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Mechanism of action of an old antibiotic revisited: Role of calcium ions in protonophoric activity of usnic acid

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ABSTRACT

Usnic acid (UA), an old antibiotic and one of the first described mitochondrial uncouplers, has demonstrated many beneficial activities, such as antimicrobial, antiviral, antitumour and anti-inflammatory properties. Here, we performed a thorough investigation of effects of usnic acid and its analogues on artificial planar bilayer lipid membrane (BLM), rat liver mitochondria and bacteria. Surprisingly enough, all of the three hydroxyl groups of UA appeared to be involved in its proton-shuttling activity on BLM. We ascribed this fact to an ability of UA to form complexes with calcium ions, aiding it in cycling protons across the membrane. Actually, the addition of calcium ions markedly stimulated the UA-induced electrical current across BLM. By using the calcium ionophore A23187, we proved the involvement of calcium ions in the UA uncoupling action on isolated rat liver mitochondria. The calcium-chelating property of UA was demonstrated here by the method of extracting metal ions into a hydrophobic phase. Modification of any of the hydroxyl groups in UA dramatically reduced not only the UA-induced current across BLM and the UA-mediated calcium extraction, but also the uncoupling activity of UA in mitochondria and the inhibiting effect of UA on the growth of *Bacillus subtilis*. The ability of UA to cause dissipation of membrane potential in isolated liver mitochondria and bacterial cells was shown here for the first time. In view of the data obtained, the protonophoric activity of UA is considered to make a significant contribution to its antibacterial action.

1. Introduction

Recently a series of biological studies have demonstrated a revival of interest to protonophores – the compounds that can transfer protons across both natural and artificial lipid membranes. The attention of researchers is due to an ability of protonophores to uncouple mitochondrial oxidative phosphorylation, i.e. to suppress ATP synthesis while accelerating the respiration. Remarkably, uncouplers have exhibited beneficial action in a variety of pathologies including cancer, obesity, and a long list of diseases associated with oxidative stress. Protonophoric activity has also proved to be crucial for the antibacterial potency of a number of effective antibiotics [1,2]. Among the first described uncouplers, following dinitrophenol and gramicidin A, was usnic acid (UA) [3], the compound isolated from lichen in 1843 [4]. Being found in early works [5,6], the antibacterial action of UA, similar to that of gramicidin A, was initially explained by protonophoric uncoupling, first demonstrated in [7–9] and much later in [10,11]. Surprisingly enough, such mechanism of antibacterial action for UA seems to be forgotten now, although the proton-carrying activity of this old antibiotic has been demonstrated in experiments with planar bilayer lipid membranes by [12]. Recent studies [13] suggest inhibition of RNA synthesis and elongation of DNA replication as main routes of UA antimicrobial action. Here we compared the abilities of UA and its analogues with modified hydroxyl groups to induce the electric current across planar bilayer lipid membranes (BLM), to dissipate mitochondrial membrane potential and to inhibit growth of *Bacillus subtilis* cells.

Abbreviations: UA, usnic acid; UA3, 8-acetyl-5,7-dihydroxy-3,4a,6-trimethylbenzofuro[3,2-f]-l,2-benzofuran-4(4a*H*)-one; UA7, (*R*)-1,1-(3,9-dihydroxy-7-methoxy-8,9b-dimethyl-1-oxo-1,9b-dihydrodibenzo[*b*,*d*]furan-2,6-diyl)diethanone; UA9, 2,6-diacetyl-l,9b-dihydro-3,7-dihydroxy-8,9b-dimethyl-9-methoxy-l-oxodibenzofuran; DNP, 2,4-dinitrophenol; TTFB, 4,5,6,7-tetrachloro-2-(trifluoromethyl)-benzimidazole; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DPhPC, diphytanoylphosphatidylcholine; TMRE, tetramethylrhodamine ethyl ester; BLM, bilayer lipid membrane; FCS, fluorescence correlation spectroscopy

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Both protonophoric and depolarizing activities of UA appeared to be highly stimulated by calcium ions. All three dissociable protons of UA proved to be important for both proton-carrying activity of this compound in model membranes and its uncoupling effect on mitochondria, as well as for its antibacterial action. Based on the present results, we reconsider the mode of UA antibacterial activity.

2. Materials and methods

2.1. Materials

Most chemicals including (*R*)-(+)-usnic acid (UA), diphytanoylphosphatidylcholine (DPhPC), tetramethylrhodamine ethyl ester (*TMRE*) were from Sigma. 8-Acetyl-5,7-dihydroxy-3,4a,6-trimethylbenzofuro[3,2-f]-l,2-benzofuran-4(4a*H*)-one (UA3) was synthesized by the procedure described in [14]. (*R*)-1,1-(3,9-Dihydroxy-7-methoxy-8,9b-dimethyl-1-oxo-1,9b-dihydrodibenzo[*b*,*d*]furan-2,6-diyl) diethanone (UA7) was synthesized by the procedure described in [15]. 2,6-Diacetyl-l,9b-dihydro-3,7-dihydroxy-8,9b-dimethyl-9-methoxy-l-oxodibenzofuran (UA9) was synthesized by the modified procedure described in [16].

2.2. Planar bilayers

Bilayer lipid membrane (BLM) was formed by the brush technique [17] from a 2% decane solution of DPhPC on a 0.6-mm aperture in a Teflon septum separating the experimental chamber into two compartments of equal 3-ml volume. Electrical parameters were measured with two AgCl electrodes placed into the solutions on the two sides of the BLM via agar bridges, using a Keithley 428 amplifier (Cleveland, Ohio, USA).

2.3. Usnic acid-mediated transfer of calcium ions from an aqueous phase into an organic phase

A chloroform/butanol solution (70:30 by volume, 0.5 ml) of usnic acid or its analogs (200 μM) and 0.5 ml of an aqueous solution of calcium chloride (200 μM in 10 mM Tris-HCl, pH 10) were shaken for 10 min in a 1-ml test tube at room temperature followed by centrifugation (3000*g*, 10 min). 0.2 ml of organic phase was evaporated (15 min at 95°C) in another test tube followed by Ca^{2+} extraction via incubation with 0.1 ml of 2 N HCl. A shift to alkaline pH was performed by the addition of 0.4 ml of 1 N KOH and 0.6 ml H_2O. Calcein (1 μM) was used as a calcium probe [18]. Fluorescence at 520 nm (excitation at 490 nm) was measured with an SM-2203 spectrofluorometer (Solar, Belarus). Concentration of calcium ions was determined by titration with EDTA (1 mM solution), fitting the fluorescence-EDTA concentration plot with a straight line and finding its intersection with the X-axis.

2.4. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation [19] in a medium containing 250 mM sucrose, 5 mM MOPS, 1 mM EGTA, pH 7.4. The final washing was performed in the medium additionally containing bovine serum albumin (0.1 mg/ml). Protein concentration was determined using the Biuret method. Handling of animals and experimental procedures were conducted in accordance with the international guidelines for animal care and use and were approved by the Institutional Ethics Committee of A.N. Belozersky Institute of Physico-Chemical Biology at the Moscow State University.

2.5. Mitochondrial respiration

Respiration of isolated mitochondria was measured using a standard polarographic technique with a Clark-type oxygen electrode (Strathkelvin Instruments, UK) at 25 °C using the 782 system software. The incubation medium contained 250 mM sucrose, 5 mM MOPS, 1 mM KH_2PO_4, and 1 mM EGTA, pH 7.4. The mitochondrial protein concentration was 0.8 mg/ml. Oxygen uptake is expressed as nmol/min·mg protein. Succinate was used as a substrate.

2.6. Membrane potential (ΔΨ) measurement in isolated mitochondria

ΔΨ was estimated using the safranine O dye [20]. The difference in the absorbance at 555 and 523 nm (ΔA) was recorded with an Aminco DW-2000 spectrophotometer in the dual wavelength mode. The following incubation medium was used: 250 mM sucrose, 5 mM MOPS, 1 mM KH_2PO_4, 1 mM EGTA, 2 μM rotenone, 5 mM succinate (pH 7.4), 1 μg/ml oligomycin, 15 μM safranine O. The mitochondrial protein content was 0.6–0.9 mg protein/ml, the temperature was 26 °C.

2.7. Measurement of calcium ions efflux from isolated mitochondria

Extramitochondrial Ca^{2+} changes were measured with Fluo-5N, a low-affinity Ca^{2+} indicator, as described previously [21]. Isolated rat liver mitochondria (protein 0.5 mg/ml) were resuspended in 250 mM sucrose, 10 mM Tris, 10 mM KH_2PO_4, pH 7.4, 1 mM succinate, 2 μM rotenone, 1 μM cyclosporine A with 1 μM Fluo-5N. Fluorescence changes (excitation 495 nm, emission 515 nm) were recorded at room temperature using the Panorama Fluorat 02 spectrofluorometer.

2.8. Swelling of mitochondria

The protonophoric ability of UA and its analogues was tested by induction of swelling of non-respiring rat liver mitochondria incubated in buffered isotonic potassium acetate in the presence of valinomycin. Under these conditions, mitochondria do not swell, because acetate can cross the membrane only as undissociated acetic acid, and the transmembrane passage of K^+ in the form of the K^+-valinomycin complex generates a charge imbalance, preventing further permeation of K^+ [22]. Intramitochondrial accumulation of potassium acetate, resulting in mitochondria swelling, becomes only possible, if H^+ is exported from the inner compartment of mitochondria, e.g., by a protonophore [23,24], thus enabling the influx of K^+. The mitochondrial swelling was recorded as a decrease in absorbance of the mitochondrial suspension at 600 nm by Amersham Ultrospec 1100 pro. Briefly, an aliquot of mitochondria was added to 1 ml of the "swelling medium" containing 145 mM potassium acetate, 5 mM Tris, 0.2 mM EDTA, 0.5 μM valinomycin and 1 μM rotenone at pH 7.4.

2.9. Bacterial growth

The standard laboratory strain *Bacillus subtilis* subs. *subtilis* Cohn 1872, stain BR151 (trpC2 lys-3 metB10) was used. Overnight bacterial cells cultures were diluted in fresh LB media. 200 μl of bacterial cell cultures ($5 * 10^5$ cells/ml) were inoculated into 96-well plates (Eppendorf AG, Hamburg, Germany) and the UA derivatives were added to reach appropriate concentrations. Cells were left to grow for 21 h at 37 °C. Optical densities at 620 nm and kinetic growth curves were measured by using a Thermo Scientific Multiskan FC plate reader with an incubator (Thermo Fisher Scientific, USA). All experiments were performed at least in triplicates.

2.10. Measurement of B. subtilis membrane potential

Membrane potential in *B. subtilis* was measured by estimating accumulation of the cationic dye TMRE in bacterial cells with a fluorescence correlation spectroscopy (FCS) setup, similar to the procedure described in [25] for isolated mitochondria. The setup allows one to record time traces of fluorescence emitted by some fluorescent particles, e.g., dye-doped bacteria, which represent sequences of peaks of variable intensity reflecting random walk of the particles through the

confocal volume. The experimental data were obtained under stirring conditions which increased the number of events by about three orders of magnitude thus substantially enhancing the resolution of the method. The setup of our own construction was described previously in [25]. Briefly, fluorescence excitation and detection were provided by a Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescence inverted microscope equipped with a 40×, NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence passed through an appropriate dichroic beam splitter and a long-pass filter and was imaged onto a 50-μm core fiber coupled to an avalanche photodiode (SPCM-AQR-13-FC, Perkin Elmer Optoelectronics, Vaudreuil, Quebec, Canada). The output signal $F(t)$ was sent to a personal computer using a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The signal was measured in Hz meaning number of photons per a second. Fluorescence traces with a sampling time of 25 μs were analyzed using the WinEDR Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). The software, originally designed for single-channel analysis of electrophysiological data, enables counting of the number of peaks ($n(F > F_0)$) of the FCS signal with amplitudes higher than the value F_0. A program of our own design with a similar algorithm (coined Saligat; provided on request) was also used.

3. Results and discussion

Bearing in mind the molecular structure of UA (Fig. 1), it was of interest to figure out which of its three hydroxyl groups are involved in the protonophoric activity. Fig. 2 displays concentration dependences of electric current generated across BLM under voltage-clamp conditions after the addition of UA (curve 1) and its three analogues UA7, UA9 and UA3 (curves 2–4), lacking the protons at positions 7, 9 and 3, correspondingly. It is seen that practically no current was observed after the addition of UA7 and UA9 in the concentration range studied, whereas the UA3 analogue was much less effective in generating the current than UA. Thus, all of the three hydroxyl groups of UA are of significance for its ionophoric activity, with the OH group at position 3 being less important than those at positions 7 and 9.

To explain this fact, we addressed metal-chelating properties of UA, taking into account its ability to form complexes with some metallic cations, such as copper and palladium [26]. Fig. 3A shows a time course

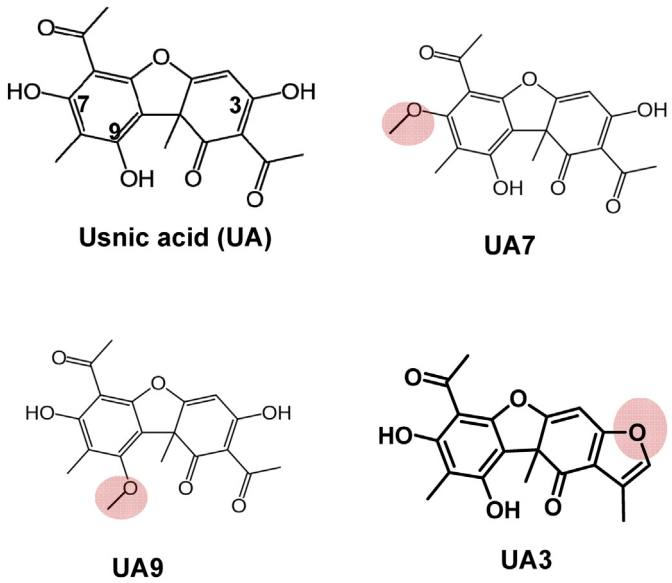

Fig. 1. Chemical structures of usnic acid (UA) and its analogs with modified hydroxyl groups at positions 3, 7, and 9. According to [40], pK_a values of hydroxyls at positions 3, 7 and 9 are equal to 4.4, 10.7 and 8.8, respectively.

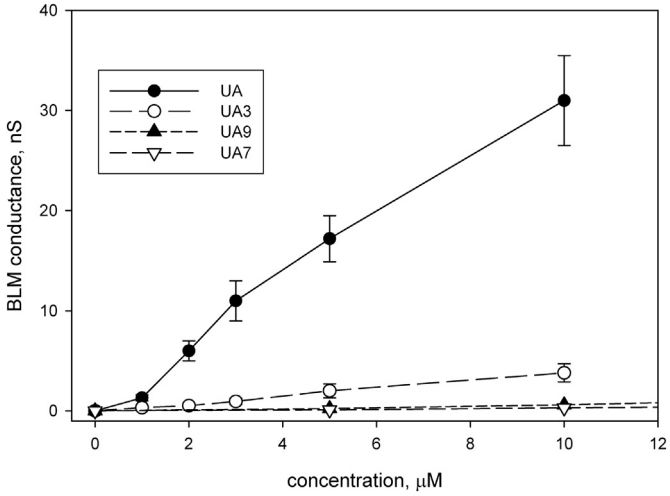

Fig. 2. Dependence of electrical conductance of a planar bilayer lipid membrane (BLM) on the concentration of UA and its analogs. The solution was 10 mM Tris, 10 mM MES, 10 mM β-alanine, 10 mM KCl, 1 mM $CaCl_2$, pH 7.0. BLM voltage 50 mV. BLM was made from DPhPC.

of UA-induced current across BLM. It is seen that the addition of 100 μM EDTA dramatically suppressed the current, whereas the subsequent addition of 0.5 mM $CaCl_2$ at both sides of the membrane led to its pronounced stimulation. The addition of calcium ions at one side of the BLM also led to an increase in the BLM current, but the stimulation was 2.5-times less, as compared to the two-side addition of $CaCl_2$ (data not shown). Importantly, the addition of the dipole modifier phloretin [27] markedly diminished the UA-induced current, thereby indicating that the membrane conductance was limited by translocation of an anionic form of the carrier. As seen from Fig. 3B, the UA-induced current showed the quadratic dependence on the UA concentration in the range of low concentrations, which is also of importance for understanding the mechanism of UA protonophoric action. The stimulating effect of calcium ions increased with raising their concentration (Fig. 3C). These data could be ascribed to an ability of UA to form complexes with calcium, which could enhance its protonophoric activity.

According to [12], the current induced by UA across BLM is proton-selective. The fact that the BLM current is a quadratic function of the aqueous concentration of the protonophore (Fig. 3B) unambiguously shows that protons are transferred across BLM by UA dimers. Taking into account the phloretin-caused suppression of the current (Fig. 3A), it can be concluded that the charged permeant species is an anionic dimeric form of UA. Earlier, the key role of anionic dimers in the protonophoric activity was shown for some classical uncouplers, such as 2,4-dinitrophenol (DNP) and 4,5,6,7-tetrachloro-2-(trifluoromethyl)-benzimidazole (TTFB) [28–30]. Fig. 4 depicts a scheme of the protonophoric action of UA assuming protonation-deprotonation of UA dimers in the form of complexes with calcium ions. We surmise that calcium ions stabilize UA dimers, which explains the inhibiting effect of EDTA on the BLM current (Fig. 3A). Obviously, the calcium-dependent formation of UA dimers is disturbed by modification of any of hydroxyl groups in UA, as evidenced by the suppressed protonophoric activity of UA3, UA7 and UA9. Of note, to the best of our knowledge, calcium-dependent protonophoric activity has not been observed so far.

The calcium-chelating properties of UA were further studied by the method of metal extraction to a hydrophobic phase [26]. Fig. 5 shows that UA was much more effective in extracting calcium ions to the hydrophobic phase, than either of the analogs lacking a single hydroxyl group. Of note, the calcium-chelating capacity was found previously for conventional uncouplers, such as DNP and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [31].

The analogs also significantly differed from the parent UA in the

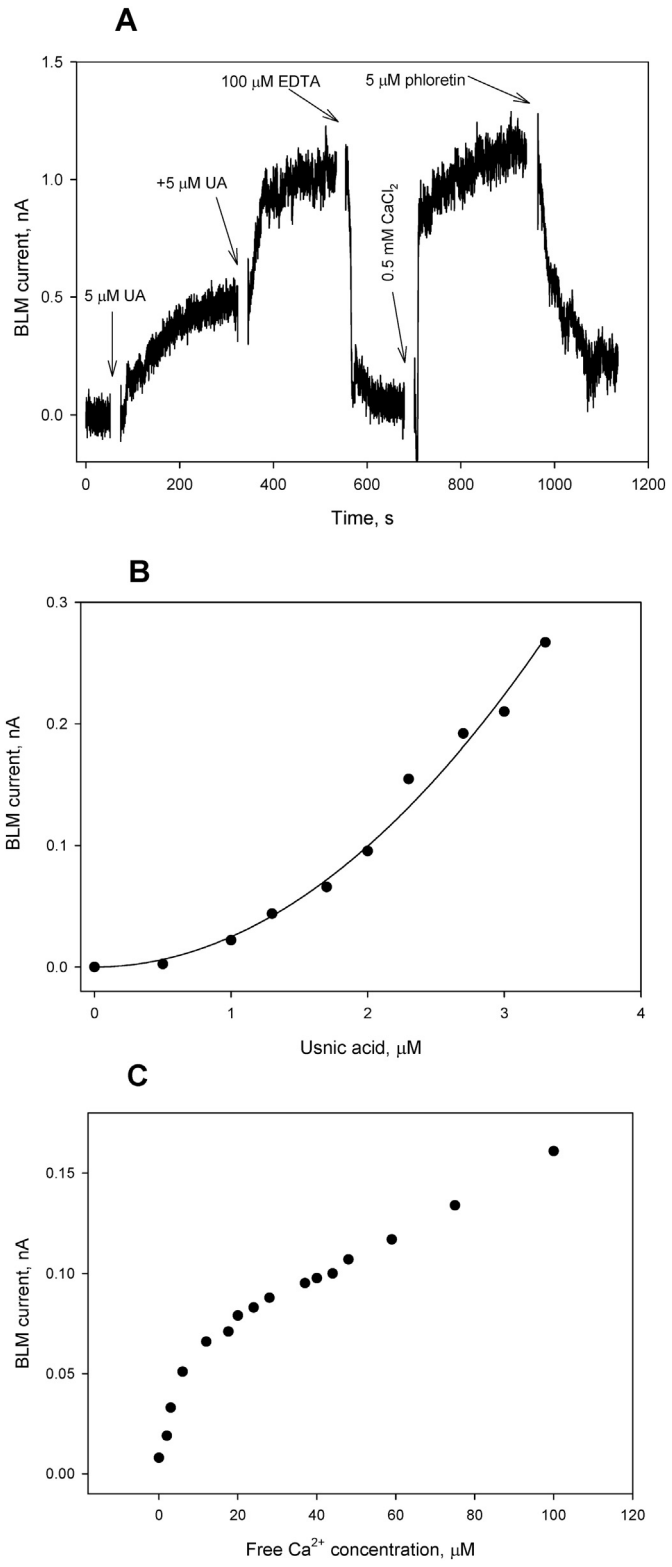

Fig. 3. A. Effect of calcium ions on the induction of electrical current through BLM by usnic acid. B. Dependence of electrical current through BLM on the concentration of usnic acid in the presence of 1 mM $CaCl_2$. The data were fitted by a quadratic equation $I = a * [UA]^2$ (black line). C. Dependence of UA-induced electrical current through BLM on the concentration of free calcium ions. The concentration of calcium ions was adjusted by variation of the concentration of nitrilotriacetic acid (NTA) and calcium ions. UA concentration was 3 μM. The solution was 10 mM Tris, 10 mM MES, 10 mM β-alanine, 10 mM KCl, pH 7.0. BLM voltage 50 mV. BLM was made from DPhPC.

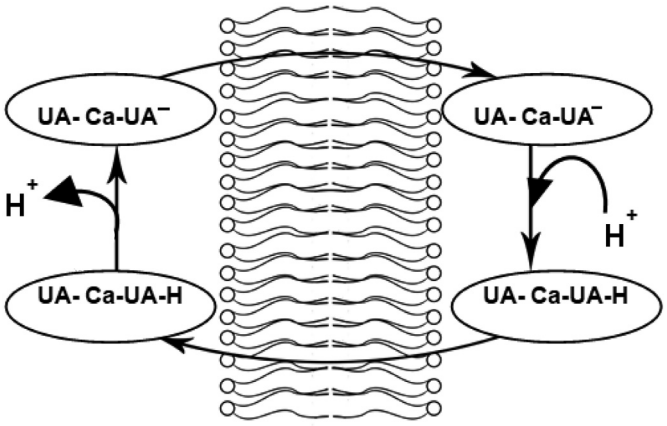

Fig. 4. A scheme of Ca^{2+}-dependent protonophoric action of UA on lipid membranes.

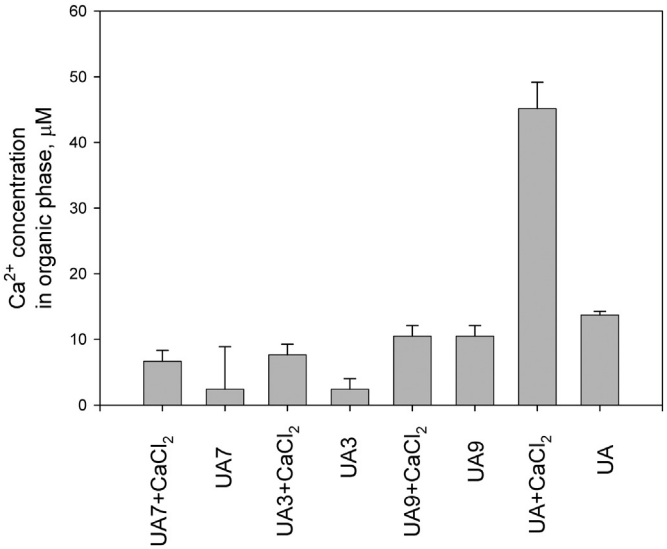

Fig. 5. Extraction of calcium ions from the aqueous phase into chloroform:butanol (3:1 v:v) by usnic acid and its analogs. The initial calcium ion concentration in the aqueous solution was 200 μM for columns marked $+CaCl_2$. The total concentration of UA and its analogs in the system was 200 μM. For other conditions see Materials and methods section. The data are represented as mean ± S.E with n = 3.

uncoupling action on rat liver mitochondria. Both UA7 and UA9 practically did not affect the mitochondrial respiration rate, whereas UA3 brought about acceleration of respiration at much higher concentrations, than UA (Fig. 6A). In line with these data, UA3 decreased the mitochondrial membrane potential much weaker, than UA, while the depolarizing effects of UA7 and UA9 were negligibly small (Fig. 6B). The data on mitochondrial swelling in the presence of potassium acetate showed that the protonophoric activity of all the analogs was substantially diminished, as compared to the parent UA (Fig. 6C).

Remarkably, the uncoupling effect of UA was markedly suppressed in the presence of the calcium ionophore A23187, as seen from measurements of both the mitochondrial respiration rate (Fig. 7A) and membrane potential (Fig. 7B). These observations could be explained by taking into account that mitochondria, usually having a high Ca^{2+} level in the matrix, can play a role of a Ca^{2+} accumulating *depot* in cells. The results shown in Fig. 7A, B indicated that mitochondrial depletion of calcium ions (upon the addition of A23187 in the presence of EDTA in the medium) diminished the uncoupling activity of UA. Based on the data obtained here, the stimulating impact of Ca^{2+} on protonophoric and uncoupling properties of UA provided evidence in favor of

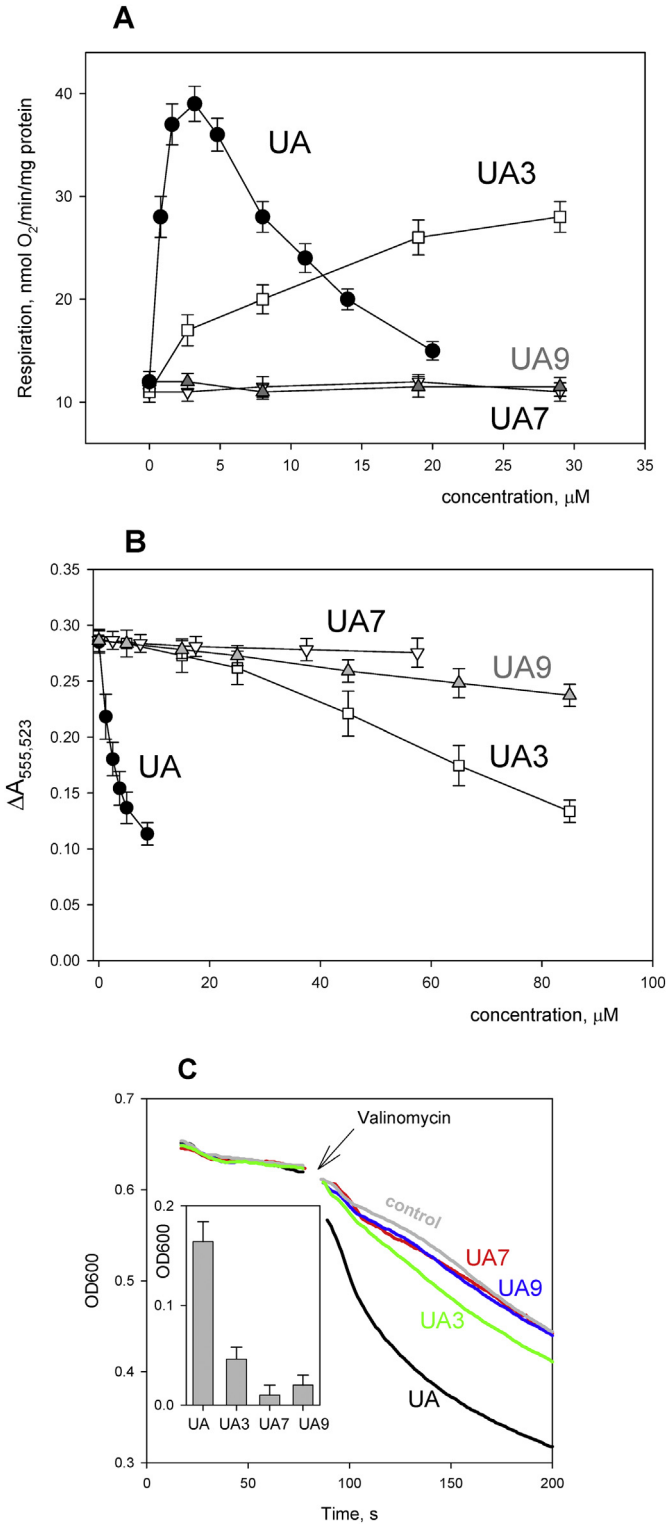

Fig. 6. Dose dependence of stimulation of the State 4 respiration (panel A) and a decrease in the membrane potential of rat liver mitochondria (panel B) by usnic acid and its analogs. Substrate: succinate. The membrane potential of mitochondria was estimated from a difference in the absorbance of the potential sensitive dye safranine O at 555 nm and 523 nm (panel B). Experimental conditions are described in "Materials and methods section". The data are represented as mean ± S.E with n = 3. C. Effect of usnic acid and its analogs at a concentration of 20 μM on the swelling of rat liver mitochondria in potassium acetate medium upon the addition of valinomycin (1 μM). Inset: OD600 values at 120 s after the addition of UA and its analogs (with respect to control swelling without UA, mean ± S.E., n = 5). Incubation mixture: 145 mM potassium acetate, 5 mM Tris, 0.2 mM EDTA, pH 7.4, 2 μM rotenone. Mitochondrial protein was 0.2 mg/ml.

involvement of UA calcium-chelating activity in the UA-mediated proton transfer. Alternatively, the enhancement of the uncoupling potency of UA by calcium ions could be associated with calcium-ionophoric properties of UA. Fig. 7C shows that 2.5 μM UA exerted a minor effect on Ca^{2+} efflux from mitochondria, as measured by fluorescence of the calcium indicator Fluo-5N, in contrast to maximal stimulation of respiration observed at this concentration of UA (Fig. 7A). It is seen from Fig. 7C that the addition of the conventional Ca^{2+} ionophore A23187 (0.1 μM) at the end of the experiment elicited a pronounced Fluo-5N response. To prevent Ca^{2+} efflux from mitochondria upon UA-induced mitochondrial depolarization, 0.5 μM Ruthenium Red, an inhibitor of mitochondrial Ca^{2+} uniporter, was supplemented here to the incubation medium (without EDTA) after mitochondria preloading with 50 μM $CaCl_2$, and 1 μM cyclosporin A inhibiting the induction of the mitochondrial permeability transition pore was also present in the medium. Based on the data presented in Fig. 7, it can be concluded that UA is a protonophore, in agreement with the data reported earlier [12].

Protonophoric uncouplers have long been known to inhibit the respiratory chain function at high concentrations, thus affecting respiration in a bell-shaped concentration manner [32,33], which is also observed for UA (Fig. 6A). Interestingly, in fully uncoupled mitochondria (in the presence of 2,4-dinitrophenol), all of the UA analogs studied here did not induce inhibition of respiration at high concentrations, in contrast to UA (data not shown), which is in line with the early data on TTFB, another classical uncoupler, where replacement of the only hydrogen atom by a methyl group suppressed both the uncoupling and the inhibiting effects of TTFB on mitochondrial respiration [33]. Earlier, the inhibiting effect of UA on mitochondrial respiration was found in [11].

Finally, we tested the effect UA and its three analogs on the growth and membrane potential of the bacteria *B. subtilis*. As seen in Fig. 8A, UA proved to be much more effective in inhibiting bacterial growth than either of its analogs, as measured by optical density at 620 nm. The addition of 100 μM UA completely suppressed the growth of *B. subtilis*, whereas UA7 and UA9 at the same concentration exerted only a slight delay in the bacterial growth. UA3 slightly affected the bacterial growth in the exponential phase, and moderately diminished the final optical density in the stationary phase. The fact that removal of deprotonatable groups by, e.g., replacing hydroxy with methoxy groups inhibited both the uncoupling and the antibacterial action of UA, in combination with early data on the reduced antimicrobial activity of diacetyl UA [6], strongly supported a significant contribution of the UA protonophoric activity to its antibacterial action.

Our preliminary experiments showed that UA interferes with fluorescence of the dye $DisC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide, conventionally used for evaluating bacterial membrane potential [34,35]. For this reason, to estimate the UA effect on the membrane potential of *B. subtilis*, we utilized the procedure of measuring accumulation of the potential-sensitive dye TMRE inside the cells, previously developed in our laboratory for isolated mitochondria [25]. This approach, being in principle similar to measuring dye accumulation in cells by flow cytometry, is more suitable for detecting submicrometer fluorescent particles due to higher sensitivity. According to our previous study [25], calculation of the number of peaks ($n(F > F_0)$) of the FCS signal with amplitudes higher than the certain value F_0 permits quantitative analysis of the binding of fluorescent molecules to dispersed particles, such as mitochondria and bacteria. As seen in Fig. 8B, C, the addition of UA led to a decrease in the number and intensity of TMRE fluorescence peaks, corresponding to single bacterial cells (red curve in Fig. 8B and circles in Fig. 8C), similar to the effect of

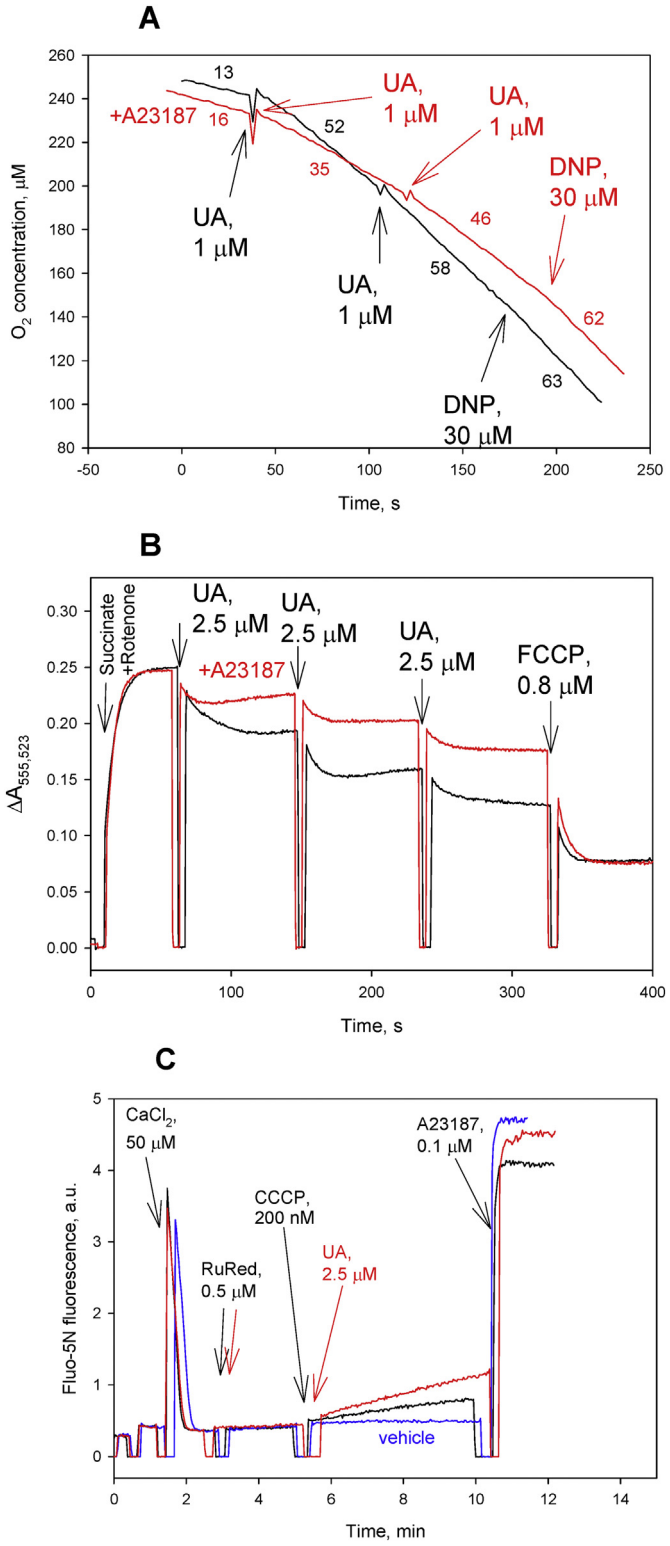

Fig. 7. A. Effect of the calcium ionophore A23187 on the stimulation of respiration of rat liver mitochondria by usnic acid. B. Effect of usnic acid on the membrane potential of mitochondria was suppressed in the presence of A23187. Red traces were recorded in the presence of 100 nM A23187. Experimental conditions are described in "Materials and methods section". C. Usnic acid did not induce efflux of calcium ions from pre-loaded mitochondria in the presence of Ruthenium Red (RuRed). Extramitochondrial Ca^{2+} was measured by Fluo-5N fluorescence. Incubation mixture: 250 mM sucrose, 10 mM Tris, 10 mM KH_2PO_4, pH 7.4, 1 mM succinate, 2 μM rotenone, 1 μM cyclosporin A. Fluo-5N 1 μM. Mitochondrial protein 0.5 mg/ml.

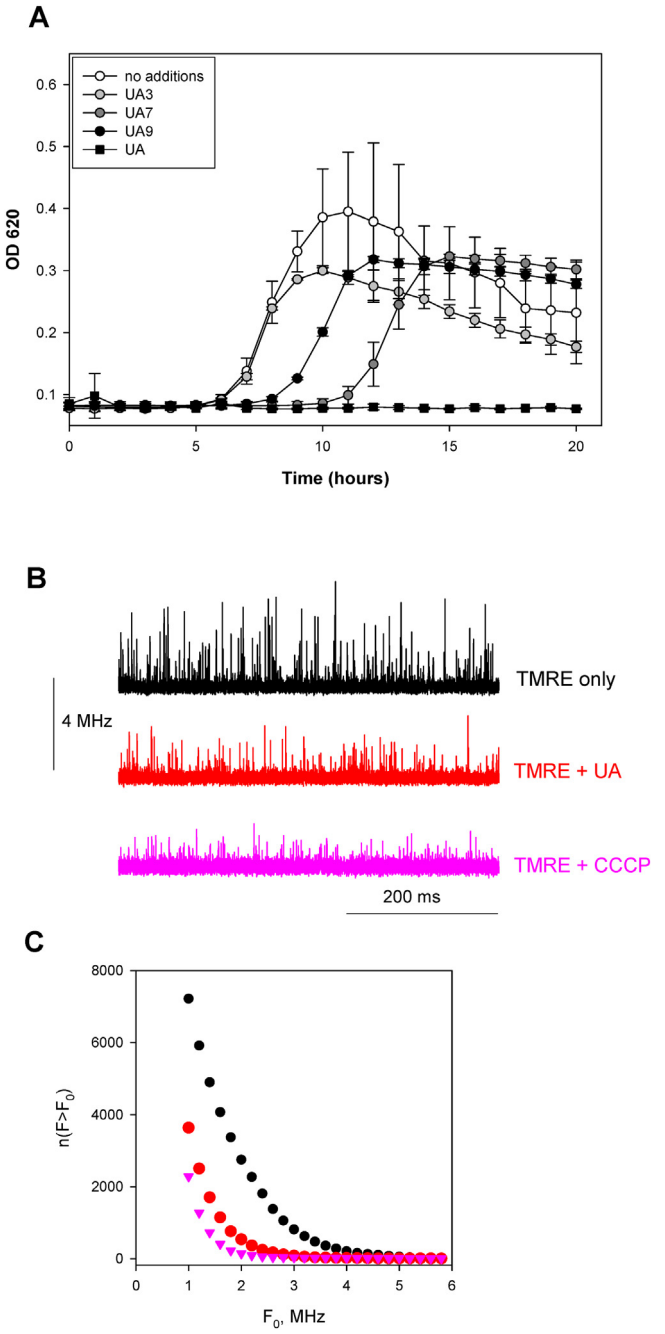

Fig. 8. A. Effect of usnic acid and its analogs (concentration 100 μM) on the growth of *Bacillus subtilis* cells. *B. subtilis* growth was evaluated from the absorbance at 620 nm at 37 °C with a 96-well Multiskan FC Microplate Reader. The data points represent mean ± SD of at least three experiments. B. Time-resolved count rates of tetramethylrhodamine ether ester (TMRE, 10 nM) fluorescence in the absence (black curve) and in the presence of 10 μM UA (red curve) or 1 μM CCCP (pink curve) measured with the FCS setup. The signal was recorded under stirring condition in PBS solution after 5 min incubation with *B. subtilis* cells (10^6 per ml of PBS). C. Corresponding dependences of the number of peaks exceeding F_0 ($n(F > F_0)$) on F_0.

the conventional protonophore CCCP (pink curve in Fig. 8B and triangles in Fig. 8C). Thus, UA actually caused dissipation of the bacterial membrane potential.

Taking into account the depolarizing effect of UA on mitochondrial membranes in cancer cell lines [36] together with the increasing evidence on the anticancer activity of various uncouplers [37,38], it should be noted that the uncoupling properties of UA could also

contribute to its long ago found antitumour activity [39].

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

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