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The Use of Dichlorofluorescein as a Probe for Monitoring the Effects of Calcium on ROS Production in Mitochondria

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Abstract

Dichlorofluorescein (DCF) is widely applied for monitoring reactive oxygen species (ROS) production in cells and isolated mitochondria. Our purpose was to compare different approaches to the use of DCF for monitoring ROS formation accompanying Ca^{2+} transport in isolated mitochondria. Ca^{2+} effects on ROS production was studied in rat brain and liver mitochondria using two modes of DCF application: the first was preloading of stock mitochondrial suspension with DCF precursor dichlorofluorescein diacetate (DCFDA), and the other was the addition of the aliquots of DCFDA directly to the incubation medium. Calcium increased ROS production in brain and liver mitochondria both under steady-state and non-equilibrium conditions caused by the gradual release of cytochrome c and the gating of the electron flow. Using DCF-preloaded mitochondria we have shown that the rate of ROS formation under steady-state conditions was linearly dependent on the rate of Ca^{2+}-cycling and Ca^{2+}-stimulated respiration. Transition to non-equilibrium state resulted in the non-linear rise in ROS formation, which too exhibited dependence on Ca^{2+} concentration. While in DCF-preloaded mitochondria DCF fluorescence closely followed the time course of Ca^{2+} transport, in unloaded mitochondria probe loading interfered with the detection of ROS formation because of continuing change of DCF concentration into matrix space. Based on the experiments, we came to the conclusion that DCF preloaded in mitochondria could serve a suitable probe for monitoring the effects of Ca^{2+} transport on ROS production.

Keywords: Dichlorofluorescein; Reactive oxygen species; Mitochondria; Oxygen consumption; Calcium transport

Introduction

Bioenergetic effects of calcium uptake and its impact on mitochondrial ROS production were widely discussed in the literature [1-3]. As it is known, the respiratory chain is one of the major sources of ROS in mitochondria. In the course of oxygen reduction ROS formation is regulated both thermodynamically and kinetically, and is dependent on the redox-potential of the sites of ROS formation, the amount, and the lifespan of these free radical products [4]. Complexes I, II and III are known to be primary sites of ROS formation in the respiratory chain. As one-electron reduction of oxygen to superoxide requires-160 mV standard redox-potential, reduced state of a number of sites within the complexes of respiratory chain (such as FMN binding site, FeS clusters, ubiquinone binding site and some others) makes the conditions energetically favourable for ROS formation [4]. Besides, the relative concentration ratio of the acceptors to the donors of electrons, the lifespan of free radical intermediates of the redox reactions, and the proportion of the rate of ROS formation to that of ROS removal also should serve as kinetic control over the rate and the amount of ROS produced in mitochondria. Worth mention too that ROS production, especially under physiological conditions, is attenuated by several matrix antioxidants, such as SOD, glutathione, thioredoxin, and many others [3,4]. On the other hand, several matrix enzymes including TCA cycle and mitochondrial NO synthase might under several conditions contribute to ROS production [3]. Thus measured rate of ROS production is a result of a steady-state balance between the rate of the formation and the removal of these species.

Calcium could affect ROS production in mitochondria in several ways, and the effects of Ca^{2+} are not simply bioenergetic [3,4], but the changes in mitochondrial energy state and the rate of respiration that accompany calcium uptake in mitochondria and calcium cycling across mitochondrial membrane [1] are eventually reflected in the change in ROS production in the electron transport chain. The following partially is a comment on the results of the authors' published works [5,6] regarding the use of DCF for monitoring ROS production in the course Ca^{2+} transport in rat brain and liver mitochondria. Dichlorofluorescein (DCF), as it is shown below, is a very convenient probe for monitoring changes in the rate of ROS production resulting from Ca^{2+} uptake in mitochondria.

Dichlorofluorescein is formed in mitochondrial matrix from a permeable compound DCFH2DA (2',7'-dichlorofluorescein diacetate) [7]. It penetrates mitochondrial membranes following the concentration gradient by a diffusion mechanism and is readily deacetylated in the matrix to form membrane-impermeable non-fluorescent derivative H2DCF (2',7'-dihydrodichlorofluorescein). This compound is then oxidized by mitochondrial ROS with the formation of highly fluorescent end product 2',7'-dichlorofluorescein (DCF [7]).

In the literature, one could find two modes of DCF application for monitoring ROS production in mitochondria. The first is direct addition of DCFDA to the cell or mitochondrial suspensions for ROS detection [8-10], the other is pre-loading of mitochondria or cell suspensions with DCFDA for 15-30 min with consequent wash-out of

the excess dye before the measurements. Our purpose was to try both approaches in order to find most appropriate conditions for monitoring the effects of Ca^{2+} transport on ROS production both in the course of Ca^{2+} uptake and under steady-state conditions in isolated rat brain and liver mitochondria respiring on Complex I and II substrates, glutamate and succinate (State 4).

Materials and Methods

Mitochondrial preparations

Adult Wistar-Kyoto male rats with body weight 180-200 g were chosen for the experiments. Animals were processed according to the European Community Council Directive 86/609/EEC approved by the Ethics Commission on Animal Experiments of A.A. Bogomoletz Institute of Physiology, NAS Ukraine. Mitochondria were isolated by differential centrifugation as described earlier [5].

Oxygen consumption study

Oxygen consumption was monitored polarographically in standard incubation medium (250 mM sucrose, 1 mM KH_2PO_4, 5 mM sodium succinate, 1 μM rotenone, 5 mM Tris-HCl buffer (pH 7.4), different concentrations of $CaCl_2$, 1 μM cyclosporine A) in a closed 1 cm^3 thermostated cell with a platinum electrode.

Calcium transport assay

Calcium transport was monitored spectrophotometrically with the indicator arsenazo III using conventional dual-wavelength technique at 654/690 nm [6].

The study of ROS formation using dichlorofluorescein

As it was described earlier [6], to study ROS formation, 200 μM DCFH2DA was added to stock mitochondrial suspension (~20 mg protein/ml) and thermostated for 30 min at 37°C in the dark. After the loading, stock suspension was washed of the excess of the probe by centrifugation and stored on ice in the cold (4°C). Alternatively, 10 μM aliquots of DCFH2DA were added directly into incubation medium; in both cases aliquots of mitochondrial suspension (1 mg protein) were sampled in 1 cm^3 of incubation medium, and DCF fluorescence was monitored at 37°C at 504/525 excitation/emission wavelengths.

All reagents were from Sigma-Aldrich, USA. Deionized water was used for medium preparations.

Statistical analysis

The data were expressed as mean ± S.E. of 3-4 independent experiments. Statistical analysis was performed using paired Student's t-test; $P<0.05$ was taken as the level of significance.

Results and Discussion

Both DCF fluorescence and mitochondrial respiration were monitored for a 12 min time interval. Time course of the changes in DCF fluorescence (Figures 1 A and 1B- 1, 2) was compared with the kinetics of Ca^{2+}-uptake (Figure 1A). Without the addition of exogenous cytochrome c, at the end of registration interval respiration was considerably suppressed because of the gradual release of endogenous cytochrome c from the intermembrane space. This was proven by the additions of exogenous cytochrome c, which restored the rate of respiration (not shown). As showed the typical data obtained in DCF-preloaded mitochondria, the time needed for DCF oxidation due to the ROS formation was comparable with the kinetics of Ca^{2+} transport, and DCF fluorescence closely followed the time course of Ca^{2+} uptake (Figure 1A).

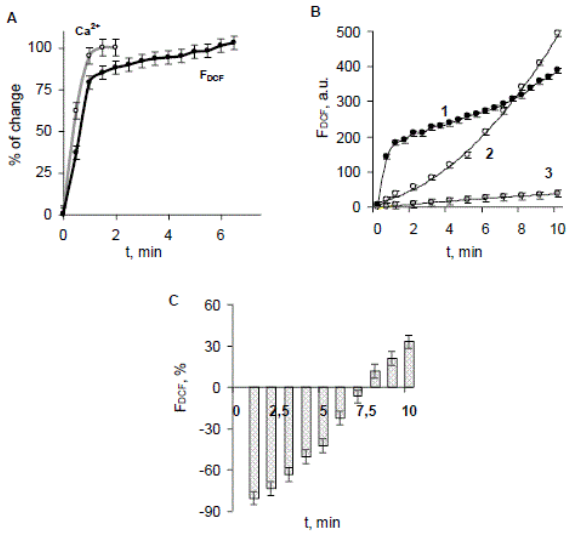

Figure 1: The typical time courses of DCF fluorescence and Ca^{2+} uptake in rat liver mitochondria. B – time courses of DCF fluorescence in preloaded (1), unloaded (2) and preloaded non-respiring mitochondria (3); in the case (2) DCF was added directly into incubation medium at 20 μM; in the case (3) rotenone was added at 10^{-6} M, without respiratory substrate; C – the level of DCF fluorescence in unloaded mitochondria as compared with loaded one; DCF fluorescence in preloaded mitochondria (Figure 1B, 1) was taken for 100% at every time of the registration. Incubation medium: 250 mM sucrose, 1 mM KH_2PO_4, 5 mM sodium glutamate, 5 mM Tris-HCl buffer (pH 7.4), 15 μM $CaCl_2$, 1 μM cyclosporine A. n = 4; M ± m.

Unlike this, with unloaded mitochondria, DCF fluorescence at first was dramatically retarded as compared with the time course of Ca^{2+} transport and showed a prolonged lag-phase with consequent steep rise of fluorescence signal (Figure 1B, 2). In this case the rise of DCF fluorescence in fact starts after the completion of Ca^{2+} accumulation in mitochondria (Figures 1A and 1B-1, 2), and the probe could not detect the changes in ROS production accompanying relatively fast kinetics of Ca^{2+} uptake. Taking the DCF fluorescence in pre-loaded mitochondria at every time for 100%, one can see that at the beginning fluorescent signal was much lower than a level reflecting DCF oxidation due to Ca^{2+} transport, and thus underestimated ROS production, and then, on the contrary, overestimated it (Figure 1C).

In respiration inhibited mitochondria the changes in DCF fluorescence were relatively negligible as compared to respiring mitochondria within the timeframes of the experiment, so observed effects of Ca^{2+} on DCF fluorescence ensued from the effects of Ca^{2+} transport on the respiratory chain (Figure 1B-1, 3).

Hence, as showed the experiment, an estimation of ROS production based on DCF fluorescence in unloaded mitochondria was largely

dependent on the time needed for the probe to reach a matrix space. Prolonged lag-phase in the kinetics of DCF fluorescence (Figure 1B, 2) was observed likewise in the literary data [8] and too could be explained by relatively slow diffusion of the probe into matrix. This interference of two different processes, fast oxidation but slow diffusion of the probe, does not allow for proper estimation of the effect of Ca^{2+} uptake on ROS formation. One more disadvantage of this mode of DCF application is a continuing loading of the probe and continuing increase of DCF concentration in mitochondria so that fluorescence should rise in parallel with accumulation of the probe even without changes in the level of ROS, which in turn should interfere with ROS detection every time when monitoring ROS production.

These disadvantages could be circumvented in DCF-preloaded mitochondria because in this case fluorescent signal closely followed the effects of Ca^{2+} uptake on the respiratory chain (Figure 1A). Similar data were obtained for K^+ uptake as well (not shown). So, DCF preloaded in mitochondria could satisfactorily respond to the changes in ROS formation, which showed a reliability of this probe to monitor ROS production evoked by transport processes.

In accordance with the purposes of our work, the next task was choosing appropriate mode of monitoring ROS production in respiring mitochondria under steady-state conditions (State 4). Generally in respiring mitochondria ROS (hydroperoxide and superoxide) were shown to be formed at a constant rate by the respiratory chain [11], thus state 4 oxygen consumption was accompanied by the steady-state rate of ROS formation (J_R). Consistent with this, experimental data showed that state 4 oxygen consumption in energized mitochondria and linear in time decrease of the oxygen concentration at a constant rate ($J_{O2} = d[O_2]/dt = const$) and stable membrane potential (ΔΨm) was accompanied by the linear increase in fluorescent signal that indicated a constant rate of DCFH2DA oxidation and reflected the amount of oxidized fluorescent product DCF formed in mitochondrial matrix: $[DCF](t) = J_R \cdot t + [DCF]_0$, where $[DCF]_0$ was the initial DCF concentration in the matrix space (Figure 2A, 1, 2; Figure 2B, part I).

The data of Figure 2C and 2D obtained in brain and liver mitochondria showed stepwise increase in the rate of respiration and ROS formation after sequential energization of non-respiring mitochondria by succinate, addition of Ca^{2+}, further activation of Ca^{2+}-cycling by Ca^{2+}-ionophore A-23187, and the abolition thereof by the removal of Ca^{2+} with EDTA, indicative of a strong dependence of the rate of ROS formation on Ca^{2+} cycling across mitochondrial membrane. Accordingly, increasing concentrations of Ca^{2+} added to the medium resulted in a concentration-dependent stimulation of both the respiration and ROS formation (Figures 3A and 3B), which increased linearly below a certain threshold caused by further inability to stimulate Ca^{2+}-cycle (not shown). This data indicated linear relationships between Ca^{2+}-stimulated oxygen consumption and ROS formation under steady-state conditions. So, measuring the rates of state 4 respiration and DCF oxidation, increasing with the increasing amounts of added Ca^{2+} (Figures 3A and 3B) we established proportionality between the rates of Ca^{2+}-stimulated respiration and ROS formation that was best approximated by quasi-linear dependences in rat brain and liver mitochondria (Figure 3C). The linearity of the obtained dependences could be well explained by the known proportionality between the rates of respiration and cation uptake [12], and constant rates of ROS formation under steady-state conditions (Figures 2A and 2B), related to the rates of respiration.

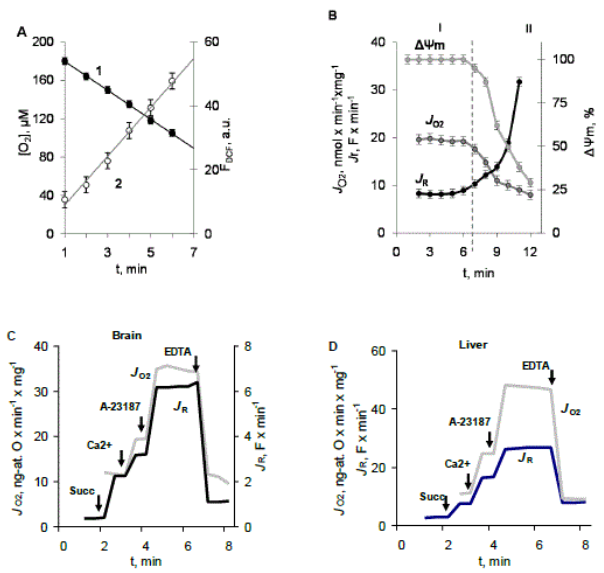

Figure 2: Oxygen consumption, ROS formation and membrane potential in DCF-preloaded rat brain mitochondria under steady-state (A, B, part I, C, D) and non-equilibrium conditions (B, part II). The changes in oxygen concentration and DCF fluorescence under steady-state conditions (A) were approximated by linear dependences. C, D – the rates of oxygen consumption and ROS formation after successive energization, addition of 30 μM Ca^{2+}, activation of Ca^{2+} cycle and its blockage by the removal of Ca^{2+} (additions are shown by the arrows). M ± m, n = 4. Incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM sodium succinate, 1 μM rotenone, 5 mM Tris-HCl buffer (pH 7.4), 30 μM $CaCl_2$, 1 μM A-23187, 1 μM cyclosporine A.

Thus proportionality between the rate of Ca^{2+} cycling and both the respiration and ROS formation (Figures 3A and 3B) on one hand, and linearity in the time courses of oxygen consumption and DCF fluorescence under steady-state conditions (Figure 2A) allowed us establish quasi-linear empirical dependences of the rate of ROS formation on the rate of Ca^{2+}-stimulated respiration dependent on the rate of Ca^{2+} cycling across mitochondrial membrane. Thus under equal conditions, provided relatively stable ΔΨm, increase in the rate of Ca^{2+}-stimulated respiration is accompanied by the proportional increase in the rate of ROS emission by the respiratory chain.

Gradual loss of cytochrome c from the intermembrane space that is common phenomena in isolated mitochondria leads to the progressive inhibition of respiration and the deviation from the equilibrium state (Figure 2B, part II). Progressive gating of the electron flow between the complexes III and IV of respiratory chain makes the conditions highly favourable for ROS formation and the prolongation of the lifespan of these species because of more reduced state of the sites of ROS release upstream and the decrease in the concentration of electron acceptors downstream the gating site. Transition to the non-equilibrium conditions was reflected in the exponential rise in DCF fluorescence, and respectively, the rate of ROS formation (Figure 2B, part II). Linearity was restored by the addition of exogenous cytochrome c (not shown).

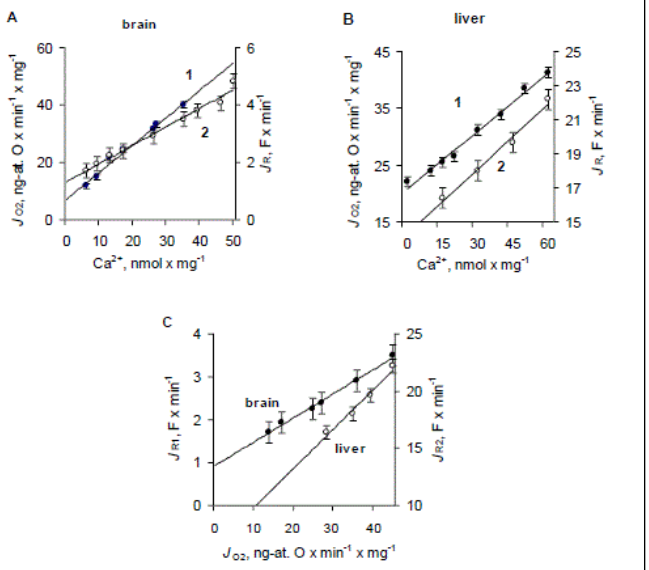

Figure 3: The effect of Ca^{2+} on the rates of state 4 respirations (1, black circles) and ROS formation (2, white circles) in DCF-preloaded rat brain and liver mitochondria (A, B); C – Dependences of the rate of ROS formation on the rate of respiration were fitted by linear functions. Incubation medium: 320 mM sucrose, 1 mM KH_2PO_4, 5 mM sodium succinate, 1 μM rotenone, 5 mM Tris-HCl buffer (pH 7.4), $CaCl_2$ was added as indicated (A, B), 1 μM cyclosporine A. M ± m, n = 4.

Under non-equilibrium conditions ROS production too exhibited dependence on Ca^{2+} concentration, and increased with the increase of the amount of added Ca^{2+} (Figure 4A). Observed time courses of DCF fluorescence were best approximated by exponential dependences of the type $F(t) = F_0ekt$, in which the rate constant (k) was dependent on Ca^{2+} concentration (Figure 4B). While the mechanisms underlying Ca^{2+} effects on ROS production under non-equilibrium conditions deserve more detailed study, we could hypothesize that ROS formation after transition to non-equilibrium state was dependent on the amount and the rate of ROS release stimulated by Ca^{2+} under steady-state conditions. Besides, increase in Ca^{2+} concentration could promote faster release of cytochrome c [13] and faster gating of the electron flow with consequent increase in ROS production.

To go back to what was said before, it worth mention that when compare DCF fluorescence in preloaded and unloaded mitochondria under conditions of equal loading, fluorescent signal reached a similar level only at the time when the deviations from the equilibrium conditions already were obvious (Figure 1C). Considering the interference of concurrent processes affecting fluorescent signal (the diffusion of the probe and non-linear rise of fluorescence and as well non-linear rise in the rate of ROS formation), it became evident that a mode of DCF application when the probe was added directly to the incubation medium could not adequately detect the effects of calcium transport on ROS production in isolated mitochondria neither in the course of Ca^{2+} uptake, nor in the course of state 4 oxygen consumption. Unlike this, in mitochondria preloaded with DCF, fluorescence satisfactorily followed both initial Ca^{2+} uptake and consequent Ca^{2+} cycling showing a linear increase of a signal accompanying the oxygen consumption at constant rate of state 4 respirations.

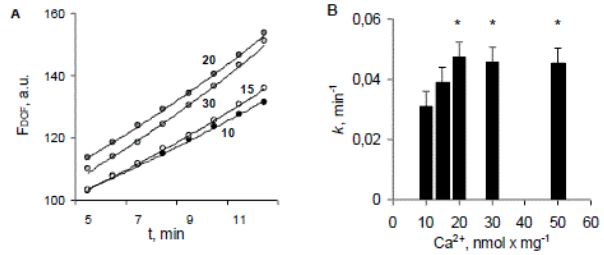

Figure 4: The effect of Ca^{2+} on ROS production in rat brain mitochondria under non-equilibrium conditions: A – typical time courses of DCF fluorescence (the numbers on the curves indicate the amount of added Ca^{2+} in nmol/mg; B – the effect of Ca^{2+} on the rate of ROS production; time courses of DCF fluorescence were fitted by exponential dependences of the type $F(t) = F_0ekt$ where the rate constant k was chosen to characterize the rate of ROS production. M ± S.D., n=4, * - $p<0.05$ as compared to control (10 nmol Ca^{2+}/mg).

Conclusion

Unlike reverse electron transport, highly sensitive to the changes in mitochondrial energy state (both ΔΨm and ΔpH components of ΔμH, ROS production caused by forward electron transport (as it was in our work) was shown to be much less sensitive to minor changes in ΔΨm [14,15] as it was the case with Ca^{2+}-cycling at low concentrations of added Ca^{2+}, which allowed for a certain proportionality between the rates of respiration and ROS formation (Figure 3). Thus, when DCFDA was directly added into medium, delayed loading of the probe and continuing increase in DCF concentration into matrix interfered with the detection of ROS production (Figures 1A, B), and besides, retarded ROS detection was coincident with the transition to non-equilibrium conditions of the functioning of the respiratory chain, and non-linear dependences of ROS production on calcium concentration (Figure 4). For this reason, fluorescent signal failed in a properly reflecting the effects of Ca^{2+} transport on ROS production. As compared with DCFDA-preloaded mitochondria, under steady-state conditions DCF signal at first underestimated the amount of ROS formed by the Ca^{2+} uptake (Figures 1A and 1B); then, with time ROS formation was overestimated because of continuing increase in DCF concentration in the matrix space. However, using mitochondria preloaded with DCFDA, it was possible to observe that DCF oxidation closely followed the kinetics of Ca^{2+} uptake, and show linear relationships between the rate of Ca^{2+} stimulated respiration and the rate of ROS production under steady-state conditions at relatively constant ΔΨm.

Thus although several disadvantages of DCF were referred to in the literature (non-selectivity respective to individual ROS species, an oxidation by cytochrome c and the sensitivity to the medium pH [7,16]), based on our experiments, we came to the conclusion that using DCF-preloaded mitochondria this probe appeared to be convenient for the detection of the changes in the kinetics of ROS formation accompanying calcium transport, starting from the very beginning of calcium uptake in mitochondria.

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