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INHIBITION OF OXIDATIVE PHOSPHORYLATION BY A Ca^{2+}-INDUCED DIMINUTION OF THE ADENINE NUCLEOTIDE TRANSLOCATOR

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The mechanism through which internal Ca^{2+} inhibits oxidative phosphorylation of rat heart mitochondria has been explored. In parallel to a Ca^{2+}-induced diminution of the activity of the adenine nucleotide translocator, an efflux of internal adenine nucleotides is observed. The efflux of adenine nucleotides depends on the amount of Ca^{2+} accumulated by the mitochondria and on the time that Ca^{2+} remains in the mitochondria; this efflux is atractyloside insensitive. These results suggest that internal Ca^{2+}, by inducing a lowering of the internal concentration of adenine nucleotides, diminishes the rate of exchange of adenine nucleotides via the translocase, and in consequence of oxidative phosphorylation. Under conditions in which the Ca^{2+}-induced release of adenine nucleotides takes place, no gross changes of the permeability properties of the membrane are observed. As revealed by studies with arsenate, respiratory activity and the function of the ATPase in the direction of ATP synthesis are not affected by internal Ca^{2+}.

Introduction

Rossi and Lehninger [1] reported that in liver mitochondria Ca^{2+} uptake predominated over oxidative phosphorylation. Years later their findings were confirmed and extended to heart mitochondria, in which a similar preference was observed [2]. Other experiments that indicate that Ca^{2+} affects oxidative phosphorylation in mitochondria from various sources have been described [3–7]. Of particular interest to the present work is the observation of Malmström and Carafoli [8] who showed that mitochondria from uterus which have a high content of endogenous Ca^{2+} exhibit a low respiratory response to ADP; along the same line, it has been found that in brain mitochondria preloaded with Ca^{2+}, oxidative phosphorylation is inhibited through a process that is reversed by inducing Ca^{2+} release [9].

In this work, the inhibiting effect of Ca^{2+} on oxidative phosphorylation of rat heart mitochondria has been studied. In agreement with reports in the literature [1,2,4,5] it was observed that the transport of Ca^{2+} inhibits oxidative phosphorylation, but in addition it has also been found that internal Ca^{2+} also affects the rate of ATP synthesis. Apparently, internal Ca^{2+} induces the release of intramitochondrial adenine nucleotides, thereby limiting the rate of the adenine nucleotide translocator and this results in lower rates of ATP synthesis.

Materials and Methods

Preparation of mitochondria

Mitochondria from the hearts of rats weighing

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

150–200 g were prepared by a combination of the methods described by Lindenmayer et al. [10] and Vercesi et al. [2]. The hearts of five rats were cut into small pieces in a small volume of 180 mM KCl/10 mM Tris-HCl/10 mM EDTA at pH 7.1. The minced tissue was incubated with Nagarse (0.5 mg/10 ml of the above mixture per heart) for 10 min under occasional stirring. The liquid was decanted and the tissue washed twice with the same mixture (without Nagarse). The minced tissue was homogenized with a loose Teflon grinding pestle, and subsequently with the commercially available tissue homogeniser. The homogenate was centrifuged at 600 × *g* for 10 min. The supernatant was centrifuged at 6660 × *g* for 10 min. The pellet was washed once and suspended in a small volume with 120 mM KCl/3 mM Tris-HCl, pH 7.1.

Ca^{2+} transport

For assays of Ca^{+} fluxes, mitochondria were incubated under the conditions described with $^{45}Ca^{2+}$ (200000–400000 cpm). At predetermined times aliquots were withdrawn and filtered through Sartorius filters of 0.45 μm. The filtrate was washed with 0.5 ml of 120 mM KCl/3 mM Tris-HCl, pH 7.1. The filter was counted for radioactivity after solubilization with 5 ml of a scintillation liquid (tritosol), which contained 3 g 2,5-diphenyloxazole, 257 ml Triton X-100, 37 ml ethylene glycol, 106 ml ethanol and 600 ml xylene.

Oxygen uptake

This was recorded with the oxygen electrode (Yellow Springs Instrument Co.) in 3 ml of incubation mixture which contained 5 mM glutamate, 120 mM KCl, 10 mM Hepes, pH 7.1, and mitochondria (2 mg) at 30°C.

Uptake of $^{32}P_i$ into ATP

Mitochondria were incubated under the indicated conditions with $^{32}P_i$ (40000–70000 cpm/μmol). At the desired times the reaction was stopped with 6% trichloroacetic acid (final concentration). After centrifugation an aliquot of the supernatant was withdrawn and $^{32}P_i$ extracted as described by Lindberg and Ernster [11]. The aqueous phase was used for assay of $^{32}P_i$ incorporated into ATP by assay of Cerenkov radiation.

Release of intramitochondrial adenine nucleotides

Mitochondria were equilibrated with [^{3}H]ADP as described by Pfaff and Klingenberg [12]. The labeled mitochondria were incubated in the desired incubation mixtures (see Results) at a concentration of 2 mg per ml. At various times aliquots of 0.5 ml were withdrawn and filtered through Sartorius filters (0.45 μm). After 2–3 min of negative pressure the unwashed filters [12] were transferred to scintillation vials and assayed for radioactivity with 5 ml tritosol.

Activity of the adenine nucleotide translocator

Mitochondria were incubated under the conditions described in Results. At predetermined times aliquots of 0.5 ml were transferred to vessels that contained [^{3}H]ADP (20–40 nmol per mg mitochondrial protein); 20 s later aliquots of this mixture were filtered. The filters were washed [12] with 1 ml of cold 125 mM KCl, 5 mM Tris-HCl, pH 7.4. The radioactivity of the filters was assayed by liquid scintillation counting.

Uptake of safranine

Mitochondria at a concentration of 1 mg/ml were incubated with 10 μM safranine according to the method of Akerman and Wikstrom [13]. The spectral shift of safranine between 511 and 533 nm was measured with a double-beam spectrophotometer in a mixture which contained 120 mM KCl, 5 mM Tris-HCl, pH 7.4.

Results

By assays of the phosphorylation of ADP by $^{32}P_i$, and by polarographic measurements, it was confirmed that during the influx of Ca^{2+} into respiring mitochondria oxidative phosphorylation is largely depressed (data not shown); this is essentially in agreement with the observations of several authors [1,2,4]. However, it was also found that after Ca^{2+} had been taken up by the mitochondria, the rate of synthesis of ATP continued to be depressed. Fig. 1A shows the time course of ATP formation in mitochondria that had been preloaded with Ca^{2+}. The rate of ATP synthesis by mitochondria that possess about 80 nmol Ca^{2+}/mg or higher is substantially lower than that of the control. The inhibition persists for at least 5 min

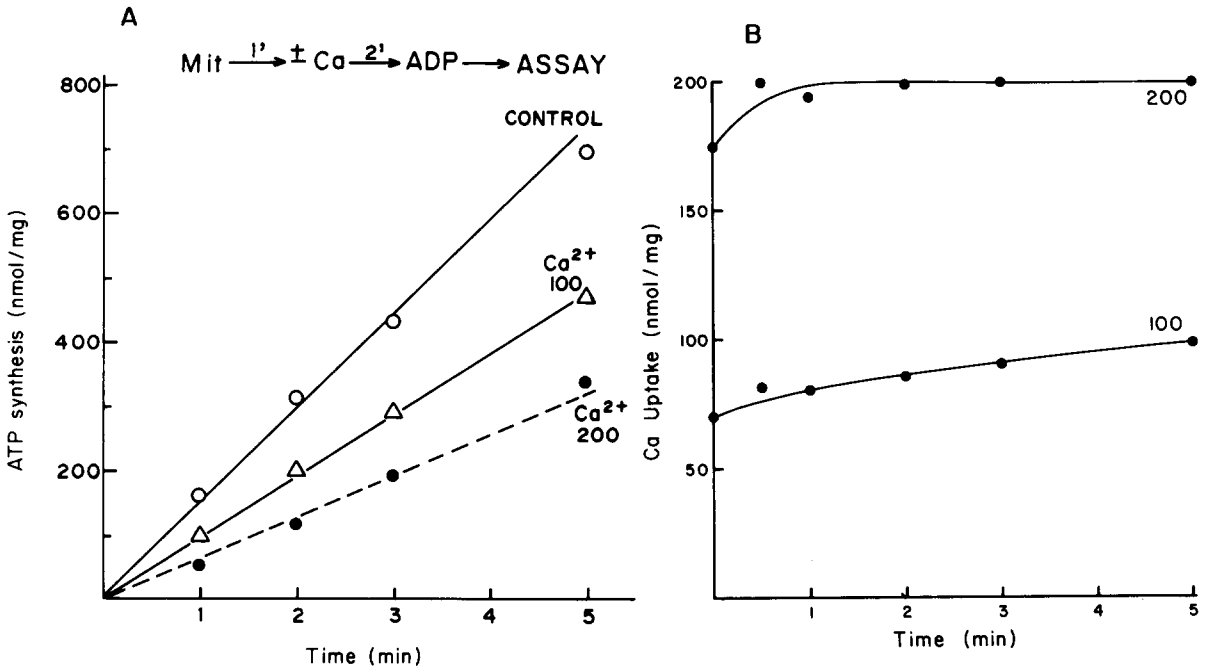

Fig. 1. Effect of internal Ca^{2+} on ATP synthesis by heart mitochondria. (A) Mitochondria (Mit) (1 mg/ml) were added to a mixture that contained 250 mM sucrose, 10 mM Hepes, 5 mM $^{32}P_i$, 5 mM succinate, 1 μg/mg rotenone and 100 and 200 nmol of Ca^{2+} as shown; the pH of the mixture was 7.1. After 2 min, 3 mM ADP was added and at the times shown the reaction was stopped with 6% trichloroacetic acid. Uptake of $^{32}P_i$ into ATP was determined. (B) Identical incubation conditions were followed, except that the mixture contained $^{45}Ca^{2+}$ and no radioactive phosphate. At the times shown after the addition of ADP, aliquots were withdrawn and filtered through sartorius filters to assay $^{45}Ca^{2+}$ in the mitochondria.

(the length of the experiment). During this time, Ca^{2+} was maintained on the inside of the mitochondria (Fig. 1B) which indicates that the inhibiting action of Ca^{2+} is not due to an inability of the mitochondria to form and maintain electrochemical gradients, otherwise Ca^{2+} would have leaked out. With concentrations of added Ca^{2+} lower than 100 nmol/mg, no effect on oxidative phosphorylation was clearly observed.

It has been reported that in mitochondria from liver and uterus [8,14,15] Ca^{2+} affects the functioning of the adenine nucleotide translocator. Therefore, it was decided to investigate whether a similar phenomenon took place in heart mitochondria. Table I shows that the exchange of external [^{3}H]ADP for internal adenine nucleotides is lower in heart mitochondria that had been exposed to Ca^{2+}. The data of Table I also indicate that as the time of exposure of mitochondria to Ca^{2+} increases, the exchange progressively diminishes. In

TABLE I

EFFECT OF Ca^{2+} ON THE ACTIVITY OF THE ADENINE NUCLEOTIDE TRANSLOCATOR OF RAT HEART MITOCHONDRIA

Mitochondria were added to the mixture detailed in the legend to Fig. 2 at a concentration of 1 mg protein/ml. After 1 min, 200 nmol Ca^{2+}/mg was added. After this addition, aliquots of 0.5 ml were withdrawn at the indicated times and added to vessels that contained 40 nmol [^{3}H]ADP. After 20 s aliquots of 0.35 ml were filtered. After washing, the radioactivity of the filter was assayed to measure the amount of adenine nucleotides exchanged.

Time (min)	[^{3}H]ADP exchanged (nmol/mg)	
	$-Ca^{2+}$	$+Ca^{2+}$
1	4.3	3.7
2	4.7	3.0
3	3.6	2.1
4	3.9	1.9
5	3.8	1.7

separate vessels, it was observed that mitochondria treated and incubated under similar conditions (except that nonradioactive ADP was added) accumulate and retain Ca^{2+} for the whole duration of the experiment (data not shown). The data of Table I indicate that the effect of Ca^{2+} is not immediate, rather, it appears that the diminution in the exchange of adenine nucleotides is due to some effect of internal Ca^{2+} and not to a direct action on the translocase per se.

There are certain reports in the literature that indicate that Ca^{2+} may induce drastic alterations of the permeability properties of the mitochondria, and that these are accompanied by release of intramitochondrial adenine nucleotides [16–20]. Therefore, it was decided to determine whether the inhibition of translocase activity by Ca^{2+} (Table I) was related to a Ca^{2+}-induced efflux of internal adenine nucleotides. For this purpose, heart mitochondria were equilibrated with [^{3}H]ADP; after washing external [^{3}H]ADP, the mitochondria were allowed to accumulate Ca^{2+}, and the possible release of labeled adenine nucleotides was assayed (Fig. 2). It may be observed that after accumulation of Ca^{2+} has taken place, a progressive loss of internal adenine nucleotides started to become apparent. In about 3 min after the addition of Ca^{2+}, approx. 30% of the internal adenine nucleotides are released to the external phase of the mitochondria, after this time the level of internal adenine nucleotide remains fairly constant (Fig. 2).

The experiments in which a Ca^{2+}-induced loss of adenine nucleotides had previously been observed have been carried out in the presence of phosphate [16–22]. Under the reported conditions, in addition to adenine nucleotides, Mg^{2+} and accumulated Ca^{2+} are also lost from the mitochondria. In parallel with these processes, some functional changes which suggest that the permeability properties of the mitochondria are drastically affected also take place. However, in the experiment of Fig. 2, mitochondria were incubated in the absence of phosphate, and Ca^{2+} was retained throughout the length of the experiment.

To assess the integrity of the mitochondria after Ca^{2+} accumulation, the ability of mitochondria to build up a membrane potential was assayed using safranine as an indicator of membrane potential [13]. Fig. 3 shows that after Ca^{2+} addition, there is

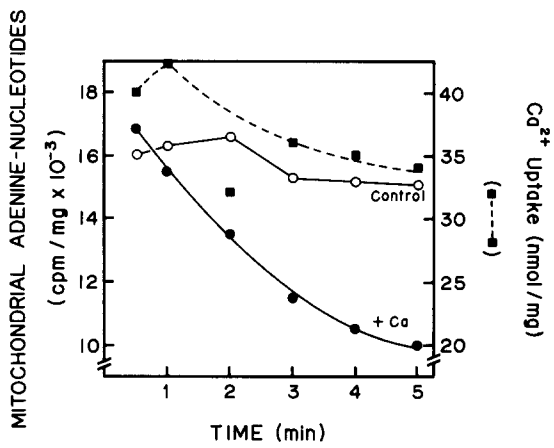

Fig. 2. Ca^{2+}-induced efflux of adenine nucleotides. Mitochondria were preloaded with [^{3}H]ADP. They were incubated at a concentration of 2 mg/ml of the mixture detailed in Fig. 1 without P_i for 1 min, and at this time 80 nmol Ca^{2+}/mg protein was added. After this addition at the times shown aliquots of 0.35 ml were filtered and washed. The radioactivity of the filters was assayed.

drop in the spectral signal which is proportional to the amount of Ca^{2+} added; the data of the figure also show that the further addition of phosphate to the mixture induces a safranine signal which is sensitive to the uncoupler FCCP. Moreover, under these conditions the extent of the safranine response in Ca^{2+}-loaded mitochondria is similar to that in which no Ca^{2+} was added. These findings

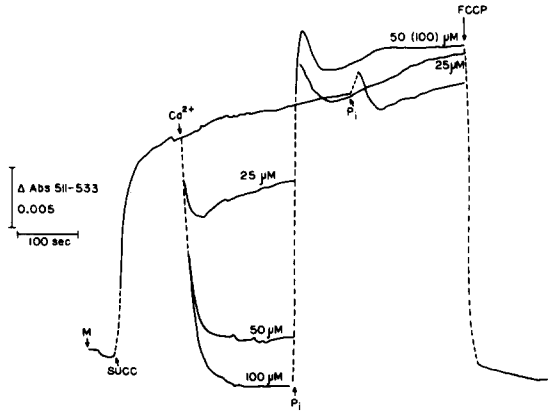

Fig. 3. Ca^{2+} and P_i effects on uptake of safranine. Mitochondria were incubated with safranine. Where indicated, additions were 5 mM succinate (succ), $CaCl_2$ at different concentrations, 5 mM P_i or 0.33 µM FCCP. Only the solid lines were registered.

indicate that mitochondria, even though containing concentrations of Ca^{2+} that induce release of adenine nucleotides, still maintain the ability to build up a membrane potential. Therefore, it would seem that their integrity is largely preserved. With respect to the data of Fig. 3, it should be recalled that Harris and Baum [23] reported that a permeant anion is required for maximal safranine response.

The Ca^{2+}-induced loss of internal adenine nucleotides depends on the concentration of added Ca^{2+}, and consequently, on the amount of Ca^{2+} that accumulates in the mitochondria (Fig. 4). Loss of internal adenine nucleotides starts to become apparent with 25 nmol Ca^{2+} added per mg mitochondrial protein, and maximum effect is observed with 100 nmol Ca^{2+}.

Fig. 5 shows that, similarly to the loss of internal adenine nucleotide, the diminution in the activity of the adenine nucleotide translocator as induced by Ca^{2+} depends on the concentration of the cation, and on the time that Ca^{2+} remains on the inside of the mitochondria. Mitochondria that had been loaded with Ca^{2+} for 0.5 min show an essentially unimpaired activity of the translocator, but after 3 min, (Fig. 5B) the activity is substantially lower.

It is important to note that the Ca^{2+}-induced decrease in translocase activity closely parallels the decrease in the level of internal adenine nucleotides. If a comparison is made of the data of Table I and Figs. 2–4, it may be observed that with respect to the time of incubation with Ca^{2+} and to the concentration of Ca^{2+} with which mitochondria are incubated, there is a very close correlation between the amount of adenine nucleotides that are released from the mitochondria, and the inhibition of translocase activity. In this respect, it has been reported that the activity of the translocator is modulated by the concentration of internal and external adenine nucleotide [24].

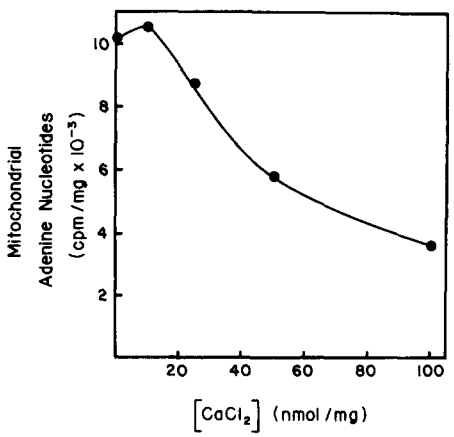

Fig. 4. Efflux of adenine nucleotides as induced by different concentrations of Ca^{2+}. The experimental conditions were as described in Fig. 2, except that the indicated concentrations of Ca^{2+} were added and the mitochondria filtered after 3 min.

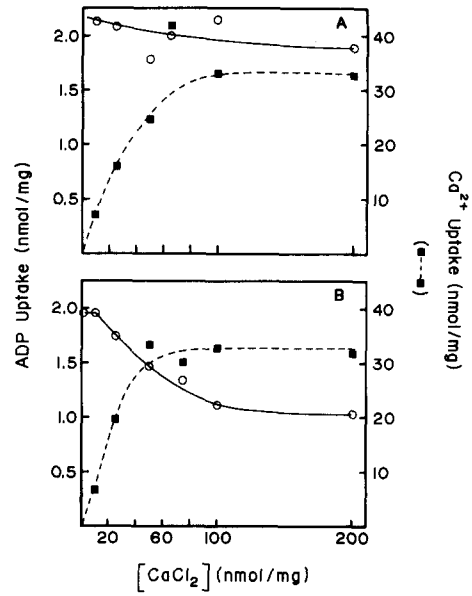

Fig. 5. Effect of Ca^{2+} on the activity of the adenine nucleotide translocator. Mitochondria were added to the incubation mixture detailed in Fig. 2 at a concentration of 2 mg protein/ml. After 1 min the indicated concentrations of Ca^{2+} were added. After 0.5 (A) or 3 (B) min, 20 nmol [^{3}H]ADP were added and after 20 s 0.35 ml was filtered to determine the amount of adenine nucleotides exchanged.

Under entirely different experimental conditions, it has been reported [25–28] that adenine nucleotides may traverse the mitochondrial membrane through a process that does not involve classical activity of the translocator. Therefore, it was interesting to explore whether the Ca^{2+}-induced efflux of adenine nucleotides was sensitive to atractyloside, a specific inhibitor of the adenine nucleotide translocator. It was found (Table II) that atractyloside at concentrations that effectively inhibit the activity of the translocator does not

TABLE II

EFFECT OF ATRACTYLOSIDE ON THE Ca^{2+}-INDUCED RELEASE OF ADENINE NUCLEOTIDES

Mitochondria were preloaded with [^{3}H]ADP as described in Materials and Methods. These mitochondria (2 mg protein/ml) were incubated as described in Fig. 2, except that the mixture contained Ca^{2+} and atractyloside as indicated. After 3 min an aliquot of 0.35 ml was filtered, the filter was washed and the radioactivity assayed.

$CaCl_2$ (nmol/mg)	Intramitochondrial adenine nucleotides (cpm/mg protein)		
Atractyloside (μM):	0	40	100
–	11306	13398	11827
25	9774	10260	10808
100	4049	2878	2743

prevent the Ca^{2+}-induced efflux of adenine nucleotides.

It was also found that Ca^{2+} induces a diminution of approx. 45% of the influx that remains in the presence of atractyloside (Table III), even though Ca^{2+} in the absence of atractyloside induces a 48% inhibition. These observations suggest that Ca^{2+} does not diminish the number of active enzymes, rather it would seem that Ca^{2+} only affects the rate at which the translocator functions.

The activity of the adenine nucleotide translocator apparently represents the rate-limiting step of oxidative phosphorylation [29–31], therefore, the diminution in activity as induced by Ca^{2+} may explain its inhibition of oxidative phosphorylation. However Ca^{2+} may affect activities other than that of the adenine nucleotide translocator, i.e., the mitochondrial ATPase or respiratory activity.

In intact mitochondria it is difficult to investigate the synthesis of ATP without a functional adenine nucleotide translocator. Nevertheless, a possible effect of Ca^{2+} on the ATPase (and respiratory activity) was examined by measuring the rate of the arsenate-stimulated oxygen uptake in the presence of ADP [32,33]. The rationale behind the experiment was that with arsenate a cyclic synthesis and breakdown of arsenylated ADP takes place on the inside of the mitochondria [34]. Thus, the increase in oxygen uptake by arsenate reflects the function of the ATPase in the direction of ATP synthesis. In this respect, it is important to stress that recently the formation of arsenylated ADP during electron transport has been directly demonstrated [35]. Accordingly, an effect of Ca^{2+} on the arsenate-stimulated oxygen uptake should reflect an action of Ca^{2+} on the events that lead to the arsenylation of ADP (which was considered analogous to phosphorylation) in the absence of translocase activity.

In mitochondria that had accumulated about 60 nmol Ca^{2+}/mg protein (100 nmol Ca^{2+} added/mg), the rate of arsenate-stimulated oxygen uptake in the presence of 0.4 μM ADP was 219 ngatom oxygen/mg per min. Approximately the same rate was obtained in mitochondria incubated in the absence of Ca^{2+}. Classical 'State 3' respiration was 233 ngatom oxygen/min per mg. These results indicate that internal Ca^{2+} does not affect the function of the ATPase, nor the activity of the respiratory chain.

TABLE III

EFFECT OF ATRACTYLOSIDE AND Ca^{2+} ON THE ACTIVITY OF THE ADENINE NUCLEOTIDE TRANSLOCATOR

The experimental conditions were as described in the legend to Fig. 4B. The amount of Ca^{2+} added was 100 nmol/mg protein, while atractyloside was used at a concentration of 100 μM.

Additions	[^{3}H]ADP exchanged (nmol/mg protein)
–	2.04
Ca^{2+}	1.04
Atractyloside	0.69
Ca^{2+} + atractyloside	0.38

Discussion

The inhibitory effect of Ca^{2+} on oxidative phosphorylation has been observed in several laborato-

ries [1–9]. Although the utilization of electrochemical gradients for the influx of Ca^{2+} seems to be the factor that depresses the rate of ATP synthesis during Ca^{2+} transport, it is not at all clear why Ca^{2+} on the internal phase of the mitochondria induces a diminution in the rate of ATP synthesis. In this work an attempt was made to explore the cause of this inhibition.

It has been found that internal Ca^{2+} induces a diminution of the exchange of adenine nucleotide mediated by the translocator. Also, it was observed that the arsenate-stimulated respiration was not affected by Ca^{2+}. The latter finding is of interest if it is considered that the increase in respiration induced by arsenate reflects all the processes involved in oxidative phosphorylation without the participation of the adenine nucleotide translocator. That is the synthesis of the highly unstable arsenylated ADP, and its subsequent hydrolysis to arsenate and ADP on the internal side of the mitochondria induce a stimulation of respiration which in a sense is analogous to oxidative phosphorylation, except that the function of the translocase is not required. On these grounds, it would appear that Ca^{2+} diminishes the activity of the adenine nucleotide translocator, and does not affect the processes that lead to the synthesis of ATP on the internal side of the mitochondria, i.e., respiratory and ATPase activities. Therefore, the inhibition of ATP synthesis by internal Ca^{2+} may be ascribed to its effect on the translocase. This, in a sense, confirms the idea that the rate-limiting step of oxidative phosphorylation is the activity of the translocase [20–31], since a diminution of its activity (as induced by Ca^{2+}) results in a lower rate of ATP synthesis.

Attempts have also been made to ascertain the mechanism through which internal Ca^{2+} diminishes the activity of the translocase. It has been observed that internal Ca^{2+} does not induce an immediate decrease in activity. Rather, the maximal effect of Ca^{2+} is observed after Ca^{2+} has been on the inside for a significant interval of time.

Simultaneous with the decrease in translocase activity, it was found that adenine nucleotides are ejected from mitochondria. A close correlation between the inhibition of translocase activity and efflux of adenine nucleotides was observed. Accordingly, it would appear that the inhibition of translocase activity is due to a diminution in the concentration of internal adenine nucleotides which results from the Ca^{2+}-induced efflux of the nucleotides. However, an additional direct effect of Ca^{2+} on the translocator that results in modification of the affinity constants for adenine nucleotides and specific inhibitors cannot be discarded as a possibility.

It may be thought that the efflux of adenine nucleotides observed in the presence of Ca^{2+} may be due to alteration of the permeability properties of the mitochondrial membrane as induced by Ca^{2+}. However, as evidenced by the ability of mitochondria to retain Ca^{2+}, to carry out additionally oxidative phosphorylation, to respond in the oxygen uptake to arsenate, and to build up a membrane potential (as monitored by safranine), it clearly appears that the integrity of the mitochondria loaded with Ca^{2+} is largely preserved.

This contrasts with previous reports in the literature which indicate that in liver mitochondria Ca^{2+} in the presence of phosphate induces drastic alterations of the permeability properties of the mitochondria, i.e., efflux of Ca^{2+}, Mg^{2+}, adenine nucleotides and pyridine nucleotides, and uncoupling of oxidative phosphorylation. The difference between our findings and those in the literature [19–22,36,37] is most likely due to the fact that heart mitochondria are much more resistant to the detrimental action of Ca^{2+} than liver mitochondria [22]. Moreover, the experiments in liver in which extensive alterations have been observed have been carried out in the presence of phosphate. In this work we have observed release of adenine nucleotides in the absence of added phosphate. Therefore, it would seem that it is possible to observe movements of adenine nucleotides in the absence of drastic alterations of permeability. The latter observation would appear to be in accordance with a movement of adenine nucleotides that occurs through a pathway different from that which occurs through the translocase, and which has been observed by other authors [25–28] under entirely different conditions. Indeed, the movement of adenine nucleotides we have observed is atractyloside insensitive which suggests that adenine nucleotides may move across the mitochondrial membrane through a process that is

not mediated by the translocase. However, this requires further analysis.

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