Studies on the Metabolism-Independent Calcium
Binding of the Rat Liver Mitochondria

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흰쥐 肝미토콘드리아의 非代謝依存性 칼슘 結合에 관한 연구

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적　요

미토콘드리아의 Ca결합은 물질대사에 의존하지 않는 初期 結合과 에너지소비를 수반하는 膜透過의 二段階로 일어 난다는 설이 있다. 본 실험에서는 이를 확인키 위하여 흰쥐의 肝에서 抽出한 미토콘드리아를 $^{45}CaCl_2$를 함유한 sucrose-tris chloride 용액에서 incubate 시키면서 Ca 흡수, 산소소비 및 ATPase 活性을 측정하였다.

미토콘드리아의 Ca 결합량은 온도의 영향을 거의 받지 않으며, succinate 나 ATP 의 존재에 의해서도 증가하지 않는다. 반면 산소소비량은 succinate 의 존재에 의하여 현저하게 증가되며 또 온도의 상승에 따라 증가된다. ATPase 活性도 온도의 상승에 따라 증가한다. 산소소비량과 Ca 의 결합량이 비례하지 않는 것으로 보아 미토콘드리아의 Ca 초기결합은 물질대사에 의존하지 않는 것으로 판단된다.

미토콘드리아의 ATPase 活性은 DNP 의 존재에 의하여 증진된다.

INTRODUCTION

Recently, numerous instances about the binding of monovalent and divalent cations to the cell membrane have been observed.

Chapell, Cohn and Greville(1963) have reported a rapid binding of Mn^{++} to the rat liver mitochondria in the presence of uncouplers or respiratory chain inhibitors. They suggested that binding of Mn^{++} to the mitochondria is nearly independent on metabolism.

Rasmussen, Chance and Ogata (1965) have described that initial binding of Ca^{++} to the rat

liver mitochondria incubated in a mannitol-sucrose-tris chloride(50mM) solution containing 3 mM tris-ATP is not blocked by olygomycin or DNP. They have denoted this binding of Ca^{++} to the mitochondria in the presence of DNP or olygomycin as nonspecific binding.

In the experiment on beef heart mitochondria, O'Brien and Brieley(1965) observed that in the absence of respiration (at O°C and in the presence of antimycin) Mg^{++} entered the mitochondria as a function of the concentration of Mg^{++} in the suspending medium which consisted of 0.33 M sucrose and 5 mM tris-chloride (pH 7.5), and that at higher concentrations of

Mg^{++} a linear relationship existed between intra-mitochondrial and extramitochondrial Mg^{++} concentrations. When the resulting line was extrapolated to zero external Mg^{++}, a value of about 60 mμmoles of intramitochondrial Mg^{++} per mg of protein was obtained. This value corresponded well to the Mg^{++} content which was observed in mitochondria which had been exposed to Mg^{++} and then washed. They designated this binding of Mg^{++} as "passive binding" and assumed that this increase in bound Mg^{++} under passive condition was due to the adsorption of the cation to various sites within the mitochondria.

Chance(1965) described that the binding of Ca^{++} to the mitochondrial surface is the preliminary step of the metabolism-dependent Ca^{++} transloaction across the mitochondrial membrane into the mitochondria. Rossi, Azzi and Azone (1967) studied a number of parameters of the metabolism-independent binding of Ca^{++} to the rat liver mitochondria. They found that a large part of the metabolism-independent binding of Ca^{++} occurs in a space which is rendered to monovalent cations by valinomycin or gramacidin. In fact, when the mitochondria were pretreated with valinomycin or gramacidin, the binding of Ca^{++} was increased in a sucrose medium and decreased in KCl or NaCl medium in consequence of the competition between Ca^{++} and the monovalent cations such as H^+ or K^+. They also showed that the amount of Ca^{++} bound to the mitochondria through a process which is independent on metabolism is increased by increasing the pH of the medium and is decreased by the addition of monovalent cations. They have concluded that the metabolism-independent Ca^{++} binding is coupled to a release of H^+ or K^+. Similar results have

also been reported by Calvalho and Leo(1967) who have observed that the binding sites for Ca^{++} in the fragments of rabbit sarcoplasmic reticulum were the same for Mg^{++}, K^+ and H^+.

Wenner and Hackney(1967) have found that the additon of Ca^{++} to the mouse liver mitochondria whose respiration is inhibited by antimycin results in a binding of Ca^{++} which is accompanied by a minor H^+ release in contrast to the results of Rossi *et al.* (1967). It seems that the difference of their results is related to the different experimental conditions.

Based upon these observations, the general conclusion reached is that Ca^{++} must be considered as a permeable cation which can be bound metabolism-independently to the outer mitochondrial membrane and that this metabolism-independent binding of Ca^{++} is an essential step for the energy-dependent accumulation of Ca^{++} inside the mitochondria. However, the relationship between the metabolism-independent Ca^{++} binding and metabolism-dependent Ca^{++} accumulation is not clear from existing data.

The present study was carried out to confirm whether the Ca^{++} could be bound to the rat liver mitochondria under metabolism-independent condition by measuring the Ca^{++} uptake, oxygen consumption and ATPase activity. The temperature effect on the Ca^{++} binding and ATPase activity was also measured.

MATERIALS AND METHODS

Mitochondria were extracted from the liver of rats according to the procedure of Schneider (1948). Extraction medium consisted of 25 mM

sucrose, 2 mM tris-chloride (pH 7.4) and 0.5 mM disodium ethylenediamine tetraacetate(EDTA). EDTA was omitted in the final suspension of the mitochondria. The amount of the mitochondrial protein was usually made 10–20 mg per ml of suspension medium and the suspension was kept at 0°C until used. All the procedures were performed at 0°– 4°C. Protein concentration was determined by micro Kjeldahl method and biuret photometry.

The binding of Ca^{++} was measured by incubating the mitochondria in the medium containing $^{45}CaCl_2$ (specific acivity, 0.8×10^5 cpm/mM Ca) and then by separating the mitochondria from the incubation medium either by rapid filtration through a Millpore filter (Type HA, 0.45 μ diameter) or, in most cases, centrifugation at 12,000 xG for 10 minutes at 0°C. The mitochondrial pellet sedimented by the centrifugation was allowed to lyse in a definite amount of deionized water and the radioactivity was measured on an aliquot of the suspension. The amount of Ca^{++} contained in the pellet was calculated from Ca^{++} present in the lysed suspension. The amount of Ca^{++} taken up into the mitochondria was calculated from the radioactivities of filter and filtrate and those of the incubation medium before and after the incubation.

Radioactivity was counted with a Tracerlab TGC-14 type, gas-flow, mica endwindow GM counter.

Oxygen consumption was measured by the direct reading method of the Warburg's manometry according to Umbreit *et al.*(1964). Incubation was done at 37°C for 30 minutes with the same basic medium as used in the Ca^{++} uptake experiment, and the oxygen consumption was expressed as μl oxygen consumed per mg

of protein per hour.

ATPase activity was assayed in various incubation conditions. The reaction was started by the addition of 0.1 mM ATP and stopped by adding trichloroacetic acid in a final concentration of 6%. The ATPase activity was expressed as μmoles inorganic phosphate produced per mg of protein per hour. Quantitative determination of the inorganic phosphate was done by Allen's method(1940) modified by Nakamura (1950).

RESULTS

1. The calcium uptake of the mitochondria.

The Ca^{++} uptake of the rat liver mitochondria was measured in various incubation media and the results were summarized in Table 1. In the control medium, which consisted of 25 mM sucrose, 2 mM tris-chloride, 0.2 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (pH 7.4), the amount of Ca^{++} bound per mg of protein was 21.85 μmoles. The addition of succinate in the final concentration of 20 mM greatly decreased the Ca^{++} uptake. As seen in Table 1, the amount of Ca^{++} taken up by the mitochondria in the presence of succinate was nearly one-third of the control. Addition of ATP(0.1 mM) to the incubation medium, on the other hand, had practically no effect on the Ca^{++} uptake. The decreasing effect of the presence of succinate in the medium was more apparent in this ATP-containing medium; when succinate was present along with ATP, the amount of Ca^{++} taken up by the mitochondria was still decreased giving a value corresponding to about one-sixth of the succinate-free, ATP-containing medium (Table 1).

The presence of DNP(0.1 mM) in the ATP medium had no effect on the Ca^{++} uptake both

Table 1. The calcium uptake, the oxygen consumption and ATPase activity of the rat liver mitochondria in various incubation media. The control medium consists of 25 mM sucrose, 2 mM tris-chloride, 0.2 mM MgCl$_2$ and 0.1 mM CaCl$_2$

Addition	Ca uptake (μMCa^{++}/ mg protein)	Oxygen consumption (μl O$_2$/mg protein/hr)	ATPase activity (μM Pi/mg protein/hr)
Control	21.85± 1.083	0.68± 0.101	1.78± 0.015
Succinate	6.85± 0.482	14.04± 0.174	3.06± 0.084
ATP	19.46± 0.929	0.32± 0.091	8.90± 0.385
Succinate+ ATP	3.47± 0.154	6.84± 0.217	10.82± 0.323
DNP+ATP	20.32± 0.887	0.34± 0.037	9.98± 0.116
ATP+DNP+ succinate	5.27± 0.129	6.56± 0.321	11.82± 0.427

in the presence and in the absence of succinate.

Table 2 shows the Ca^{++} concentration in and outside of mitochondria after 1 minute incubation in various incubation medium at 37°C. As shown in the table, Ca^{++} concentrations inside of the mitochondria were larger than those of outside of the mitochondria in every conditions studied. In the control medium, the ratio of Ca^{++} concentration inside of the mitochondria to Ca^{++} concentration outside of the mitochondria was 1.89 and the ratios in other incubation media were less than the ratio in control medium. These results indicate that the Ca^{++} uptake was undertaken in every incubation medium and show the concordance with the data presented in Table 1.

2. The oxygen consumption of the mitochondria.

Table 2. Calcium concentration of inside and outside of the mitochondria after incubation in various incubation media. The control medium consists of 25 mM sucrose, 2mM tris-chloride, 0.2 mM MgCl$_2$ and 0.1mM CaCl$_2$

Addition	Ca^{++} concentration after incubation		ratio, Ca$_i$/ Ca$_0$
	inside (Ca$_i$)	outside (Ca$_0$)	
	μmoles/ml	μmoles/ml	
Control	7.26±0.312	3.84±0.114	1.9
succinate	6.05±0.209	4.95±0.222	1.2
ATP	6.85±0.197	4.15±0.240	1.7
succinate+ ATP	5.75±0.043	5.25±0.081	1.1
ATP+DNP	6.99±0.284	4.01±0.205	1.7
ATP+DNP+ succinate	5.84±0.125	5.16±0.117	1.1

In order to correlate the Ca^{++} uptake with the respiratory activity, the oxygen consumption of the mitochondria was measured in the same incubation media with those employed in the study of the Ca^{++} uptake (Table 1 & Fig. 1).

In the control medium, the amount of oxygen consumed was on average 0.68 μl/mg protein/ hr. The oxygen consumption was highly stimulated by the presence of succinate in the medium. By adding succinate in the concentration of 20 mM to the incubation medium, the oxygen consumed by the mitochondria was increased up to nearly twenty times that of control as can be revealed from Table 1. The addition of ATP to the medium, on the other hand, decreased the oxygen consumption to approximately half that of control both in the presence and in the absence of succinate. The addition of DNP along with ATP showed essentially the same degree of oxygen consumption with that of ATP-medium again both in the presence and in the absence of succinate. Thus, it is apparent

that succinate highly increases the mitochondrial respiration while it significantly lowers the uptake of Ca^{++} by the mitochondria in the same medium. It is also interesting to note that the addition of ATP has no effect on the Ca^{++} uptake while it decreased the oxygen consumption remarkably. The presence of DNP along with ATP seems to have no meaning both in the Ca^{++} uptake and the oxygen consumption.
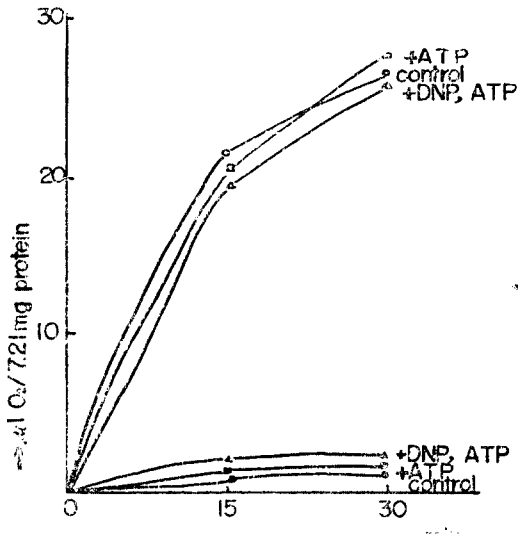

Fig. 1. The oxygen consumption of the rat liver mitochondria in the presence and absence of succinate. The control medium consisted of 25 mM sucrose, 2 mM tris-chloride, 0.1mM $MgCl_2$ and 0.1mM $CaCl_2$. The upper three curves are in the presence of 20 mM succinate and lower three ones are in the absence of succinate. The mitochondrial protein was 7.21 mg and incubation temperature, 37°C; total incubation volume, 2.0 ml.

3. The ATPase activity of the mitochondria.

Since the ATPase activity of the cell membrane is widely known to participate with the active transport of various ions across the membrane, we have measured this activity residing on the mitochondrial membrane in the same incubation media which were employed

in the determination of the oxygen consumption and of the calcium uptake, and the results were summarized in Table 1.

The ATPase activity in this experiment is expressed as the amount of inorganic phosphate produced per mg of protein after the incubation for 1 hour. The activity in the control medium was found to be about 1.8 μmoles, whereas a nearly double amount of activity was measured in the succinate-containing medium. The addition of ATP to the medium highly increased, as was expected, the activity up to nearly five times that of control. The presence of succinate in this case caused a much more increased activity. The co-existence of DNP with ATP slightly increased the ATPase activity, the increment in this system being about 10% of the value found in the medium where ATP is the only addition. This degree of increment was also found in the system where succinate had been present. It is, therefore, considered that DNP stimulates the ATPase activity of the mitochondria.

4. The effect of temperature on the Ca binding of the mitochondria.

If the binding of Ca^{++} to the mitochondria is a process of active transport which involves an expenditure of energy, the amount of Ca^{++} bound to the mitochondria will increase as the temperature of the system increases to a certain limit. Contrary, if the binding is a metabolism-independent process, increasing the temperature will cause little change in the amount of the Ca^{++} uptake.

Table 3 and Fig. 2 show the effect of temperature on the Ca^{++} binding of the mitochondria in various media. In the control medium, the amount of Ca^{++} taken up by the mitochondria was nearly constant at every temperature mea-

sured in the range between 0°C to 37°C. Whne the mitochondria were treated with 0.1 mM DNP, the amount of Ca⁺⁺ bound to the mito-chondria was less than that of the control by about 20% and was constant. as in the case of the control, at all the temperature range studied.

Table 3. The effect of temperature on Ca⁺⁺ binding of the mitochondria. The mitochondria (10mg of protein) were incubated in the control medium (25 mM sucrose, 2 mM tris-chloride, pH 7.4) containing either 0.1mM DNP, 0.1mM ATP, or 20 mM succinate, for 1 minute at various temperature after the addition of ^{45}CaCl₂. The mitochondria were separated from the medium by rapid filtration through a Millipore filter. The data in the table represent an average of triplicate de-terminations. The amount of Ca⁺⁺ bound was expressed as μM Ca⁺⁺/mg protein.

| Conditions | Temp. °C | | | | | |
	0	10	20	25	30	37
Control	17.75	18.15	17.80	18.33	17.84	17.75
DNP	14.46	14.46	14.55	14.50	14.50	14.51
Succinate	3.74	4.06	6.53	6.76	8.51	8.20
ATP	3.02	3.11	3.15	3.60	4.06	3.70

The addition of succinate in the medium caused a great decrease in the amount of Ca⁺⁺ taken up, especially at low temperatures. As the temperature increases the amount of bound Ca⁺⁺ increases, in the contrary with the control system, up to 30°C. Similar results were obs-erved when the mitochondria were treated with ATP, but to a lesser extent.

The saturation of Ca⁺⁺ uptake by the mito-chondria occurs rapidly; it took less than 30 seconds after the addition of ^{45}CaCl₂ as deter-mined at 20°C in the control medium.
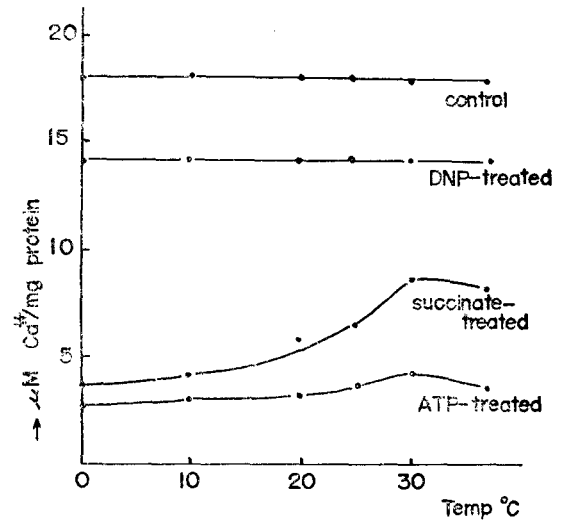

Fig. 2. The effect of temperature on the Ca binding of the mitochondria. Experimental condition was the same as in Table 3.

5. Effect of the temperature on the ATPase
   activity of the mitochondria.

The ATPase activity of the mitochondria was measured at varying temperatures beween O°C and 30°C and the results were shown in Fig. 3.
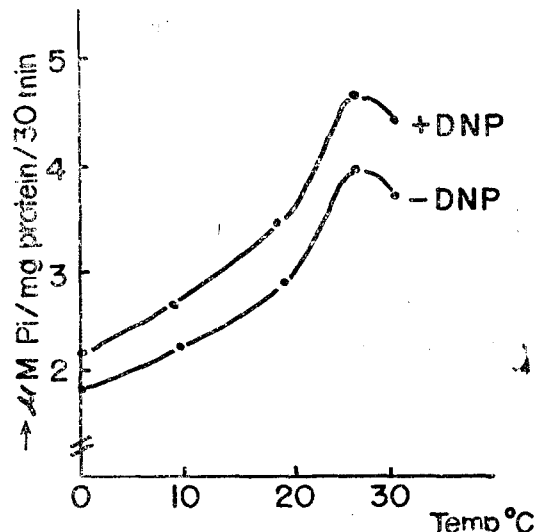
The activity increased as the temperature

Fig. 3. Effect of DNP on the ATPase activity of the rat liver mitochondria.

increases up to 25°C with a Q_{10} approximately 1.5 (in the range between 20°C and 25°C). At temperatures higher than 25°C, the activity tends to decrease slowly.

The presence of DNP in the medium stimulated the ATPase activity at all the temperatures, but did not change the pattern of the curve as seen in Fig. 3.

DISCUSSION

In the present paper we have presented the evidence that Ca^{++} can be bound to the mitochondria by a metabolism-independent process. The evidence supporting this conclusion is as follows.

First, the amount of Ca^{++} bound per mg of protein is not increased, but contrary, decreased in the presence of succinate, while the amount of oxygen consumed per mg of protein was highly increased as shown in Table 1. In other words, our preparation of mitochondria oxidized succinate consuming oxygen through the respiratory chain, nevertheless the Ca^{++} uptake is not increased. Of course there exists the possibility that the decrease of Ca^{++} uptake in the presence of succinate may be due to the formation of Ca-succinate complex lowering the concentration of the available free Ca^{++} in the medium. The possibility, however, seems to have but little meaning because as the succinate is oxidized more and more, an equivalent amount of Ca^{++} would be liberated progressively as free available Ca^{++} in the medium.

Second, the addition of ATP did not increase the Ca^{++} bound in the presence of succinate, whereas the ATPase activity was increased highly. This result indicates that the mitochondria hydrolyzes ATP but the energy liberated

from the hydrolysis is not used in the Ca^{++} uptake. Consequently, it could be concluded that the Ca^{++} binding of mitochondria must be independent on metabolism and furthermore, the enrgy-linked Ca^{++} binding does not occur in the initial uptake of Ca^{++} by mitochondria.

In the absence of succinate, the addition of ATP or DNP practically does not change the amount of Ca^{++} uptake. This result also supports the metabolism-independent Ca^{++} binding. It can be confirmed that the Ca^{++} uptake was occurred in every incubation conditions by the fact that the ratios of Ca^{++} concentration inside and outside of the mitochondria after the incubation were always larger than 1 as presented in Table 2.

O'Brien and Brieley(1965) have reported that 60 μmoles of Mg^{++} per mg of protein binds to the heart mitochondria incubated in 33 mM sucrose solution containing 5 mM tris-chloride (pH 7.5), and Rossi et al.(1967) have described that the rat liver mitochondria incubated in 25 mM sucrose solution containing 2.5 mM tris-chloride(pH 7.4) binds 20 to 30 μmoles of Ca^{++} per mg of protein. The data reported in this paper are in a substantial agreement with those of O'Brien and Brieley(1965) and Rossi et al. (1967).

Addition of DNP to the incubation medium resulted in the decrease of the amount of Ca^{++} bound, but the amount of bound Ca^{++} is constant at various incubation temperature in the range 0°–30°C. In the case of succinate- or ATP-treated mitochondria, on the other hand, the amount of Ca^{++} bound decreased significantly and increased as the temperature increased. These results are in concordance with the report described by Lehninger and Reynafarje (1969).

Korean J. Zool.

Comparing Table 1 with Table 3, it may be observed that the amount of Ca^{++} uptake in the presence of Mg^{++} was higher than that found in the absence of Mg^{++}(21.85 μmoles/mg vs. 17.75 μmoles/mg). The amount of mitochondrial protein was 7.21 mg in the former case and was 10.0 mg in the latter. This difference in the amount of bound Ca^{++} might be due to the effect of increasing protein concentration as reported by Rossi et al.(1967). It was also reported that Mg^{++} does not inhibit the Ca^{++} uptake(Rossi et al. 1967; Scarpa et al. 1968 ; and Lehninger and Reynafarje, 1969). On the other hand, Calvalho and Leo (1967) reported that Mg^{++} ions share the same binding sites with the Ca^{++} ions in the membrane of fragmented sarcoplasmic reticulum. According to their observations more Ca^{++} was bound to the membranes of sarcoplasmic reticulum in the absence of Mg^{++}, a result completely contrast with mitochondria. Thus, there seems to be a significant difference in the property of cation binding between these two organelles.

SUMMARY

Measurements were made of the Ca^{++} uptake, oxygen consumption and ATPase activity of mitochondria extracted from the rat liver in sucrose-tris chloride medium.

Ca^{++} binding of mitochondria was not affected by the incubation temperature in the range of 0°-37°C.

Succinate did not increase the amount of Ca^{++} bound while it increased oxygen consumption ; highly. The presence of ATP in the incubation medium did not enhance the Ca^{++} uptake either. Therefore, it is concluded that the initial binding of Ca^{++} is independent on metabolism.

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