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Characterization of the mitochondrial respiratory chain of *Leishmania tarentolae* promastigotes

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submitted by

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1. INTRODUCTION

With approximately one million new cases every year, *Leishmania* parasites play a critical role in the spread of the zoonotic disease leishmaniasis (1). The disease mainly occurs in tropical and subtropical regions, mostly affecting developing countries and their low-income populations (2). As a result of climate change and global warming, changes in the spreading and geographical expansion of the disease-carrying sandflies are noticeable, putting more and more regions at risk (1). Despite this development, leishmaniasis receives very little funding for research and is classified as a neglected tropical disease (2). There is an urgent need to develop more effective antileishmanial treatments as more than one billion people in nearly 90 countries worldwide are currently at risk of infection (3).

Three clinical forms of the disease can be distinguished: visceral, cutaneous and mucocutaneous leishmaniasis (4). Among these three types, visceral leishmaniasis is considered the most dangerous. Characteristic symptoms include irregular febrile episodes, weight loss, abdominal pain, diarrhoea, swollen lymph nodes, enlarged liver and spleen and severe anaemia. Without treatment, this type of leishmaniasis usually has a fatal outcome. Cutaneous leishmaniasis is characterized by the development of skin lesions, which can vary in size and evolve into large skin ulcers. Those ulcers can lead to scar formations and disfigurements that mainly affect exposed body parts such as face and hands. Mucocutaneous leishmaniasis makes itself noticeable through destructed mucous membranes, mostly affecting mouth, throat and larynx. The clinical presentation varies depending on the species of *Leishmania* involved. More than 30 different species are known. The main species responsible for visceral leishmaniasis are *Leishmania donovani*, *Leishmania infantum* and *Leishmania chagasi*. Pathogens of cutaneous leishmaniasis include *Leishmania tropica*, *Leishmania major*, *Leishmania aethiopica* and *Leishmania mexicana*. In contrast, infections with *Leishmania braziliensis* can cause mucocutaneous leishmaniasis (5).

Treatment of leishmaniasis is complicated due to drug-resistant strains (6) and the difference of clinical symptoms, depending on immune response and *Leishmania* species (7). There is no vaccination available for this disease and patients are usually treated with conventional chemotherapeutics (2). Along with pentavalent antimonials as first-line drugs, agents such as amphotericin B and pentamidine are often used for the chemotherapy of leishmaniasis (8). Although the disease can be treated by these conventional chemotherapeutics to a certain extent, factors such as high toxicity, severe side effects, drug resistance and costs, all emphasize the need to find new and effective drug treatments (9). For the development of

specific antileishmanial drugs, it is important to better understand the biological function and bioenergetic activities of *Leishmania* parasites (2).

Leishmania is a genus of obligate intracellular parasites, which can be transmitted by using sandflies as their vectors and belong to the family of *Trypanosomatidae* (10). Two developmental forms of *Leishmania* can be distinguished: promastigotes and amastigotes (Fig. 1). During the transmission, the flagellated extracellular form in the vector (promastigotes) transforms into the non-flagellated intracellular form in mammalian host macrophages (amastigotes) (11).

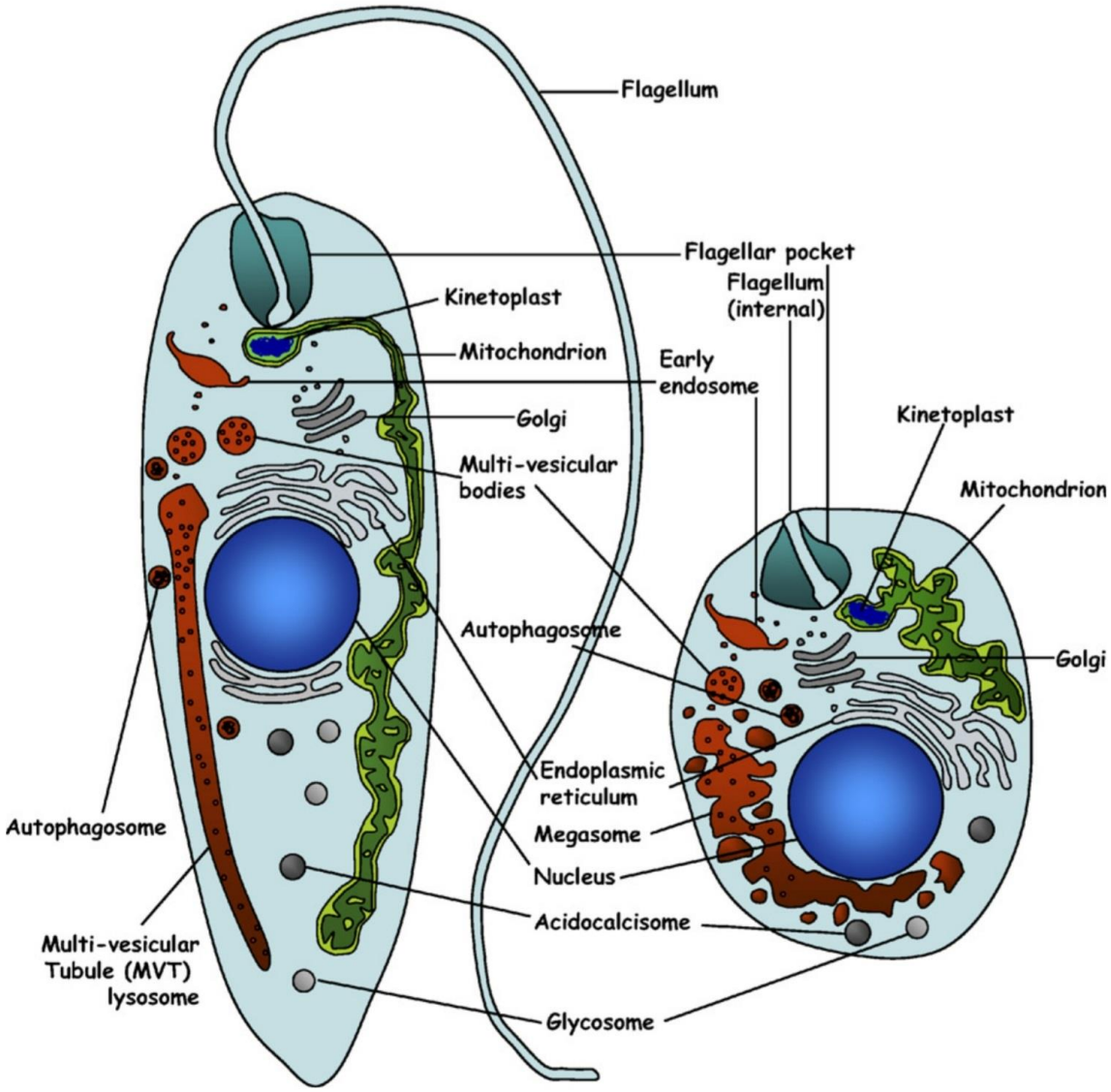

Figure 1 Main intracellular organelles from *Leishmania* promastigotes (left) or amastigotes (right) (11).

Normally, studying *Leishmania* is complicated by the need for special safety precautions due to their pathogenicity to humans, and not all laboratories are able to provide facilities with high biosafety levels. This difficulty does not apply to *Leishmania tarentolae*, that were used in the present study, as they only infect lizards and are non-pathogenic to humans. Therefore, *Leishmania tarentolae* are considered to be well suited for *in vitro* testing under biosafety level 1 conditions. The promastigote form of *Leishmania tarentolae* is particularly preferred as it is easily obtained *in vitro* (12).

Both developmental forms of *Leishmania* protozoa only have one mitochondrion per cell. This large mitochondrion occupies up to 12 % of the cell volume and provides the vast majority of the energy for *Leishmania*. In research, this fact can be exploited by focusing on this single organelle for the development of antileishmanial drugs. Drugs that interfere with the mitochondrial function of *Leishmania*, preferably with high selectivity in comparison to host cells, can lead to the desired cell death of the parasites. For this purpose, the inner mitochondrial membrane is particularly important, as this is where oxidative phosphorylation, leading to the generation of adenosine triphosphate (ATP), takes place in the four respiratory chain complexes and the additional ATP synthase complex (2).

Within this respiratory chain, a series of subsequent redox reactions takes place, ultimately providing energy for the production of ATP. Coenzymes reduced within the citric acid cycle, such as reduced nicotinamide adenine dinucleotide (NADH), donate electrons for the respiratory chain. At complex I (NADH:ubiquinone oxidoreductase) of the respiratory chain, NADH is oxidised to NAD^+. The electrons are then transferred to coenzyme Q (ubiquinone). Electrons are also transferred to coenzyme Q via complex II (succinate:ubiquinone oxidoreductase). In the further course of the respiratory chain, cytochrome c receives the electrons that have been transported by coenzyme Q through complex III (ubiquinol:cytochrome c oxidoreductase). At complex IV (cytochrome c oxidase), cytochrome c is oxidised and the electrons are used to reduce oxygen to water. A proton gradient is created across the inner mitochondrial membrane by the redox energy released by the respiratory chain. The protons, which then flow back through the proton channel of the F_OF_1-ATP synthase, cause the enzyme to produce ATP when adenosine diphosphate (ADP) is available (13). The end product, ATP, provides *Leishmania* parasites with the energy they need to survive (10).

Various substances have been shown to interfere with certain parts of the respiratory chain, leading to reduced oxygen consumption. Classical inhibitors (Fig. 2) used in this work include:

rotenone, malonate, antimycin A and cyanide. The presence of rotenone can have an inhibitory effect on complex I of the respiratory chain. Malonate inhibits complex II. Antimycin A is known to inhibit complex III and cyanide is a mitochondrial complex IV inhibitor (14). Some previous studies on *Leishmania* have confirmed the presence of mitochondrial respiratory complexes I-IV and their susceptibility to classical mitochondrial inhibitors (15,16). But some have failed to show inhibition with rotenone for example, raising questions about the presence and/or classic functional activity of complex I in *Leishmania* (10,14). Overall, in order to efficiently use the respiratory chain as a target in drug development, more information on the leishmanial respiratory chain and its molecular mechanisms is needed.

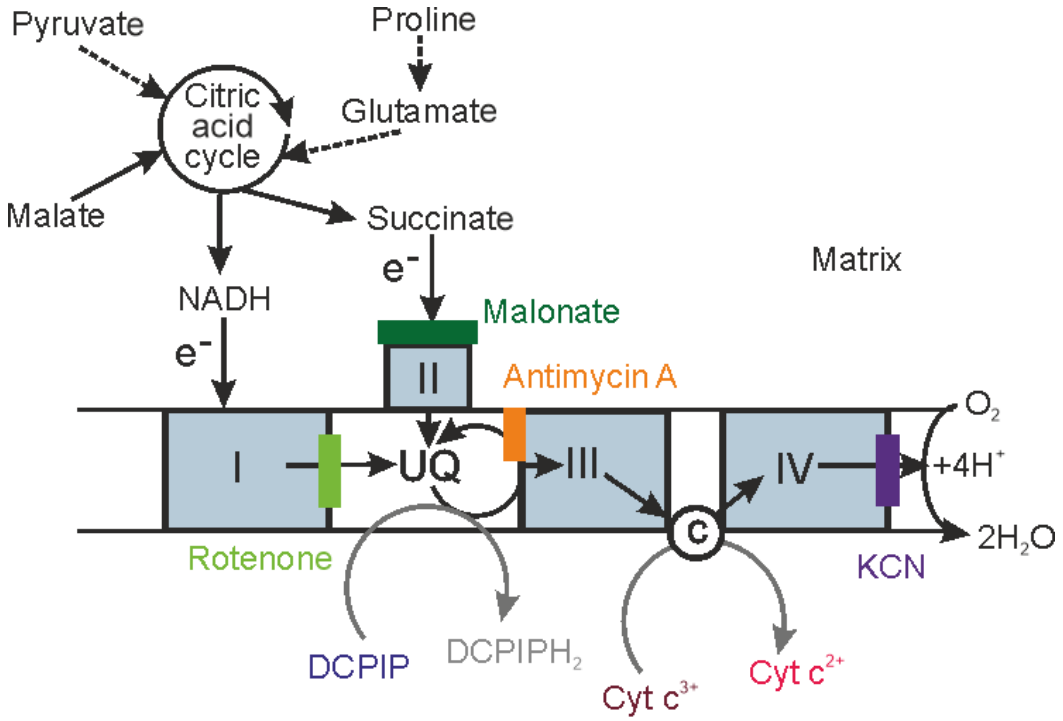

Figure 2 Mitochondrial respiratory chain with its classical respiratory substrates and inhibitors.

For such studies, the isolation of mitochondria is the most commonly used method. This method is complicated by several factors and cannot be always carried out successfully. The first hurdle is to disrupt the cells without damaging and affecting the mitochondrial membranes. In practice, this is very difficult because *Leishmania* only have one large mitochondrion per cell, which is highly branched and connected to the plasma membrane by microtubule networks. This makes isolating the mitochondria very difficult, as the mitochondria could be easily damaged under the usual harsh conditions (15). Another problem with mitochondrial

isolation is the relatively large number of cells required, as only a small percentage of mitochondria is recovered in the isolation process (17).

Given these difficulties, it would be advantageous to circumvent the problems of isolating mitochondria by using other techniques. One possible strategy is cell permeabilization. Non-ionic mild detergents, such as digitonin, will be used in this work to disrupt the leishmanial plasma membrane in such a way that different substrates and ADP are able to pass through the porous plasma membrane and reach the mitochondria. The addition of ADP in the context of oxygen consumption measurements allows to check whether the inner mitochondrial membrane is still intact and the permeabilised *Leishmania* are still capable of oxidative phosphorylation. Ideally, the detergents should be mild enough to ensure that the mitochondria in the cell suspensions are still intact after the permeabilization process. It is important to accurately determine the concentration of the detergent used, as too low concentrations would not sufficiently permeabilise the plasma membrane, whereas too high concentrations could lead to complete destruction of mitochondria (14,15,18).

Building on previous data on permeabilization of *Leishmania donovani* with digitonin, in this study we aimed to optimise the permeabilization of intact *Leishmania tarentolae* promastigotes (LtP) and create a good *in vitro* model for oxygen consumption measurements that would allow us to test the mitochondrial bioenergetic activities of the parasites. Oxygen consumption rates as well as activities of mitochondrial respiratory complexes of LtP will be measured in the presence of different respiratory substrates (succinate, glutamate/malate, proline, pyruvate, NADH) and mitochondrial inhibitors (14,19). The activities of mitochondrial respiratory complexes will be determined spectrophotometrically using artificial (e.g. 2,6-dichlorophenolindophenol; DCPIP) or natural electron acceptors (e.g. cytochrome c) (10).

This study therefore aims to:

1) optimise permeabilization of intact *Leishmania tarentolae* promastigotes as an *in vitro* model for studying mitochondrial function of *Leishmania*, avoiding the critical difficulties in the isolation of mitochondria,
2) investigate the mitochondrial oxidative phosphorylation of detergent-permeabilised LtP in the presence and absence of different mitochondrial respiratory substrates and mitochondrial inhibitors,
3) determine the activities of mitochondrial respiratory complexes in detergent-permeabilised LtP in the presence and absence of different mitochondrial inhibitors.

2. MATERIALS AND METHODS

2.1. Chemicals

The following chemicals were used to carry out the experiments.

Table 1 Used chemicals.

Chemicals	Purity	Manufacturer
Adenosine 5'-diphosphate (ADP), dihydrate, potassium salt	–	Boehringer Mannheim
Antimycin A from S*treptomyces sp.*	–	Sigma
Bovine serum albumin, fraction V	> 96 %	Fluka
Brain Heart Infusion Broth (No.53286)	for microbiology	Sigma-Aldrich
$CuSO_4$, pentahydrate	per analysis	Merck
Cytochrome c (Equine Heart)	–	Merck
2,6-Dichlorophenolindophenol (DCPIP), sodium salt	–	Sigma
Digitonin	–	Sigma
Dimethyl sulfoxide (DMSO)	> 99.8 %	VWR (prolabo chemicals)
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	per analysis	Merck
D-(+)-Glucose, monohydrate	for biochemical purposes	Merck
L-Glutamic acid	–	Merck
Haemin (porcine)	≥ 98 %	Sigma

KCl	per analysis	Merck
KCN	≥ 98 %	Sigma
K_2HPO_4	per analysis	Merck
KH_2PO_4	per analysis	Merck
KOH	per analysis	Merck
L-Malic acid	–	Sigma
Malonic acid	per synthesis	Merck
D-Mannitol	≥ 98 %	Sigma-Aldrich
$MgCl_2$, hexahydrate	per analysis	Merck
NaCl	per analysis	Merck
Na_2HPO_4	per analysis	Merck
NaOH	per analysis	Merck
β-Nicotinamide adenine dinucleotide reduced (NADH), disodium salt, hydrate	–	Sigma
Penicillin (20,000 U/ml)/streptomycin (20,000 µg/ml)	–	Lonza
Potassium iodide	per analysis	Merck
Potassium sodium tartrate, tetrahydrate	per analysis	Merck
DL-Proline	–	Sigma
Pyruvic acid, sodium salt	> 99 %	Sigma
Rotenone	–	Sigma

Sodium dithionite	per analysis	Merck
Succinic acid	> 99.5 %	Fluka
Trichloroacetic acid	per analysis	Merck
Triethanolamine hydrochloride (TEA)	per analysis	Fluka
Triton X-100	–	Sigma-Aldrich

Aqueous solutions were prepared using MQ-water from a Milli-Q® Advantage A10 water purification system (Merck Millipore, Darmstadt, Germany). Stock solutions of the following chemicals were made in DMSO: haemin (2.5 mg/ml), antimycin A (40 µM), rotenone (0.2 M; 0.1 M) and digitonin (5 mg/ml; 20 mg/ml).

2.2. Cultivation of *Leishmania tarentolae* promastigotes

All experiments were performed using *Leishmania tarentolae* promastigotes (LtP) that are non-pathogenic to humans (LEXSY host strain P10, biosafety level 1, Jena Bioscience GmbH, Jena, Germany).

A culture medium consisting of 37 g/l Brain Heart Infusion Broth, 5 mg/l haemin, 25,000 U/l penicillin and 25 mg/l streptomycin was prepared for the growth of the cells. The LtP suspension culture was cultured in sterile 50 ml polypropylene filter tubes (TubeSpin® Bioreactor 50, TPP, Trasadingen, Switzerland) and passaged three times a week (Monday, Wednesday and Friday). For the duration of the cultivation process, the tubes were placed on a shaker (0.05 s^{-1}) in an incubator (Ehret GmbH Life Science Solutions, Emmendingen, Germany) at around 26 °C.

2.3. Cell number determination of *Leishmania tarentolae* promastigotes

On each measurement day, the cell suspension was placed into a centrifuge (Sorvall LYNX 6000 centrifuge, Thermo Fisher GmbH, Vienna, Austria) for 10 min at 25 °C and 1900 × g. After the first centrifugation, the supernatant was removed while the LtP pellet remained at the bottom of the tube. Following the initial suspension volume, the pellet then was resuspended

in the same amount of mannitol medium (0.3 M mannitol, 20 mM TEA, 1 mM EGTA, 5 mM K_2HPO_4, pH 7.4). The now mannitol-medium-resuspended LtP were centrifuged a second time with the same technique and parameters. For experiments that required cells, which were resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), the same procedure was followed.

In order to determine the cell number, the optical density (OD) was measured at a wavelength of 600 nm using a U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan) and 1.5 ml semi-micro cuvettes (BRAND GmbH, Wertheim, Germany). For this purpose, 950 µl of the appropriate medium and 50 µl of the LtP was pipetted creating a diluted suspension in the cuvette. For reference cuvettes, only the medium was used. After measuring the cuvettes against the blank, the number of cells per ml of the undiluted LtP suspension was calculated using the following formula according to Fritsche (20):

$$\text{Cell number } (10^6 \text{ cells/ml}) = OD_{600\ nm} \times \text{dilution factor} \times 0.969 \times 124$$

0.969 … conversion factor of g/l dry weight

124 … 1 g dry weight/l corresponds to 124 × 10^6 cells/ml

The LtP suspension was then diluted to a cell number of approximately 2×10^8 LtP per ml medium to carry out the daily experiments. Aliquots of the mannitol-medium-washed or PBS-washed LtP suspensions (500 µl duplicates) were frozen at around -20 °C until later measurements of protein concentration.

2.4. Determination of protein concentration

The protein concentrations were determined by the Biuret method and used for eventually normalising oxygen consumption rates and spectrophotometric data.

500 µl MQ-H_2O and 200 µl of 3 M trichloroacetic acid were pipetted onto the thawed LtP suspensions (500 µl aliquots), which were then incubated at room temperature for about 10 min. After that, they were centrifuged in a Z 366 K centrifuge (Hermle, Wehingen, Germany) for 10 min at 25 °C and 2500 × g. The supernatants were discarded, leaving only the protein pellets behind. Then, 1000 µl of Biuret solution (12.02 mM $CuSO_4$, 31.89 mM potassium sodium tartrate, 30.12 mM potassium iodide and 0.2 M NaOH) was added to each tube. The dissolved pellets were incubated at room temperature for 10 minutes and were then decanted into 1.5 ml semi-micro cuvettes (BRAND GmbH, Wertheim, Germany). Afterwards, the

extinction of each sample was measured at a wavelength of 546 nm, using a U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan). MQ-H_2O served as a reference. Both the blank values of the Biuret solution and the sample values were determined as the difference of extinctions in the absence and presence of KCN. Using the tip of a spatula, KCN grains were added to the cuvettes with the aim of decolourising the blue copper-protein complexes and eliminating turbidity errors caused by interfering pigments (21). By subtracting the blank value from the sample value, the difference of extinction (ΔE) was calculated. The final protein concentrations of the samples were determined using following formulas (21):

$$\Delta E = (E_{sample-KCN} - E_{sample+KCN}) - (E_{blank-KCN} - E_{blank+KCN})$$

$$c = \frac{\Delta E}{\varepsilon \times d} \times V_f$$

c protein concentration in the sample [mg/ml]

ε 0.20666 $mg^{-1} \times ml \times cm^{-1}$ (extinction coefficient determined from a calibration curve using bovine serum albumin as a standard)

d layer thickness of the cuvette (1 cm)

V_f dilution factor (µl total volume/µl sample volume)

2.5. Oxygen consumption measurements

For measuring the oxygen consumption rates of LtP, a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, United Kingdom) was used consisting of a silver anode, a platinum cathode and a 50 % saturated KCl solution as electrolyte. The Clark-type oxygen electrode is covered by an oxygen-permeable polytetrafluoroethylene (PTFE) membrane. The PTFE membrane and the electrolyte had to be renewed on a regular basis to ensure unobstructed measurements. To guarantee an optimal measuring environment of 25 °C, a thermostat (mgw Lauda, Lauda-Königshofen, Germany) kept the oxygen electrode disc and the electrode chamber (DW1, Hansatech Instruments) at a constant temperature. While measuring, the oxygen-dependent current changes were registered under the use of a magnetic stirrer and were converted into voltage signals, using an oxygen electrode control box (CB1D, Hansatech Instruments). The received signals were recorded by a computer through an analog-digital converter and the MCREC software (provided by Prof. Lars Gille).

On each measurement day, the oxygen electrode was calibrated before usage (Fig. 3). For this purpose, 580 µl air-saturated MQ-water (oxygen concentration 256 µM O_2 at 25 °C) was pipetted into the electrode chamber. After the registration of the upper calibration value, a small amount of sodium dithionite ($Na_2S_2O_4$) was added which chemically removed oxygen from the electrode chamber. The chamber then was closed immediately with an air-tight acrylic glass plug for maintaining an oxygen concentration of 0 µM O_2. The just described calibration procedure was carried out a second time before continuing the daily experiments. Using the MCREC software, the upper and lower calibration values (256 µM O_2 and 0 µM O_2) were then selected during the evaluation of the recorded oxygen consumption rates.

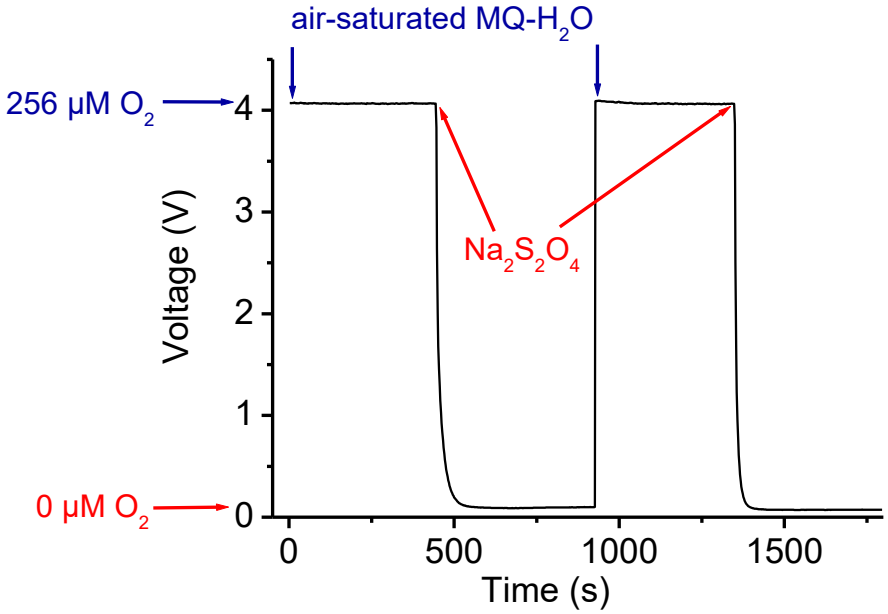

Figure 3 Representative calibration curve of the oxygen electrode.

2.6. Selection of resuspension medium and detergent

In order to determine the optimal resuspension medium for our experimental setup, the following tests were performed with PBS-washed and mannitol-medium-washed *Leishmania tarentolae* promastigotes. The LtP suspension was pipetted into the electrode chamber and was supplemented with additives. For PBS-washed LtP, the calcium chelator EGTA (1 mM[1]) and $MgCl_2$ (5 mM) were added. Since mannitol medium already contains EGTA, mannitol-

[1] All indicated following concentrations are given as final concentrations.

medium-washed LtP were only supplemented with $MgCl_2$ (5 mM). Then, the basal oxygen consumption of LtP was recorded for 5 minutes at 25 °C, after closing the electrode chamber with an air-tight plug. With the additional aim of determining the optimal detergent and detergent concentration for permeabilising LtP, either digitonin or Triton X-100 was added next. In particular, the following detergent concentrations were tested with the respective medium:

- Digitonin: 10 µg/ml, 25 µg/ml or 50 µg/ml
- Triton X-100: 0.005 %, 0.01 %, 0.015 % or 0.02 %

After incubation of LtP with the detergent (digitonin 10 minutes; Triton X-100 2 minutes), succinate (10 mM) was pipetted into the electrode chamber to stimulate the mitochondrial respiration. In order to stimulate oxidative phosphorylation, ADP (250 µM) was added 5 minutes later. After recording the oxygen consumption rates for another 5 minutes, cyanide (250 µM), an inhibitor of mitochondrial complex IV, was added.

2.7. Determination of O_2 consumption in LtP with different substrates and inhibitors

As described above, LtP were resuspended in mannitol medium (selected optimal resuspension medium) and were diluted to a concentration of around 2×10^8 LtP per ml for the execution of the experiments. The prepared LtP suspension was stored at around 25 °C throughout the experiments in order to reduce the amount of endogenous substrates.

The experimental procedure started with checking the metabolic condition of the cells by pipetting 568 µl LtP to the electrode chamber and feeding them with 10 mM glucose (11.6 µl of a 0.5 M stock solution) before closing it. After 5 minutes of recording the oxygen consumption, cyanide (250 µM) was added to inhibit the mitochondrial respiration. If no anomalies were registered, the tests could be continued.

Experiments for measuring the oxygen consumption rates and activities of mitochondrial respiratory complexes, were carried out in the presence of appropriate respiratory substrates (succinate, glutamate/malate, proline, pyruvate, NADH) and mitochondrial inhibitors (cyanide, antimycin A, malonate, rotenone).

All measurements were performed by pipetting a total of 580 µl LtP including 5 mM $MgCl_2$ into the electrode chamber, before closing it with an air-tight plug. The basal oxygen consumption was recorded for 5 minutes, after closing the electrode chamber. Next, digitonin (25 µg/ml) was added, which was previously determined to be optimal for permeabilising the cells. An

incubation of 10 minutes followed, ensuring the possible permeation of respiratory substrates and ADP through the plasma membrane of LtP. In the next step, one of the appropriate substrates was pipetted into the electrode chamber to stimulate the mitochondrial respiration: succinate (10 mM) or glutamate (5 mM)/malate (5 mM) or proline (10 mM) or pyruvate (10 mM) or NADH (1 mM). Additionally, ADP (250 µM) was added after 5 minutes to prove that the inner mitochondrial membrane was still intact and that the cells were still capable of oxidative phosphorylation. At the end, the sensitivity to one of the respective mitochondrial inhibitors was recorded for another 5 minutes: cyanide (250 µM, complex IV) or antimycin A (40 nM, complex III) or malonate (20 mM, complex II) or rotenone (100 µM, complex I).

2.8. Reduction of cytochrome c by LtP supplemented with different substrates and inhibitors

Cytochrome c is known to be a natural electron acceptor/donor in the mitochondrial respiratory chain, by taking electrons from complex III (ubiquinol:cytochrome c oxidoreductase) and shuttling them to complex IV (cytochrome c oxidase), where oxygen is reduced and cytochrome c is oxidised (13). The reduction of exogenously added cytochrome c can be used as a parameter for the activity of mitochondrial respiratory complexes. In the presence of KCN, an inhibitor of complex IV, the reoxidation of reduced cytochrome c by the cytochrome c oxidase is prevented, thereby avoiding an underestimation of the mitochondrial complex activities under aerobic conditions.

Time scans for the reduction of cytochrome c were monitored with a U-3300 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The measurements were performed in quartz cuvettes with 1 cm layer thickness at a wavelength of 550 nm (extinction coefficient for cytochrome c 21 mM^{-1} cm^{-1}), 2 nm slit width, under magnetic stirring and a constant temperature of 25 °C. Mannitol medium was pipetted into a reference cuvette and closed with a plug to minimise potential vaporisation of the medium over time.

Daily prepared LtP suspension (2×10^8 LtP per ml mannitol medium) was diluted to a concentration of 5×10^7 LtP per ml mannitol medium in the cuvette. After pipetting the detergent (either 10; 25; 50; 100 µg/ml digitonin or 0.005; 0.01; 0.015; 0.02 % Triton X-100), the LtP suspension was magnetically stirred in the spectrophotometer for the duration of the incubation period (10 min with digitonin; 2 min with Triton X-100). Then, the permeabilised LtP

were supplemented with $MgCl_2$ (5 mM) and KCN (1 mM). Following the addition of cytochrome c (50 µM), the monitoring started.

After 5 minutes of recording the time scan, an appropriate substrate was pipetted: 10 mM succinate, 10 mM proline or 5 mM glutamate/5 mM malate to stimulate cytochrome c reduction. Finally, one of the respective mitochondrial inhibitors was added and monitored for another 5 minutes: rotenone (100 µM, complex I), malonate (20 mM, complex II) or antimycin A (40 nM, complex III).

For measurements with NADH (1 mM) as a substrate, the same experimental setup was used with slight changes in the order of pipetting the additives: The respective inhibitor was added right after the LtP were permeabilised by the detergent. After pipetting the supplements $MgCl_2$ (5 mM) and KCN (1 mM), cytochrome c was added and recorded for 5 minutes. Then, NADH (1 mM) was pipetted and monitored for another 5 minutes.

2.9. Reduction of 2,6-dichlorophenolindophenol by LtP supplemented with different substrates and inhibitors

2,6-dichlorophenolindophenol (DCPIP) is a blue redox dye which decolourises when it is reduced to $DCPIPH_2$ (22). It can be used as an artificial electron acceptor for the determination of mitochondrial complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) activities (23).

Reduction of DCPIP (extinction coefficient 19.1 mM^{-1} cm^{-1}) was measured in quartz cuvettes with 1 cm layer thickness at a wavelength of 600 nm and 2 nm slit width using a U-3300 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Mannitol medium (1000 µl) was pipetted into the reference cuvette and closed with a plug.

5×10^7 LtP per ml mannitol medium were added to the measuring cuvette by diluting the daily prepared LtP suspension (2×10^8 LtP per ml mannitol medium). The detergent (10 µg/ml digitonin) was added next. The cuvette was then put into the spectrophotometer under magnetic stirring at 25 °C. After incubating LtP with digitonin for 10 minutes, permeabilised LtP were supplemented with $MgCl_2$ (5 mM) and KCN (1 mM). The addition of KCN was necessary to avoid reoxidation of reduced DCPIP by the respiratory chain. Scanning started after the addition of DCPIP (50 µM). 5 minutes later, one of the respective substrates was added: 10 mM succinate, 10 mM proline or 5 mM glutamate/5 mM malate. After another 5 minutes,

rotenone (100 µM, complex I inhibitor), malonate (20 mM, complex II inhibitor) or antimycin A (40 nM, complex III inhibitor) was pipetted into the cuvette in order to inhibit the reduction of DCPIP.

2.10. Data analysis and statistics

The oxygen consumption rates were recorded and evaluated using the MCREC software. Spectrophotometric data were recorded and evaluated using the UV Solutions 2.2 software (Hitachi Ltd., Tokyo, Japan). Further processing of the data and the statistical calculations were carried out using the software programme MicroCal Origin® 6.1 (OriginLab Corp., Northampton, MA, USA). Diagrams and figures were generated using MicroCal Origin® 6.1, with all data in bar diagrams being shown as means ± standard error of mean (SEM). To determine statistically significant differences between the respective experimental groups ($p < 0.05$, $p < 0.01$, $p < 0.001$), unpaired and paired two-tailed Student's t- tests were applied as appropriate. To determine statistically significant differences between one experimental group and a certain test mean ($p < 0.05$, $p < 0.01$, $p < 0.001$), the t-test for one population was used.

3. RESULTS

3.1. Selection of resuspension medium and detergent for the permeabilization of LtP

Figure 4 illustrates a representative oxygen consumption curve. The initial O_2 consumption was primarily caused by endogenous respiratory substrates, that were still available in the LtP suspension. A decrease of the initial oxygen consumption of LtP was observed 5 minutes after the addition of digitonin, making the plasma membranes porous and permeable for respiratory substrates and ADP. Even 10 minutes after the addition of digitonin the O_2 consumption did not completely cease, probably due to endogenous respiratory substrates still available in intact mitochondria.

A slight increase was seen after adding succinate to stimulate the mitochondrial respiration by shuttling electrons through complex II of the respiratory chain. Addition of ADP, to stimulate oxidative phosphorylation, resulted in a further increase. The almost horizontal line, after adding KCN, an inhibitor of mitochondrial complex IV, indicates that the observed substrate-stimulated O_2 consumption originates from the mitochondrial respiratory chain.

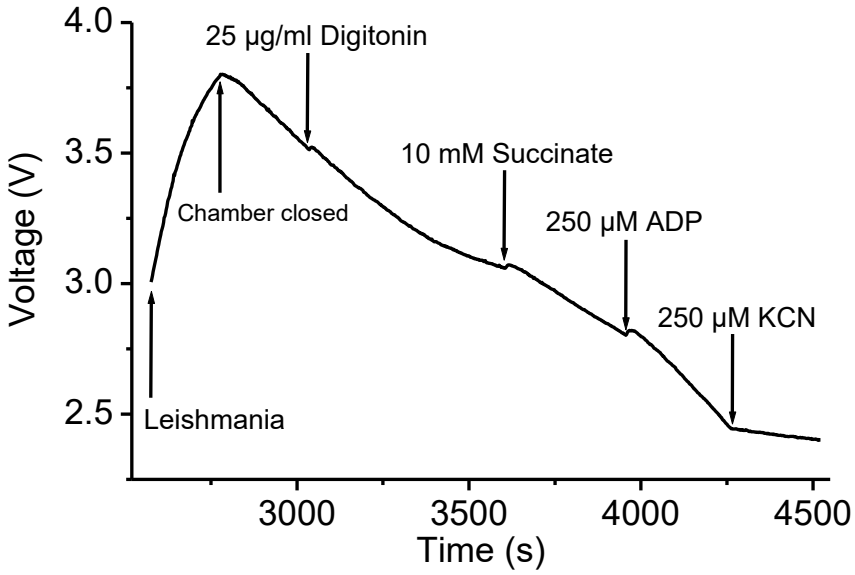

Figure 4 Representative oxygen consumption curve of digitonin-permeabilised *Leishmania tarentolae* promastigotes (2.02×10^8 LtP/ml = 0.6 mg protein/ml) in mannitol medium supplemented with 5 mM $MgCl_2$.

For the selection of the resuspension medium and the detergent for permeabilization of LtP, oxygen consumption rates in PBS and mannitol medium were compared (Fig. 5). As can be seen from this figure, the highest three Triton X-100 concentrations in PBS (Fig. 5C) and the highest Triton X-100 concentrations in mannitol medium (Fig. 5D) showed significant differences between O_2 consumption before and after adding the detergent. However, with Triton X-100, in both media, no statistically significant stimulation of oxygen consumption by both succinate and ADP seemed possible.

In LtP, permeabilised with the smallest digitonin concentration in PBS (Fig. 5A), no significant stimulation of the mitochondrial respiration was detected through the addition of succinate. Although stimulation of O_2 consumption was possible by succinate in LtP, permeabilised with the highest digitonin concentration, LtP appeared to be unaffected by the further addition of ADP. LtP, permeabilised with 25 µg/ml digitonin in PBS, showed both stimulation with succinate and ADP and could be inhibited with KCN.

The data in Fig. 5B indicate that an increase of mitochondrial respiration by succinate and a stimulation of oxidative phosphorylation by ADP, was possible with all three digitonin concentrations in mannitol medium.

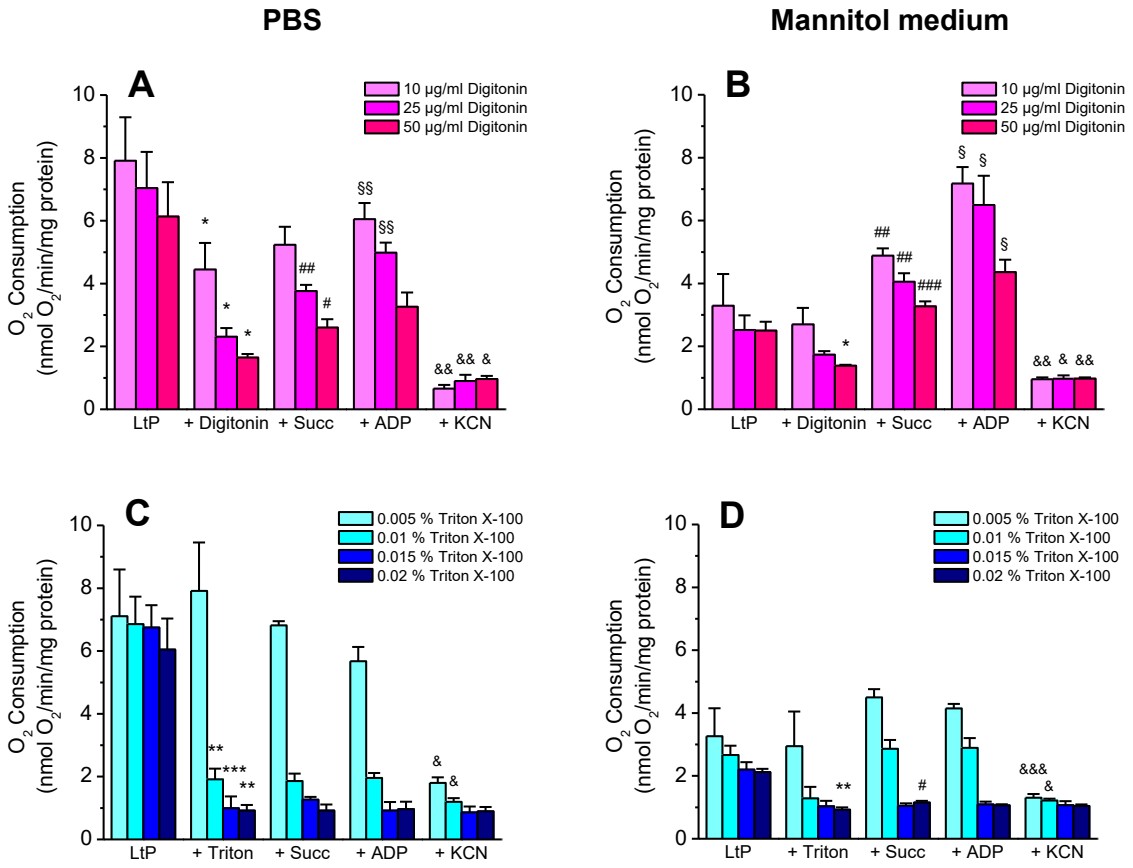

Figure 5 Oxygen consumption rates of *Leishmania tarentolae* promastigotes (LtP) in PBS (**A**, **C**; 2.015 ± 0.025 × 10^8 LtP/ml = 0.407 ± 0.001 mg protein/ml; n = 4) or mannitol medium (**B**, **D**; 1.915 ± 0.066 × 10^8 LtP/ml = 0.663 ± 0.015 mg protein/ml; n = 4) supplemented with 5 mM $MgCl_2$. LtP were permeabilised either with digitonin (**A**, **B**) or Triton X-100 (**C**, **D**). Afterwards, oxygen consumption was stimulated with 10 mM succinate (Succ), 250 µM ADP and subsequently inhibited with 250 µM KCN. Data represent means ± SEM. *, #, §, &; **, ##, §§, &&; ***, ###, &&& indicate significant differences at the level of $p < 0.05$, 0.01 and 0.001 (paired t-test) between corresponding measurements at the same detergent concentration: * Digitonin or Triton X-100 vs. LtP; # Succ vs. digitonin or Triton X-100; § ADP vs. Succ; & KCN vs. ADP.

From the measured O_2 consumption rates, respiratory control values of permeabilised LtP were calculated as the ratio of O_2 consumption after the addition of ADP to O_2 consumption in the presence of respiratory substrate (e.g. succinate) but in the absence of ADP. Higher

respiratory control values are indicative of a higher coupling of the mitochondrial respiratory chain to ATP synthesis, i.e. oxidative phosphorylation, in the permeabilised LtP.

As shown in Fig. 6A, the highest respiratory control values were observed in LtP, which were permeabilised with digitonin (25 µg/ml) in mannitol medium.

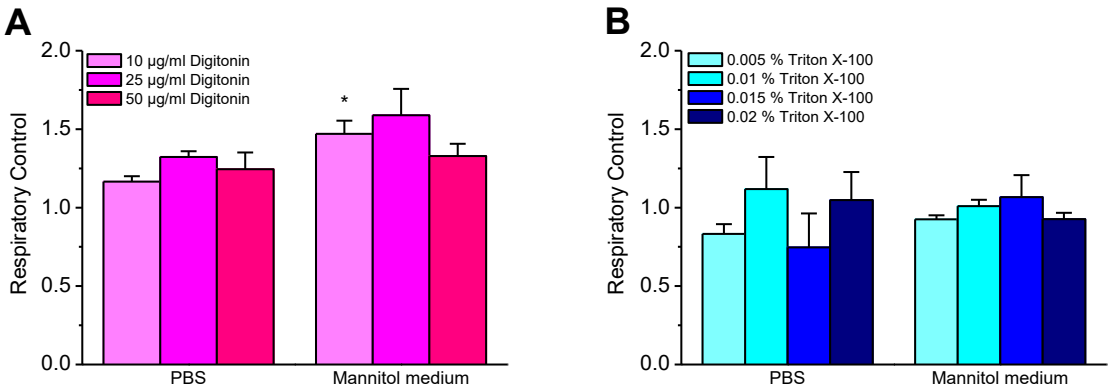

Figure 6 Respiratory control values of *Leishmania tarentolae* promastigotes (LtP) in PBS ($2.015 \pm 0.025 \times 10^8$ LtP/ml = 0.407 ± 0.001 mg protein/ml; n = 4) or mannitol medium ($1.915 \pm 0.066 \times 10^8$ LtP/ml = 0.663 ± 0.015 mg protein/ml; n = 4) supplemented with 5 mM $MgCl_2$. LtP were permeabilised either with digitonin (**A**) or Triton X-100 (**B**) and supplemented with 10 mM succinate followed by the addition of 250 µM ADP. Data represent means ± SEM. * indicates significant difference at the level of $p < 0.05$ (unpaired t-test) between corresponding measurements in PBS and mannitol medium at the same digitonin concentration.

The percentage of inhibition of O_2 consumption by the respective mitochondrial inhibitor (e.g. KCN) was calculated by subtracting the residual percental activity after the addition of the mitochondrial inhibitor from O_2 consumption before its addition which was set to 100 %.

As shown in Fig. 7, the inhibition of O_2 consumption by KCN is higher in digitonin-permeabilised LtP (Fig. 7A) than in LtP, which were permeabilised with Triton X-100 (Fig. 7B). However, in LtP permeabilised with 50 µg/ml digitonin a comparable inhibition of O_2 consumption by KCN was observed as with LtP permeabilised with 0.005 % Triton X-100.

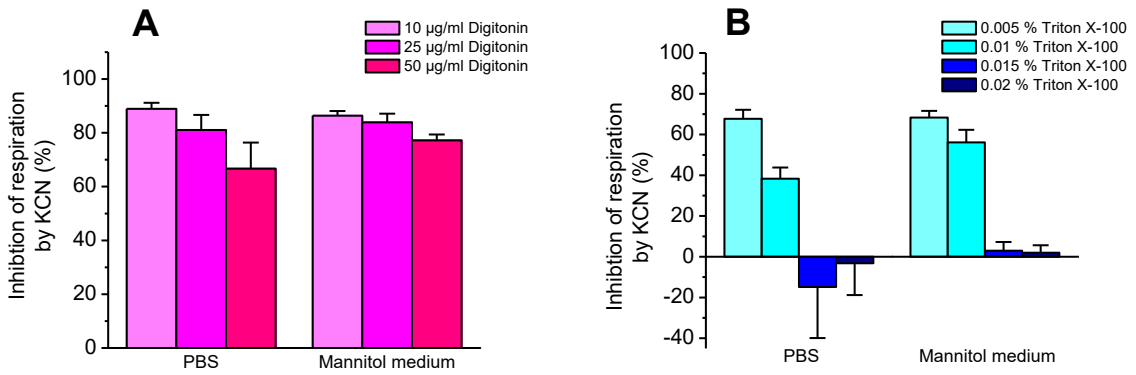

Figure 7 Inhibition of oxygen consumption of *Leishmania tarentolae* promastigotes (LtP) by KCN in PBS ($2.015 \pm 0.025 \times 10^8$ LtP/ml = 0.407 ± 0.001 mg protein/ml; n = 4) or mannitol medium ($1.915 \pm 0.066 \times 10^8$ LtP/ml = 0.663 ± 0.015 mg protein/ml; n = 4) supplemented with 5 mM $MgCl_2$. LtP were permeabilised either with digitonin (**A**) or Triton X-100 (**B**) and supplemented with 10 mM succinate, 250 µM ADP followed by the addition of 250 µM KCN. Data represent means ± SEM.

Taken together, these results suggested that mannitol medium and the detergent digitonin were optimal for further experimental setups.

3.2. Oxygen consumption of digitonin-permeabilised LtP with different substrates

Figure 8 illustrates O_2 consumption rates and respiratory control values in the presence of different respiratory substrates and the inhibition of respiration by KCN. A significant stimulation of the mitochondrial respiration was observed with all substrates, except NADH (Fig. 8A). However, only LtP, that were stimulated with succinate and glutamate/malate, showed significantly increased oxygen consumption rates after the addition of ADP (Fig. 8A). Comparing the respiratory control values in Fig. 8B, it can be seen that significant effects were only present when succinate, glutamate/malate or proline were used. Inhibition of respiration by KCN was also highest with these three substrates (Fig. 8C).

Because of these results, it was decided to use succinate, glutamate/malate and proline as respiratory substrates for following analysis of different mitochondrial inhibitors.

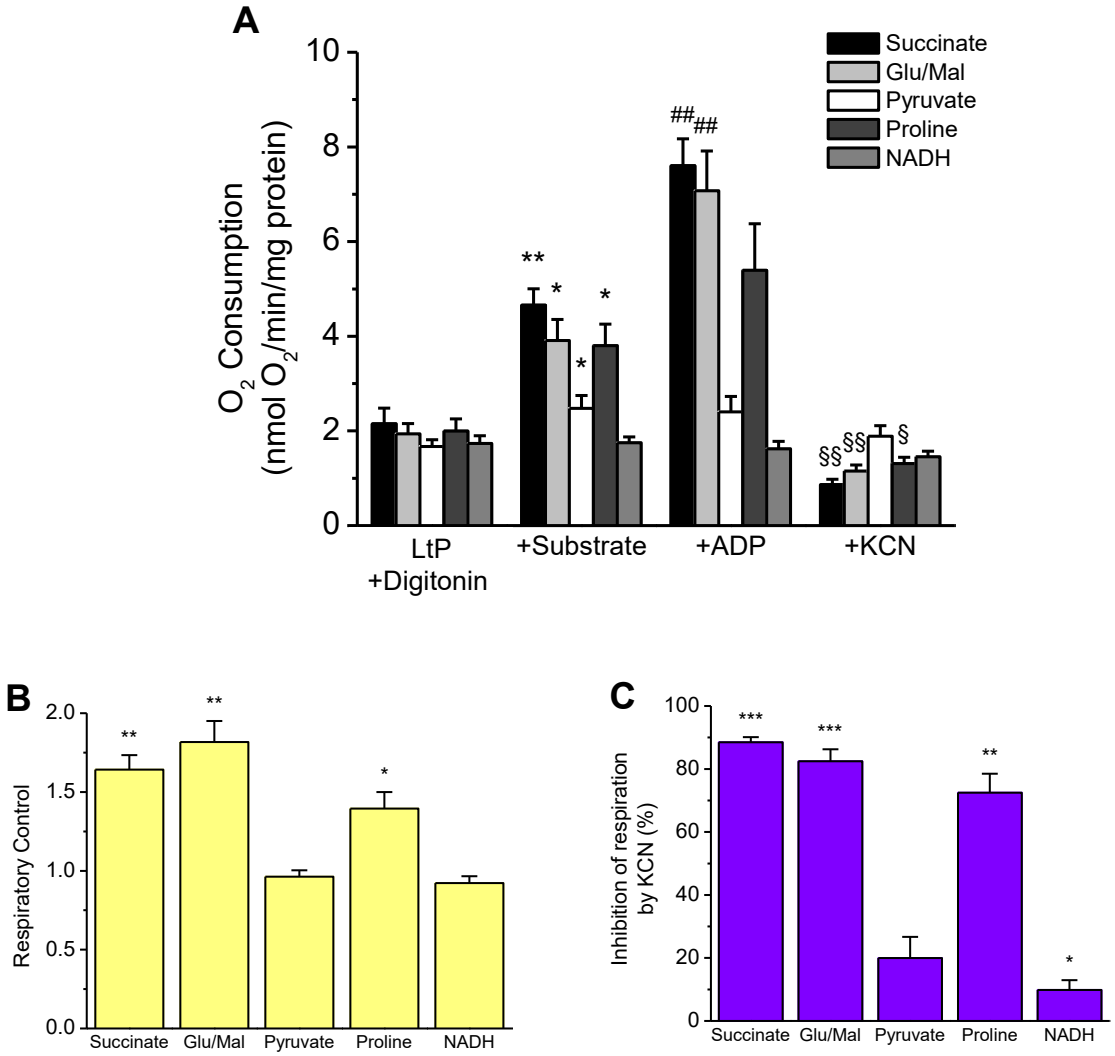

Figure 8 Oxygen consumption rates (**A**), respiratory control values (**B**) and inhibition of respiration by KCN (**C**) of *Leishmania tarentolae* promastigotes ($2.008 \pm 0.038 \times 10^8$ LtP/ml = 0.730 ± 0.047 mg protein/ml; n = 4) in mannitol medium supplemented with 5 mM $MgCl_2$. LtP were permeabilised with 25 µg digitonin/ml. Afterwards, 10 mM succinate, 5 mM glutamate/5 mM malate (Glu/Mal), 10 mM pyruvate, 10 mM proline or 1 mM NADH were added. Oxygen consumption was stimulated with 250 µM ADP and consequently inhibited with 250 µM KCN. Data represent means ± SEM.

*, §; **, ##, §§ indicate significant differences at the level of $p < 0.05$ and 0.01 (paired t-test; **A**) between corresponding measurements with the same respiratory substrate: * Substrate vs. LtP + digitonin; # ADP vs. substrate; § KCN vs. ADP. *, **, *** indicate significant differences at the level of $p < 0.05$, 0.01 and 0.001 (one population t-test, **B**: vs. a respiratory control value of 1.0; **C**: vs. 0 % inhibition of respiration).

3.3. Oxygen consumption of digitonin-permeabilised LtP with different substrates and inhibitors

The results obtained from the analysis of mitochondrial inhibitors in the presence of the respective respiratory substrates are summarised in Fig. 9 and Fig. 10.

As confirmed by the previous results (Fig. 8A, 8B), a stimulation of the mitochondrial respiration by all three tested substrates and further stimulation of O_2 consumption by ADP could be achieved (Fig. 9A, 9B and 9C). As expected, the respective respiratory control values were high as well (Fig. 9D).

The results in Fig. 9 already indicate that rotenone hardly had any inhibitory effect on mitochondrial respiration of LtP with all tested substrates, as the O_2 consumption rates were still high in the presence of 100 µM of rotenone. Compared to the percentage of respiratory inhibition (Fig. 10A), it is apparent that the respective substrate-stimulated LtP appeared to be unaffected by rotenone. In contrast, high percentages of inhibition could be reached by addition of antimycin A in all tested substrate-stimulated LtP (Fig. 10C). The highest inhibition of over 90 % was seen with malonate in prior succinate-stimulated LtP, followed by glutamate/malate- and proline-stimulated LtP (Fig. 10B).

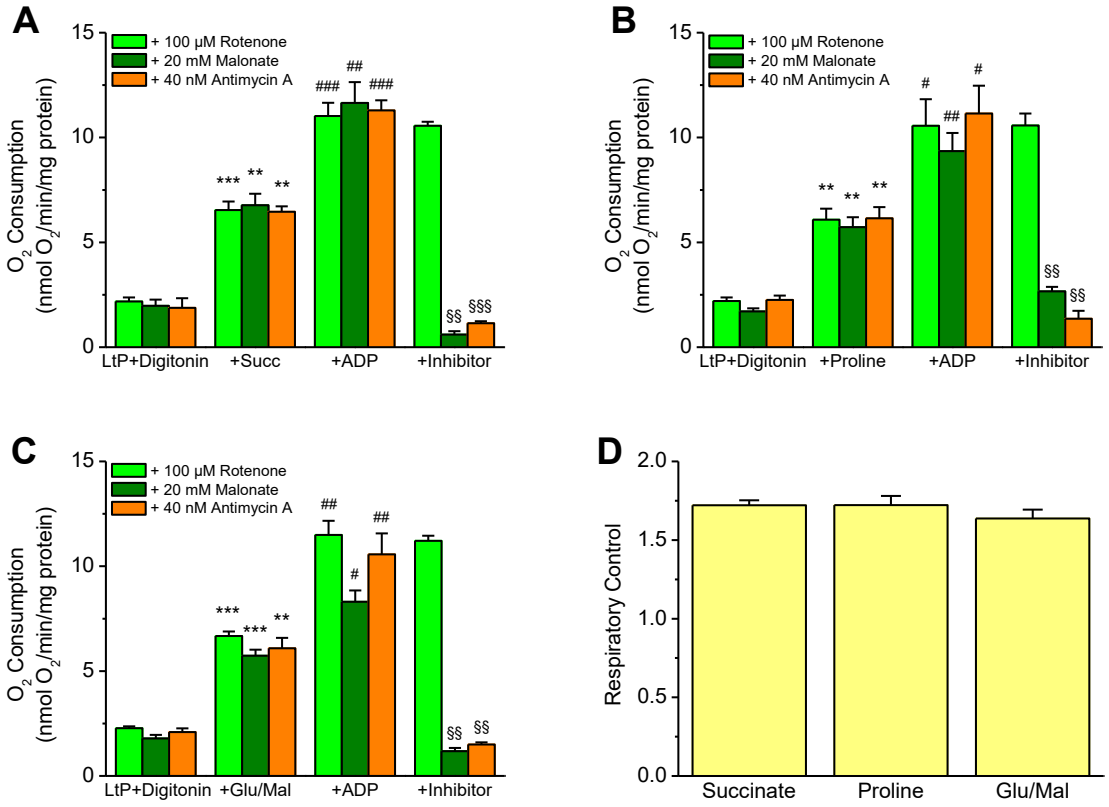

Figure 9 Oxygen consumption rates (**A**, **B**, **C**) and respiratory control values (**D**) of *Leishmania tarentolae* promastigotes (2.035 ± 0.059 × 10^8 LtP/ml = 0.610 ± 0.008 mg protein/ml; n = 4) in mannitol medium supplemented with 5 mM $MgCl_2$. LtP were permeabilised with 25 µg digitonin/ml followed by the addition of 10 mM succinate (Succ; **A**), 10 mM proline (**B**) or 5 mM glutamate/5 mM malate (Glu/Mal; **C**). Afterwards, oxygen consumption was stimulated with 250 µM ADP and subsequently inhibited with rotenone, malonate or antimycin A. Data represent means ± SEM. #; **, ##, §§; ***, ###, §§§ indicate significant differences at the level of $p < 0.05$, 0.01 and 0.001 (paired t-test; **A – C**) between corresponding measurements with the same inhibitor: * Substrate (Succ, proline, Glu/Mal) vs. LtP + digitonin; # ADP vs. substrate; § inhibitor vs. ADP.

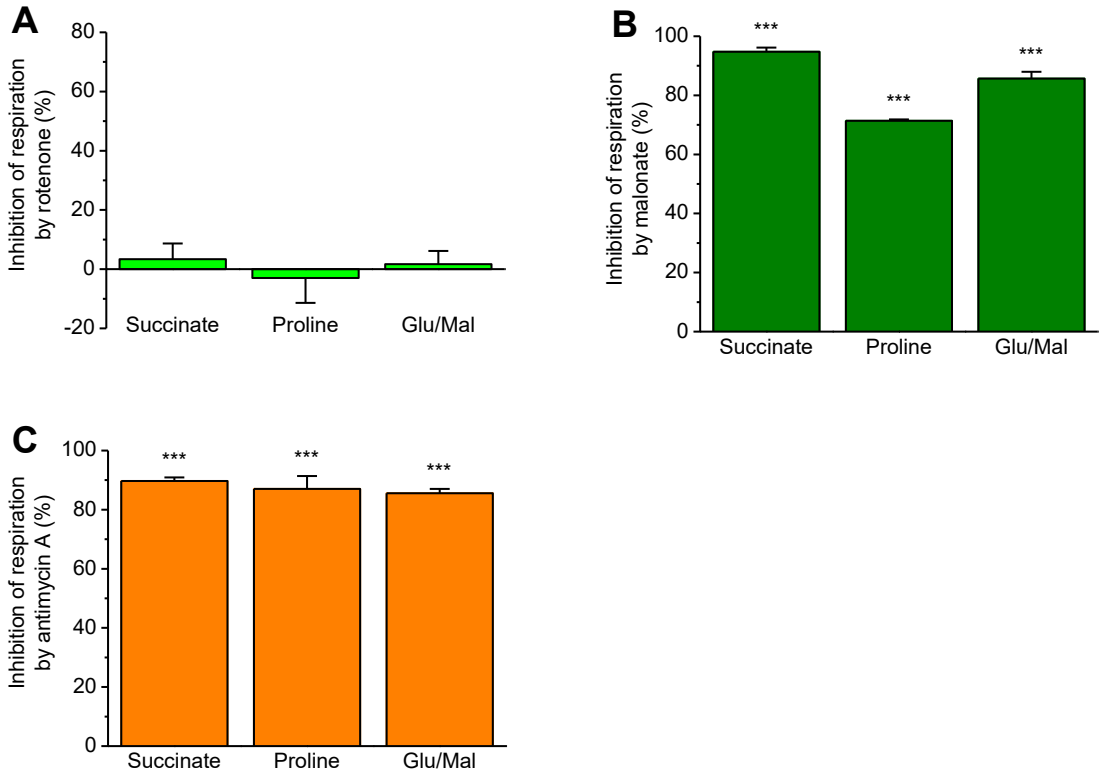

Figure 10 Inhibition of respiration of *Leishmania tarentolae* promastigotes ($2.035 \pm 0.059 \times 10^8$ LtP/ml = 0.610 ± 0.008 mg protein/ml; n = 4) in mannitol medium supplemented with 5 mM $MgCl_2$. LtP were permeabilised with 25 µg digitonin/ml followed by the addition of 10 mM succinate, 10 mM proline or 5 mM glutamate/5 mM malate (Glu/Mal). Finally, oxygen consumption was inhibited with 100 µM rotenone (**A**), 20 mM malonate (**B**) or 40 nM antimycin A (**C**). Data represent means ± SEM. *** indicate significant differences at the level of $p < 0.001$ (one population t-test vs. 0 % inhibition of respiration).

3.4. Reduction of cytochrome c by LtP supplemented with different substrates and inhibitors

As can be seen from the spectra in Fig. 11A, reduced cytochrome c shows an absorption peak at 550 nm. In Fig. 11B a representative time scan of cytochrome c reduction by digitonin-permeabilised LtP can be seen at a measured wavelength of 550 nm. As shown in the time scan (Fig. 11B), cytochrome c reduction could be stimulated with the addition of succinate and successfully inhibited with malonate afterwards.

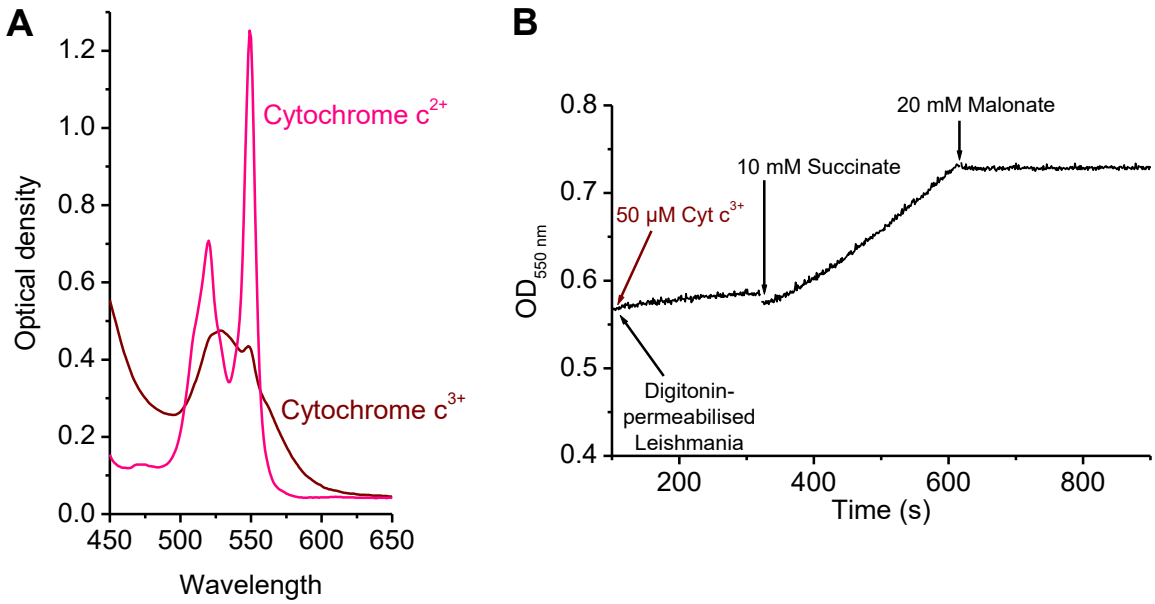

Figure 11 A) Spectra of oxidised cytochrome c (c^{3+}, 50 µM) and cytochrome c reduced by sodium dithionite (c^{2+}, 50 µM) in mannitol medium. **B)** Representative time scan of cytochrome c reduction by *Leishmania tarentolae* promastigotes (5×10^7 LtP/ml = 0.161 mg protein/ml) permeabilised by digitonin (100 µg/ml) in mannitol medium supplemented with 5 mM $MgCl_2$ and 1 mM KCN.

Figure 12 illustrates the reduction of cytochrome c by succinate-consuming LtP that were permeabilised with different concentrations of either digitonin (Fig. 12A) or Triton X-100 (Fig. 12B). The highest significant stimulation of cytochrome c reduction was achieved with LtP, which were treated with 100 µg/ml digitonin beforehand (Fig. 12A). The results suggested that for these spectrophotometric measurements, digitonin with a concentration of 100 µg/ml was optimal for further analysis with different substrates and inhibitors.

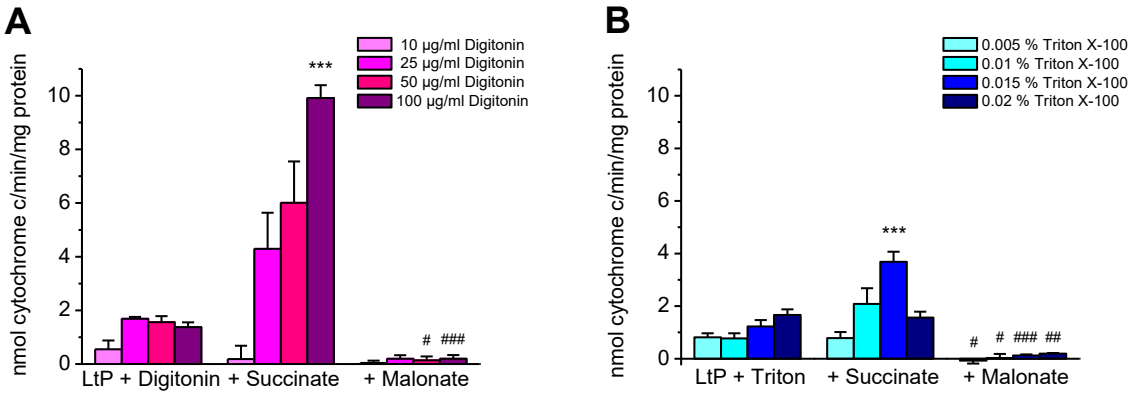

Figure 12 Reduction of cytochrome c (50 µM) by *Leishmania tarentolae* promastigotes (5×10^7 LtP/ml = 0.171 ± 0.007 mg protein/ml; n = 5) in mannitol medium supplemented with 5 mM $MgCl_2$ and 1 mM KCN. LtP were permeabilised either with digitonin (**A**) or Triton X-100 (**B**). Cytochrome c reduction was stimulated with 10 mM succinate and subsequently inhibited with 20 mM malonate. Data represent means ± SEM of four independent experiments, each. #; ##; ***, ### indicate significant differences at the level of $p < 0.05$, 0.01 and 0.001 (paired t-test) between corresponding measurements at the same detergent concentration: * Succinate vs. LtP + digitonin or Triton X-100; # malonate vs. succinate.

While the addition of glutamate/malate seemed to not have any stimulatory effect (Fig. 13C), reduction of cytochrome c could be stimulated with the substrates succinate (Fig. 13A), proline (Fig. 13B) and NADH (Fig. 13D).

Rotenone did not show any inhibitory effect on the cytochrome c reduction neither in glutamate/malate-supplemented LtP (Fig. 13C; Fig. 14A), succinate-supplemented LtP (Fig. 13A; Fig. 14A) nor in NADH-supplemented LtP (Fig. 13D; Fig. 14A). Although analysis indicates significant differences with proline-consuming LtP, overall only a small percentage of inhibition (~20 %) could be observed (Fig. 13B; Fig. 14A).

Antimycin A showed a high inhibition of cytochrome c reduction in succinate- and proline-stimulated LtP, but not in LtP that were supplemented with glutamate/malate or NADH (Fig. 14C).

The highest inhibition of cytochrome c reduction was observed in succinate-consuming cells after the addition of malonate (Fig. 14B). Cytochrome c reduction by glutamate/malate- and proline-stimulated LtP could be significantly inhibited by malonate as well (Fig. 14B).

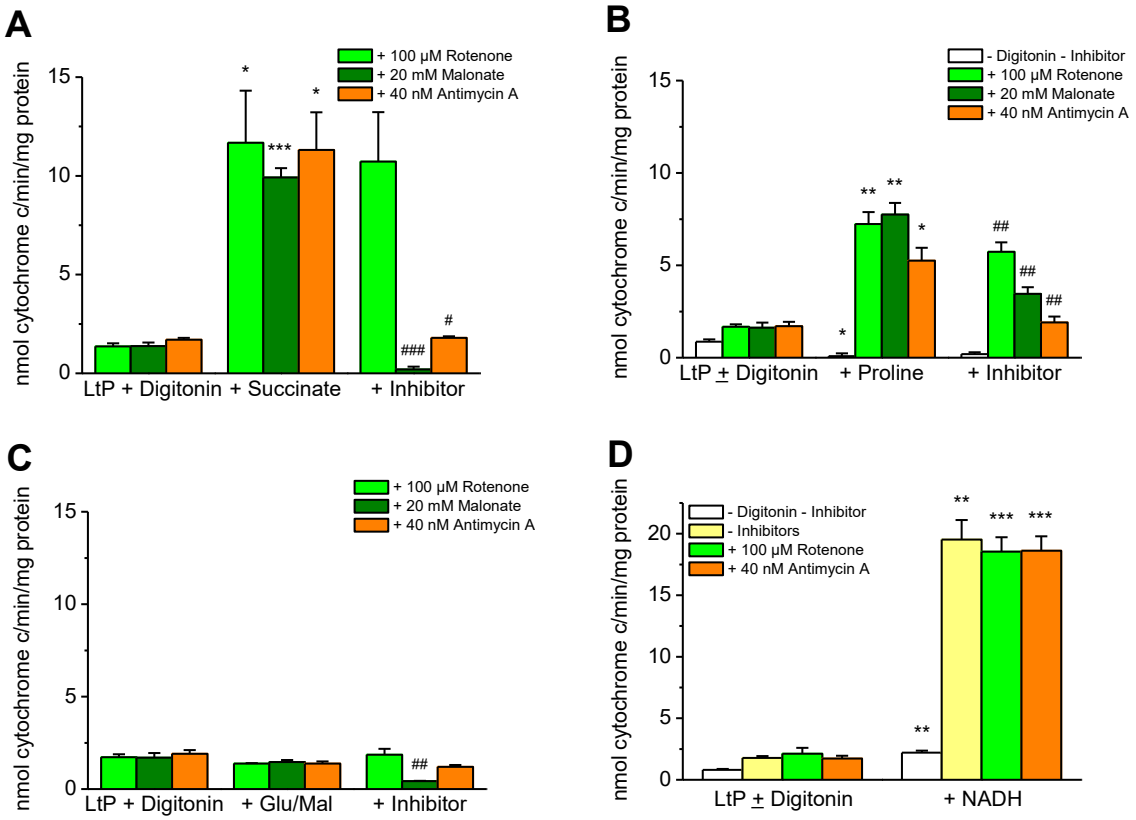

Figure 13 Reduction of cytochrome c (50 µM) by *Leishmania tarentolae* promastigotes (5×10^7 LtP/ml = 0.158 ± 0.004 mg protein/ml; n = 11) in mannitol medium supplemented with 5 mM $MgCl_2$ and 1 mM KCN. LtP were permeabilised with 100 µg digitonin/ml followed by the addition of 10 mM succinate (**A**), 10 mM proline (**B**), 5 mM glutamate/5 mM malate (Glu/Mal, **C**) or 1 mM NADH (**D**). Finally, cytochrome c reduction was inhibited with rotenone, malonate or antimycin A. Data represent means ± SEM of four independent experiments, each. *, #; **, ##; ***, ### indicate significant differences at the level of $p < 0.05$, 0.01 and 0.001 (paired t-test, **A – C**) between corresponding measurements with the same inhibitor: * Substrate (succinate, proline, Glu/Mal) vs. LtP + digitonin; # inhibitor vs. substrate. **, *** indicate significant differences at the level of $p < 0.01$ and 0.001 (unpaired t-test, **D**) between corresponding measurements with the same inhibitor.

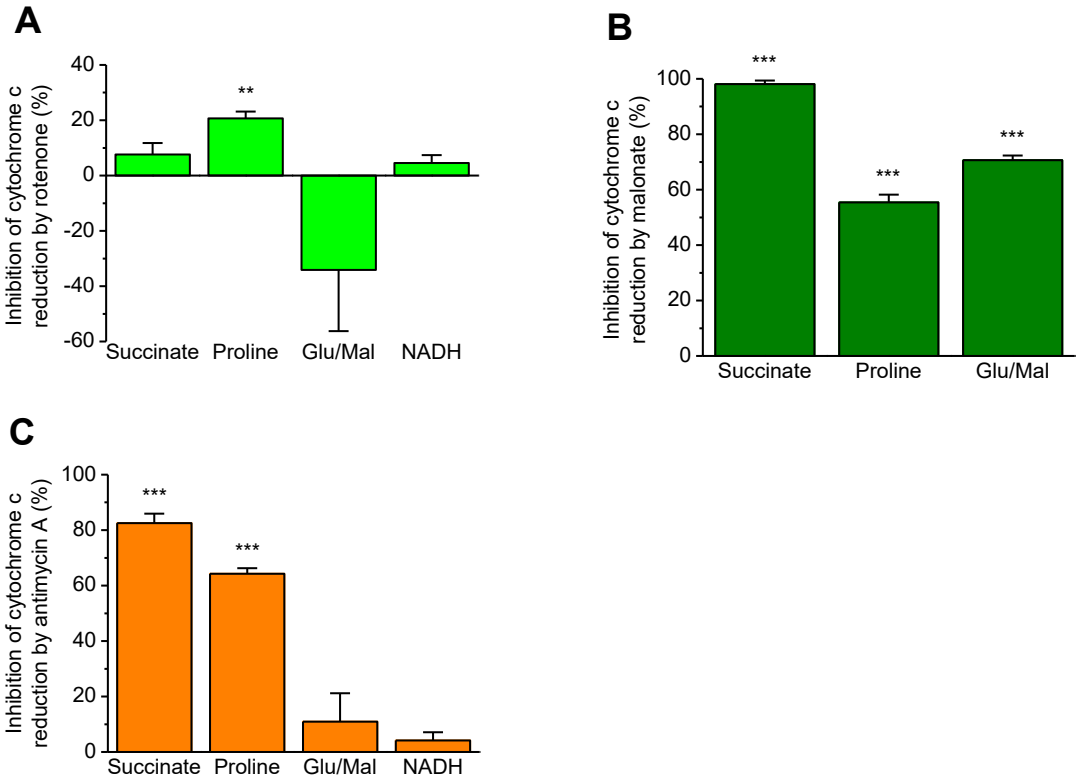

Figure 14 Inhibition of cytochrome c (50 µM) reduction by *Leishmania tarentolae* promastigotes (5×10^7 LtP/ml; 0.158 ± 0.004 mg protein/ml; n = 11) in mannitol medium supplemented with 5 mM $MgCl_2$ and 1 mM KCN. LtP were permeabilised with 100 µg digitonin/ml followed by the addition of 10 mM succinate, 10 mM proline, 5 mM glutamate/5 mM malate (Glu/Mal) or 1 mM NADH. Finally, cytochrome c reduction was inhibited with 100 µM rotenone (**A**), 20 mM malonate (**B**) or 40 nM antimycin A (**C**). Data represent means ± SEM of four independent experiments, each. **, *** indicate significant differences at the level of $p < 0.01$ and 0.001 (one population t-test vs. 0 % inhibition of cytochrome c reduction).

3.5. Reduction of 2,6-dichlorophenolindophenol by LtP supplemented with different substrates and inhibitors

The spectra below (Fig. 15A) illustrate the maximal absorption of oxidised 2,6-dichlorophenolindophenol (DCPIP) at a wavelength of 600 nm. In Fig. 15B an example of a representative time scan of DCPIP reduction is shown. The addition of succinate stimulated

the DCPIP reduction by digitonin-permeabilised LtP. Following the addition of malonate, an inhibition of DCPIP reduction was recorded.

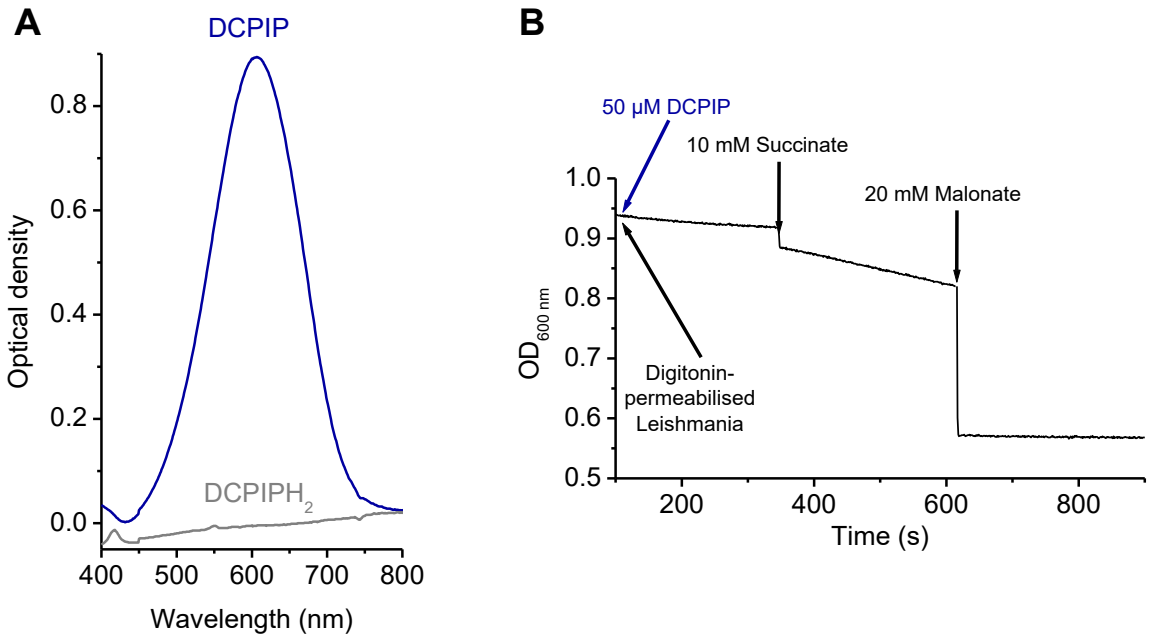

Figure 15 A) Spectra of oxidised 2,6-dichlorophenolindophenol (DCPIP, 50 µM) and DCPIP reduced by sodium dithionite ($DCPIPH_2$, 50 µM) in mannitol medium. **B)** Representative time scan of DCPIP reduction by *Leishmania tarentolae* promastigotes (5×10^7 LtP/ml = 0.128 mg protein/ml) permeabilised by digitonin (10 µg/ml) in mannitol medium supplemented with 5 mM $MgCl_2$ and 1 mM KCN.

Figure 16 indicates that both succinate (Fig. 16A) and proline (Fig. 16B) successfully stimulated DCPIP reduction, while hardly any difference could be achieved with the addition of glutamate/malate (Fig. 16C).

No significant inhibition of DCPIP reduction was possible with the addition of rotenone (Fig. 17A). Antimycin A only had an inhibiting effect on the DCPIP reduction of proline-stimulated LtP (Fig. 17C). As shown in Fig. 17B, the highest inhibition of DCPIP reduction was achieved by malonate in succinate-consuming LtP, followed by proline- and glutamate/malate-supplemented LtP.

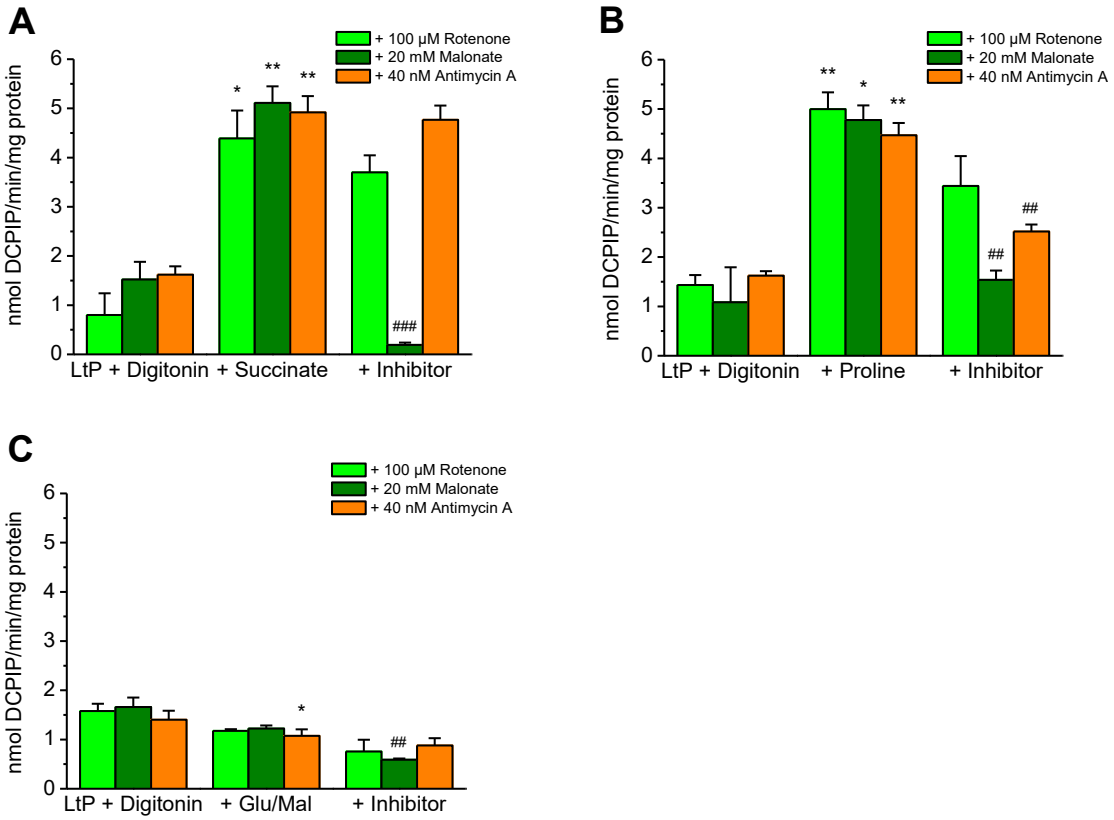

Figure 16 Reduction of 2,6-dichlorophenolindophenol (DCPIP; 50 µM) by *Leishmania tarentolae* promastigotes (5×10^7 LtP/ml) in mannitol medium supplemented with 5 mM $MgCl_2$ and 1 mM KCN. LtP were permeabilised with 10 µg digitonin/ml followed by the addition of 10 mM succinate, 10 mM proline or 5 mM glutamate/5 mM malate (Glu/Mal). Finally, DCPIP reduction was inhibited with malonate (0.141 ± 0.006 mg protein/ml; n = 4), rotenone or antimycin A (0.147 ± 0.004 mg protein/ml; n = 4, each). Data represent means ± SEM. *; **, ##; ### indicate significant differences at the level of $p < 0.05$, 0.01 and 0.001 (paired t-test) between corresponding measurements with the same inhibitor: * Substrate (succinate, proline, Glu/Mal) vs. LtP + digitonin; # inhibitor vs. substrate.

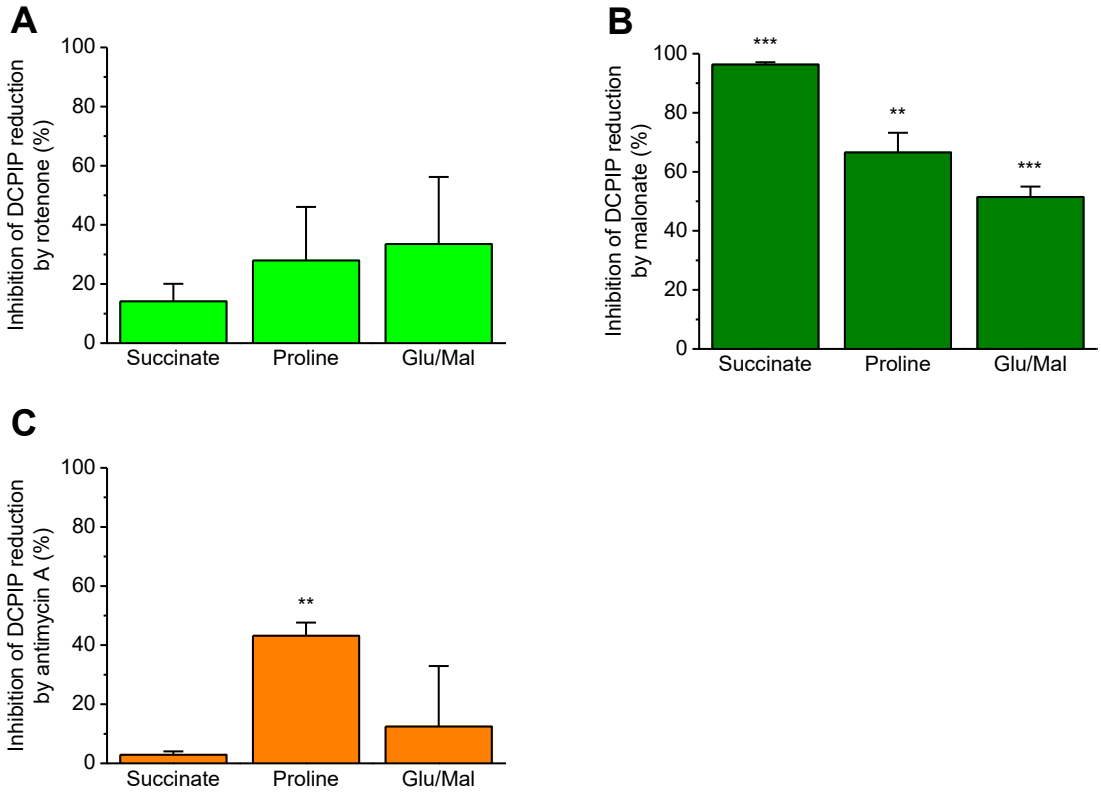

Figure 17 Inhibition of 2,6-dichlorophenolindophenol (DCPIP; 50 µM) reduction by *Leishmania tarentolae* promastigotes (5×10^7 LtP/ml; 0.147 ± 0.004 mg protein/ml; n = 4; **A**, **C**; 0.141 ± 0.006 mg protein/ml; n = 4; **B**) in mannitol medium supplemented with 5 mM $MgCl_2$ and 1 mM KCN. LtP were permeabilised with 10 µg digitonin/ml followed by the addition of 10 mM succinate, 10 mM proline or 5 mM glutamate/5 mM malate (Glu/Mal). Finally, DCPIP reduction was inhibited with 100 µM rotenone (**A**), 20 mM malonate (**B**) or 40 nM antimycin A (**C**). Data represent means ± SEM. **, *** indicate significant differences at the level of $p < 0.01$ and 0.001 (one population t-test vs. 0 % inhibition of DCPIP reduction).

4. DISCUSSION

Leishmania parasites are the main cause of the neglected tropical disease leishmaniasis. This disease is a growing public health concern worldwide, as climate change causes its sandfly vectors to spread in more countries (1). Considering multi-drug resistance, there is an increasing need to develop new and effective drugs against leishmaniasis (24). However, investigating the mitochondrial respiratory chain for this purpose is hampered by the difficulty of isolating mitochondria (15,17). For this study, it was of interest to broaden the current knowledge about detergent-permeabilised *Leishmania* as an alternative study method and their mitochondrial respiratory chain.

Mitochondria from different sources can be studied either in non-ionic or ionic media used for osmotic support. Sucrose-based medium is one of the most frequently used non-ionic media (25). However, *Leishmania* possess an intracellular sucrase activity splitting sucrose into fructose and glucose (26). Therefore, for studying the mitochondrial respiratory chain of *Leishmania* in the absence of glucose, sucrose should be avoided.

Mannitol, in combination with a suitable buffer and other necessary additives (e.g. calcium chelator), is well established in many mitochondrial isolation protocols (25). In the present study, we investigated whether PBS (ionic), normally used for washing cells, or mannitol (non-ionic) medium is more suitable as a resuspension medium for measurements with permeabilised LtP. For oxygen consumption measurements with permeabilised LtP, mannitol medium was shown to be a better resuspension medium than PBS resulting in higher respiratory control values. It can therefore be assumed that mannitol medium can be used for analysing mitochondrial function with permeabilised LtP in the same way as it is used for isolating mitochondria.

In order to make the *Leishmania* accessible to the respective mitochondrial substrates and inhibitors, the cells were permeabilised with the mild non-ionic detergents digitonin and Triton X-100. In the present study, for oxygen consumption measurements in *Leishmania tarentolae* promastigotes a final concentration of 25 µg/ml digitonin (37 µg digitonin/mg cell protein) turned out to be optimal for stimulating oxidative phosphorylation by the addition of ADP, indicative of an intact inner membrane. Chakraborty et al. worked with *Leishmania donovani* promastigotes and used a comparable concentration to permeabilise the cells by applying 30 µg digitonin/mg cell protein (19). In contrast, for our spectrophotometric determination of cytochrome c reduction, a higher final concentration of 100 µg/ml digitonin

(81.3 µM; 585 µg digitonin/mg cell protein) was necessary in order to allow the diffusion of externally added cytochrome c through the outer mitochondrial membrane, which is otherwise impermeable for cytochrome c. Although this concentration was higher than that used in the oxygen consumption measurements, Vercesi et al. claim in their study of *Trypanosoma cruzi* that only higher concentrations of digitonin (higher than 120 µM) could have a negative effect on the inner mitochondrial membrane (18). Regarding detergent incubation time, Mondal et al. added respiratory substrates to *Leishmania donovani* promastigotes 3 minutes after adding digitonin (24). In the case of *Leishmania tarentolae* promastigotes 10 minutes were required for sufficient permeabilization.

In the present study, succinate, glutamate/malate and proline were found to cause the highest respiratory control values in digitonin-permeabilised *Leishmania tarentolae* promastigotes, while pyruvate and NADH did not show neither an ADP-dependent stimulation of O_2 consumption nor a pronounced KCN-dependent inhibition of O_2 consumption. While a comparable stimulatory effect of ADP on oxygen consumption was observed in isolated succinate-respiring mitochondria or membrane-rich fractions of *Leishmania donovani* promastigotes (14,19), a smaller stimulation of oxidative phosphorylation by ADP was found in isolated glutamate/malate-respiring mitochondria of *Leishmania donovani* promastigotes by Chakraborty et al. (19). Their respiratory control values were lower in comparison to digitonin-permeabilised glutamate/malate-respiring *Leishmania tarentolae* promastigotes (1.16 vs. 1.82).

Furthermore, our study demonstrates that 100 µM rotenone (complex I inhibitor) has little to no inhibitory effect on O_2 consumption rates, cytochrome c reduction and DCPIP reduction in permeabilised LtP, which were supplemented with succinate, glutamate/malate and proline. This finding of insensitivity of leishmanial O_2 uptake to rotenone was also reported by previous studies (10,14). Even at high concentrations of 320 µM, O_2 consumption of intact *Leishmania donovani* promastigotes was not inhibited by rotenone (14). Contrary to this outcome, a partial inhibition of O_2 consumption could be achieved with 40 µM rotenone in glutamate-respiring, but surprisingly also in succinate-respiring, digitonin-permeabilised *Leishmania donovani* promastigotes (19). In another study, Bermudez et al. found 60 µM rotenone to be effective in inhibiting O_2 uptake (71.3 %) of pyruvate/malate-respiring mitochondrial preparations of *Leishmania mexicana* promastigotes (15). This inconsistency in literature about the presence or more specifically the classic functional activity of complex I in *Leishmania* parasites, shows that this topic remains to be studied (10,14,27).

In our study, the mitochondrial complex III inhibitor antimycin A (40 nM) was found to have a strong inhibitory effect on the oxygen consumption of succinate-, glutamate/malate- and proline-supplemented LtP as well as the cytochrome c reduction measured in succinate- and proline-supplemented LtP. These results support previous research of Bermudez et al. and Chakraborty et al. on antimycin A and its inhibiting effect in *Leishmania* (15,19). Comparison of this finding with those of earlier studies indicates the presence of a mitochondrial complex III in *Leishmania* promastigotes.

Our results revealed that the complex II inhibitor malonate not only has influence on oxygen consumption rates, but also on cytochrome c reduction and DCPIP reduction in digitonin-permeabilised succinate-, glutamate/malate- and proline-consuming LtP. This result is in line with those of previous studies which suggest inhibition of succinate oxidation through the addition of malonate (14–16). Although we observed comparable inhibition of proline-stimulated oxygen consumption by 20 mM malonate in LtP as Santhamma and Bhaduri with 25 mM malonate in *Leishmania donovani* promastigotes (71 % vs. 55 %), Martin and Mukkada failed to detect any inhibition of oxygen consumption with 1.7 mM malonate in proline-stimulated *Leishmania tropica* promastigotes (14,16).

In summary, permeabilizing LtP with digitonin has shown promising results and this could be a suitable way to perform studies on mitochondrial function in *Leishmania*. Effects of the respectively tested mitochondrial substrates and complex inhibitors demonstrate that identification and direct studying of mitochondrial drug targets seem to be possible, without isolating the mitochondria. Despite these promising results, several questions remain open regarding the respiratory chain of *Leishmania tarentolae* promastigotes, e.g. the activity and presence of complex I. Additional studies on the current topic are therefore recommended to draw a full picture of the mitochondrial respiratory chain of *Leishmania tarentolae* promastigotes and its possible targets for antileishmanial drugs.

5. SUMMARY

Leishmania are responsible for visceral and (muco)cutaneous leishmaniasis in humans and animals. In view of severe side effects of approved drugs and development of resistance, new antileishmanial therapeutic approaches are needed. *Leishmania* exist in two forms: as flagellated extracellular promastigotes and as aflagellated intracellular amastigotes (in the phagolysosomes of mammalian macrophages). Both forms contain a single mitochondrion per cell. For the identification of direct mitochondrial drug targets, usually isolation of mitochondria is required. This is complicated by the large size and ramification of the mitochondrion and microtubule arrays adhering to the inner side of the leishmanial cell membrane.

The aim of this bachelor thesis was to find out whether detergent-permeabilised (digitonin, Triton X-100) *Leishmania tarentolae* promastigotes (LtP) are a suitable experimental model for studying mitochondrial function.

The respiratory chain was assessed in permeabilised LtP by means of a Clark-type O_2 electrode. The activities of mitochondrial electron transfer complexes were determined spectrophotometrically using artificial (2,6-dichlorophenolindophenol) or natural electron acceptors (cytochrome c). O_2 consumption rates and activities of mitochondrial complexes were measured in the presence of appropriate substrates (succinate, glutamate/malate, proline, pyruvate, NADH) and mitochondrial inhibitors (cyanide, antimycin A, malonate, rotenone).

The highest respiratory control values were observed in digitonin-permeabilised LtP (25 µg digitonin/ml mannitol medium) consuming succinate, glutamate/malate and proline, while pyruvate and NADH did not show neither an ADP-dependent stimulation of O_2 consumption nor a pronounced KCN-dependent inhibition of O_2 consumption.

While antimycin A (complex III inhibitor) and malonate (complex II inhibitor) have shown a significant inhibition, rotenone (complex I inhibitor) has little influence on O_2 consumption rates and cytochrome c reductase activities in succinate-, glutamate/malate- and proline-consuming digitonin-permeabilised LtP.

In summary, digitonin-permeabilised LtP could be a suitable model for studies on mitochondrial function avoiding the difficulties in isolation of mitochondria from *Leishmania*.

6. ZUSAMMENFASSUNG

Leishmanien sind für die viszerale und (muko)kutane Leishmaniose bei Mensch und Tier verantwortlich. Angesichts der schweren Nebenwirkungen zugelassener Medikamente und der Entwicklung von Resistenzen sind neue Therapieansätze gegen *Leishmanien* erforderlich. *Leishmanien* kommen in zwei Formen vor: als extrazelluläre Promastigoten mit Geißeln und als intrazelluläre Amastigoten ohne Geißeln (in den Phagolysosomen von Säugetiermakrophagen). Beide Formen enthalten ein einziges Mitochondrium pro Zelle. Für die Identifizierung direkter mitochondrialer Wirkstoffziele ist in der Regel die Isolierung der Mitochondrien erforderlich. In *Leishmanie*n wird dies durch die Größe und Verzweigung des Mitochondriums und die Mikrotubuli-Arrays erschwert, welche an der Innenseite der Zellmembran haften.

Ziel dieser Bachelorarbeit war es, herauszufinden, ob mit Detergenzien (Digitonin, Triton X-100) permeabilisierte *Leishmania tarentolae* Promastigoten (LtP) ein geeignetes experimentelles Modell zur Untersuchung der Mitochondrienfunktion darstellen.

Die Atmungskette wurde in permeabilisierten LtP mit Hilfe einer Clark O_2-Elektrode evaluiert. Die Aktivitäten der mitochondrialen Elektronentransfer-Komplexe wurden spektrophotometrisch mit künstlichen (2,6-Dichlorphenolindophenol) oder natürlichen Elektronenakzeptoren (Cytochrom c) bestimmt. Die O_2-Verbrauchsraten und Aktivitäten der mitochondrialen Komplexe wurden in Gegenwart geeigneter Substrate (Succinat, Glutamat/Malat, Prolin, Pyruvat, NADH) und mitochondrialer Inhibitoren (Cyanid, Antimycin A, Malonat, Rotenon) gemessen.

Die höchsten Atmungskontrollwerte wurden in Digitonin-permeabilisierten LtP (25 µg Digitonin/ml Mannitolmedium) beobachtet, die Succinat, Glutamat/Malat und Prolin konsumierten. Pyruvat und NADH hingegen zeigten weder eine ADP-abhängige Stimulation des O_2-Verbrauchs, noch eine ausgeprägte KCN-abhängige Hemmung des O_2-Verbrauchs.

Während Antimycin A (Komplex-III-Inhibitor) und Malonat (Komplex-II-Inhibitor) eine signifikante Hemmung zeigten, hatte Rotenon (Komplex-I-Inhibitor) nur einen geringen Einfluss auf die O_2-Verbrauchsraten und die Cytochrom-c-Reduktase-Aktivitäten in Succinat-, Glutamat/Malat- und Prolin-verbrauchenden Digitonin-permeabilisierten LtP.

Zusammenfassend lässt sich sagen, dass Digitonin-permeabilisierte LtP ein geeignetes Modell für Studien zur Untersuchung der mitochondrialen Funktion darstellen könnten, welches die Schwierigkeiten bei der Isolierung von Mitochondrien aus *Leishmanie*n vermeidet.

7. LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DCPIP	2,6-Dichlorophenolindophenol
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
LtP	*Leishmania tarentolae* promastigotes
NADH	Nicotinamide adenine dinucleotide reduced
OD	Optical density
PBS	Phosphate-buffered saline
SEM	Standard error of mean
TEA	Triethanolamine hydrochloride

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9. LIST OF FIGURES