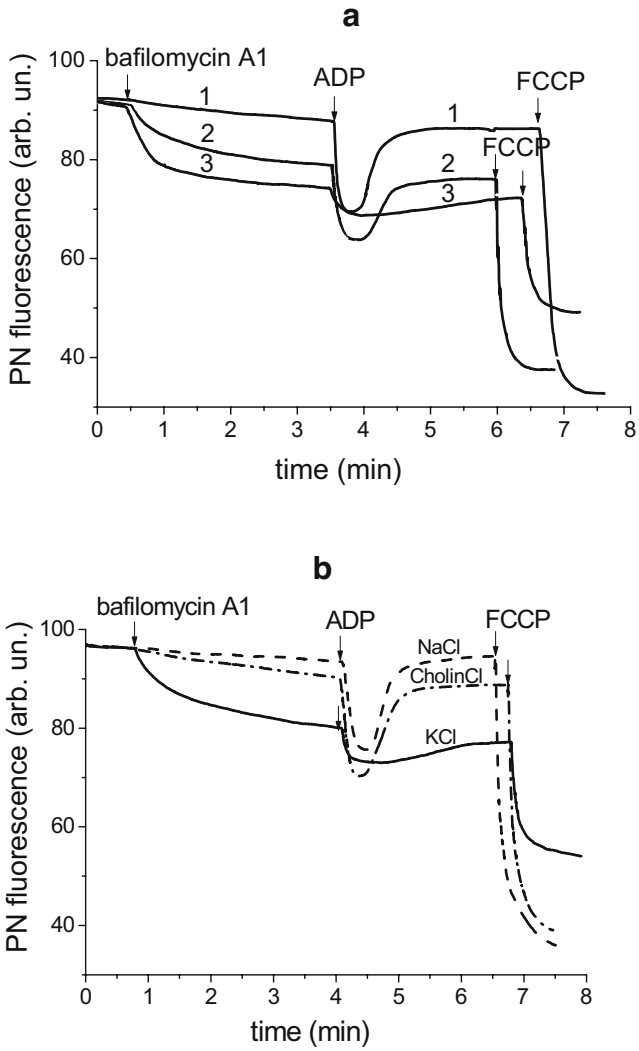


Fig. 1 The effect of bafilomycin A1 on the redox state of pyridine nucleotides (*PN*) and oxidative phosphorylation of mitochondria. **a** Mitochondria (0.5 mg protein/ml) were incubated in standard medium containing 120 mM KCl, 2 mM KH_2PO_4, 10 mM HEPES (pH 7.4), 5 mM malate *plus* 5 mM glutamate. Trace 1 shows the solvent (methanol) control. Bafilomycin A1 was added to concentrations of 50 nM (trace 2) and 100 nM (trace 3). The final concentrations of other additions were 160 μM ADP, and 1 μM FCCP. **b** Mitochondria (0.5 mg protein/ ml) were incubated in medium containing 120 mM different cations as chloride (K^+ as cation, *solid trace*; Na^+ as cation, *dashed trace*; *cholin* as cation, *dashed dot trace*), 2 mM cation (K^+ or Na^+)H_2PO_4, 10 mM HEPES (pH 7.4), 5 mM malate *plus* 5 mM glutamate. Bafilomycin A1 was added to concentration of 100 nM. The final concentrations of the other additions were 160 μM ADP and 1 μM FCCP. The traces shown are representative for three separate experiments

at 460 nm. The traces shown in the figures are representative for three separate experiments. Details are given in the respective figure legends.

Other assays

Protein was measured by the Biuret method with bovine serum albumin (BSA) as standard. Conductance in bilayer black lipid membranes (BLM) was measured with BLM formed in a 0.5 mm diameter hole between two Teflon chambers from soybean phosphatidylcholine (20 mg/ml in heptane) by the brush-painting method (Mueller et al. 1963) as described earlier (Mikkola et al. 1999). Bafilomycin A1, dissolved in methanol, was added both to the *cis* and *trans* compartments. An operational electrometer amplifier OPA 2111 (Burr-Brown Corp., Tucson, Arizona) connected as a current-voltage converter was used for measuring membrane current by the voltage-clamp method. Details are given in the figure captions.

Results

The effects of balifomycin A1 on mitochondrial functions

We investigated the action of bafilomycin A1 on isolated RLM. The intactness of mitochondria was measured by the redox state of the pyridine nucleotide (PN) pool, mitochondrial membrane potential ($\Delta\Psi_m$), and oxygen consumption in different respiratory states.

The effects of bafilomycin A1 on the PN fluorescence of mitochondria oxidizing glutamate *plus* malate and oxidative phosphorylation are shown in Fig. 1. Bafilomycin A1 at concentrations of 50 nM (100 pmol/mg of protein, trace 2) and 100 nM (trace 3) induced a clear oxidation of PN and extended the time of oxidative phosphorylation (Fig. 1a). With higher concentrations of bafilomycin A1 the PN fluorescence was only slightly further decreased, but the time of phosphorylation of the same amount of ADP was significantly prolonged (data not shown). Moreover, bafilomycin A1 decreased the oxidation of PN induced by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) in a concentration-dependent manner. In order to clarify whether these effects of bafilomycin A1 are cation selective, RLM were incubated in the presence of different monovalent cations. It was observed (Fig. 1b) that 100 nM of bafilomycin A1 induced strong PN oxidation and inhibited oxidative phosphorylation in the KCl medium (continuous trace) but not in NaCl (dashed trace) and only slightly in choline chloride (dashed dot trace) media.

The effects of bafilomycin A1 on $\Delta\Psi_m$ and the oxygen consumption are shown in Fig. 2. The measurement of oxygen consumption (Fig. 2a) was carried out simultaneously with the recording of $\Delta\Psi_m$ by a TPP^+-electrode (Fig. 2b). Bafilomycin A1 was found to increase the rate of glutamate *plus* malate oxidation and to induce decrease of $\Delta\Psi_m$ in dose-dependent manner from 65 nM to 260 nM (Fig. 2a and b). Bafilomycin A1 inhibited both the respiratory rate V_3 after ADP addition as well as the uncoupled respiration (Fig. 2a). The effect of bafilomycin A1 on V3 is likely to be caused by inhibition of the respiratory chain, but not by H^+-ATPase, since addition of ADP induced a significant drop of $\Delta\Psi_m$, (Fig. 2b). It may thus be concluded that bafilomycin A1 acted both as an uncoupler of oxidative phosphorylation and as an inhibitor of the respiratory chain.

In order to test whether the effects of bafilomycin A1 on $\Delta\Psi_m$ also were cation-selective, the mitochondria were incubated in the presence of different monovalent cations. As shown in Fig. 3a, bafilomycin A1 lowered $\Delta\Psi_m$ (shown as increased rodamine 123 fluorescence) and inhibited oxidative phosphorylation in a concentration-dependent

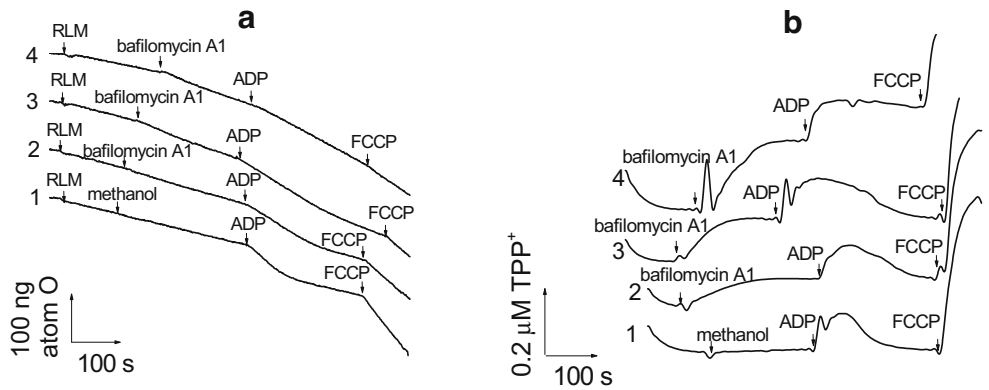


Fig. 2 Concentration-dependent effects of bafilomycin A1 on mitochondrial respiration (**a**) and mitochondrial membrane potential (**b**). Experimental conditions were as in Fig. 1a, but the concentration of mitochondria was 1 mg protein/ml and 0.7 μM TPP^+ was present. *Trace 1* shows the solvent (methanol) control. Bafilomycin A1 was added to concentrations of 65 nM (*trace 2*), 130 nM (*trace 3*), and 260 nM (*trace 4*). The final concentrations of the other additions were 200 μM ADP and 0.4 μM FCCP. The traces shown are representative for three separate experiments

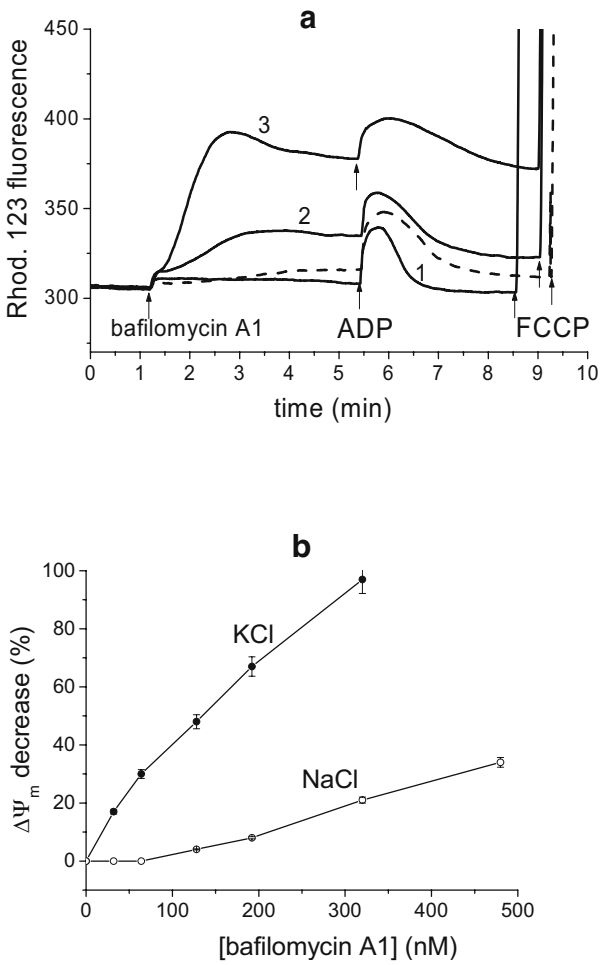


Fig. 3 The comparison of the effects of bafilomycin A1 on the membrane potential of mitochondria incubated in KCl or NaCl media. Mitochondria (0.5 mg protein/ ml) were incubated in medium contained 120 mM different cations as chloride (K^+ as cation, *solid traces*, or Na^+ as cation, *dashed trace* in **a**), 2 mM cation (K^+ or Na^+) H_2PO_4, 10 mM HEPES (pH 7.4), 5 mM malate *plus* 5 mM glutamate. Rhodamine 123 at concentration 20 nM was used for measurements of changes in $\Delta\Psi_m$. **a** The solvent (methanol) was added in the control (*solid trace 1*). Bafilomycin A1 was added to concentrations of 50 nM (*solid trace 2*) or 100 nM (*solid trace 3* and *dashed trace*). The final concentrations of the other additions were 160 μM ADP, and 1 μM FCCP. The traces shown are representative for three separate experiments. **b** Shows the dose-dependent effects of bafilomycin A1 on the $\Delta\Psi_m$ in KCl and NaCl media

way in the KCl medium (continuous traces 2 and 3), but not in the NaCl medium (dashed trace). The concentration dependences of bafilomycin A1-induced decreases in $\Delta\Psi_m$ of the mitochondria incubated in KCl or NaCl media are presented in Fig. 3b. It is seen, that large decrease in $\Delta\Psi_m$ was induced by 30–60 nM bafilomycin A1 in the KCl medium but in the NaCl medium the effect was small and occurred only at high concentrations of bafilomycin A1 (300–500 nM). These results demonstrate that the effect of bafilomycin A1 on $\Delta\Psi_m$ is dependent on the ion composition of the incubation medium similarly to the effect observed on PN oxidation.

In case the lowering of $\Delta\Psi_m$ and uncoupling of oxidative phosphorylation by bafilomycin A1 in KCl medium is due to K^+ influx into the mitochondrial matrix, bafilomycin A1 should induce mitochondrial swelling in K^+-containing media. Recordings of the absorbance changes in the presence of bafilomycin A1 in media containing 120 mM KCl or 120 mM NaCl are shown in Figs. 4 and 5, respectively. Bafilomycin A1 (25 nM) induced a clear decrease in absorbance in the KCl medium (Fig. 4a). The swelling was of low amplitude, but a subsequent addition of $CaCl_2$ induced high amplitude swelling due to opening of the mitochondrial permeability transition pore. This was confirmed by its inhibition by 1 μM cyclosporin A as shown in Fig. 4b. The high amplitude swelling induced by $CaCl_2$ in the control experiment (solid trace 1 in Fig. 4b) was inhibited by 1 μM cyclosporin A (dashed trace 2). One micromole cyclosporin A had no effect on the mitochondrial swelling induced by bafilomycin A1 but inhibited swelling induced by following addition of $CaCl_2$ (solid trace 3 and dashed trace 4). Increasing the concentration of bafilomycin A1 induced an increase of the amplitude of the swelling and disturbed Ca^{2+}-transport. The maximal swelling induced by different concentrations of bafilomycin A1 is shown in Fig. 4c. Figure 4d shows that swelling induced by 600 nM bafilomycin A1 was similar to the swelling induced by 20 nM valinomycin. The swelling induced by valinomycin was also not prevented by cyclosporin A.

As shown in Fig. 5a in the NaCl medium bafilomycin A1 induced weak swelling of mitochondria but only at high concentrations (about 600 nM).

Figure 5b displays the selectivity of bafilomycin A1 for potassium, when the swelling assay was carried out in isotonic media that contained 120 mM of NaCl plus KCl, with varying ratios of Na^+/K^+. The amplitude of swelling was highly dependent on the Na^+/K^+ ratio in the incubation medium. The mitochondrial swelling induced by 300 nM bafilomycin A1 in 10 mM KCl was ca 50% of the maximal achieved at 100 mM K^+. The swelling assays in Figs. 4 and 5 thus show that bafilomycin A1 acted as a K^+-selective ionophore.

Transport of potassium ions by bafilomycin A1

In order to further explore the ionophoric properties of bafilomycin A1, assays with a K^+-selective electrode were carried out. As shown in Fig. 6, the addition of bafilomycin A1 to energized mitochondria respiring on glutamate *plus* malate induced a decrease in $[K^+]$ in the external medium, indicating K^+ influx into the mitochondrial matrix.

Effects of bafilomycin A1 on the conductance in BLM were measured in absence of any protein in order to find out if the K^+ ionophoric activity was an independent property of the plecomacrolide molecule bafilomycin A1, or if it depended on interaction with mitochondrial proteins/compo-

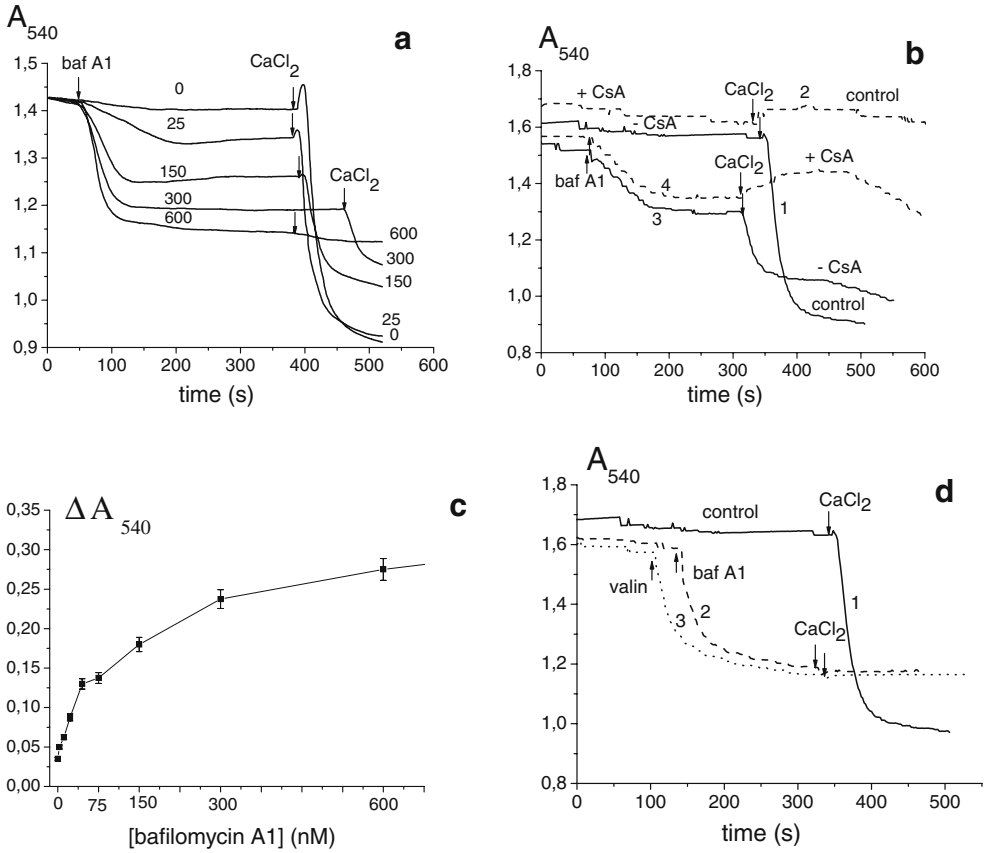


Fig. 4 Mitochondrial swelling induced by different concentrations of bafilomycin A1 in 120 mM KCl. Mitochondria (1 mg protein/ml) were incubated in standard KCl (120 mM) medium as in Fig. 1a. **a** Shows mitochondrial swelling (decreasing absorbance) induced by varying concentrations of bafilomycin A1. Trace 0 shows the solvent (methanol) control. At the end of the experiments, 200 μM $CaCl_2$ was added to concentration to induce full swelling. **b** Shows the effect of cyclosporin A on swelling induced by 200 μM $CaCl_2$ (traces 1 and 2) and by 100 nM bafilomycin A1 (traces 3 and 4). **c** Shows the maximal mitochondrial swelling at different of concentrations of bafilomycin A1. **d** Shows mitochondrial swelling induced by 600 nM bafilomycin A_1 (trace 2) and 20 nM valinomycin (trace 3). Trace 1 shows the solvent (methanol) control with addition of 200 μM $CaCl_2$

nents (Fig. 7). The results show that conductance of BLM to 100 mM KCl increased at increasing of concentrations of bafilomycin A1 (Fig. 7a). The conductance of the lipid bilayer was 2,000 nS/cm^2 at 7.5 nM of bafilomycin A1. This conductance is more than two orders of magnitude above the background conductance 4 nS/cm^2 but lower than the conductance obtained with valinomycin (indicated in Fig. 7). In Fig. 7b the starting concentrations of KCl at the *cis*- and *trans*-sides of the bilayer were 100 mM, the concentration of bafilomycin A1 was 7.5 nM. With 100 mM KCl on both sides of the BLM, the ΔΨ signal was zero as it should be. Then NaCl was added to give a concentration of 156 mM at the *cis*-side, i.e. a gradient for NaCl across the membrane was created. This did not change

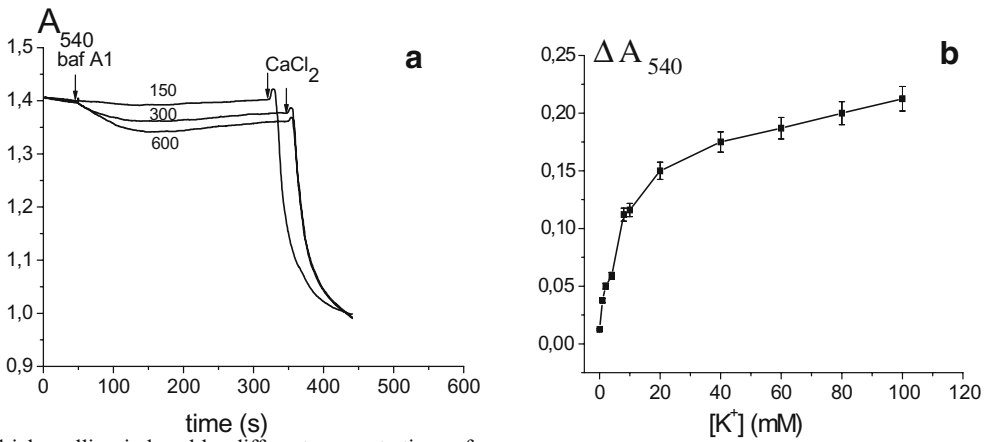


Fig. 5 Mitochondrial swelling induced by different concentrations of bafilomycin A1 in medium with different potassium concentrations. Mitochondria (1 mg protein/ml) were incubated in standard medium as in Fig. 1a, but in **a** the KCl was replaced by NaCl (120 mM). **b** The medium contained a mixture of NaCl and KCl (sum 120 mM) in order to obtained different K^+ concentrations. **a** Shows mitochondrial swelling induced by 150, 300, and 600 nM bafilomycin A1 in 120 mM NaCl medium. At the end of the experiments, 200 μM $CaCl_2$ was added to induce full swelling. **b** Shows the swelling induced by 300 nM bafilomycin A1 at different concentrations of K^+

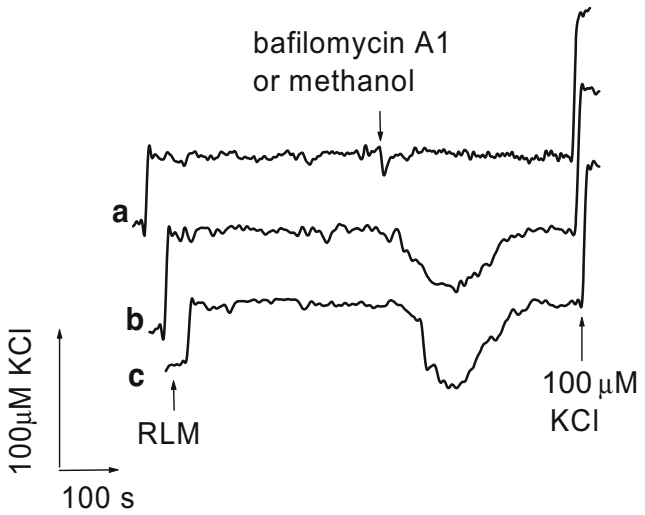
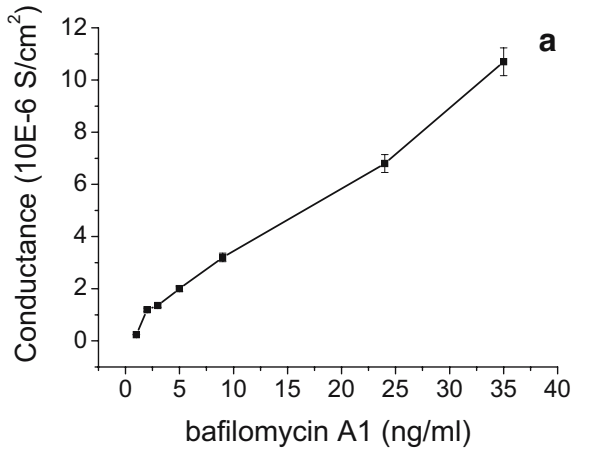
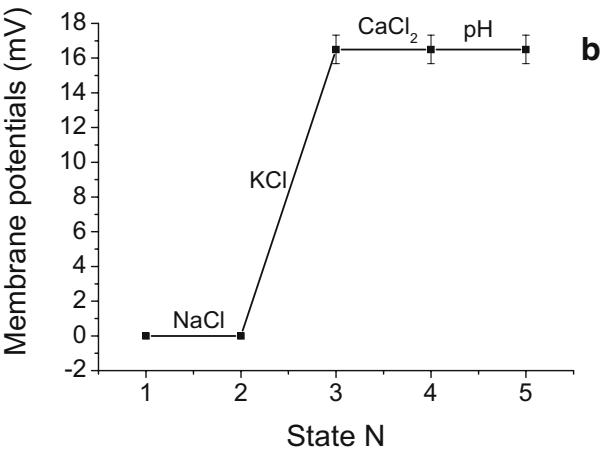
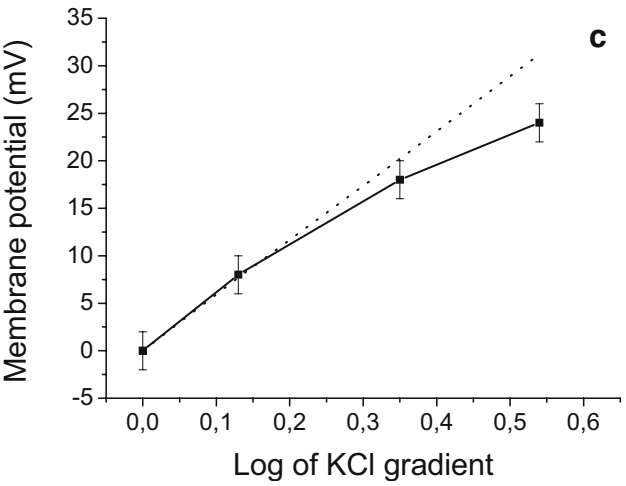


Fig. 6 Bafilomycin A1-induced influx of potassium in energized mitochondria. Mitochondria (1 mg/ml) were incubated in medium containing 116 mM NaCl, 4 mM KCl, 2 mM NaH_2PO_4, 10 mM HEPES (pH 7.4), 5 mM glutamate *plus* 5 mM malate. The potassium contents were determined with a K^+-selective electrode. *Trace a* shows the solvent (methanol) control. Bafilomycin A1 was added to concentrations of 300 nM (*trace b*) and 600 nM (*trace c*). At the end of the experiments, KCl was added to the concentration of 100 µM for calibration. The traces shown are representative for three separate experiments







the $\Delta\Psi$. The subsequent addition of KCl to a total concentration of 221 mM, increased the $\Delta\Psi$ to 16.1 mV, which corresponds to the theoretical value given by Nernst equation for this gradient. Finally, an addition of $CaCl_2$ to a concentration of 5 mM to the same side, or changing of the pH from 6.2 to 5.5 in this compartment did not change the $\Delta\Psi$ any further, indicating that neither Na^+, Ca^{2+} nor H^+ were carried by bafilomycin A1. The bafilomycin A1-induced increase in conductance was thus specific for K^+.

Figure 7c shows the dependence of the $\Delta\Psi$ as a function of the KCl gradient across the membrane. With 100 mM KCl on both sides of the membrane the $\Delta\Psi$ naturally was 0 V. Then the [KCl] at the *cis*-side was changed by addition of KCl as dry salt and the potentials were recorded. These results show that the $\Delta\Psi$ of the BLM increased in response to an increasing gradient of KCl almost following the theoretical values. The conductance measured in the presence of bafilomycin A1 at high sensitivity did not show any abrupt changes with time as seen on channel openings and closings (data not shown). This indicates that bafilomycin A1 did not form any membrane channels.

Assays on mitochondrial effects and ionophoricity of other macrolides

The results explained above convincingly showed that the macrolide bafilomycin A1 possesses ionophoric activity with high selectivity for K^+. This was unexpected, as macrolide antibiotic have not been described to possess such activity. We therefore checked four macrolide antibiotic substances (currently medically used as antibacterial drugs) for K^+ ionophoric properties or effects on the mitochondrial functions, using the same assays as described above for bafilomycin A1. It was found that erythromycin,

◀ **Fig. 7** BLM studies on bafilomycin A1-induced membrane permeability to K^+ and Na^+. The experimental conditions for formation of BLM and measurement of conductance and $\Delta\Psi$ see Materials and methods. **a** The membrane conductance induced by varying concentrations of bafilomycin A1. Conditions: 100 mM KCl, applied voltage 50 mV. Valinomycin, 1 , 3, and 5 ng/ml, used as a positive reference, induced conductance of 25, 50 and 80 μS/cm^2, respectively. **b** Changes of $\Delta\Psi_m$ upon changes of the ion composition of the electrolyte at the *cis*-side of the membrane. Initially 100 mM KCl was present at the *cis*- and the *trans*- sides of the membrane, then NaCl was added to concentration of 156 mM (point 1), KCl was added to final concentration of 221 mM (point 2), $CaCl_2$ was added to concentration of 5 mM (point 3), at point 4 pH was change from 6.2 to 5.4. C. Zero-current potential as a function of the KCl gradient across the membrane. Conditions: starting KCl concentration at *cis*- and *trans*- sides 100 mM, bafilomycin A1 was added to 5 ng/ml then KCl gradient was formed by increasing [KCl] on the *cis* side. The *dotted line* is the theoretical curve calculated by the Nernst equation for a case of ideal cation over anion selective membrane

azithromycin, clarithromycin and roxithromycin, when tested at concentrations from 20 nM to 20 μM, had no effect on the $\Delta\Psi_m$ and / or the mitochondrial respiration (Fig. 8). Nor did they induce mitochondrial swelling or any effects on BLM (data not shown).

Discussion

In the present study bafilomycin A1, known as the inhibitor of the V-ATPase, was found to induce lesions of basic mitochondrial functions in nanomolar concentrations. Our findings indicate that bafilomycin A1 acted on isolated RLM and in BLM as a K^+-specific ionophore. Bafilomycin A1 induced K^+ influx into the mitochondrial matrix, oxidation of PN, decrease of $\Delta\Psi_m$, and mitochondrial swelling. It is known that K^+ is an osmotically active ion and the predominant cation in both the cytosol and mitochondrial matrix and the increase in permeability to K^+ may aberrate the mitochondrial functions (Garlid and Paucek 2003).

Similar to the potassium ionophores valinomycin and cereulide (Teplova et al. 2006) bafilomycin A1 inhibited the mitochondrial electron transport chain but not the F_1F_0-ATPase. The affinity of bafilomycin A1 to potassium ions appeared low compared to valinomycin and cereulide: valinomycin (5 nM) needed 20–30 mM KCl for a substantial effect, cereulide (5 nM) only 1–3 mM KCl (Teplova et al. 2006), whereas 300 nM of bafilomycin A1 in 20–40 mM [K^+] was required for induction of high-amplitude swelling.

The bafilomycin A1 activity as a K^+ selective ionophore was also demonstrated as increased conductance in BLM based on carrier mode (no channel formation). The effect of valinomycin on conductance in BLM was 1–2 orders of magnitude higher than that of bafilomycin A1 at the lower end of concentrations. This molecule thus possessed ionophoric carrier activity independently of the presence of any mitochondrial components. The plecomacrolide bafilomycin A1 thus is similar in mitochondrial effects and ionophoric properties to the cyclic peptide ionophores valinomycin and cereulide (Mikkola et al. 1999; Teplova et al. 2006; Hoornstra et al. 2003), in spite of its very different chemical structure. The effect of bafilomycin A1 in stimulating mitochondrial

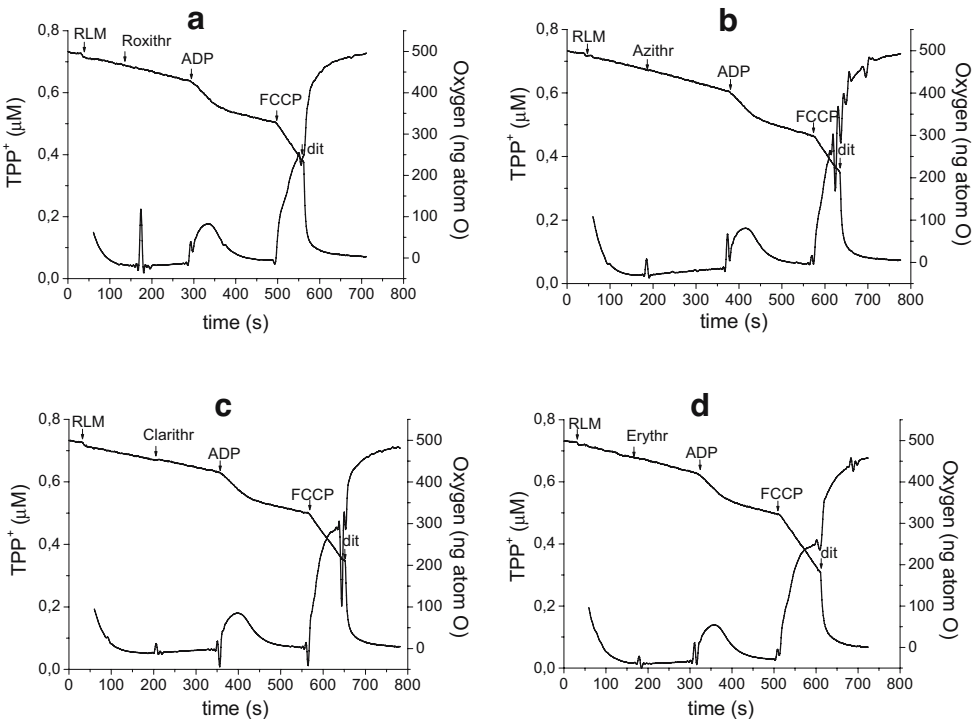


Fig. 8 The absence of any effects of roxithromycin, azithromycin, claritromycin and erythromycin on mitochondrial respiration and membrane potential. Experimental conditions were as in Fig. 2 with 0.7 μM TPP^+ present. Roxithromycin (Roxithr, **a**), azithromycin (Azithr, **b**), clarithromycin (Clarithr, **c**), and erythromycin ((Erythr, **d**) were added to concentrations 2 μM. The final concentrations of the other additions were 200 μM ADP and 0.4 μM FCCP. The traces shown are representative for three separate experiments

swelling is thus due mainly to its ionophoretic properties for K^+. This was supported by the absence of an effect of Cyclosporin A, a well known inhibitor of the Ca^{2+}-induced swelling due to opening of the permeability transition pore (Crompton et al. 1988; Broekemeyer et al. 1989).

Bafilomycin A1 appears to be the first macrolide antibiotic with reported ionophoric activity and there is no literature on its physicochemical properties to explain the molecular mechanism of this activity. It is known that the effects of immunosuppressive macrolides involve interference with the function of Ca^{2+} channels (Takahashi 2002). These macrolides possess 21 to 29 membered macrolide ring structures possessing multiple functional oxygens. Bafilomycin A1 belongs the group of plecomacrolides, with a 16-membered macrolactone ring with a side chain connected by a C3-spacer to the carbon that bears the ring lactone oxygen (Shomi and Omura 2002). It is known that oxygen atoms have affinity for cations. The cation selectivity of bafilomycin A1, demonstrated in this paper, could connect to the four oxygen atoms around the C3 spacer (carbons 1, 15, 17, 19) of the bafilomycin A1 molecule. The medically used antibacterial macrolide drugs are 14-membered ring structures. In our study the four macrolides, erythromycin, roxithromycin, and clarithromycin, azithromycin, appeared void of any ionophoric activity. These substances possess a dimethylamine group in the side chains (Sunazuka et al. 2002). Dimethylamine groups will at pH 7.4 be protonated and positively charged and thus would electrostatically repel a cation. Bafilomycin A1 is more hydrophobic (log K_{ow} 3.9) than the four antibacterial macrolide drugs (log K_{ow} value 2.8 for erythromycin and for roxithromycin, 3.2 for clarithromycin and 3.3 for azithromycin, Scifinder 2006; Clarke's Analysis of Drugs and Poisons 2006).

Until now it was believed that the biological activities of bafilomycin A1 depend on its capacity to inhibit V-ATPase activity (Ohkuma et al. 1993; Hishita et al. 2001; Nakashima et al. 2003). The present results revealed a new target of bafilomycin A1 action: the cellular ion homeostasis system. The K^+ ionophores valinomycin, cereulide, beauvericin are known to induce apoptosis (Inai et al. 1997; Paananen et al. 2002; Abdalah et al. 2006; Lin et al. 2005). Our demonstration that bafilomycin A1 also acts as K^+ ionophore may thus explain its apoptotic activity, observed in different cells (Kinoshita et al. 1996; Okahashi et al. 1997; Ohta et al. 1998; Hong et al. 2006; Boya et al. 2005), rather than the V-ATPase inhibitory activity. We previously reported that bafilomycin A1 decreased the $\Delta\Psi_m$ in sperm cells and inhibited sperm motility, which is strongly dependent on mitochondrial functions (Hoornstra et al. 2003). Mitochondrial depolarization, an index of apoptosis, was observed in the mouse leukaemia monocyte cell line RAW 264.7, in which bafilomycin A1 decreased growth and survival (Hong et al. 2006). The decrease of $\Delta\Psi_m$ induced by bafilomycin A1 may be due to its K^+ ionophoric activity.

A recent study has shown that neuroprotective effects of macrolide antibiotics against stress-induced cell death may be limited to the plecomacrolide subclass (Shacka et al. 2006a). The treatment with macrolide antibiotics, which are not members of the plecomacrolide subclass, did not alter the death-inducing effects of chloroquine and exhibited no neuroprotection. Bafilomycin A1-induced neuroprotection suggests a mechanism independent of its ability to inhibit V-ATPase (Shacka et al. 2006b).

Our present data are the first indication on the ionophoric properties of bafilomycin A1.

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