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Reduced capacity of Ca^{2+} retention in liver as compared to kidney mitochondria. ADP requirement

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Abstract Ca^{2+} loading in mitochondria promotes the opening of a non-selective transmembrane pathway. Permeability transition is also associated with the interaction of cyclophilin D at the internal surface of the non-specific transmembrane pore. This interaction is circumvented by cyclosporin A and ADP. Our results show that, in the absence of ADP, liver mitochondria were unable to retain Ca^{2+}, they underwent a fast and large amplitude swelling, as well as a rapid collapse of the transmembrane potential. In contrast, in the absence of ADP, kidney mitochondria retained Ca^{2+}, swelling did not occur, and the collapse of the membrane potential was delayed. Ca^{2+} efflux was reversed by the addition of ADP and cyclosporin A. Our findings indicate that the differences between liver and kidney mitochondria are due to the low association of cyclophilin D to the ADP/ATP carrier found in kidney mitochondria as compared to liver mitochondria.

Keywords Mitochondria · Calcium · Kidney · Liver · Permeability transition · ADP

Introduction

Mitochondrial permeability transition is a membrane process that allows the release of matrix content, through the opening of a non-specific pore with a diameter of up to 3 nm, in response to several stimuli (Zoratti et al. 2005). Although the chemical nature of the pore has not been elucidated yet, current consensus confers adenine nucleotide translocase (ANT) a central role in the configuration and regulation of the pore open/closed cycles (Halestrap and Brenerb 2003; García et al. 2005; Di Paola and Lorusso 2006; Halestrap 2009). From several reports, it is known that by locking ANT at the external side of the inner membrane the pore becomes open, whereas locking ANT at the internal side brings the pore into a closed configuration (Haworth and Hunter 2000; Pestana et al. 2009). A *sine qua non* prerequisite to initiate pore opening is massive Ca^{2+} accumulation (Chávez et al. 1997; Brookes et al. 2004; Bernardi et al. 2006; Lemasters et al. 2009). In this respect, it has been argued that the cation binds to negative charges of cardiolipin, surrounding the adenine nucleotide translocase (Brustovetsky and Klingenberg 1996). Such an interaction generates structural modifications in the architecture of ANT, in such a way that it becomes located at the external side of the membrane and the pore opens (Chávez et al. 1998; Pestana et al. 2009). Retention of the bivalent cation inside the matrix requires the presence of ADP (Hunter and Haworth 1976; Haworth and Hunter 2000). The binding of this nucleotide to the carrier holds this protein towards the matrix side of the inner membrane, circumventing the Ca^{2+}-induced opening of the non-specific transmembrane pore (Halestrap et al. 1997). In addition to Ca^{2+}, another important prerequisite to pore opening is the binding of the isomerase enzyme cyclophilin D (Cyp D) to an internal ANT site (Crompton et al. 1998; Woodfield et al. 1998; Basso et al. 2005). The activity of this isomerase on the carrier protein allows for the change to the open configuration (Tanver et al. 1996; Halestrap 2009). Thus, it must be considered that the open/closed configuration of the pore depends on ADP, Ca^{2+}, and cyclophilin D content, as well as on the inhibition of this

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enzyme by cyclosporin A (Fournier et al. 1987; Crompton et al. 1988; Bernardi et al. 1994). Since there are differences in mitochondrial sensitivity to permeability transition, these differences would be due to the density of such factors.

This work was performed, in liver and kidney mitochondria, with the aim of studying whether or not a different sensitivity exists for pore opening from that depending on ADP, Ca^{2+} addition, and cyclophilin D content. The results show that, in the absence of added ADP, kidney mitochondria were able to take up a higher amount of Ca^{2+} than liver mitochondria. In addition, without added ADP, Ca^{2+} remained accumulated in kidney mitochondria for a longer time, in contrast to what occurred in liver mitochondria. Similar results were observed when mitochondrial swelling or the maintenance of a high level of membrane potential was analyzed, i.e., kidney mitochondria were more resistant to membrane leakage than liver mitochondria. Interestingly, we found that the association between cyclophilin D and ANT was lower in kidney mitochondria than in liver mitochondria. We propose that this diminution could be the cause for the differences found between liver and kidney mitochondria to undergo permeability transition.

Materials and methods

Mitochondria from liver and kidney, from 24-h fasted rats, were prepared by homogenizing the tissue in 0.25 M sucrose-1 mM EDTA, adjusted to pH 7.3, and following the standard centrifugation procedure. Proteins were determined by the Lowry method (Lowry et al. 1951). Mitochondrial Ca^{2+} movement was analyzed spectrophotometrically at 675–685 nm, using the indicator Arsenazo III. Transmembrane potential was assayed using the dye safranine, at 511–535 nm. Mitochondrial swelling was followed at 540 nm (Garlid and Beavis 1985). The basic incubation mixture contained 125 mM KCl, 10 mM HEPES, pH 7.3, 10 mM succinate, 2 mM phosphate, 2 μg oligomycin, and 5 μg rotenone. Chellex 100 was added to the incubation medium to diminish as much as possible Ca^{2+} contamination. Cyclophilin and ANT content in mitochondria was analyzed as described in the legends of the respective figures.

Results

Figure 1 shows Ca^{2+} movements in liver and kidney mitochondria. Figure 1a illustrates the ADP dependence of liver mitochondria on the accumulation of different concentrations of Ca^{2+}. As shown, in the presence of 100 μM ADP, Ca^{2+} remained accumulated within the matrix for a longer period of time, regardless from the addition of 50 μM Ca^{2+}. In contrast, when the medium was not supplemented with ADP, liver mitochondria were not able to accumulate Ca^{2+}, even at the low concentration of 25 μM and, as observed, a fast release reaction took place. In opposition to the above, the data in Fig. 1b show that kidney mitochondria were able to accumulate Ca^{2+} in spite of the absence of ADP. However it is worthwhile to mention that, these mitochondria did not retain Ca^{2+} when this cation reached up to 75 μM concentration in the incubation mixture. As widely known, membrane leakage is brought about in response to massive Ca^{2+} accumulation and does not occur with another bivalent cation. In agreement, Fig. 1c shows that, even in the absence of ADP, liver and kidney mitochondria accumulate Sr^{2+} regardless of the fact that the concentration of this cation in the incubation mixture reached 50 μM.

Next, we decided to explore whether the addition of ADP, after the Ca^{2+} efflux phase, would reverse pore opening; thus, initiating a Ca^{2+} reuptake reaction. The results are shown in Fig. 2. Figure 2a reports that the addition of 100 μM ADP after Ca^{2+} release failed to induce the reuptake of the cation. Nevertheless, this reaction took place, at a faster rate, after the addition of 0.5 μM cyclosporin A (CSA). Figure 2b shows that the behavior of kidney mitochondria was similar. Remarkably, a fast and partial reuptake of Ca^{2+} was achieved after ADP addition as compared to that observed in liver mitochondria. As in liver mitochondria, the addition of CSA completely closed the pore and the Ca^{2+}reuptake seemed to be almost total. The order of addition of ADP or CSA, after Ca^{2+} efflux, did not influence the results obtained with liver or kidney mitochondria. In addition, it is important to point out that in A the medium contained 30 μM Ca^{2+}, whereas in B the medium contained 50 μM Ca^{2+}.

In Fig. 3, we investigated whether N-ethylmaleimide (NEM) would substitute for ADP, therefore acting as an ADP-like molecule promoting Ca^{2+} accumulation. The proposal of this experiment emerged from the knowledge that, in ANT, NEM binds to the same site as ADP, that is Cys^{159} (Majima et al. 1994). Figure 3a shows that the addition of 30 μM NEM to liver mitochondria induced Ca^{2+} retention, regardless of the absence of ADP in the incubation mixture. As shown in Fig. 3b, NEM behaved similarly in Ca^{2+} uptake by kidney mitochondria. Thus, NEM prevented Ca^{2+} efflux, acting in a similar manner, as does ADP in permeability transition.

Pore opening can be mirrored also as an increase in mitochondrial volume. The data in Fig. 4 show typical traces of mitochondrial swelling in response to Ca^{2+} addition. Figure 4a shows that, in the absence of added ADP, when increasing Ca^{2+}concentration from 25 to 75 μM, liver mitochondria underwent a fast and large amplitude swelling. In contrast to the behavior of liver mitochondria, Fig. 4b

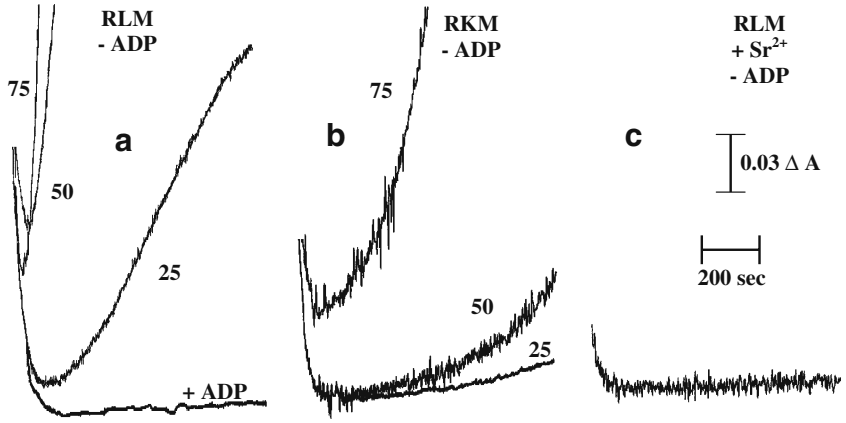

Fig. 1 The effect of increasing concentrations of Ca^{2+} on membrane permeability in the absence of ADP. Mitochondria (2 mg protein) were added to 3 ml of the basic incubation medium as described in Materials and methods. In **a**, Ca^{2+} movement in liver mitochondria was assayed. The concentrations of Ca^{2+} that were added are indicated at the side of the traces. Where indicated, 100 μM ADP was added. In **b**, Ca^{2+} movement in kidney mitochondria was analyzed. The numbers indicate the added Ca^{2+} concentrations. **c** and **d** show Sr^{2+} accumulation, in the absence of ADP, by liver and kidney mitochondria, respectively. Both incubation mixtures contained 50 μM Sr^{2+}. Temperature 25 °C

demonstrates that kidney mitochondria do not require ADP to be more resistant to the swelling-inducing effect of Ca^{2+}, since, regardless of the addition of 75 μM Ca^{2+}, the increase in mitochondrial volume was lower than that observed in liver mitochondria.

When the effect of Ca^{2+} on the transmembrane electric gradient (Δψ) was assayed in a medium lacking ADP, the behavior of liver mitochondria was almost similar to that of kidney mitochondria. Figure 5a shows the response of liver mitochondria to Ca^{2+} addition. As seen, a fast collapse of membrane potential followed. It also shows that addition of ADP and CSA failed to restore membrane potential. Figure 5b shows that, in kidney mitochondria, after a short lag period, Ca^{2+} addition initiated a fast collapse of Δψ; nevertheless, in these mitochondria the addition of ADP plus CSA partially restored Δψ. Figure 5c and d show that, similarly to what occurred with Ca^{2+} movements, in the absence of ADP, NEM delayed the Ca^{2+}-induced collapse of Δψ, this was more apparent in kidney than in liver mitochondria.

At this stage of the experimental work, the possibility emerged that the differences in sensitivity to permeability transition, between liver and kidney mitochondria, could be due to a different Cyp D content. However, as shown in Fig. 6, kidney mitochondria have a similar amount of cyclophilin D as liver mitochondria. Nevertheless, when the association of Cyp D to ANT was analyzed in both types of mitochondria, a higher amount of Cyp D associated to ANT was found in liver mitochondria than in kidney mitochondria (Fig. 7). Figure 7a shows the Coomasie-stained blue

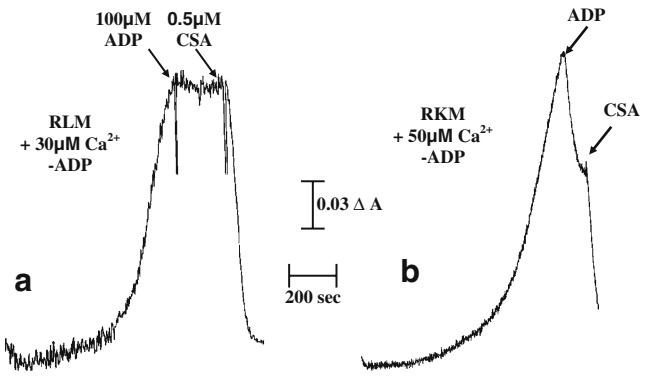

Fig. 2 The effect of ADP and cyclosporin A on Ca^{2+} reuptake. In panel **a**, 2 mg of liver mitochondrial protein was added. The basic medium contained, in addition, 30 μM Ca^{2+}, and, where indicated, 100 μM ADP and 0.5 μM cyclosporin A (CSA) were added. In panel **b**, 2 mg of protein from kidney mitochondria was added. In addition the basic medium contained 50 μM Ca^{2+}, and, as indicated, 100 μM ADP and 0.5 μM CSA were added. Final volume 3 ml. Temperature 25 °C

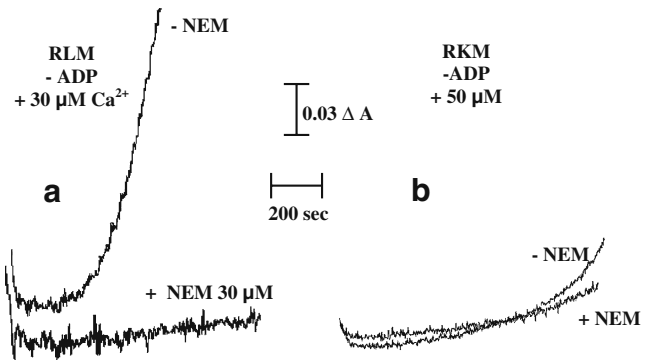

Fig. 3 The effect of N-ethylmaleimide on Ca^{2+} retention in liver and kidney mitochondria. In panel **a**, liver mitochondria (2 mg protein) were added to the incubation mixture that contained, in addition, 30 μM Ca^{2+}; where indicated, 20 μM NEM was added to the incubation medium. In panel **b**, 2 mg protein from kidney mitochondria was added to a basic medium containing 50 μM Ca^{2+}. Where indicated, the medium contained 20 μM NEM. Volume 3 ml. Temperature 25 °C

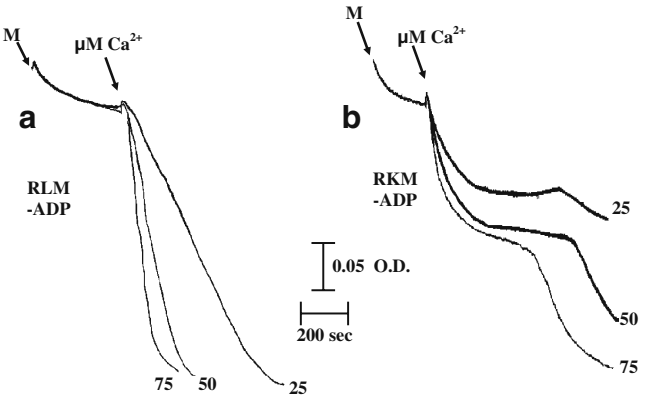

Fig. 4 The effect of Ca^{2+} on mitochondria swelling in media lacking ADP. Panel **a** depicts the behavior of liver mitochondria in media containing increasing concentrations of Ca^{2+}, from 25 to 75 μM. In trace C, the medium contained 100 μM ADP. Panel **b** shows the behavior of kidney mitochondria incubated under increasing Ca^{2+} concentrations, from 25 to 75 μM. Final volume 3 ml. Temperature 25 °C

gel of membrane proteins, electrophoresed in a non-reduction system, from mitochondria treated in the presence or absence of ADP, and plus or minus Ca^{2+}. Figure 7b shows the increased amount of ANT in kidney as compared to liver mitochondria. Figure 7c indicates that a higher amount of cyclophilin D bound to ANT was found in liver mitochondria than in kidney mitochondria. Figure 7c clearly depicts the relative intensity, in pixels, of the relationship between cyclophilin D and ANT.

Cardiolipin is another membrane constituent involved in the process of pore opening (Schlame et al. 1991; Chávez et al. 2008). Brustovetsky and Klingenberg (1996) postulated that this phospholipid is the target site for the binding of Ca^{2+}. Thus, a different content of membrane cardiolipin in liver and kidney mitochondria would result in a different sensitivity to Ca^{2+}. However, we found similar values of cardiolipin content for liver and kidney mitochondria (not shown).

Discussion

The results reported in this work establish that, in the absence of ADP, mitochondria isolated from rat liver are more sensitive to Ca^{2+}-induced membrane leakage than those isolated from rat kidney. The findings are closely similar to those of Endlicher et al. (2009), who reported that liver mitochondria are more sensitive than heart mitochondria to swelling induced by Ca^{2+}. It is well established that ADP inhibits the opening of the non-specific pore. The hypothesis sustaining such inhibition rests on the finding that the nucleotide locks ANT on the internal side of the inner membrane (Halestrap et al. 1997). This conformation of ANT confers a closed pattern to the pore. Our findings confirm the actual participation of ADP in the protection against Ca^{2+}-induced membrane leakage. As demonstrated, mitochondria were unable to retain matrix Ca^{2+} when the incubation medium lacked ADP. Similarly, in the absence of the nucleotide, mitochondria lost their ability to sustain a high value of membrane potential, and underwent a fast swelling after Ca^{2+} addition. However, a notable contrast regarding membrane damage was observed, depending on the source of mitochondria. Whereas liver mitochondria were more susceptible to the membrane deleterious action of

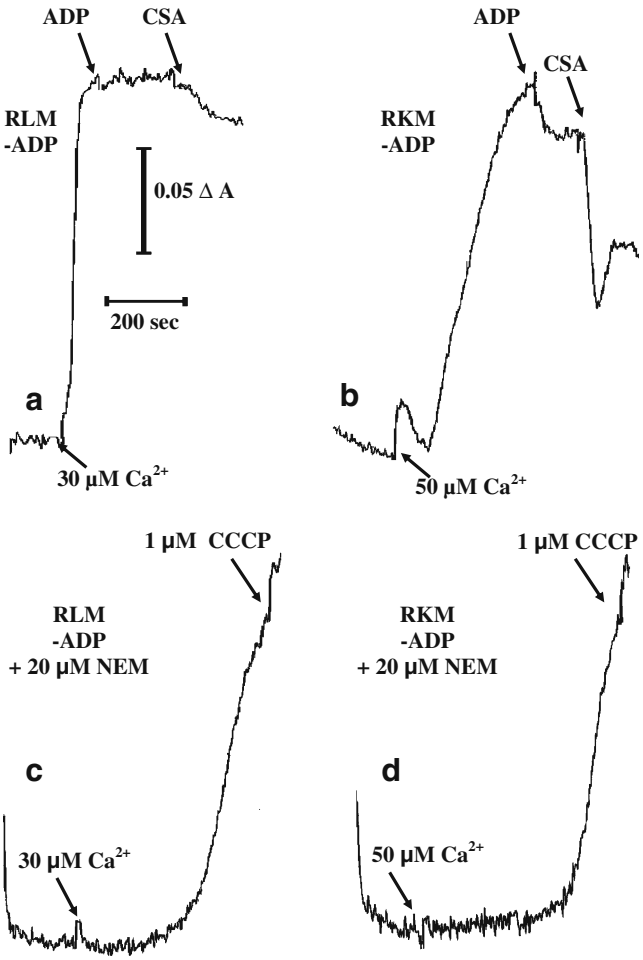

Fig. 5 Effect of ADP and NEM on Ca^{2+}-induced collapse of the transmembrane electric gradient. The basic medium contained 10 μM safranine. In **a**, liver mitochondria (2 mg protein) were added to 3 ml of the basic medium. The additions were: 30 μM Ca^{2+}, 100 μM ADP, and 0.5 μM CSA. In **b**, 2 mg protein from kidney mitochondria was added; 50 μM Ca^{2+}, 100 μM ADP, and 0.5 μM CSA were added as indicated. In panel **c**, liver mitochondria (2 mg protein) were incubated in the basic medium lacking ADP, containing 20 μM NEM. The additions were 30 μM Ca^{2+} and 1 μM carbonyl cyanide p-chlorophenylhydrazone (CCCP). In panel **d**, 2 mg protein from kidney mitochondria was added to the basic medium lacking ADP and containing 20 μM NEM. Where indicated, 50 μM Ca^{2+} and 1 μM CCCP were added. Temperature 25 °C

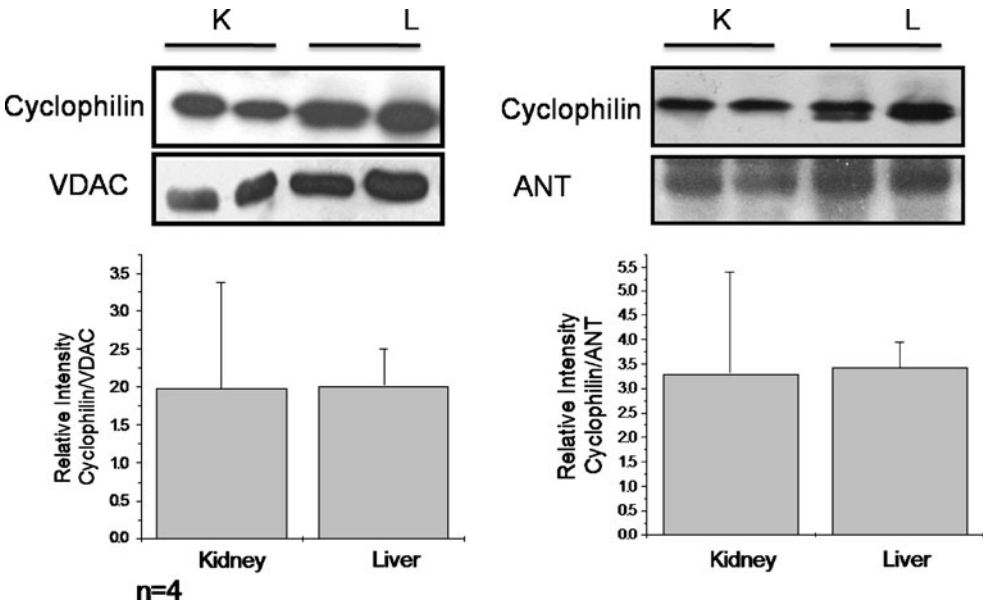

Fig. 6 Cyclophilin D content in liver and kidney mitochondria. Mitochondrial fractions (50 μg protein) were mixed with gel loading buffer containing 0.19 M Tris, pH 6.2, 30% glycerol, 6% SDS, 0.1% bromophenol blue, 100 mM DTT, and boiled for 5 min. The samples were separated into 12.5 acrylamide gels and transferred onto PVDF membranes, and blocked overnight with PBS-Tween, plus 5% fat-free milk. Then the membranes were incubated alternatively during 2 h with the following primary antibodies: mouse anti-cyclophilin (1:1,000), goat anti-ANT (1:500), or rabbit anti-VDAC (1:500), then for 1 h with the respective AP-conjugated anti-mouse, antigoat, or anti-rabbit IgG (H+L) secondary antibodies (1:25,000). The signal was detected by chemiluminescense using the Immobilon Western AP substrate

Ca^{2+} accumulation, kidney mitochondria appeared to be quite resistant to that process. Another finding that deserves to be pointed out is the fact that, in liver mitochondria, the addition of ADP during the Ca^{2+} release phase did not reverse the course of the reaction; in other words, it did not induce closing of the pore with the subsequent reuptake of Ca^{2+}. As shown, to reverse Ca^{2+} efflux, it was necessary to add CSA together with ADP. It should be pointed out that the picture was completely different when ADP was added, together with Ca^{2+}, at the start of the reaction. ADP and Ca^{2+} accumulated rapidly

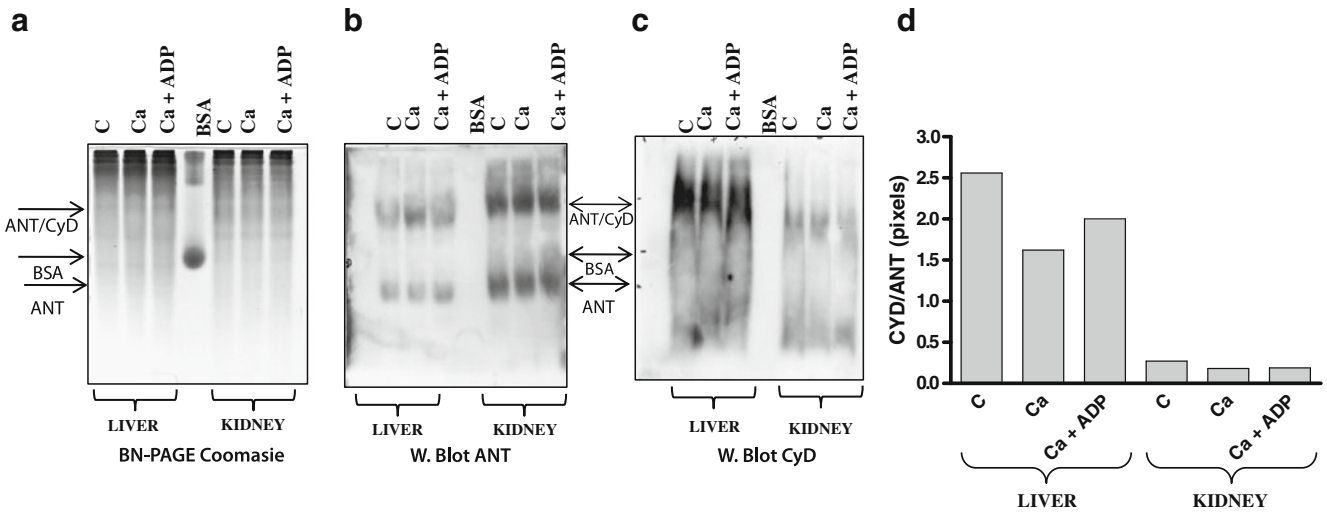

Fig. 7 Different levels of cyclophilin-ANT interaction in liver and kidney mitochondria. Mitochondrial protein (1 mg) was incubated in 1 ml of basic medium, and in the absence or presence of 50 μM Ca^{2+}, as well as in the absence or presence of 100 μM ADP. After 10 min incubation, the samples were centrifuged during 10 min at 10,000 rpm. The resulting pellets were solubilized in a medium containing 1.5 mg of digitonin 0.75 mM aminocaproic acid, 50 mM Tris, pH 7.0, and 1 mM PMSF, and maintained 30 min at 4 °C. Then, the samples were centrifuged at 14,000 rpm/15 min. The supernatant was treated with 10 μl aminocaproic acid 0.5 M-5%, Coomassie blue, pH 7.0. Aliquots of 100 μg were electrophoresed onto 5–12% acrylamide non-reduction gels. Then, the gels were transferred to PVDF membranes and revealed with anti-CyD and anti-ANT antibodies

and the cation remained, more than 30 min, within the matrix. A plausible explanation could be as follows: in the absence of ADP, Ca^{2+} may induce a conformational change in ANT in such a way that it facilitates the binding of cyclophilin D to the carrier, thus, the pore opens. If ADP is present previously in the incubation mixture, the binding of the nucleotide to the carrier avoids the binding of Ca^{2+} to its target site. Therefore, ADP circumvents the change in ANT configuration, as induced by Ca^{2+}, as well as the binding of cyclophilin D; thus, the pore cannot be opened. The above would be in concordance with the model proposed by Tanver et al. (1996). These authors discuss that ADP prevents the formation of Ca^{2+}• targeted-protein•cyclophilin complex by binding to the free target protein, hypothetically ANT. As shown, once the pore has been opened by the binding of Ca^{2+} and cyclophilin D to the pore, the addition of ADP was unable to reverse Ca^{2+} efflux in liver mitochondria. Probably cyclophilin D was tightly bound to its target site; thus, addition of CSA was necessary to remove it. However, in kidney mitochondria, ADP partially reversed pore opening. This may be explained considering that, in these mitochondria, a lower interaction between cyclophilin D and ANT was found.

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