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Stimulation of ATP synthase by Ca^{2+} in heart mitochondria

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We have shown that Ca^{2+} ions within the physiological range of concentrations activate the succinate oxidation rate in rat heart mitochondria at 28 °C, but have no effect at 37 °C. The ability of Ca^{2+} to stimulate only state 3 respiration at 28 °C but not the respiration in the uncoupled state confirms that the respiratory subsystem is not sensitive to Ca^{2+} and that the stimulatory effect of Ca^{2+} resides within the phosphorylation subsystem. The results of modular kinetic analysis are in line with this conclusion. Since the only component of the phosphorylation subsystem exerting an appreciable control over respiration rate in state 3 at 28 °C is ATP synthase, we conclude that Ca^{2+} directly stimulates ATP synthase. This effect is responsible for activation of the phosphorylation subsystem by calcium, however, it is manifested only under conditions where ATP synthase contributes to the control of respiration (at 28 °C but not at 37 °C).

Key words: heart mitochondria, calcium, oxidative phosphorylation, ATP synthase, metabolic control analysis

INTRODUCTION

In most tissues the mitochondrial ATP synthase generates the bulk of ATP for fueling energy consuming processes. Mitochondrial respiration and ATP production are tightly regulated in response to energy demand [1–3]. It has been suggested that *in vivo* heart respiration is regulated not by adenine nucleotide levels but by calcium [2]. Calcium increases NADH supply to the respiratory chain by stimulating matrix dehydrogenases: pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase (reviewed in [4]). There are also indications that Ca^{2+} stimulates the mitochondrial respiratory chain [2], ATP synthase [5, 6] and the adenine nucleotide translocator (ANT) [7], but the evidence for such actions is relatively poor. Using metabolic control analysis, we demonstrated that calcium had no significant direct kinetic effects on the ANT [8], although it clearly stimulated the phosphorylation subsystem in heart mitochondria [9]. Another potential site in the phosphorylation subsystem that might be activated by Ca^{2+} is ATP synthase [5]. Therefore in this study we experimentally investigated Ca^{2+} effects on mitochondrial respiration with succinate at two different temperatures (28 °C and 37 °C) with the aim to check the hypothesis that calcium activates ATP synthase in rat heart mitochondria.

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MATERIALS AND METHODS

Mitochondria were isolated from the heart of male Wistar rats as described previously [9]. Mitochondria were suspended in the buffer containing 180 mM KCl, 20 mM Tris-HCl, 3 mM EGTA (pH 7.35) and stored on ice. The protein concentration was estimated by a modified biuret method [10]. The quality of mitochondrial preparations was determined by the respiratory control index RCI equal to the ratio of respiratory rates (V_3/V_2) of mitochondria in state 3 (V_3) and state 2 (V_2) (according to the common terminology [11]). Mitochondrial respiration and transmembrane difference in electric potential ($\Delta\Psi$ were measured in a closed, stirred and thermostatically controled 1.5 ml vessel as described previously [9] in a medium containing 30 mM Tris-HCl, 5 mM KH_2PO_4, 100 mM KCl, 50 mM creatine, 10 mM NaCl, 3 mM EGTA. The concentration of free Ca^{2+} and Mg^{2+} in the incubation medium was stabilized by Ca^{2+}/EGTA buffers and calculated using the stability constants and the program published by Fabiato and Fabiato [12]. Either 0.09 mM $CaCl_2$ (5 nM free Ca^{2+}) plus 1.5 mM $MgCl_2$ (1 mM free Mg^{2+}) or 2.56 mM $CaCl_2$ (1 μM

Table. **The functional and control parameters of heart mitochondria oxidizing 5 mM succinate (+ rotenone) at different temperature and Ca^{2+} concentration**

Temperature / Ca^{2+} concentration	28 °C		37 °C	
	5 nM	1 μM	5 nM	1 μM
V_2	73 ± 5	76 ± 6	155 ± 23**	151 ± 18**
V_3	213 ± 10	249 ± 18*	385 ± 32**	385 ± 23**
V_{CCCP}	235 ± 24	235 ± 15	379 ± 19**	380 ± 23**
RCI	3.0 ± 0.2	3.3 ± 0.2*	2.7 ± 0.2**	2.75 ± 0.06**
$C_{ATP\text{-}synthase}$	0.26 ± 0.04	–	0	0
C_{ANT}	0	–	0	0
$C_{Pi\ carrier}$	0	–	–	–

The rate of respiration is expressed in nmolO/min per mg protein. Mitochondrial respiration was measured in the medium described in Methods. The rate of uncoupled respiration was registered after addition of 0.2 μM CCCP. The flux control coefficient of ATP synthase ($C_{ATP\ synthase}$) was determined by titration of the respiratory rate with oligomycin (0–60 nmol/mg protein), the flux control coefficient of ANT (C_{ANT}) – by titration with CAT (0.1–1.2 μM) and the flux control coefficient of phosphate carrier – ($C_{Pi–carrier}$) – by titration with mersalile (0–10 μM). * – statistically significant effect of Ca^{2+}, ** statistically significant effect of temperature.

free Ca^{2+}) plus 1.3 mM $MgCl_2$ (1 mM free Mg^{2+}) were used. Modular Kinetic Analysis (MKA) was performed as described in detail earlier [12]. The system of mitochondrial oxidative phosphorylation was divided into 3 subsystems connected by a common intermediate $\Delta\Psi$ (as proposed in [13]): the proton leak subsystem (L), the respiratory subsystem (R), and the phosphorylation subsystem (P). We estimated the kinetics of these subsystems from the dependencies of the fluxes (J_L, J_R, and J_p, respectively) through these subsystems on $\Delta\Psi$: J_R kinetics was determined by titrating mitochondrial respiration in state 3 with an inhibitor of phosphorylation system (oligomycin); J_L kinetics was determined by malonate titration under the conditions of complete inhibition of phosphorylation by excess oligomycin (1 μg/mg mitochondrial protein); J_p kinetics was determined by titrating mitochondrial respiration in State 3 with an inhibitor of respiration system (malonate) in the conditions of active respiration (without oligomycin). J_p at any given $\Delta\Psi$ is calculated as $J_P = J_R – J_L$ at the same $\Delta\Psi$ [14].

The control of ATP-synthase, ANT or P_i carrier on the respiratory rate was determined with non-competitive, tight binding inhibitors (oligomycin, carboxyatractiloside (CAT) and mersalile, respectively) as described in [8].

Statistical analysis was performed using Student's t test. Data are expressed as means ± S.E.M. of at least 3 separate experiments.

RESULTS

Metabolic Control Analysis [15–17] provides means for analyzing the response of multienzyme systems to pleiotropic effectors. According to this approach (reviewed in [16]), the total response of flux J through the pathway to a multi-site effector P (or the flux response coefficient R^J_p) is described as the sum of the individual responses of each enzyme affected: $R^J_p = \Sigma\ C^J i\ \varepsilon^i_p$, where $C^J i$ is the sensitivity of the pathway to the activity of the enzyme that is the target for the effector (the enzyme's E_i flux control coefficient, C_i), and ε^i_p is the sensitivity of the individual enzyme to the effector P (elasticity coefficient). That means that an effector cannot act on a pathway by changing the activity of an enzyme with a near zero flux control coefficient, even if it affects the enzyme. Therefore we experimentally investigated how the overall response of the oxidative phosphorylation system to Ca^{2+} in mitochondria oxidizing succinate depends on the contribution of ATP synthase to the control of oxygen consumption rate.

Under the experimental conditions used, increase of calcium concentration in the medium from 5 nM to 1 μM stimulated oxidation of NAD^+-dependent substrates, however, it had no effect on succinate oxidation in state 3 [7, 8]. In contrast to our data, Moreno-Sanchez has reported [18] that Ca^{2+} ions stimulate succinate oxidation in heart mitochondria; however, their experiments were carried out at a lower non-physiological temperature (30 °C). Therefore we have compared the effect of Ca^{2+} on succinate oxidation in state 3 at 37 °C and at 28 °C on the same preparations of heart mitochondria.

The results of these experiments (Table) show that Ca^{2+} ions stimulate the rate of succinate oxidation in state 3 by 17% at 28 °C, however, neither the rate of uncoupled respiration with succinate at 28 °C nor respiration in state 3 and uncoupled respiration at 37 °C are affected by Ca^{2+}. The ability of Ca^{2+} to stimulate only state 3 respiration at 28 °C but not the respiration in the uncoupled state confirms that the respiratory subsystem in mitochon-

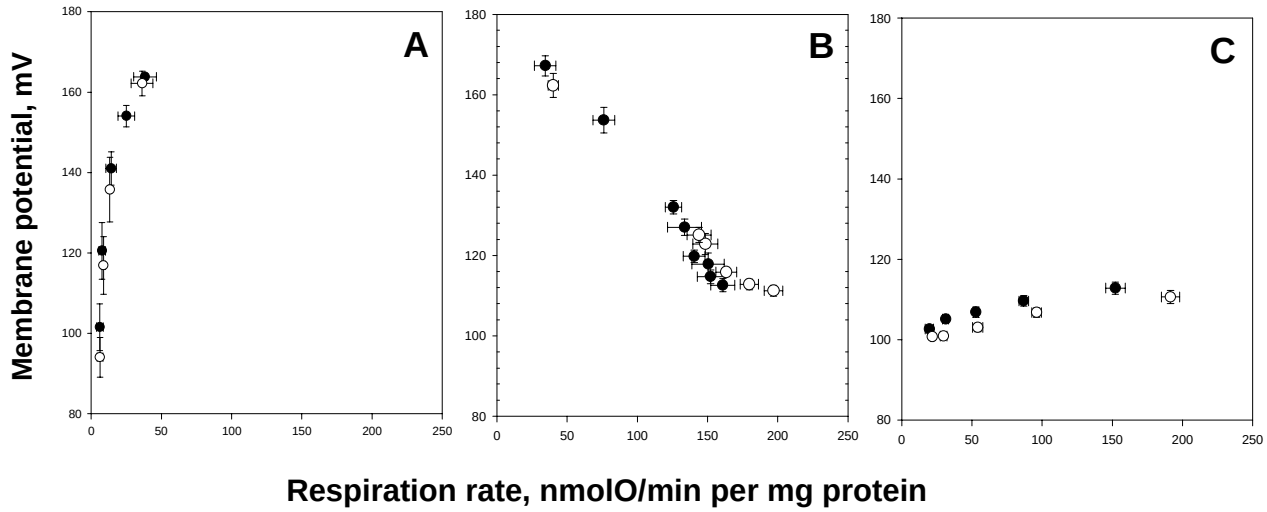

Figure. Effect of calcium on the relationship between membrane potential and mitochondrial respiration with 5 mM succinate in state 3 at 28 °C. A – kinetics of the membrane leak, B – kinetics of the respiration subsystem, C – kinetics of the phosphorylation subsystem (n = 5); ● – 5 nM, ❍ – 1 μM extramitochondrial free Ca^{2+}

dria oxidizing succinate is not sensitive to Ca^{2+} and the stimulatory effect of Ca^{2+} resides within the phosphorylation subsystem.

The stimulation of succinate oxidation in state 3 at 28 °C and the absence of the effect at 37 °C should be caused by a shift of the flux control towards Ca^{2+}-sensitive components of the phosphorylation subsystem with a decrease of the temperature. In order to determine differences in the contribution of the processes involved in the phosphorylation subsystem (*i.e.* ATP synthase, ANT or phosphate (P_i) carrier) to the control of respiration flux in state 3 at 28 °C and 37 °C, we used titration with specific inhibitors (oligomycin, CAT and mersalile, respectively). The results (Table) show that the only component of the phosphorylation subsystem exerting an appreciable control over respiration rate in state 3 at 28 °C was ATP synthase. The oligomycin titration curve has a sigmoidal shape at 37 °C, indicating that under these conditions ATP synthase does not control the rate of respiration, therefore Ca^{2+} does not stimulate oxygen consumption, even though it stimulates ATP synthase.

To obtain a more convincing evidence that stimulation of succinate oxidation is determined by Ca^{2+} effects on the phosphorylation subsystem only, we performed MKA [14]. Using this approach, a complex process is simplified by dividing it into a small number of modules connected by common intermediates. In our case, we divided oxidative phosphorylation into three subsystems (the proton leak, the respiratory and the phosphorylation subsystems) that are connected by the membrane potential $\Delta\Psi$ [9] To detect the components of the system that are influenced by an effector, the effector-induced shift in kinetic dependencies of each module on the $\Delta\Psi$ is determined.

Direct measurement of ATP synthase and ANT activity in mitochondria is complicated, since the kinetics of ADP phosphorylation is ultimately dependent on the rest part of the oxidative phosphorylation system that produces $\Delta\Psi$, *i.e.* the respiratory chain that mostly controls the phosphorylation flux [9, 14, 19]. Any factor disturbing the activity of the respiratory subsystem (or the membrane leak) via induced changes in $\Delta\Psi$ elicits secondary effects on the phosphorylation flux. Measurement of ATP synthase activity *per se* is hardly technically possible and is conventionally estimated by the reverse reaction (ATP hydrolytic ability), however, the conditions for ATPase kinetic measurements are far from being relevant to physiological conditions of mitochondria in state 3.

In MKA, the flux of phosphorylation is estimated by subtracting the portion of oxygen consumption determined by the membrane leak J_L from the flux through the respiratory subsystem J_R at a certain value of $\Delta\Psi$ (*i.e.*, $J_P = J_R - J_L$). Thus, this approach provides a valuable tool for the detection of changes in J_P kinetics *per se* induced by an effector. The kinetic dependencies for all three subsystems measured at 5 nM and 1 μM $Ca^{2+}$and 37 °C completely coincided (data not shown), and at 28 °C Ca^{2+} induced an appreciable shift only in the kinetics of the phosphorylation subsystem (Figure, C), implying that at both temperatures (28 °C and 37 °C) an increase in the concentration of Ca^{2+} does effect the kinetics of the proton leak (J_L) and the respiratory subsystem (J_R), and that it stimulates the phosphorylation subsystem only at 28 °C. The results of these experiments provide additional evidence that the stimulation of mitochondrial respiration by Ca^{2+} at 28 °C is determined by the components of the phosphorylation subsystem.

Thus, we conclude that the effect of Ca^{2+} on succinate oxidation occurs via interaction of Ca^{2+} with ATP synthase, but it is manifested only under conditions where ATP synthase contributes to the control of respiration. Together with the obtained evidence that Ca^{2+} has no direct kinetic effects on ANT [9] and that the phosphate carrier is not important in the flux control (Table), our data indicate that Ca^{2+} ions stimulate ATP synthase.

Ca^{2+} ions are the key regulators integrating cellular metabolic mashinery and determining the concerted response to external and internal signals. Besides involvement in multiple other signalling pathways, interaction of Ca^{2+} with mitochondrial enzymes is important as a tool for a tight manipulation between energy production and demand. The stimulation of ATP synthase by Ca^{2+} was intuitively suspected by many researchers [5, 6, 20], however, attempts to prove the effect by direct evidences confirmed that it is hardly identifiable. Data indicating the existence of special Ca^{2+}-dependent inhibitor protein of ATP synthase [20] have not been reproduced by other authors. More recently, Balaban's group [6] has presented a very complicated experimental evidence that Ca^{2+} stimulates ATP synthetic activity. Our data confirm their main conclussion, however, we derive it in a different, simpler and more direct way.

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Ca^{2+} JONAI AKTYVINA ÐIRDIES MITOCHONDRIJØ ATP SINTAZÆ

S a n t r a u k a

Nustatëme, kad Ca^{2+} jonai, didëjant jø koncentracijai fiziologiniø reikðmiø ribose, aktyvina sukcinato oksidacijà þiurkës ðirdies mitochondrijose esant 28 °C temperatûrai, taèiau nepaveikia ðio proceso greièio, kai temperatûra 37 °C. Tai, kad Ca^{2+} gali stimuliuoti sukcinato oksidacijà (28 °C) tik esant treèiai metabolinei bûsenai, o ne atskirtai ðirdies mitochondrijø bûsenai rodo, jog kvëpavimo grandinë yra nejautri Ca^{2+} jonams, ir stimuliuojantis poveikis paaiðkinamas efektoriaus sàveika su fosforilinimo posistemës sandais. Moduliø kinetinës analizës rezultatai patvirtino ðià prielaidà. Vienintelë fosforilinimo posistemës grandis, turinti akivaizdþià átakà kvëpavimo greièio kontrolei, esant treèiai metabolinei mitochondrijø bûsenai 28 °C temperatûroje, yra ATP sintazë. Tai leidþia daryti iðvadà, kad Ca^{2+} jonai tiesiogiai stimuliuoja ATP sintazê. Šis efektas lemia ir fosforilinimo aktyvinim¹ veikiant Ca^{2+}, taèiau jis gali pasireikðti tik tuomet (esant 28 °C, bet ne 37 °C temperatûrai), kai ATP sintazë kontroliuoja kvëpavimà.