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Ubiquinol (QH_2) functions as a negative regulator of purine nucleotide inhibition of *Acanthamoeba castellanii* mitochondrial uncoupling protein

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ABSTRACT

We compared the influence of different adenine and guanine nucleotides on the free fatty acid-induced uncoupling protein (UCP) activity in non-phosphorylating *Acanthamoeba castellanii* mitochondria when the membranous ubiquinone (Q) redox state was varied. The purine nucleotides exhibit an inhibitory effect in the following descending order: GTP>ATP>GDP>ADP≫GMP>AMP. The efficiency of guanine and adenine nucleotides to inhibit UCP-sustained uncoupling in *A. castellanii* mitochondria depends on the Q redox state. Inhibition by purine nucleotides can be increased with decreasing Q reduction level (thereby ubiquinol, QH_2 concentration) even with nucleoside monophosphates that are very weak inhibitors at the initial respiration. On the other hand, the inhibition can be alleviated with increasing Q reduction level (thereby QH_2 concentration). The most important finding was that ubiquinol (QH_2) but not oxidised Q functions as a negative regulator of UCP inhibition by purine nucleotides. For a given concentration of QH_2, the linoleic acid-induced GTP-inhibited H^+ leak was the same for two types of *A. castellanii* mitochondria that differ in the endogenous Q content. When availability of the inhibitor (GTP) or the negative inhibition modulator (QH_2) was changed, a competitive influence on the UCP activity was observed. QH_2 decreases the affinity of UCP for GTP and, *vice versa*, GTP decreases the affinity of UCP for QH_2. These results describe the kinetic mechanism of regulation of UCP affinity for purine nucleotides by endogenous QH_2 in the mitochondria of a unicellular eukaryote.

1. Introduction

Acanthamoeba castellanii is a free-living amoeba that has been used frequently as a model organism to study mitochondrial energy-dissipating systems, such as a cyanide-resistant alternative oxidase [1–3], an ATP-sensitive potassium channel [4], and an uncoupling protein (UCP), AcUCP (*A. castellanii* UCP) [5–10]. UCPs catalyse a proton conductance that dissipates the H^+ electrochemical gradient ($\Delta\mu H^+$) built up by the mitochondrial respiratory chain in animal, plant, and some fungal and protist mitochondria (for review, see Refs. 11–16). UCPs fulfil a physiological function by a free fatty acid (FFA)-activated purine nucleotide (PN)-inhibited H^+ cycling process driven by membrane potential ($\Delta\Psi$) and ΔpH, which together constitute $\Delta\mu H^+$. In unicellular organisms, as well as in non-thermogenic plant and animal tissues, the physiological role of this energy-dissipating system has not yet been established. UCPs (with the exception of thermogenic UCP1 of mammalian brown adipose tissue) have been proposed to play a central role in limiting the production of mitochondrial reactive oxygen species and in the maintenance of the cell energy metabolism balance related to the regulation of ATP production as well as control of the NADH/NAD^+ ratio [11–16].

The evidence for the presence of UCPs in unicellular eukaryotes is mainly functional and immunological [16]. To date, evidence proving that genes identified by comparative sequence analysis as encoding UCPs in fact function as UCPs is lacking. In mammalian mitochondria, FFA-induced mitochondrial uncoupling can be mediated, at least in part, by several other members of the mitochondrial anion carrier protein family. There is no evidence that FFA-induced PN-inhibited UCP-independent uncoupling could occur in the mitochondria of unicellular eukaryotes. Results suggest that neither the adenine nucleotide carrier, the aspartate/glutamate antiporter, nor the dicarboxylate carrier participate in the GTP-inhibited protonophoric action of FFA in the mitochondria of the parasitic protist *Plasmodium berghei* [17]. Similarly, the GTP-inhibited uncoupling effect of fatty acids is insensitive to carboxyatractyloside (an inhibitor of the adenine nucleotide carrier) in the mitochondria of *A. castellanii* [6,10], the parasitic protist *Plasmodium yoelii*, and the fungus *Aspergillus fumugatus* [18,19]. Therefore, it seems that in the mitochondria of unicellular eukaryotes, FFA-dependent GTP-inhibited

Abbreviations: AcUCP, uncoupling protein of *Acanthamoeba castellanii* mitochondria; BSA, bovine serum albumin; FFA, free fatty acids; LA, linoleic acid; PN, purine nucleotide; TPP^+, tetraphenylphosphonium; UCP, uncoupling protein; UCP1, uncoupling protein of brown adipose tissue; Q, ubiquinone; Qox, oxidised Q; QH_2, reduced Q (ubiquinol); Qtot, total pool of endogenous Q in the inner mitochondrial membrane; QH_2/Qtot, reduction level of Q; $\Delta\Psi$, mitochondrial membrane electrical potential; H^+, proton electrochemical gradient

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proton conductance can be attributed only to UCP activity. In the case of *A. castellanii* mitochondria, in addition to the functional link to AcUCP, there are immunological data indicating that the protein (~32 kDa) detected using antibodies raised against mammalian UCP3 follows changes in FFA-induced GTP-inhibited mitochondrial uncoupling in cold-treated cells and throughout the growth of amoeba cells [6,7,20].

In mitochondria of the amoeboid protozoan *A. castellanii*, the action of AcUCP has been shown to mediate FFA-activated, PN-inhibited H^+ reuptake driven by $\Delta\mu H^+$ that in phosphorylating (state 3) respiration can divert energy from oxidative phosphorylation [5,21]. The fatty acid efficiency profile in uncoupling of *A. castellanii* mitochondria has been described [9]. It has been also shown that a cold treatment of amoeba cell culture increases AcUCP activity and protein level, indicating that UCP could be a cold response protein in unicellular eukaryotes [6]. Moreover, we have shown that UCPs of unicellular organism mitochondria, such as of amoeba *A. castellanii* mitochondria, may play a role in decreasing reactive oxygen species production leading to its constant level throughout the growth cycle [8,20].

Because UCPs are specialised proteins for $\Delta\mu H^+$ dissipation, their activity must be finely regulated. Taking into account the apparent affinity of reconstituted UCPs for PNs [22,23] and the concentration of nucleotides *in vivo* (at millimolar concentrations inside the cells), UCPs should be permanently inhibited under *in vivo* conditions, even in the presence of FFA, unless a regulatory factor or mechanism could alleviate the inhibition by PNs [11]. Unlike the proton conductance of the adenine nucleotide carrier that is inhibited by GDP [24,25], UCPs are strongly inhibited not only by GDP but also by GTP, ATP, and ADP. Because of this difference in specificity, inhibition of H^+ conductance by PNs other than GDP may be considered diagnostic of UCP function. For UCP1 homologues, the FFA-induced H^+ conductance has been shown to be differently sensitive to PNs under nonphoshorylating (state 4) and phosphorylating (state 3) conditions. It has been proposed that the membranous ubiquinone (coenzyme Q, Q) redox state could be a metabolic sensor that modulates PN inhibition of FFA-activated UCP1 homologues; such is the case in isolated skeletal muscle (UCP3 and UCP2), potato tuber (plant UCP), and *A. castellanii* (protist UCP) mitochondria respiring under phosphorylating conditions [7,26,27]. Moreover, a Q redox state-dependent level of UCP inhibition by PNs has also been recently observed in isolated non-phosphorylating mitochondria of *A. castellanii* (AcUCP) and rat brown adipose tissue (UCP1) [10,28]. This indicates a likely universal regulation of UCPs. So far, this regulation has been studied with GTP or GDP. The question therefore arises as to whether the Q redox state-dependent inhibition of UCP involves other PNs (GMP and adenine nucleotides).

The aim of the present study was to compare the influence of different adenine and guanine nucleotides on the FFA-induced AcUCP activity in nonphosphorylating *A. castellanii* mitochondria when the membranous Q redox state was varied. Moreover, it was found that ubiquinol (QH_2) but not oxidised Q (Qox) directly influences PN-inhibition of AcUCP. When availability of the inhibitor (GTP) or the negative inhibition modulator (QH_2) was changed, a competitive influence on the AcUCP activity was observed. These results describe the kinetic mechanism of regulation of AcUCP affinity for PNs by endogenous QH_2 level.

2. Materials and methods

2.1. Cell culture and isolation of mitochondria

The soil amoeba, *A. castellanii* strain Neff, was cultured as described by Jarmuszkiewicz et al. [20]. Cells were inoculated to a final density of approximately $5–6\times10^5$ cells/ml. Trophozoites of the amoeba were collected between 44 and 46 h following inoculation at the late exponential phase (at a density of about $4.5–5.0\times10^6$ cells/ml) or between 22 and 24 h following inoculation at the middle exponential phase (at a density of about $2.5–3.0\times10^6$ cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (28%) as previously described earlier [1]. The presence of 0.3% bovine serum albumin (BSA) in the isolation media allowed endogenous FFA to be chelated from the mitochondrial suspension. Mitochondrial protein concentration was determined by the biuret method.

Most experiments were performed with mitochondria isolated from the late exponential phase of *A. castellanii* cell growth except experiments (Fig. 5) investigating the form of Q (QH_2 or Qox) interacting with AcUCP when mitochondria isolated from the middle and late exponential phases were used.

2.2. Mitochondrial oxygen consumption and mitochondrial membrane potential measurements

Oxygen uptake was measured polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) in 2.8 ml of incubation medium (25 °C), with 1–1.4 mg of mitochondrial protein. As a control, state 3 (phosphorylating) respiration was measured to check coupling parameters. Only high quality mitochondria preparations, i.e., with an ADP/O value of around 1.40 (with succinate as a respiratory substrate) and a respiratory control ratio of around 2.5–3.0, were used in all experiments. State 3 respiratory rate was 269 ± 29 nmol of O per minute per milligram of protein ($n=13$) for mitochondria isolated from the exponential or late exponential phases of growth. Experiments described in this study were performed under nonphosphorylating conditions. Values of O_2 uptake are given in nanomoles of O per minute per milligram of protein.

The mitochondrial membrane electrical potential ($\Delta\Psi$) was measured simultaneously with oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode according to Kamo et al. [29]. The TPP^+-electrode was calibrated with four sequential additions (0.4, 0.4, 0.8, and 1.6 μM) of TPP^+. After each run, 0.5 μM FCCP was added to release TPP^+ for baseline correction. For calculation of the $\Delta\Psi$ value, the matrix volume of amoeba mitochondria was assumed to be 2.0 μl/mg protein. The calculation assumes that TPP^+ distribution between mitochondria and medium followed the Nernst equation. The values of $\Delta\Psi$ were corrected for TPP^+ binding using apparent external and internal partition coefficients of TPP [30,31]. The correction shifted calculated $\Delta\Psi$ values to lower values (approx. 30-mV shift), but it did not influence changes in the resulting membrane potential (relative changes). Values of $\Delta\Psi$ are given in millivolts.

2.3. Proton leak measurements

The proton conductance response to its driving force can be expressed as the relationship between the oxygen consumption rate and $\Delta\Psi$ (flux–force relationship) when varying the potential by titration with respiratory chain inhibitors. Respiration rate and $\Delta\Psi$ were measured simultaneously using electrodes sensitive to oxygen and TPP^+. Proton leak rates can be calculated from respiration rates by multiplying by an H^+/O ratio of 6. Mitochondria (0.36–0.5 mg of protein/ml) were incubated in a standard incubation medium (25 °C) containing: 120 mM KCl, 20 mM Tris–HCl pH 7.4, 3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.5 mM EGTA, and 0.025% BSA. Measurements were performed in the absence of exogenous ADP, i.e., in resting state (nonphosphorylating respiration, state 4). To inhibit the activity of an ATP/ADP antiporter and ATP synthase, 1.8 μM carboxyatractyloside and 0.5 μg/ml oligomycin were used, respectively. Benzohydroxamate (1.5 mM) was used to inhibit alternative oxidase activity. Glybenclamide (1 μM) was used to inhibit the mitochondrial ATP-regulated potassium channel. The oxidisable substrate was succinate (7 mM, pH 7.4) in the presence of rotenone (4 μM) to block electron input from

complex I. When we compared the influence of different adenine and guanine nucleotides on FFA-induced AcUCP activity (Figs. 1–3 and Table 1), succinate dehydrogenase was activated by up to 180 μM ATP. Under control conditions (no AcUCP activators or inhibitors), addition of ATP to isolated *A. castellanii* mitochondria oxidising succinate leads to dehydrogenase activation that is evidenced by increases in both state 4 respiration (by approx. 10–12%) and $\Delta\Psi$ (by approx. 1–1.5 mV) [4,5,10]. For a given mitochondrial preparation, we estimated the minimal amount of ATP (150–180 μM) needed to activate the dehydrogenase (until no further changes in respiration or $\Delta\Psi$ were observed). Therefore, the effect of adding ATP to 2 mM (where indicated) on FFA-induced AcUCP activity could be attributed to an inhibitory effect on the carrier (Figs. 1–3 and Table 1). Experiments describing the influence of GTP on FFA-induced AcUCP activity (Figs. 4–6) were performed in the absence of ATP. To induce AcUCP activity-mediated respiration, measurements were made in the presence of linoleic acid (LA) (9 μM). LA-induced AcUCP activity was inhibited by the addition of guanine and adenine nucleotides (GTP, GDP, GMP, ATP, ADP, and AMP) (Sigma). Superoxide dismutase (SOD) was used to remove endogenously produced superoxide radicals from mitochondrial suspension.

Respiratory rate, $\Delta\Psi$, and Q redox state (QH_2 or Qox concentrations) were varied by modulating the Q-reducing or QH_2-oxidising pathways. To decrease the rate of the Q-reducing pathway during state 4 respiration (thereby decreasing a steady-state resting respiration), succinate dehydrogenase activity was titrated by increasing the concentration of malonate (a competitive inhibitor; up to 2 mM). To decrease the rate of the QH_2-oxidising pathway during state 4 respiration, complex IV was inhibited with cyanide (up to 100 μM). To avoid possible errors due to non-steady-state conditions such as when sequential additions of inhibitors were applied and to assess the Q redox state for a given steady state, data from separate measurements with different (given) inhibitor concentrations were combined to generate common curves.

2.4. Measurements of the quinone reduction level

The redox state of Q in steady-state respiration was determined by an extraction technique followed by HPLC detection as previously described [10]. A completely oxidised extract was obtained during incubation in the absence of substrate using an evaporation/ventilation step and a completely reduced extract was obtained upon anaerobiosis and in the presence of respiratory substrate (succinate), 1.5 mM cyanide, and 1.5 mM benzohydroxamate. The endogenous ubiquinone in *A. castellanii* mitochondria is Q_9 [2]. Commercial Q_9 (Sigma) was used for peak calibration. Q reduction levels are expressed as percentage of total Q (QH_2/Qtot).

Table 1
The effect of various PNs on the LA-induced proton leak (AcUCP activity) during uninhibited (initial) state 4 respiration.

PNs	Respiratory rate inhibition [%]	$\Delta\Psi$ recovery [%]	QH_2/Qtot recovery [%]
GTP	100	100	100
ATP	78 ± 5	85 ± 6	80 ± 6
GDP	67 ± 3	60 ± 5	62 ± 5
ADP	47 ± 5	46 ± 5	46 ± 4
GMP	11 ± 2	17 ± 2	26 ± 3
AMP	10 ± 2	10 ± 1	20 ± 2

Assay conditions as in Fig. 1. Measurements were carried out with 2 mM PNs. Results are given as percentage of effect caused by GTP. GTP decreased respiratory rate by 15 ± 2 nmol of O per minute per milligram of protein (from 26 nmol of O per minute per milligram of protein of LA-induced respiration), increased $\Delta\Psi$ by 3.3 ± 0.2 mV (from 5 mV of LA-induced $\Delta\Psi$ drop), and increased QH_2/Qtot by 7.4% (from 9% of LA-induced QH_2/Qtot change). Values are means ± SD of three to six independent experiments.

3. Results

3.1. The effect of various PNs on the LA-induced proton leak (AcUCP activity) during uninhibited (initial) state 4 respiration

AcUCP-containing *A. castellanii* mitochondria are activated by FFA, among which LA is the most efficient [9]. The influence of 2 mM adenine and guanine nucleotides on AcUCP activity induced with 9 μM LA was measured in isolated *A. castellanii* mitochondria. Representative traces of these measurements using GTP and GMP are shown in Fig. 1. The effects of PNs on the change in respiration, $\Delta\Psi$, and Q redox state caused by LA-induced proton leak (AcUCP activity) during initial (not inhibited by respiratory chain inhibitors) state 4 respiration were measured. After the addition of PN, inhibition of the respiratory rate as well as restoration of $\Delta\Psi$ and the Q redox state indicated that the LA-induced H^+ leak was inhibited. The inhibitory effect of GTP was much stronger compared to that of GMP. However, neither GTP nor GMP restored the three parameters to the level observed before LA addition. This indicated incomplete inhibitory effects under initial state 4 respiration and therefore at higher $\Delta\Psi$ and redox state of Q. Table 1 summarises the effect of different purine nucleoside phosphates on LA-induced AcUCP activity in *A. castellanii* mitochondria. In general, guanine nucleotides inhibit AcUCP activity to a greater level than adenine nucleotides with the same degree of phosphorylation and nucleoside triphosphates are more inhibitory than nucleoside di- or monophosphates. The inhibitory effect of nucleoside monophosphates (GMP or AMP) was quite slight. The PNs exhibit an inhibitory effect in the following descending order: GTP>ATP>GDP>ADP≫GMP>AMP. AcUCP activity was not sensitive to pyrimidine nucleoside mono-, di-, and triphosphates (data not shown).

3.2. The increasing inhibitory effect of PNs on the LA-induced proton leak (AcUCP activity) when the Q-reducing pathway is decreased

In *A. castellanii* mitochondria, it has been shown that the inhibitory effect of 1 mM GDP on the LA-induced uncoupling during state 4 respiration can be enhanced when endogenous Q is sufficiently

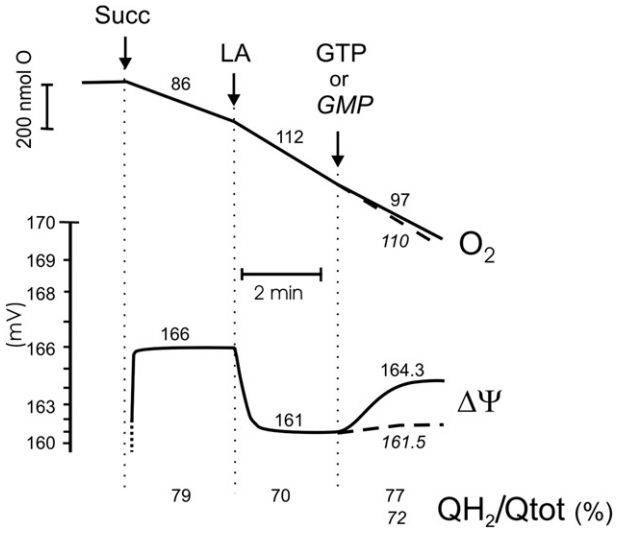

Fig. 1. The effect of GTP and GMP on the change in respiration, membrane potential and Q redox state caused by LA-induced proton leak (AcUCP activity) during uninhibited (initial) non-phosphorylating respiration. Additions: 7 mM succinate (Succ), 9 μM LA, 2 mM GTP (solid line) or GMP (dashed line). Numbers on the traces refer to O_2 consumption rates in nanomoles of O per minute per milligram of protein or to $\Delta\Psi$ values in millivolts. The Q redox state values (in %) corresponding to given experimental steady-state conditions are shown. An example of measurements using mitochondria from six different preparations is shown.

oxidised [10]. Therefore, we investigated the effect of other PNs when the rate of the Q-reducing pathway is decreased in nonphosphorylating conditions (Fig. 2). To investigate the inhibitory effects with the weakest inhibitors (GMP and AMP), a PN concentration of 2 mM was chosen. Fig. 3 shows proton–conductance curves (Figs. 2A and C) and the relationship between respiratory rate versus Q reduction level (Figs. 2B and D) in the presence or absence of 9 μM LA and/or 2 mM guanine or adenine nucleotides, during titration with three concentrations of malonate. The sensitivity profile of AcUCP to various PNs observed for initial (without malonate) state 4 respiration (Table 1) was maintained when Q reduction level was decreased with Q-reducing pathway titration (Fig. 2).

Figure 2 shows that when the rate of the Q-reducing pathway was progressively decreased during succinate oxidation, the inhibitory effect of tested PNs on the LA-induced H^+ leak increased with decreasing $\Delta\Psi$ (Figs. 2A and C) and Q redox state (Figs. 2B and D). Data with ATP and AMP are shown only for uninhibited (without malonate) conditions. With a 2-mM concentration of PNs, the strongest inhibitory effect was reached at approximately 10% of Q reduction level corresponding to approximately 125 mV, when the points obtained in the presence of LA and PNs approached the control curve without LA and PNs (Fig. 2). Complete inhibition was observed with GTP and GDP. The LA-induced H^+ leak decreases, and the relationship between $\Delta\Psi$ and Q reduction level is no longer linear below a $\Delta\Psi$ value of approximately 125 mV and a Q reduction level of approximately 10% for all experimental conditions (data not shown). Therefore, succinate oxidation was not further decreased with higher concentrations of malonate.

These results indicate that the efficiency of guanine and adenine nucleotides in inhibiting AcUCP-sustained uncoupling in nonphosphorylating *A. castellanii* mitochondria depends on the Q redox state. Inhibition by PNs can be increased with decreasing Q reduction level in the range of respiration in which the $\Delta\Psi$–Q redox state relationship is linear. With decreasing Q reduction level, strong inhibition of LA-induced AcUCP-sustained uncoupling can be reached even with purine nucleoside monophosphates that are very weak inhibitors at the initial state 4 respiration.

3.3. The decreasing inhibitory effect of PNs on LA-induced AcUCP-sustained proton leak when the Q redox state is increased

To exclude the role of respiratory rate and $\Delta\Psi$ in the modulation of the PN inhibitory effect, they were gradually decreased using an inhibitor of the QH_2-oxidising pathway (the cytochrome pathway) leading to an increase in the Q reduction level. Fig. 3 shows proton–conductance curves (Figs. 3A and C) and the relationships between

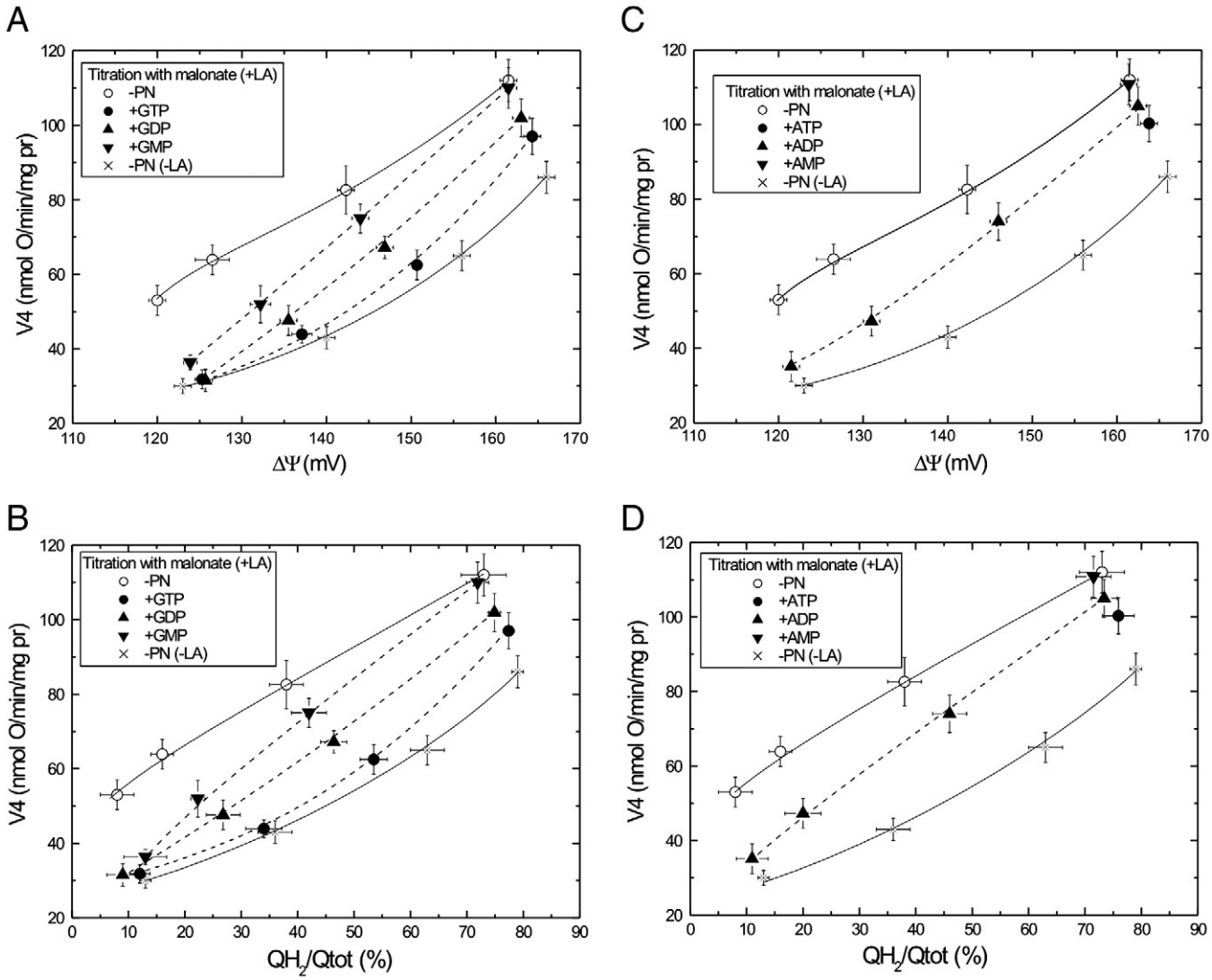

Fig. 2. The inhibitory effect of PNs on the LA-induced proton leak when the Q-reducing pathway is decreased during state 4 respiration. The relationships between (A, C) the respiratory rate (V4) and membrane potential (proton leak kinetics) and (B, D) V4 and Q redox state are shown. Succinate oxidation was gradually decreased by increasing the concentration of malonate (0.6, 1.3, and 2 mM) in the absence or presence of 9 μM LA and in the absence or presence of 2 mM guanine (A, B) and adenine (C, D) nucleotides. In the case of adenine nucleotides, titration with malonate is shown only with ADP. Values are means ± SD from three to six independent experiments.

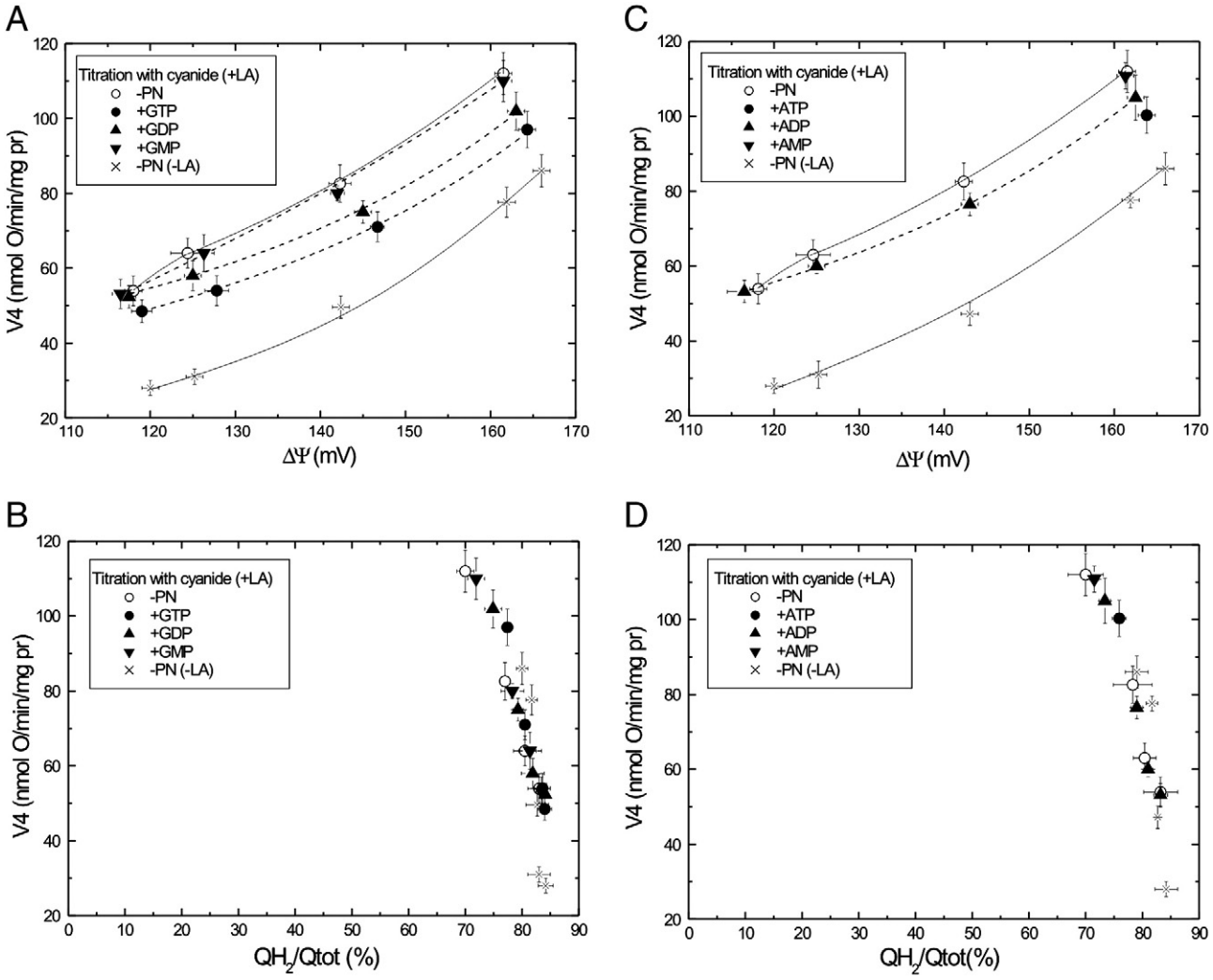

Fig. 3. The effect of PNs on the LA-induced proton leak when the QH_2-oxidising pathway is decreased during state 4 respiration**.** The relationships between (A, C) the respiratory rate (V4) and membrane potential (proton leak kinetics) and (B, D) V4 and Q redox state are shown. Titration with cyanide (0, 60, 80, 100 μM) was performed in the absence or presence of 9 μM LA and in the absence or presence of 2 mM guanine (A, B) and adenine (C, D) nucleotides. In the case of adenine nucleotides, titration with cyanide is shown only with ADP. Values are means ± SD from three to six independent experiments.

respiratory rate versus Q reduction level (Figs. 3B and D) in the presence or absence of 9 μM LA and/or 2 mM PNs during titration with three increasing concentrations of cyanide. The flux/force relationships indicated that inhibition of the LA-induced H^+ conductance by PNs progressively diminished, especially in the case of nucleoside tri- and diphosphates that displayed higher level of initial inhibition of the LA-induced AcUCP activity in the absence of cyanide compared to purine nucleoside monophosphates (Figs. 3A and C). The points obtained in the presence of LA and PNs progressively came forward to the points obtained in the presence of LA only. Because, during titration with cyanide, the Q redox state progressively increased within a very narrow range (from 70% to 84%), alleviation of the PN inhibitory effect is less visible within this steep relationship between the respiratory rate and the Q reduction level (Figs. 3B and D). At a given cyanide concentration, points obtained in the presence of LA and PNs came progressively closer to the points obtained in the presence of LA alone. Although titration of succinate oxidation with cyanide (Fig. 3) comprised of the respiratory rate and $\Delta\Psi$ ranges at which PN sensitivity progressively increased during titration of the Q-reducing pathway with malonate (Fig. 2), under these conditions, the inhibitory effect of PN was progressively cancelled. These results clearly indicate that the attenuation of the PN inhibitory effect on LA-induced H^+ leak cannot be attributed to changes in the $\Delta\Psi$ or respiratory rate. Inhibition by guanine and adenine nucleotides can be alleviated with increasing Q reduction level.

We studied the effect of superoxide dismutase on the LA-induced GTP-inhibited proton leak when the QH_2-oxidising pathway or the Q-reducing pathway was decreased during state 4 respiration**.** Fig. 4 shows proton–conductance curves (Figs. 4A and C) and the relationships between respiratory rate and Q reduction level (Figs. 4B and D) during titration with increasing concentrations of cyanide or malonate in the presence of superoxide dismutase (40 U/mg of mitochondrial protein). The results suggest that independently of superoxide dismutase, the endogenous Q redox state has no effect on basal or FFA-induced UCP-catalysed H^+ conductance in the absence of GTP, but it affects its sensitivity to inhibition by GTP. Addition of the enzyme did not significantly affect respiration rate, $\Delta\Psi$, or the Q redox state (for all conditions), indicating that modulation of GTP-mediated AcUCP inhibition by the endogenous Q redox state does not involve endogenous superoxide. Moreover, comparison of the titrations with malonate (lower Q redox state and likely lower superoxide formation) (Figs. 4A and B) and cyanide (high Q redox state and likely higher superoxide formation) (Figs. 4C and D) reveals that stimulation of AcUCP activity by endogenously formed superoxide can be excluded under our experimental conditions, i.e., during 10–12 min of measurements.

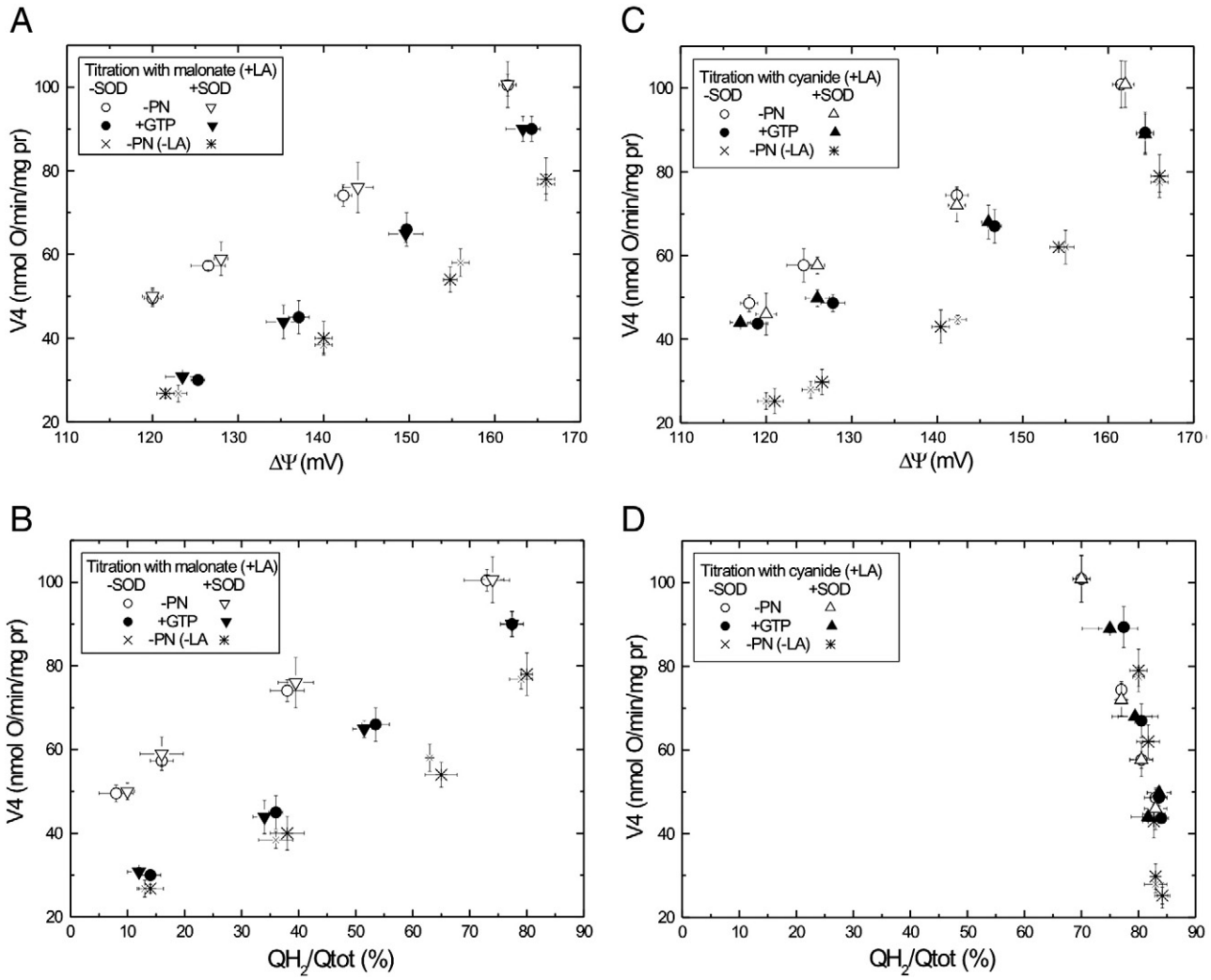

Fig. 4. The effect of superoxide dismutase on the LA-induced GTP-inhibited proton leak when the QH_2-oxidising or Q-reducing pathways are decreased during state 4 respiration. The relationships between (A, C) the respiratory rate (V4) and membrane potential (proton leak kinetics) and (B, D) V4 and Q redox state are shown. Titration with malonate (0, 0.6, 1.3 and 2 mM) (A, B) or cyanide (C, D) (0, 60, 80, 100 μM) was performed in the absence or presence of 9 μM LA, 2 mM GTP, and in the absence or presence of superoxide dismutase (SOD) (40 U/mg of mitochondrial protein). Values are means ± SD from three to four independent experiments.

3.4. The reduced form of Q (QH_2) but not oxidised Q (Qox) modulates AcUCP sensitivity to PNs

To elucidate whether the reduced or oxidised form of Q binds to AcUCP and influences its sensitivity to PNs, we designed the experiment in which the concentrations of GTP and LA as well as AcUCP protein content in mitochondrial membrane were constant while the content of membranous reducible Q was varied. Application of exogenous Q (Q9) (in various solvents) to change membranous reducible Q content in *A. castellanii* mitochondria was unsuccessful. Therefore, we used *A. castellanii* mitochondria isolated from two growth phases that differed significantly in the total mitochondrial membranous Q content. LA-induced GTP-inhibited H^+ leak was measured in mitochondria isolated from the middle and late exponential growth phases containing 1.4–1.5 and 1.0–1.1 nmol Qtot/mg of mitochondrial protein, respectively. A slight increase in state 4 respiration (at the same phosphorylating respiration rate) has been previously observed in *A. castellanii* mitochondria isolated from the late exponential growth phase compared to mitochondria from middle growth phase. However, mitochondria from these growth phases are characterised by having the same capacity of cytochrome pathway-dependent respiration as well as the activity and protein levels of AcUCP [20].

Our aim was to find the form of Q for which the LA-induced GTP-inhibited H^+ is the same for two types of mitochondria that differ in the endogenous Q content, for a given Q redox state or for a given concentration of QH_2 or Qox. The LA-induced AcUCP-catalysed H^+ conductance (the difference between H^+ leak in the presence and absence of LA) was the same (204 ± 11 nmol H^+/min/mg of mitochondrial protein with 9 μM LA) and not dependent on Q redox state, QH_2 concentration, or Qox concentration in both types of mitochondria, at a given LA concentration, in the absence of GTP (Fig. 5). Thus, the LA-induced H^+ leak remained constant as the Q reduction level was decreased despite a slight shift of curves obtained for all conditions towards higher H^+ leak rates in mitochondria isolated from the late exponential phase of growth with lower Q content. Fig. 5 shows the relationships between the LA-induced GTP-inhibited H^+ leak (calculated as difference between LA-induced H^+ leak in the absence and presence of GTP) and Q redox state (Fig. 5B), membranous QH_2 concentration (Fig. 5C), or membranous Qox concentration (Fig. 5D). In both types of mitochondria, when the rate of the Q-reducing pathway was progressively decreased during inhibition of succinate oxidation with malonate in the absence or presence of 9 μM LA and/or 2 mM GTP, the LA-induced GTP-inhibited H^+ leak increased with decreasing Q redox state (Fig. 5B) and QH_2 concentration (Fig. 5C) and decreased with decreasing Qox

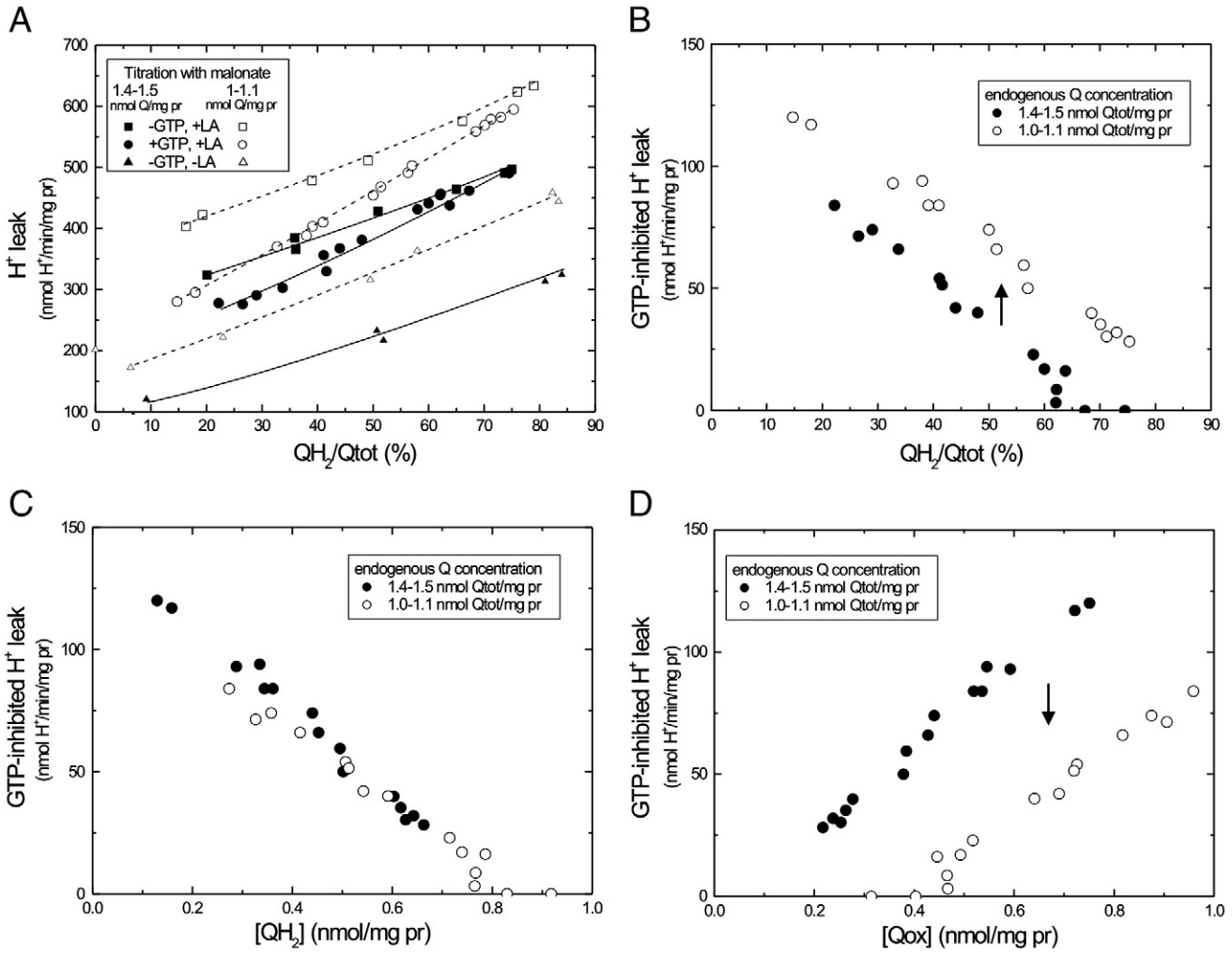

Fig. 5. The effect of Q redox state, reduced Q (QH_2) concentration, and oxidised Q (Qox) concentration on proton leak in mitochondria with different endogenous Q content. (A) The relationship between the H^+ leak (in the absence or presence of 9 μM LA and in the absence or presence of 2 mM GTP) and Q redox state. (B, C, D) The relationships between the GTP-inhibited H^+ leak and (B) Q redox sate (QH_2/Qtot) or (C) QH_2 concentration or (D) Qox concentration. Mitochondria isolated from the middle (1.4–1.5 nmol Qtot/mg of mitochondrial protein) and late (1.0–1.1 nmol Qtot/mg of mitochondrial protein) exponential growth phases were used. To calculate concentrations of reducible QH_2 and Qox, the content of nonreducible Q in total membranous Q content was taken into account. Succinate oxidation was gradually decreased by increasing the concentration of malonate (0–2 mM). GTP-inhibited H^+ leak rates (B, C, D) were calculated from A as difference between LA-induced H^+ leak in the absence and presence of GTP for a given Q redox state (or QH_2 concentration or Qox concentration, respectively). Data represent three different mitochondria preparations of each phase of growth.

concentration (Fig. 5D). When the leak in mitochondria with a higher total (and reducible) Q content was plotted versus Q redox state or Qox concentration, a shift of the LA-induced GTP-inhibited H^+ leak to higher or lower levels was observed. This shift is not observed when the LA-induced GTP-inhibited H^+ leak was plotted versus QH_2 concentration (Fig. 5C). Thus, mitochondria with different endogenous Q content appeared to have a single relationship only when the LA-induced GTP-inhibited H^+ leak is plotted versus QH_2 concentration. This clearly indicates that ubiquinol (QH_2) but not the oxidised form of Q binds to AcUCP and influences its sensitivity to PNs.

3.5. The negative allosteric interaction of GTP and QH_2

To investigate the inhibition of AcUCP activity by PNs and attenuation of this inhibition by QH_2, we determined the apparent concentrations of the inhibitor GTP (an apparent $I_{0.5}$) and the negative effector of this inhibition, QH_2 (an apparent $E_{0.5}$), that gave a half maximal effect on LA-induced H^+ leak by varying the concentrations of these effectors (Fig. 6). This established the dependence of inhibition of LA-induced AcUCP-mediated H^+ conductance in *A. castellanii* mitochondria on the concentration of GTP or QH_2. As PNs interact with UCPs when they are free and not complexed with Mg^{2+}, free GTP concentrations were calculated and applied to this analysis. Fig. 6A shows the LA-induced H^+ leak rates in the absence or presence of three different GTP concentrations when QH_2 concentration was varied with malonate during succinate oxidation. As above, in the absence of GTP, the LA-induced AcUCP-catalysed H^+ conductance was the same (218 ± 17 nmol H^+/min/mg of mitochondrial protein with 9 μM LA) and not dependent on QH_2 concentration.

Figure 6B shows concentration-dependent effect of GTP on GTP-inhibited H^+ leak, calculated as the difference between LA-induced H^+ leak in the absence and presence of GTP for a given GTP concentration, at four different concentrations of QH_2 (common for all GTP concentrations). The inhibitory effect of GTP concentration on LA-induced H^+ leak was observed as the GTP-inhibited H^+ leak increased with GTP concentration. With increasing concentrations of QH_2 (0.31, 0.43, 0.53, and 0.68 nmol/mg protein), the slope of the double-reciprocal plot was increased and the apparent $I_{0.5}$ values for GTP increased (0.56, 0.69, 1.24, and 1.95 mM, respectively), while the maximal H^+ leak rate did not change. These results confirm that QH_2 behaves as negative effector of AcUCP inhibition by GTP. QH_2 clearly decreases the affinity of AcUCP for GTP. Moreover, this regulatory effect indicates an opposite influence of both effectors, GTP and QH_2, on AcUCP activity.

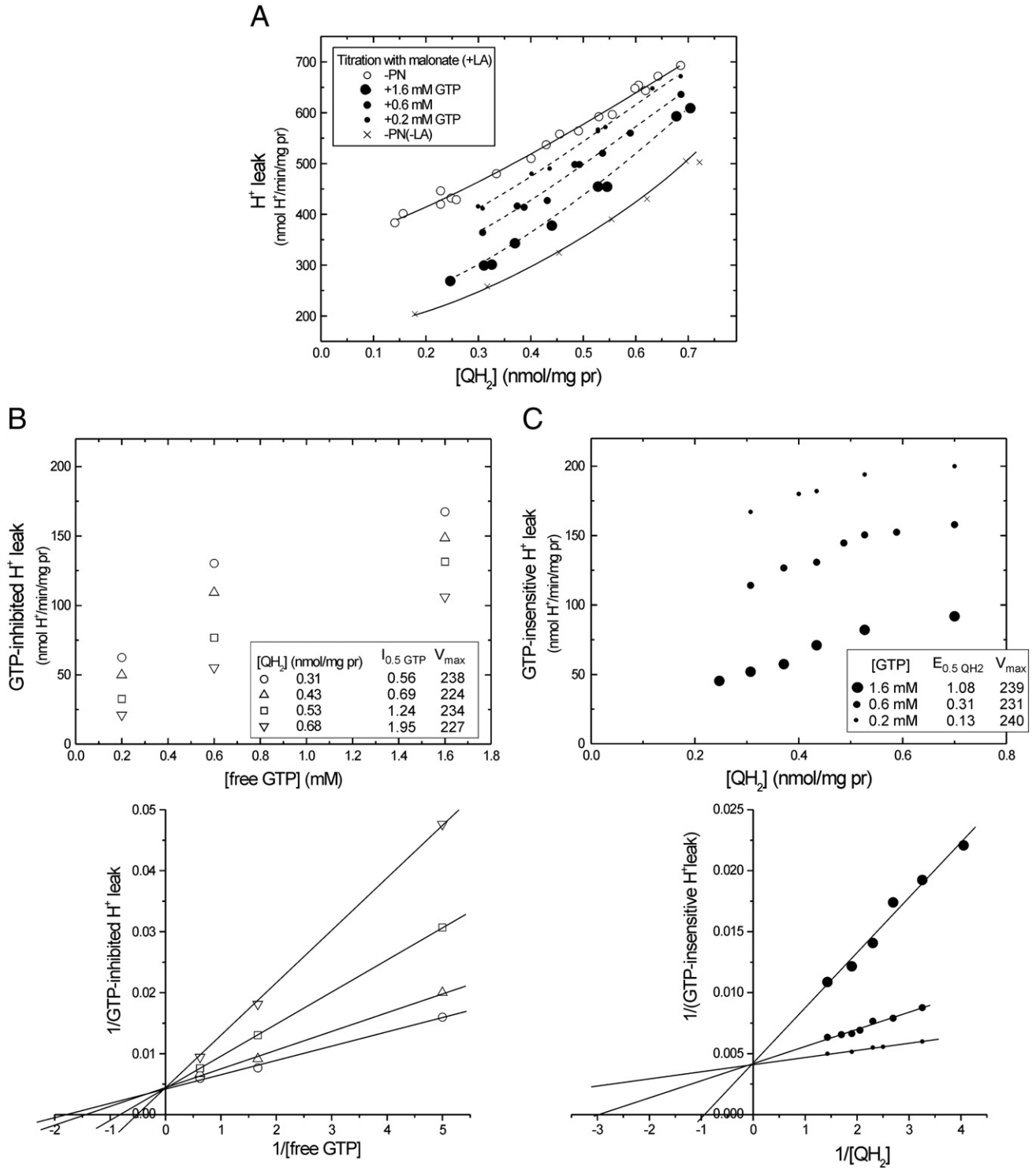

Fig. 6. AcUCP-specific interaction between GTP and QH_2. (A) The relationship between the H^+ leak (in the absence or presence of 9 μM LA and in the absence or presence of various GTP concentrations) and QH_2 concentration. Calculated free GTP concentrations are indicated. (B) The inhibitory effect of GTP concentration on LA-induced H^+ leak (AcUCP activity) in the presence of four different concentrations of QH_2. GTP-inhibited H^+ leak rates (B) were calculated from A as the difference between LA-induced H^+ leak in the absence and presence of GTP for a given GTP concentration is shown. For each GTP concentration, a separate trace was performed (no sequential additions). The double reciprocal plot of GTP-inhibited H^+ leak versus GTP concentration is presented. Values of the concentration required for 50% of maximum inhibition by GTP ($I_{0.5\ GTP}$) and of the rates of maximal GTP-inhibited H^+ leak (V_{max}), calculated by linear regression, are shown. Linear regressions ($y = A + BX$) revealed: for 0.31 nmol QH_2/mg protein, $A = 4.20\times10^{-3} \pm 5.01\times10^{-4}$, $B = 2.34\times10^{-3} \pm 1.63\times10^{-4}$, $r = 0.998$, $n = 3$; for 0.43 nmol QH_2/mg protein, $A = 4.46\times10^{-3} \pm 5.43\times10^{-4}$, $B = 3.09\times10^{-3} \pm 1.77\times10^{-4}$, $r = 0.998$, $n = 3$; for 0.53 nmol QH_2/mg protein, $A = 4.27\times10^{-3} \pm 6.01\times10^{-5}$, $B = 5.28\times10^{-3} \pm 1.96\times10^{-5}$, $r = 0.999$, $n = 3$; for 0.68 nmol QH_2/mg protein, $A = 4.41\times10^{-3} \pm 3.03\times10^{-4}$, $B = 8.59\times10^{-3} \pm 9.9\times10^{-5}$, $r = 0.999$, $n = 3$. Similar results were obtained from three independent mitochondria isolations, yielding similar LA-induced H^+ leaks. (C) The negative effect of QH_2 concentration on inhibition of LA-induced H^+ leak (AcUCP activity) by GTP in the presence of three different concentrations of GTP. GTP-insensitive H^+ leak rates (C) were calculated from A as difference between H^+ leak in the presence of GTP (plus LA) and basal H^+ leak (in the absence of GTP and LA) for a given QH_2 concentration. Decreasing concentrations of QH_2 were obtained with a given concentration of malonate (no sequential additions). The double reciprocal plot of GTP-insensitive H^+ leak versus QH_2 concentration is shown. Values of the concentration required for 50% of the maximum effect by a negative allosteric effector of inhibition by GTP ($E_{0.5\ QH2}$) and of the rates of maximal GTP-insensitive H^+ leak (V_{max}), calculated by linear regression, are shown. Linear regressions ($y = A + BX$) revealed: for 1.6 mM GTP, $A = 4.19\times10^{-3} \pm 9.23\times10^{-4}$, $B = 4.52\times10^{-3} \pm 3.36\times10^{-4}$, $r = 0.991$, $n = 6$; for 0.6 mM GTP, $A = 4.33\times10^{-3} \pm 2.81\times10^{-4}$, $B = 1.37\times10^{-3} \pm 1.25\times10^{-4}$, $r = 0.979$, $n = 7$; for 0.2 mM GTP, $A = 4.17\times10^{-3} \pm 9.07\times10^{-5}$, $B = 5.58\times10^{-4} \pm 3.85\times10^{-5}$, $r = 0.993$, $n = 5$.

Figure 6C shows the concentration-dependent effect of QH_2 on GTP-insensitive H^+ leak, calculated as the difference between H^+ leak in the presence of GTP (and LA) and basal H^+ leak (in the absence of GTP and LA) for a given QH_2 concentration, in the presence of three different concentrations of GTP. The negative effect of QH_2 concentration on inhibition of LA-induced H^+ leak by GTP was observed as GTP-insensitive H^+ leak increased with QH_2 concentration. With increasing concentrations of GTP (0.2, 0.6, and 1.6 mM), the slope of the double-reciprocal plot was increased and the apparent $E_{0.5}$ values for QH_2 increased (0.13, 0.31, and 1.08 nmol/mg protein, respectively), while the maximal H^+ leak rate did not change. Increasing GTP concentration decreases the affinity of AcUCP for QH_2. This regulatory effect indicates that both effectors, GTP (as the inhibitor) and QH_2 (as negative effector of the inhibition by GTP), competitively influence on the AcUCP activity.

4. Discussion

The results presented in this study obtained with nonphosphorylating *A. castellanii* mitochondria show that the guanine and adenine nucleotides exhibit an inhibitory effect on the FFA-induced AcUCP-sustained uncoupling in the following descending order: GTP>ATP>GDP>ADP≫GMP>AMP. The affinity of UCP1 of mammalian brown adipose tissue for different purine nucleotides decreases in a similar order. Guanine nucleotides exhibit higher affinities than adenine nucleotides, while the monophosphates are poor ligands [32]. The PN sensitivity profile found in *A. castellanii* mitochondria is maintained when the redox state of membranous Q is varied. Our results suggest that the endogenous Q redox state has no effect on the basal and FFA-induced AcUCP-catalysed H^+ conductance in the absence of PNs but affects its sensitivity to inhibition by nucleotides. Thus, AcUCP has an inhibitor specificity that is dependent on nucleobase type and number of phosphate groups and this inhibition can be regulated by the redox state of membranous Q (particularly by QH_2 concentration). At a given LA concentration and at a given PN concentration, inhibition by guanine and adenine nucleotides increases when Q is sufficiently oxidised and conversely diminishes when Q is sufficiently reduced. By decreasing the Q reduction level, LA-induced AcUCP-sustained uncoupling can be strongly inhibited even with nucleoside monophosphates that are very weak inhibitors in the initial state 4 respiration. The results suggest that when there is low respiratory substrate availability corresponding to a low Q reduction level (a low QH_2 concentration), AcUCP can be inhibited by all guanine and adenine nucleotides. This mechanism likely preserves ATP synthesis yield. Conversely, when the cytochrome pathway is impaired corresponding to increased Q reduction level (a higher QH_2 concentration), inhibition of AcUCP is diminished likely in order to limit the reactive oxygen species production by mitochondria.

In *A. castellanii* mitochondria, fatty acids seem to be essential for AcUCP function, as in their absence no (or weak), inhibition by PNs is observed with succinate or external NADH as respiratory substrates. The decrease in the Q redox state (and thus the QH_2 concentration) in the presence of FFA [10] could allow inhibition by PNs. One important step in elucidating the kinetic mechanism of AcUCP inhibition by PNs was finding whether the reduced or oxidised form of Q influences AcUCP sensitivity to PNs. Our present results show that the relationship between the LA-induced GTP-inhibited H^+ leak and QH_2 concentration (but not Qox concentration or Q redox state) does not depend on the membranous Q content at given FFA and PN concentrations, at a given AcUCP protein mitochondrial content and activity but at a different endogenous mitochondrial Q contents. This is clear kinetic evidence that ubiquinol (QH_2) but not oxidised Q (Qox) directly influences the PN inhibition of AcUCP. Thus, our model [16,28] describing the function of reduced ubiquinone (QH_2) in modulating PN inhibition of AcUCP (Fig. 7) now has direct

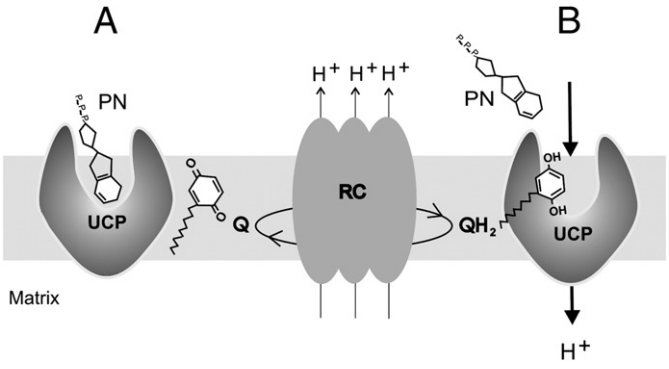

Fig. 7. A model for negative regulation of UCP purine nucleotide inhibition by QH_2. At a given fatty acid concentration, (A) an increased amount of QH_2 could lead to decrease in the binding affinity of PN thereby alleviating inhibition of UCP activity by the nucleotide. UCP-mediated H^+ reuptake is enhanced. (B) Conversely, at a lower QH_2 concentration, no negative regulation occurs and proton conductance through UCP is inhibited by PN. RC, respiratory chain.

experimental support. At a given FFA concentration, an increased concentration of membranous QH_2 could lead to a decreased AcUCP sensitivity to PN, thereby alleviating inhibition of UCP. QH_2 plays a role as a negative regulator of AcUCP inhibition by PNs. Conversely, at a lower QH_2 concentration, PN may bind to AcUCP, inhibiting UCP-mediated proton conductance.

In view of the possibility that PNs and QH_2 may act competitively on AcUCP, we investigated the dependence of H^+ conductance inhibition on the concentration of GTP or QH_2. Changes in the availability of the inhibitor (GTP) or the negative inhibition modulator (QH_2), resulted in a competitive-like influence on AcUCP activity. QH_2 clearly decreases the affinity of AcUCP for GTP and, *vice versa*, GTP decreases affinity of AcUCP for QH_2. Therefore, PNs and QH_2 may interact dependently but in opposite ways with AcUCP. Simple competitive interaction is most easily understood as two agents interacting at one site. Although PNs and QH_2, which is more hydrophilic than Qox, have very different structures, some similarity could be found as they both are composed of aromatic rings. The structure of the PN-binding site is fairly well established for mammalian UCPs [33], but no attempts have been made to elucidate that of QH_2. Therefore, further studies are necessary to determine whether PNs and QH_2 could bind to the same binding site.

Studies with mammalian UCPs have yielded conflicting results concerning the possibility that Q may be an obligatory cofactor for their action. Oxidised Q (Qox) has been shown to activate PN-sensitive FFA-dependent H^+ transport through reconstituted UCP1-3 [22,34]. On the other hand, other studies have shown that Qox has no significant activating effect on FFA-dependent H^+ translocation or any effect on the inhibition by PN in reconstituted UCP1-3 [35]. Studies with mitochondria isolated from yeast mutants expressing mouse UCP1 but lacking ubiquinone have also shown that Q is not required for proton conductance by UCP1 [36]. These latter observations are consistent with the present study showing that the endogenous Q redox state (thereby Qox and QH_2) has no effect on the basal and FFA-induced AcUCP-catalysed H^+ conductance in the absence of PNs and that Qox does not affect its sensitivity to inhibition by nucleotides in contrast to QH_2. For kidney mitochondria, it has been postulated that exogenous Q, likely in its reduced form, activates proton conductance in mitochondria through the production of superoxide, as superoxide dismutase inhibits Q-induced mitochondrial uncoupling [37]. However, it was concluded that the amount of endogenous Q reduced during titration with myxothiazol or cyanide is not sufficient to induce superoxide-stimulated proton leak until exogenous Q is added [37]. Our results suggest that endogenous QH_2 has no effect on the basal and FFA-induced AcUCP-catalysed H^+ conductance in the absence of

PNs but affects its sensitivity to inhibition by nucleotides. This conclusion can be made as the concentration of membranous QH_2 was determined when H^+ leak curves were established with inhibitors of the Q-reducing or QH_2-oxidising pathways.

The current model for the activation of UCPs by superoxide through the initiation of lipid peroxidation [38,39] assumes that superoxide generated within mitochondria and a high membranous Q reduction level (as required for superoxide formation) work indirectly as UCP activators by generating lipid peroxidation products in the mitochondrial inner membrane. However, in our opinion, these indirect effects could be a late response of UCPs as flux–force studies with isolated mitochondria of any type, plant, animal or protozoan, do not reveal any effect of endogenously generated superoxide or the endogenous Q redox state on basal or FFA-induced activity of UCP1 or its homologues in the absence of PNs. To observe the indirect activation of UCPs by superoxide (or a high Q redox state), exogenous lipid peroxidation products such as hydroxy-nonenal (a few minutes of incubation at a high concentration, ~35 μM) or an exogenous system that generates superoxide such as xanthine plus xanthine oxidase (which leads to much more superoxide formation than mitochondria) must be applied. In our opinion, activation of UCPs by endogenous lipid peroxidation products requires a prolonged period of increased endogenous superoxide formation. Interestingly, it has been shown recently that endogenous activation of proton conductance in isolated mammalian mitochondria (including UCP-mediated uncoupling) increases with incubation time and requires a high membrane potential [40]. Our results obtained with AcUCP-containing *A. castellanii* mitochondria ([7,10] and this study) and those results previously described for plant and mammalian UCP1 homologues [26,27] and UCP1 in rat brown adipose tissue mitochondria [25] indicate that the quick response through the endogenous Q redox state (particularly through the endogenous QH_2 concentration as determined in this study) can directly regulate UCP activity and that this quick response does not involve endogenous superoxide formation or arisen lipid peroxidation products. Because the presence of superoxide dismutase did not affect the negative regulation of PN inhibition of AcUCP by the Q reduction level (Fig. 4), the involvement of endogenous superoxide can be excluded from the quick response of UCPs, but it could be important at longer time periods when lipid peroxidation products accumulate (the late response). Thus, in our model for the activation of UCPs involving the quick response to a high Q reduction level in mitochondria [16,28], at a given FFA concentration, an increased concentration of QH_2 (at a higher Q reduction level) activates UCP by relieving inhibition from PNs. On the other hand, at a lower QH_2 concentration (at a low endogenous Q reduction level), PN may bind to UCP and proton conductance through UCP would be inhibited. It should be emphasised that the described regulation of UCP by the endogenous Q redox state has so far been observed only for FFA-induced UCP-mediated uncoupling. Therefore, further studies are needed to determine if regulation of the sensitivity of UCP to PNs through the membranous QH_2 concentration can also be observed under conditions when uncoupling is activated by lipid peroxidation products, and thus if it concerns the mentioned late response of UCP.

Our finding that QH_2, and not Qox, directly influences PN inhibition of AcUCP and that it functions as a competitive regulator for the PN inhibitory effect represent a significant step towards understanding one of the mechanisms of UCP activity regulation in unicellular eukaryote mitochondria.

Acknowledgments

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