Ca²⁺ stimulates both the respiratory and phosphorylation subsystems in rat heart mitochondria

Vida MILDAZIENE*, Rasa BANIENE*, Zita NAUCIENE*, Ausra MARCINKEVICIUTE*, Ramune MORKUNIENE*, Vilmante BORUTAITE*, Boris KHOLODENKO† and Guy C. BROWN‡

* Institute for Biomedical Research, Kaunas Medical Academy, 3007 Kaunas, Lithuania, † A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia and ‡ Department of Biochemistry, University of Cambridge, Cambridge, U.K.

Stimulation of mitochondrial respiration by physiological concentrations of Ca²⁺ was studied to determine which components of oxidative phosphorylation are affected by Ca²⁺. The kinetic dependence of the respiratory chain, phosphorylation subsystem and proton leak on the mitochondrial membrane potential in isolated rat heart mitochondria respiring on 2-oxoglutarate or succinate was measured at two different concentrations of external free Ca²⁺. The results show that proton leak is not directly affected by Ca²⁺, but that both the respiratory and phosphorylation systems can be directly stimulated by Ca²⁺

depending on conditions. Although Ca²⁺ directly stimulates the phosphorylation system, this has relatively little effect on respiration rate with 2-oxoglutarate in States 3 and 4 because the subsystem has little control over respiration. However, in intermediate states, the phosphorylation system has greater control and Ca²⁺ stimulation of this system contributes substantially to the stimulation of respiration and phosphorylation. In the case of succinate oxidation neither the respiratory subsystem nor the phosphorylation system is stimulated by Ca²⁺.

INTRODUCTION

The mechanism by which the cell achieves tight coupling between ATP demand and ATP production remains a topic of intensive study (reviewed in refs. [1–3]). Recent ³¹P-NMR data, particularly those obtained on heart [4,5], clearly indicate that an increased work rate causes an increased respiration rate via regulation other than ADP, ATP and phosphate. Intramitochondrial free Ca²⁺ concentration appears to be the most likely candidate for an additional metabolic regulator to supplement ADP, ATP and phosphate [6]. Mitochondria possess a Ca²⁺-transport system that allows rapid access of Ca²⁺ to the possible sites of action: three dehydrogenases (the pyruvate dehydrogenase complex, NAD⁺-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase [7–9]), the components of the respiratory chain [10], the F₀F₁-ATPase [11] and the adenine nucleotide translocator [12,13]. In addition, Ca²⁺ cycling by the Ca²⁺-transport system itself may increase effective proton permeability, causing uncoupling [6].

In heart mitochondria, the 2-oxoglutarate dehydrogenase is supposed to be the most important target of direct activation by Ca²⁺ [14]. The evidence suggesting Ca²⁺ effects on mitochondrial function at several other sites between NADH oxidation and ADP phosphorylation is relatively slight. Nevertheless, there is the clear possibility that Ca²⁺ in the submicromolar to low micromolar range stimulates mitochondrial respiration and ATP synthesis more effectively by acting in a co-ordinated manner on several steps. In this study, we investigated the relative importance of Ca²⁺ effects on different parts of the oxidative phosphorylation system for overall flux through the system.

EXPERIMENTAL

Isolation of mitochondria

Hearts from several male Wistar rats were removed and washed with ice-cold 0.9% KCl. Subsequently the tissue was cut

into small pieces and homogenized in buffer containing 160 mM KCl, 10 mM NaCl, 20 mM Tris/HCl, 5 mM EGTA and 1 mg/ml BSA (pH 7.7). The homogenate was centrifuged at 450 g, and the supernatant obtained was centrifuged at 6300 g. The mitochondrial pellet was resuspended in buffer containing 180 mM KCl, 20 mM Tris/HCl and 3 mM EGTA (pH 7.35) to approximately 50 mg/ml protein and stored on ice. The whole procedure was performed at 4 °C. The protein concentration was determined by the biuret method.

Measurement of mitochondrial respiration and membrane potential

Respiration and membrane potential of mitochondria were measured in a closed, stirred and thermostatically controlled 1.5 ml vessel fitted with both a Clark-type oxygen electrode and a tetraphenylphosphonium (TPP⁺)-selective electrode. The experiments were performed at 37 °C using 2-oxoglutarate (1 or 10 mM) or 5 mM succinate (plus 1 μM rotenone) as oxidizable substrate, and 0.5 mg/ml mitochondria. Free Ca²⁺ and Mg²⁺ concentrations in the incubation media were stabilized by Ca²⁺ EGTA buffers and calculated using the stability constants and the program published by Fabiato and Fabiato [15]. In all experiments with these buffers care was taken to adjust pH as precisely as possible to 7.2 at 37 °C after addition of Ca²⁺ (either 5 nM free or 1 μM free) and Mg²⁺ (1 mM free). All solutions added to the vessel were prepared in such a way that they did not change the pH on addition.

The [ATP]/[ADP] ratio was clamped by adding excess creatine kinase (0.1 mg/ml), 1 mM ATP and varying the ratio of creatine and phosphocreatine, while their sum was kept constant at 50 mM [16]. The intermediate rate of mitochondrial respiration (called ‘State 3.5’ here) comprising approximately 40–60% of the rate in State 3 was achieved in medium containing 30 mM Tris/HCl, 5 mM KH₂PO₄, 80 mM KCl, 10 mM NaCl, 3 mM EGTA, 1 mM dithiothreitol, 1 mM ATP, 30 mM phosphate

Abbreviations used: TPP⁺, tetraphenylphosphonium; Δ*p*, protonmotive force; ΔΨ, membrane potential; Δ*p*H, pH gradient across the mitochondrial inner membrane; *J*_R, rate of respiration; *J*_P, rate of phosphorylation; *J*_L, rate of proton leak; *J*_O, rate of oxygen consumption.

§ To whom correspondence should be addressed.

creatine, 20 mM creatine (and either 0.09 mM CaCl_2 plus 2.79 mM MgCl_2 or 2.6 mM CaCl_2 plus 3.0 mM MgCl_2). For the maximal rate of respiration corresponding to the rate in State 3 the same medium was used except that phosphocreatine was absent, the concentration of creatine was 50 mM and KCl was 110 mM (0.09 mM CaCl_2 plus 2.3 mM MgCl_2 or 2.6 mM CaCl_2 plus 2.2 mM MgCl_2). Mitochondrial respiration in state 3 and State 3.5 was measured after the addition of 1 mM ATP after 3–3.5 min of preincubation of mitochondria in the respective incubation medium for equilibration with Ca^{2+} [9], otherwise the rate of respiration with 1 mM 2-oxoglutarate and 5 nM free Ca^{2+} decreased with time. Mitochondrial membrane potential was measured as described by Brown and Brand [17] using a TPP⁺-binding correction factor of 0.162 ($\mu\text{l}/\text{mg}$). The binding correction factor was determined from the ratio of Rb^+ to TPP⁺ accumulation as a function of mitochondrial volume [17]. The TPP⁺-binding correction factor obtained by the same method in mitochondria uncoupled by valinomycin and K^+ was much lower (0.03) and very close to the binding constant reported by La Noue et al. [18].

We found that TPP⁺ concentrations in the micromolar range commonly used to measure the membrane potential of mitochondria (1–5 μM) inhibited respiration of heart mitochondria with 2-oxoglutarate as well as with pyruvate + malate, particularly at low Ca^{2+} concentration, although it had no effect on respiration with succinate. Therefore the concentration of TPP⁺ in our experiments was reduced to the much lower value of 200 nM. This concentration did not affect 2-oxoglutarate oxidation.

Determination of mitochondrial volume and ΔpH

Mitochondrial volume was determined from the distribution of $^3\text{H}_2\text{O}$ and [^{14}C]sucrose, and ΔpH from the distribution of [^3H]acetate and [^{14}C]sucrose [17]. Mitochondrial volumes at 5 nM and 1 μM external free Ca^{2+} concentrations respectively (with 1 mM 2-oxoglutarate as substrate) were: in State 4, 1.01 ± 0.08 and 1.11 ± 0.09 ; in State 3, 0.80 ± 0.02 and 0.95 ± 0.06 ; in State 3.5, 0.87 ± 0.03 and 1.04 ± 0.02 $\mu\text{l}/\text{mg}$. The values for ΔpH in mitochondria under the same conditions were: at 5 nM and 1 μM external free Ca^{2+} concentration respectively, in State 4, 14.5 ± 1.4 and 15.6 ± 0.9 ; in State 3, 23.4 ± 1.7 and 27.4 ± 0.4 ; in State 3.5, 26.1 ± 2.5 and 28.7 ± 4.5 mV. The effect of Ca^{2+} on mitochondrial volume and ΔpH was not statistically significant.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. for at least three experiments carried out on three different mitochondrial preparations (each point for every individual preparation averaged for two to three repetitive runs).

RESULTS

The dependence of the rate of mitochondrial respiration in State 3 with 1 mM 2-oxoglutarate on the concentration of free Ca^{2+} in the incubation medium is presented in Figure 1. 2-Oxoglutarate oxidation is stimulated nearly twofold when the concentration of free Ca^{2+} increases from 0.1 to 2 μM , and is inhibited in a time-dependent manner when free Ca^{2+} concentration rises further above 2 μM . Although the initial rate of mitochondrial respiration at 2 μM external free Ca^{2+} is slightly higher than at 1 μM , the rate decreases with time showing that inhibition starts to prevail over stimulation. The dependence of 2-oxoglutarate oxidation on Ca^{2+} concentration under our experimental conditions (Figure 1) is essentially the same as reported previously by

other authors [18a]. Similar dependence on Ca^{2+} concentration was also obtained under these conditions for pyruvate + malate (1 mM + 1 mM) and palmitoylcarnitine (15 μM); however, the maximal stimulation of mitochondrial respiration with these substrates by Ca^{2+} did not reach more than 30 % (results not shown). It is well established that Ca^{2+} stimulates 2-oxoglutarate dehydrogenase directly by reducing its K_m for 2-oxoglutarate [7,8]. Therefore in the experiments described below we used a saturating and subsaturating concentration of 2-oxoglutarate at 5 nM and 1 μM external free Ca^{2+} .

The system under consideration consisted of isolated heart mitochondria oxidizing 2-oxoglutarate or succinate. We divided the system into three parts connected by the common intermediate Δp [19]. Δp is produced by the respiratory subsystem (i.e. the subsystem including substrate transporters, dehydrogenases and the complexes of the respiratory chain) and consumed by the phosphorylation subsystem (consisting of ATP synthase, ADP/ATP translocator and phosphate carrier) and the proton leak (consisting of various components mediating proton permeability including Ca^{2+} cycling). The rates of respiration were set at different values by changing the ratio of creatine and phosphocreatine in the incubation medium in the presence of excess of creatine kinase.

The dependence of the flux through the respiratory system (J_R) on Δp was determined by titrating Δp with oligomycin (to inhibit phosphorylation and thus raise Δp) and measuring the dependence of the respiration rate ($J_O = J_R$) on Δp (Figure 2B). The dependence of the flux through the phosphorylation system (J_P) on Δp was determined by titrating Δp with rotenone (to inhibit the respiratory chain and thus lower Δp) and measuring the dependence of the coupled respiratory flux ($J_P = J_R - J_L$) on Δp (Figure 2C). The dependence of the proton leak J_L on Δp was determined by titrating Δp with an inhibitor of the respiratory chain in the presence of excess oligomycin (2 $\mu\text{g}/\text{mg}$ mitochondrial protein) to completely inhibit phosphorylation (in this case $J_L = J_R = J_O$); see Figure 2(A). The aim of our experiments was to measure the kinetic dependences of these fluxes on Δp at two different concentrations of external free Ca^{2+} . A shift in the kinetic curves indicates that the system is affected by Ca^{2+} . We were able to measure changes in Δp as changes in $\Delta\Psi$ without introducing significant error under conditions when the ΔpH value is small and changes in ΔpH are negligible. Direct measurement of ΔpH in our experiments (see the Experimental section) confirmed that this was so.

Figure 2 shows the kinetic responses of the three different subsystems to $\Delta\Psi$ during oxidation of 1 mM 2-oxoglutarate at the maximal rate of mitochondrial respiration (corresponding to the rate in State 3). Under these conditions, Ca^{2+} stimulated the rate of respiration from 107 ± 9 to 239 ± 21 ng-atom of O/min per mg of protein. The rotenone titration when the phosphorylation subsystem is completely inhibited by oligomycin [as in Figure 2(A)] reflects the dependence of the proton leak on $\Delta\Psi$. The curves obtained for the two concentrations of Ca^{2+} superimpose (Figure 2A) indicating that there is no Ca^{2+} effect on proton leak. However, the dependences representing the kinetics of the respiratory chain diverge substantially, particularly in State 3 (Figure 2B), showing a much higher rate of oxygen consumption at the same value of $\Delta\Psi$ at the high Ca^{2+} concentration. This means that the respiratory subsystem is stimulated by Ca^{2+} to a large extent, most probably as a result of activation of the 2-oxoglutarate dehydrogenase complex [7–9]. Note that the mitochondrial membrane potential is approximately the same at the maximal respiration rate for both concentrations of Ca^{2+} in spite of the fact that the rate of respiration is increased more than twofold by Ca^{2+} . This indicates

Figure 1 Effect of Ca²⁺ concentration on mitochondrial respiration with 1 mM 2-oxoglutarate as substrate

Respiration was measured in the medium giving maximal rates corresponding to that in State 3 (see the Experimental section) and containing different concentrations of free Ca²⁺ calculated according to the program [15]. The rates are presented as percentages of that in State 3 measured after addition of 2 mM ADP to the medium containing 30 mM Tris/HCl, 5 mM KH₂PO₄, 125 mM KCl, 10 mM NaCl, 3 mM EGTA, 1 mM free Mg²⁺ and 5 nM free Ca²⁺; the rate of this set of experiments was 98 ± 7 ng-atom of O/min per mg ($n = 4$). Since the rate of respiration was not linear with time as free Ca²⁺ concentration reached $2 \mu\text{M}$ and above, the open symbols represent the rate during the second minute of the registration curve after addition of ATP (creatine kinase ADP-regenerating system used), whereas the closed symbols show the rate after 1 min; there was no change in rate with time below $2 \mu\text{M}$ free Ca²⁺.

that, in addition to the respiratory subsystem, the $\Delta\Psi$ -consuming subsystem must also be stimulated by Ca²⁺. The data in Figure 2(C) confirm that the phosphorylation subsystem is significantly stimulated, since the phosphorylation flux at the same magnitude of $\Delta\Psi$ is obviously larger at $1 \mu\text{M}$ than at 5 nM extramitochondrial free Ca²⁺. Similar results showing a stimulation of the phosphorylation system and the absence of a Ca²⁺ effect on the proton leak (Figure 3A and 3B) were obtained in the intermediate state between State 3 and State 4 (State 3.5).

The stimulation of the 2-oxoglutarate dehydrogenase complex by the increase in 2-oxoglutarate concentration from 1 to 10 mM is followed by a rise in the respiratory rate at both low and high Ca²⁺ concentration (from 107 ± 9 to 207 ± 18 and from 239 ± 21 to 301 ± 12 ng-atom of O/min per mg of protein at 5 nM and

Figure 3 Effect of Ca²⁺ on the relationship between membrane potential and mitochondrial respiration with 1 mM 2-oxoglutarate in State 3.5

(A) Kinetics of the proton leak (rotenone titration in the presence of oligomycin); (B) kinetics of the phosphorylation subsystem (rotenone titration: ○, 5 nM extramitochondrial free Ca²⁺; ●, $1 \mu\text{M}$ extramitochondrial free Ca²⁺).

$1 \mu\text{M}$ Ca²⁺ respectively). Although the effect of Ca²⁺ on the rate of mitochondrial respiration with saturating concentrations of 2-oxoglutarate (10 mM) is less pronounced (+45%) than with 1 mM 2-oxoglutarate (+123%), this stimulation is important bearing in mind that Ca²⁺ has been shown not to stimulate heart 2-oxoglutarate dehydrogenase at saturating concentrations of substrate [8,14]. In a separate set of experiments we determined the rate dependence of mitochondrial respiration on 2-oxoglutarate concentration at 5 nM free Ca²⁺. With 1, 5, 10 and 15 mM 2-oxoglutarate the measured rates were 112 ± 12 , 178 ± 20 , 219 ± 2 and 225 ± 5 ng-atom of O/min per mg respectively ($n = 3$). This indicates that there is no increase in the rate of 2-oxoglutarate oxidation with an increase in the substrate concentration above 10 mM under the experimental conditions used.

The kinetic curves determined for mitochondria oxidizing 10 mM 2-oxoglutarate show that in this case the proton leak (Figure 4A) is not affected by Ca²⁺; however, the phosphorylating subsystem (Figure 4C) is stimulated by Ca²⁺. Stimulation of the respiratory subsystem at 10 mM 2-oxoglutarate seems to be small or absent (Figure 4B). These results are in agreement with the observations of other authors [14], who found no increase in NADH fluorescence (reflecting the stimulation of 2-oxoglutarate dehydrogenase) on addition of Ca²⁺ to heart mitochondria oxidizing 10 mM 2-oxoglutarate, whereas a substantial increase

Figure 2 Effect of Ca²⁺ on the relationship between membrane potential and mitochondrial respiration with 1 mM 2-oxoglutarate in State 3

(A) Kinetics of the proton leak (rotenone titration in the presence of oligomycin); (B) kinetics of the respiratory subsystem (oligomycin titration); (C) kinetics of the phosphorylation subsystem (rotenone titration). ○, 5 nM extramitochondrial free Ca²⁺; ●, $1 \mu\text{M}$ extramitochondrial free Ca²⁺.

Figure 4 Effect of Ca^{2+} on the relationship between membrane potential and mitochondrial respiration with 10 mM 2-oxoglutarate in State 3

(A) Kinetics of the proton leak (rotenone titration in the presence of oligomycin); (B) kinetics of the respiratory subsystem (oligomycin titration); (C) kinetics of the phosphorylation subsystem (rotenone titration). $\circ$, 5 nM, extramitochondrial free Ca^{2+} ; $\bullet$, 1 μM extramitochondrial free Ca^{2+} .

Figure 5 Effect of Ca^{2+} on the relationship between membrane potential and mitochondrial respiration with 5 mM succinate in State 3

(A) Kinetics of the proton leak (malonate titration in the presence of oligomycin); (B) kinetics of the phosphorylation subsystem (malonate titration). $\circ$, 5 nM extramitochondrial free Ca^{2+} ; $\bullet$, 1 μM extramitochondrial free Ca^{2+} .

in NADH fluorescence was registered when a non-saturating concentration (0.6 mM) of 2-oxoglutarate was used. In order to obtain additional information we carried out a separate set of experiments to compare the effects of Ca^{2+} on mitochondrial respiration oxidizing 10 mM 2-oxoglutarate in the uncoupled rate with that in State 3. The results show that the uncoupled respiration (measured as in [20] after addition of 0.2 μM carbonyl cyanide *m*-chlorophenylhydrazone to mitochondria preincubated for 3 min with 1 μg of oligomycin/mg of mitochondrial protein in the presence of 1 mM ATP and the creatine kinase ADP-regenerating system, i.e. in the same medium as State 3 respiration) is stimulated from 240 ± 34 to 253 ± 34 (an average increase of 6% which is not significant), whereas State 3 respiration rate measured in parallel increases from 235 ± 12 to 301 ± 15 ng-atom of O/min per mg (an average increase of 28%; $P < 0.001$, $n = 3$). This is consistent with a requirement for the phosphorylation system for significant stimulation of respiration by Ca^{2+} at saturating concentrations of 2-oxoglutarate.

In contrast with 2-oxoglutarate oxidation, heart mitochondrial respiration with succinate is not sensitive to Ca^{2+} . The rate of respiration with 5 mM succinate in State 3 is 393 ± 23 and 404 ± 8 ng-atom of O/min per mg of protein at 5 nM and 1 μM Ca^{2+} respectively. The results presented in Figure 5 show that the kinetic dependences coincide for both proton leak (Figure 5A) and the phosphorylation subsystem (Figure 5B)

Table 1 Distribution of the control over respiration and phosphorylation fluxes at different Ca^{2+} concentrations with 1 mM 2-oxoglutarate in State 3

$C_{L^0}^J$, $C_{C^0}^J$, $C_{P^0}^J$, $C_{L^p}^J$, $C_{C^p}^J$, $C_{P^p}^J$, control coefficients of proton leak, respiratory chain and phosphorylation subsystem over the rate of mitochondrial respiration and phosphorylation respectively. *Statistically significant effect of Ca^{2+} , $P < 0.05$.

Control coefficients		
	Ca^{2+} concentration 5 nM	Ca^{2+} concentration 1 μM
$C_{L^0}^J$	0.014 ± 0.004	$0.029 \pm 0.002^*$
$C_{C^0}^J$	0.974 ± 0.009	$0.776 \pm 0.05^*$
$C_{P^0}^J$	0.015 ± 0.005	$0.195 \pm 0.05^*$
$C_{L^p}^J$	-0.478 ± 0.092	$-0.093 \pm 0.015^*$
$C_{C^p}^J$	1.250 ± 0.047	$0.809 \pm 0.093^*$
$C_{P^p}^J$	0.169 ± 0.007	0.284 ± 0.081

indicating that Ca^{2+} has no effect on the kinetics of the proton leak, phosphorylation and respiratory subsystem under these conditions. There is also no effect of Ca^{2+} on respiration rate, either when the succinate concentration is decreased from 5 to 0.5 mM or when the concentration of phosphate is reduced from 5 to 1 mM (results not shown). Titration with carboxyatractyloside (carried out as described previously [20]) of the rate of 5 mM succinate oxidation in State 3 at 5 nM Ca^{2+} gives a strongly sigmoidal curve of respiration against inhibitor concentration; the same type of curve is obtained with oligomycin titration ($n = 3$; results not shown) indicating that neither the adenine nucleotide carrier nor the ATP synthase has control over the rate of respiration in State 3 with succinate.

Thus our data show that the phosphorylation subsystem is stimulated by Ca^{2+} when 2-oxoglutarate (1 mM, State 3 and State 3.5; 10 mM, State 3) is used as respiratory substrate (Figures 2C, 3B and 4C), but it is not affected by this ion in the case of succinate oxidation.

The relative control over respiration rate exerted by the respiratory, phosphorylation, and proton leak varied with conditions. The data in Figures 2–5 were used to calculate elasticities and control coefficients of these three blocks of reactions using equations described by Hafner et al. [19]. With low 2-oxoglutarate and low Ca^{2+} concentrations in State 3, all the control was located in the respiratory subsystem (Table 1), consistent

Table 2 Distribution of the control over respiration and phosphorylation fluxes at different Ca²⁺ concentrations with 10 mM 2-oxoglutarate in state 3

$C_{L}^{j_0}$, $C_{C}^{j_0}$, $C_{P}^{j_0}$, $C_{L}^{j_p}$, $C_{C}^{j_p}$, $C_{P}^{j_p}$, control coefficients of proton leak, respiratory chain and phosphorylation subsystem over the rate of mitochondrial respiration and phosphorylation respectively. *Statistically significant effect of Ca²⁺, $P < 0.05$.

	Control coefficient	
	Ca ²⁺ concentration 5 nM	Ca ²⁺ concentration 1 μ M
$C_{L}^{j_0}$	0.007 $\pm$ 0.004	0.008 $\pm$ 0.001
$C_{C}^{j_0}$	0.929 $\pm$ 0.005	0.916 $\pm$ 0.016
$C_{P}^{j_0}$	0.061 $\pm$ 0.004	0.076 $\pm$ 0.015
$C_{L}^{j_p}$	-0.162 $\pm$ 0.002	-0.106 $\pm$ 0.015
$C_{C}^{j_p}$	1.083 $\pm$ 0.040	1.000 $\pm$ 0.023
$C_{P}^{j_p}$	0.079 $\pm$ 0.022	0.105 $\pm$ 0.015*

Table 3 Distribution of the control over respiration and phosphorylation fluxes at different Ca²⁺ concentration with 1 mM 2-oxoglutarate in State 3.5

$C_{L}^{j_0}$, $C_{C}^{j_0}$, $C_{P}^{j_0}$, $C_{L}^{j_p}$, $C_{C}^{j_p}$, $C_{P}^{j_p}$, control coefficients of proton leak, respiratory chain and phosphorylation subsystem over the rate of mitochondrial respiration and phosphorylation respectively. *Statistically significant effect of Ca²⁺, $P < 0.05$.

	Control coefficient	
	Ca ²⁺ concentration 4 nM	Ca ²⁺ concentration 1 μ M
$C_{L}^{j_0}$	0.185 $\pm$ 0.040	0.158 $\pm$ 0.05
$C_{C}^{j_0}$	0.597 $\pm$ 0.110	0.435 $\pm$ 0.055
$C_{P}^{j_0}$	0.218 $\pm$ 0.071	0.408 $\pm$ 0.006*
$C_{L}^{j_p}$	-0.480 $\pm$ 0.055	-0.089 $\pm$ 0.012*
$C_{C}^{j_p}$	1.021 $\pm$ 0.082	0.360 $\pm$ 0.078*
$C_{P}^{j_p}$	0.459 $\pm$ 0.004	0.729 $\pm$ 0.071*

with (but not demonstrating) a high level of control by 2-oxoglutarate dehydrogenase. The fact that the phosphorylation subsystem has little control over the respiration rate in this state is interesting, implying that even though Ca²⁺ stimulates the phosphorylation system in these conditions, it contributes little to the stimulation of respiration. Increasing the Ca²⁺ concentration in these conditions decreases the control exerted by the respiratory system, consistent with a stimulation of this system by Ca²⁺. The respiratory subsystem also exerts very tight control over respiration with 10 mM 2-oxoglutarate, and the control pattern in this case remains unchanged or changes very slightly when mitochondrial respiration is stimulated by Ca²⁺ (Table 2).

In State 3.5 with 1 mM 2-oxoglutarate (which may be a more physiological state for heart mitochondria, as it is unlikely that mitochondria achieve the equivalent of State 3 or State 4 *in vivo* [21]), control is shared between the respiratory chain, phosphorylation and proton leak subsystems (Table 3). Thus stimulation of any of these systems will have a significant effect on respiration rate.

DISCUSSION

Ca²⁺ has no detectable effect on the kinetics of the proton leak in any of the conditions studied (Figures 2A, 3A, 4A and 5A).

Indeed, under the conditions used, we found no detectable effect on these kinetics up to 70 μ M external free calcium (not shown). This has two important implications: (1) Ca²⁺ cycling does not significantly contribute to the proton leak or metabolic rate at physiological levels of Ca²⁺, contrary to the suggestions of some authors [22] and in line with others [23]; (2) Ca²⁺ does not regulate proton leak in heart mitochondria.

Ca²⁺ stimulated the respiratory subsystem in some conditions (low 2-oxoglutarate in State 3) but not others (State 4 or succinate). With high 2-oxoglutarate this stimulation is small or non-existent. We did not attempt to determine which components in the respiratory system were directly activated by Ca²⁺, but the findings that the stimulation with low 2-oxoglutarate in State 3 was eliminated with high 2-oxoglutarate concentrations, or in State 4, or with succinate replacing 2-oxoglutarate as substrate, are consistent with the stimulation being due to Ca²⁺ decreasing the K_m of 2-oxoglutarate dehydrogenase for Ca²⁺ [7–9]. This activation and the consequent stimulation of respiration would decrease (a) with high 2-oxoglutarate because the dehydrogenase would already be saturated, (b) with succinate since the dehydrogenase would not be involved, and (c) in State 4 because a decrease in ADP levels decreases the Ca²⁺ sensitivity of the dehydrogenase, and because the dehydrogenase would have less control over respiration in State 4 [19,24].

Ca²⁺ stimulated the phosphorylation subsystem in some conditions (respiring on 2-oxoglutarate) but not in others (respiring on succinate). We did not attempt to determine which components of the phosphorylation subsystem were activated by Ca²⁺. Two potential sites have been suggested: the adenine nucleotide translocator [12,13] and the ATP synthase [11]. In a previous paper [20] we have shown that the adenine nucleotide translocator has a higher control coefficient over respiration rate in State 3.5 during respiration on succinate rather than on 2-oxoglutarate, and that Ca²⁺ increases this coefficient during respiration on 2-oxoglutarate, but has no effect on the coefficient during respiration on succinate. This indicates that Ca²⁺ did not directly stimulate the adenine nucleotide translocator [20]. In State 3 the adenine nucleotide translocator had no control over respiration at either 1 or 10 mM 2-oxoglutarate [20], indicating that, even if Ca²⁺ did stimulate the translocator, it would not affect respiration rate. Thus it appears that the component of the phosphorylation system that is most likely to be the cause of the stimulation is the ATPase, but we do not have any direct evidence for this. The Ca²⁺ effect on the phosphorylating subsystem does not reveal itself in the case of succinate oxidation, and this might be because the ATPase has no control over the phosphorylation system under these conditions.

The mitochondrial membrane potential did not change with the increase in respiration rate caused by Ca²⁺ with 1 mM 2-oxoglutarate in State 3 (Figure 1). This fact indicates an equal activation by Ca²⁺ of the $\Delta\Psi$ -producing subsystem and the $\Delta\Psi$ -consuming subsystem under these conditions. Since almost all the control belongs to $\Delta\Psi$ producers (Table 1), the increase in respiration rate is almost entirely due to the activation of the respiratory subsystem. The control over respiration rate is shared in State 3.5 with 1 mM 2-oxoglutarate (Table 3) and therefore the increase in respiration rate is due partly to the activation of the respiratory system and partly to the activation of the phosphorylation subsystem. Because the phosphorylation subsystem has substantial control over respiration, and even greater control over the rate of phosphorylation in this state (Table 3), the Ca²⁺ stimulation of the phosphorylation system contributes significantly to the increased rate, and has the added advantage of indirectly decreasing the proton leak (by decreasing $\Delta\Psi$) and thus increasing the efficiency of oxidative phosphorylation.

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