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EFFECTS OF ADENINE NUCLEOTIDE TRANSLOCASE INHIBITORS ON DINITROPHENOL-INDUCED Ca^{2+} EFFLUX FROM PIG HEART MITOCHONDRIA

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SUMMARY

Bongkrekic acid and atractyloside, inhibitors of adenine nucleotide translocase, do not inhibit Ca^{2+} uptake and H^+ production by pig heart mitochondria. However, bongkrekic acid, but not atractyloside, inhibits dinitrophenol-induced Ca^{2+} efflux and H^+ uptake. Conversely, ruthenium red blocks Ca^{2+} uptake and H^+ production but does not prevent dinitrophenol-induced Ca^{2+} efflux and H^+ uptake by mitochondria. These results suggest that mitochondrial Ca^{2+} uptake and release exist as two independent pathways. The efflux of Ca^{2+} from mitochondria is mediated by a bongkrekic acid sensitive component which is apparently not identical to the ruthenium red sensitive Ca^{2+} uptake carrier.

INTRODUCTION

Although Ca^{2+} uptake by mitochondria is known to be supported by respiration or ATP hydrolysis [1] and is mediated by a ruthenium red and La^{3+}-sensitive carrier [2–4], the mechanism of Ca^{2+} efflux from mitochondria is not as well characterized. The respiration-linked accumulation of Ca^{2+} and ejection of H^+ by isolated intact mitochondria has been studied in two different experimental conditions; the presence or absence of permeant anions such as inorganic phosphate (P_i) [1, 5, 6]. A small amount of Ca^{2+} is transported into the mitochondria in the absence of P_i and remains bound to anionic sites on the inside of the inner membrane [6, 7]. The subsequent release of Ca^{2+} induced by uncouplers or respiratory inhibitors in the absence of P_i is presumably a dissociation of bound form. On the other hand, in the presence of P_i much larger amounts of Ca^{2+} are accumulated as a calcium phosphate complex within the mitochondrial matrix which is counterbalanced by an electro-neutral H^+ efflux [1, 6–8]. Efflux of accumulated Ca^{2+} under these conditions is activated by high P_i concentration [5, 9], or with low P_i concentration in the presence of phosphoenolpyruvate [9–12], as well as by uncoupling agents, i.e., dinitrophenol [1, 5]. Activation of Ca^{2+} efflux by P_i and by low P_i plus phosphoenolpyruvate have been shown to be inhibited by bongkrekic acid or atractyloside [9–12]. Since these two inhibitors

are known to block adenine nucleotide and phosphoenolpyruvate transport [11–15], these results have been interpreted in terms of blocking an efflux of internal adenine nucleotides which in some way stabilizes the accumulated calcium phosphate [1, 5]. On the other hand, the phenomenon of dinitrophenol-induced efflux of Ca^{2+} has not been similarily investigated. The studies described here were carried out to characterize the effects of adenine nucleotide translocase inhibitors on dinitrophenol-activated Ca^{2+} efflux.

MATERIALS AND METHODS

Pigs weighing 80–120 pounds were fasted overnight and anesthetized with 6 % phenobarbital administered intravenously. The heart was removed and taken to the cold room where samples were excised, minced and homogenized in a medium containing 0.25 M sucrose, 20 mM Tris buffer, pH 7.5 with a polytron PT10 homogenizer. Mitochondria were isolated by a method similar to that previously described [16]. Mitochondria were suspended in a medium containing 0.2 mM Hepes, potassium (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.45 and 250 mM sucrose after the final washing. Mitochondrial protein was determined by the biuret method [17]. Oxygen uptake was determined polarographically with the Clark oxygen electrode [18]. The production and uptake of H^+ during the influx and efflux of Ca^{2+} were followed with a sensitive recording pH electrode as previously described [19]. Appropriate corrections were made for the buffering capacity of the medium and suspended mitochondria by addition of standardized HCl or NaOH in each experiment. Ca^{2+} uptake and efflux were followed with $^{45}Ca^{2+}$ as described previously [10]. Aliquots of the incubation mixture were withdrawn at selected time intervals after the addition of $^{45}CaCl_2$ and rapidly filtered through a millipore filter [20]. The radioactivity of $^{45}Ca^{2+}$ of each sample was determined with a Beckman liquid scintillation spectrometer, Model 1650. Mitochondrial ATP content was determined parallel to Ca^{2+} transport studies. In all cases, the reaction mixture was removed and added to a tube containing 0.5 ml 20 % trichloroacetic acid 1 min after dinitrophenol-induced Ca^{2+} efflux occurred. The TCA extracts were then used to determine the amount of internal ATP by the firefly luciferase reaction [21]. All reagents were analytical grade. Atractyloside, dinitrophenol and ruthenium red were obtained from Sigma Chemical Co. Bongkrekic acid was a gift from Professor W. Berends, Biochemistry Laboratory of the Technological University of Delft, Delft, Holland. A23187 was the gift of Dr. Hamill, Eli Lilly and Co., Indianapolis, Indiana. $^{45}CaCl_2$ was purchased from New England Nuclear Co.

RESULTS

A typical oxygen and pH electrode tracing of mitochondrial respiration and H^+ production is shown in Fig. 1. The addition of Ca^{2+} stimulates mitochondrial respiration and causes H^+ ejection from mitochondria. The stimulated-respiration and H^+ production ceased when Ca^{2+} uptake was complete. The addition of 0.2 mM dinitrophenol following the completion of Ca^{2+} uptake by mitochondria caused indefinite stimulation of mitochondrial respiration (uncoupled respiration) and H^+ uptake (Fig. 1a). The rate and the extent of this H^+ uptake is much lower than that of H^+

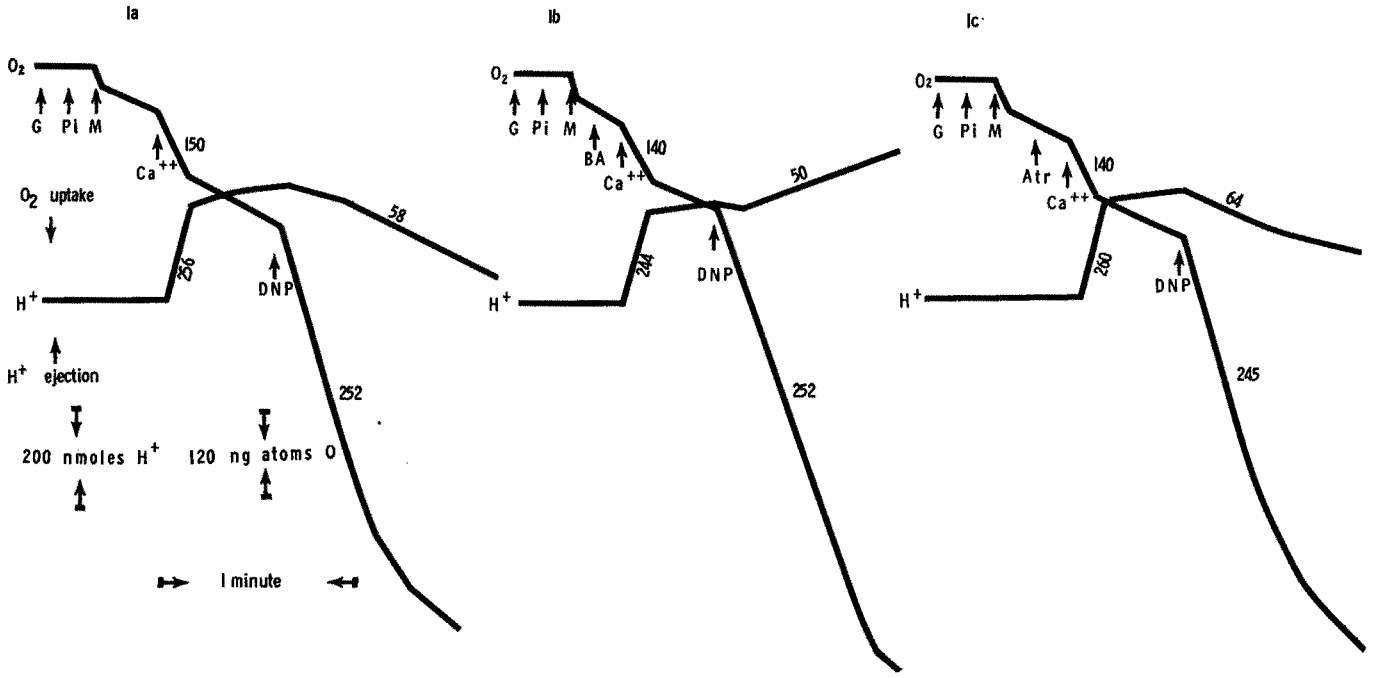

Fig. 1. Oxygen and pH electrode tracing showing the effect of bongkrekic acid and atractyloside on mitochondrial oxygen consumption and H^+ movement. Experimental system containing 0.2 mM Hepes buffer, pH 7.45; sucrose (250 mM), G (sodium glutamate, 2.5 mM), P_i (potassium phosphate, 1 mM), M (mitochondria, 4.07 mg protein), BA (bongkrekic acid, 44 μM), Atr (atractyloside, 110 μM), Ca (400 nmol) and DNP (dinitrophenol, 0.2 mM) was added to the incubation mixture in a final volume of 2.2 ml at 30 °C as indicated. The number adjacent to each oxygen trace represents the rate of oxygen consumption by mitochondria (ngatoms O/mg/min). The number adjacent to each pH trace represents the rate of H^+ movement/mg/min (ejection or uptake).

ejection during Ca^{2+} uptake. In the presence of bongkrekic acid, the Ca^{2+}-induced oxygen uptake and H^+ ejection from mitochondria were not altered, and the addition of dinitrophenol following the completion of Ca^{2+} uptake by mitochondria also caused uncoupled respiration. However, H^+ uptake by mitochondria following dinitrophenol addition did not occur in the presence of bongkrekic acid. Instead, continuous H^+ ejection took place (Fig. 1b). The addition of atractyloside to the incubation mixture, on the other hand, did not affect Ca^{2+}-induced oxygen uptake and H^+ ejection from mitochondria,nor did it alter respiration and H^+ uptake by mitochondria following dinitrophenol addition (Fig. 1c). Thus, in contrast to the effect caused by bongkrekic acid atractyloside has no effect on dinitrophenol-induced H^+ uptake.

It has been noted that adenine nucleotides may be required in maintaining accumulated Ca^{2+} in mitochondria [1, 5]. Therefore, the effect of adenine nucleotides

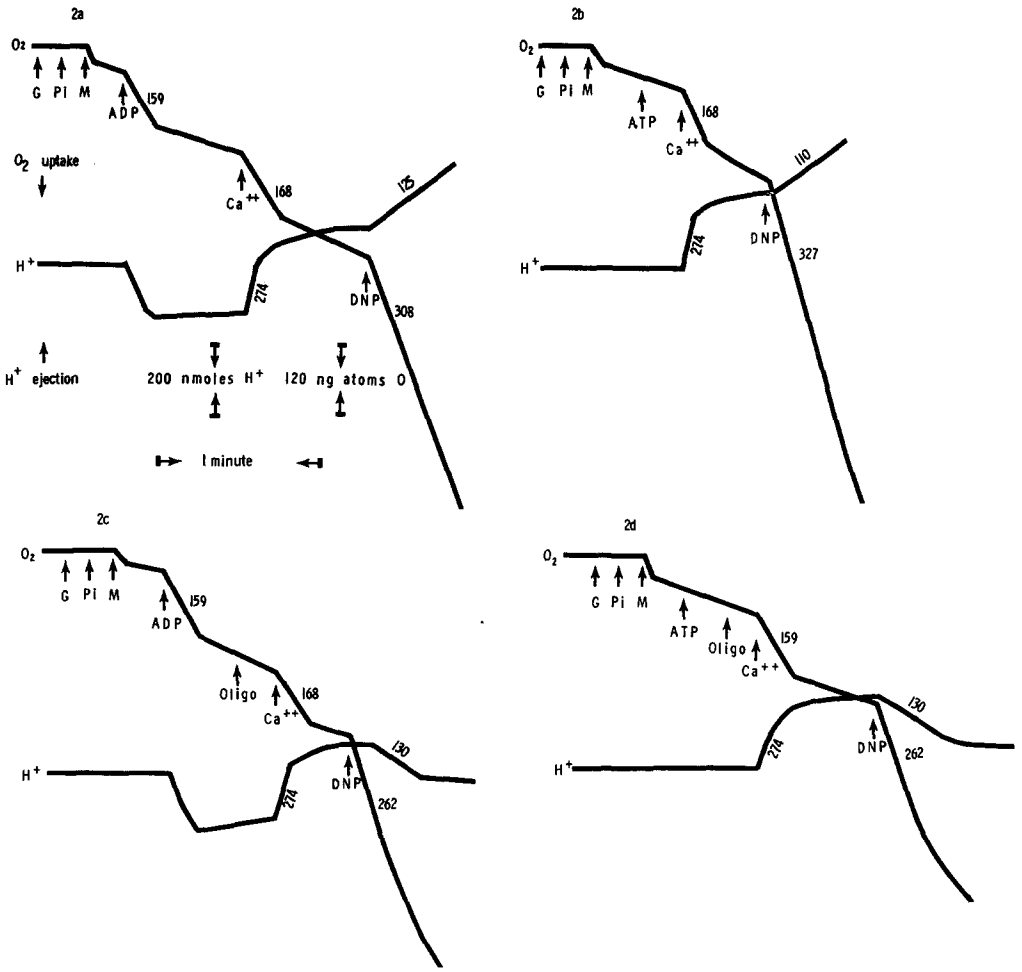

Fig. 2. Oxygen and pH electrode tracing showing the effect of ADP, ATP and oligomycin on mitochondrial oxygen consumption and H^+ movement. Experimental conditions were the same as in Fig. 1. ADP (300 nmol), ATP (300 nmol) and oligomycin (4 μg) were added as indicated.

on dinitrophenol-induced Ca^{2+} efflux and H^+ uptake was studied. Fig. 2 demonstrates that dinitrophenol-induced uncoupled respiration is not altered by the addition of ATP; however, continuous H^+ ejection occurs, instead of H^+ uptake, following the dinitrophenol addition in the presence of adenine nucleotides (Figs. 2a and b). This dinitrophenol-induced H^+ ejection in the presence of adenine nucleotides is abolished by oligomycin (Figs. 2c and d). Thus, the dinitrophenol-induced H^+ uptake which accompanies Ca^{2+} efflux is masked by the H^+ production from ATP hydrolysis.

Table I shows the effects of bongkrekic acid and atractyloside on Ca^{2+} transport as determined by measurement of $^{45}Ca^{2+}$. The uptake of Ca^{2+} by mitochondria was not altered by the addition of the adenine nucleotide translocase inhibitors or adenine nucleotides themselves. However, as would be expected from the proton shift in the previous results dinitrophenol-induced Ca^{2+} efflux was inhibited by bongkrekic acid but not by atractyloside or by 300 nmol exogenous ATP. Fig. 3 shows that Ca^{2+} efflux induced by 0.1 mM dinitrophenol is totally inhibited by concentrations of bongkrekic acid of 44 μM.

Since exogenous ATP does not prevent dinitrophenol-activated Ca^{2+} efflux, the internal ATP pool size may be important to stabilize the calcium phosphate complex inside the mitochondria. The endogenous mitochondrial ATP content was thus examined in relation to Ca^{2+} efflux. Table II shows that mitochondria contain 5.56 nmol ATP after the Ca^{2+} accumulation was completed. The addition of dinitrophenol immediately caused the hydrolysis of ATP and the efflux of Ca^{2+}. Neither bongkrekic acid nor atractyloside inhibited this dinitrophenol-induced ATP hydrolysis, yet bongkrekic acid inhibited Ca^{2+} efflux at the same concentration.

The efflux of mitochondrial Ca^{2+} was also studied in the presence of ruthenium

TABLE I

EFFECT OF BONGKREKIC ACID AND ATRACTYLOSIDE ON RESPIRATION LINKED Ca^{2+} UPTAKE AND ON DINITROPHENOL-INDUCED Ca^{2+} EFFLUX

Incubation system is the same as described in Fig. 1. Glutamate (2.5 mM), potassium phosphate (1 mM), mitochondria (3.4–4.0 mg protein), and $^{45}CaCl_2$ (400 nmol) were added to the incubation mixture at 30 °C as indicated in Fig. 1. Bongkrekic acid (44 μM), atractyloside (110 μM), ATP (300 nmol) or ATP plus oligomycin (4 μg) were added before the addition of Ca^{2+} if they were used. $^{45}Ca^{2+}$ uptake by mitochondria was measured 30 s after Ca^{2+} was added. In Ca^{2+} efflux studies, dinitrophenol (0.2 mM) was added after Ca^{2+} uptake was complete (30 s after Ca^{2+} was added) and the reaction was terminated one minute after the addition of dinitrophenol by filtering the reaction mixture through a millipore filter as described in Methods.

Inhibitor	N	Ca^{2+} uptake by mitochondria (nmol/mg protein)	Ca^{2+} remaining in mitochondria 1 min after dinitrophenol was added (nmol/mg)*
None	7	105±15	25±6
Bongkrekic acid	7	100±13	77±18
Atractyloside	7	101±10	24±7
ATP	7	102±8	19±10
ATP+ oligomycin	7	101±10	19±7

* Data expressed as mean values ± 1 standard error of mean.

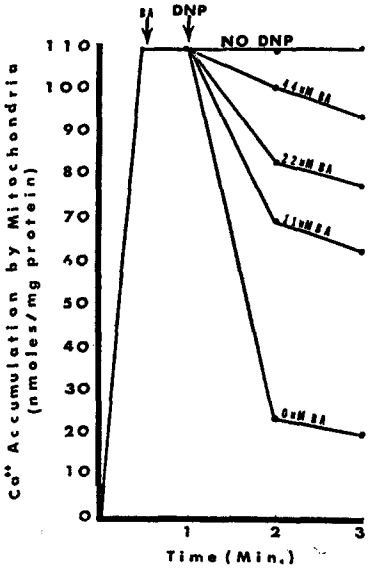

Fig. 3. Effect of bongkrekic acid on dinitrophenol-induced Ca^{2+} efflux. Experimental conditions are as shown in Figure 1. Mitochondria (3.4–4.0 mg), glutamate (2.5 mM), P_i (1 mM) were used in each experiment. Indicated amounts of BA (bongkrekic acid) were added before the addition of dinitrophenol. The Ca^{2+} retained by mitochondria was measured 1 and 2 min after the addition of dinitrophenol. Incubation medium contains indicated amounts of bongkrekic acid and 0.1 mM dinitrophenol.

TABLE II

MITOCHONDRIAL ATP CONTENTS

Experimental conditions were the same as described in Fig. 1. Glutamate, malate and pyruvate (2.5 mM each), potassium phosphate (1 mM), mitochondria (2.9–3.0 mg protein) were incubated at 30 °C. Ca^{2+} (400 nmol) was added to initiate the reaction. Bongkrekic acid (44 μM), or atractyloside (110 μM) was added before the addition of Ca^{2+} when they were used. Dinitrophenol (0.1 mM) was added to the reaction mixture after Ca^{2+} uptake was complete. The entire reaction mixture was withdrawn 1 minute after dinitrophenol was added, and ATP content was determined as described in Methods.

Experimental condition	Amount of ATP in mitochondria (nmol/mg protein)*
Control (−dinitrophenol)	5.56±0.23 (3)
dinitrophenol	1.05±0.1 (3)
BA+dinitrophenol	1.44±0.47 (3)
ATR+dinitrophenol	1.39±0.16 (3)

* Data expressed as mean values ± standard error. Numbers in parentheses represent the number of determinations.

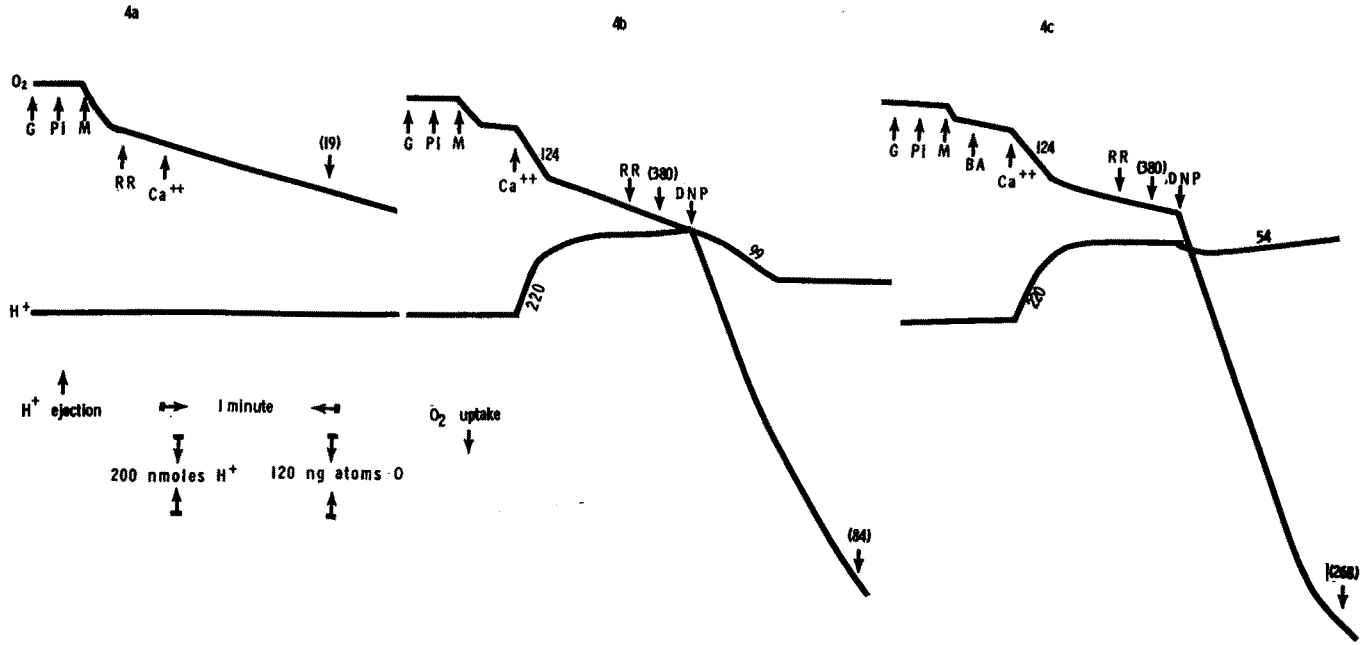

Fig. 4. Effect of ruthenium red on respiration linked Ca^{2+} uptake, H^+ ejection and on dinitrophenol-induced Ca^{2+} efflux. Experimental conditions were the same as in Fig. 1. RR (ruthenium red, 3 nmol) was added as indicated. Numbers included in parentheses indicate the amount of Ca^{2+} accumulated by mitochondria after the addition of 400 nmol $^{45}Ca^{2+}$ and/or BA (bongkrekic acid) and DNP (dinitrophenol). The number adjacent to oxygen tracing and H^+ tracing represent the rate of oxygen consumption and H^+ movement respectively.

red. Fig. 4 shows that in the absence of ruthenium red 380 nmol Ca^{2+} was accumulated 1 min after 400 nmol Ca^{2+} were added. The Ca^{2+} uptake, H^+ ejection and Ca^{2+}-induced oxygen consumption were totally inhibited when 3 nmol ruthenium red was included in the reaction mixture (4a). Conversely, dinitrophenol-induced respiration, H^+ uptake and Ca^{2+} efflux were not inhibited by the addition of ruthenium red after the initial uptake (4b). One minute after dinitrophenol was added, mitochondrial Ca^{2+} was decreased from 380 nmol to 84 nmol. (Mitochondria retained 380 nmol Ca^{2+} in the absence of dinitrophenol in the same time period). However, dinitrophenol-induced H^+ uptake and Ca^{2+} efflux were inhibited when bongkrekic acid was included in the reaction system (4c). 268 nmol Ca^{2+} still remains in the mitochondria in the presence of bongkrekic acid after dinitrophenol was added. These results indicate that Ca^{2+} efflux is inhibited by bongkrekic acid but not by ruthenium red.

Ionophore A23187 is known to cause Ca^{2+} efflux from mitochondria by acting as an artificial carrier for Ca^{2+}. The effect of bongkrekic acid and atractyloside on A23187-induced Ca^{2+} efflux can be seen in Fig. 5. Mitochondrial Ca^{2+} also decreased from 388 nmol to 84 nmol one minute after A23187 was added (5a). Neither bongkrekic acid (Fig. 5b) nor atractyloside (data not shown) affected Ca^{2+} efflux and H^+ uptake induced by A23187. These results suggest that there exists a component of the mitochondrial membrane involved in Ca^{2+} efflux that is sensitive to bongkrekic acid, and that ionophores can bypass its effect.

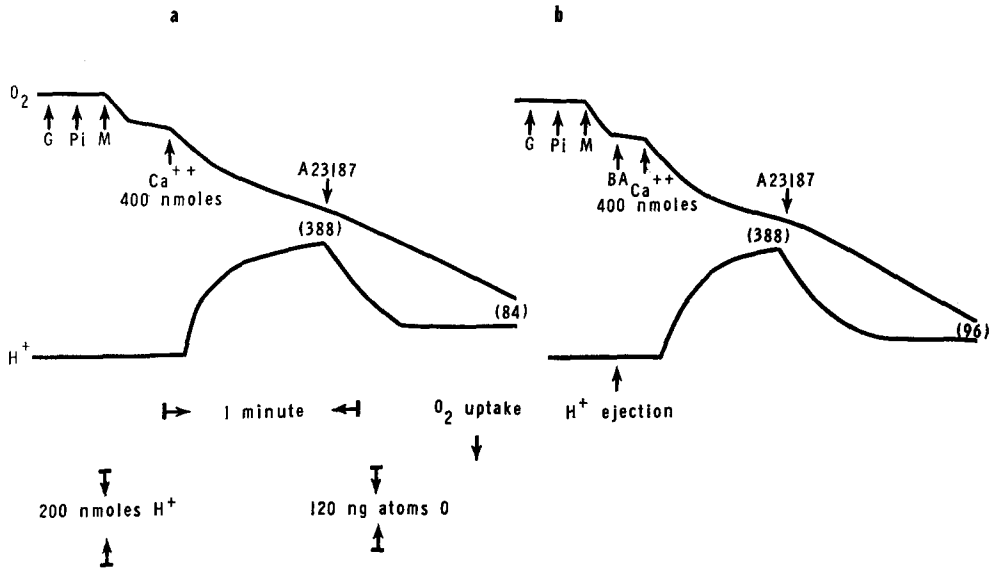

Fig. 5. Effect of bongkrekic acid on A23187-induced Ca^{2+} efflux and H^+ uptake. Experimental conditions were the same as in Fig. 1. G (sodium glutamate 2.5 mM), P_i (1 mM), M (mitochondria 4.07 mg protein) were used in each experiment. $^{45}Ca^{2+}$ (400 nmol) was added to initiate respiration and H^+ ejection. Numbers included in parentheses indicate the amount of Ca^{2+} accumulated by mitochondria after the addition of $^{45}Ca^{2+}$ and/or A23187. Bongkrekic acid (44 μM) and A23187 (1 μg) were added to the reaction mixture as indicated.

DISCUSSION

This study demonstrates that bongkrekic acid inhibits the efflux of Ca^{2+} which has been accumulated in the presence of inorganic phosphate (P_i). Bongkrekic acid, however, has no effect on respiration-linked Ca^{2+} uptake and H^+ ejection in the presence of P_i, and therefore must primarily influence the efflux process. These results suggest that uptake and efflux of Ca^{2+} occur by separate pathways. This conclusion is supported by the finding that ruthenium red inhibits Ca^{2+} uptake in the presence of P_i but does not inhibit dinitrophenol-activated Ca^{2+} efflux under the same conditions.

Recently, it was reported that high P_i-activated [5, 9] or low P_i plus phosphoenolpyruvate-activated Ca^{2+} [9–12] efflux from mitochondria was inhibited by the addition of ATP [5, 9], bongkrekic acid [9, 10] or atractyloside [11, 12]. Since bongkrekic acid and atractyloside are known to inhibit adenine nucleotide and phosphoenolpyruvate transport across the mitochondrial membrane [11–15], the participation of the adenine nucleotide carrier in Ca^{2+} efflux has been suggested [12]. Thus, the inhibition of phosphoenolpyruvate influx or the inhibition of internal mitochondrial ATP efflux can prevent the efflux of accumulated Ca^{2+} in the presence of P_i. In contrast to these results, this study shows that ATP does not inhibit Ca^{2+} efflux activated by dinitrophenol. Henderson and Lardy [22] have shown that bongkrekic acid does not inhibit dinitrophenol-stimulated ATPase activity in submitochondrial particles but does inhibit in intact mitochondria. This implies that the hydrolysis of internal mitochondrial ATP by dinitrophenol is not inhibited by bongkrekic acid. This implication is confirmed by the results demonstrated in this study. In addition, oligomycin inhibits mitochondrial ATP hydrolysis but does not prevent Ca^{2+} efflux activated by dinitrophenol [1, 5]. These observations together indicate that mitochondrial ATP is not a modulator for dinitrophenol-activated Ca^{2+} efflux and that bongkrekic acid must thus act directly on the Ca^{2+} efflux carrier. However, it remains unclear whether bongkrekic acid inhibits a specific Ca^{2+} carrier as well as adenine nucleotide translocase, or the binding of bongkrekic acid to the latter indirectly influences the functional state of the Ca^{2+} efflux carrier. The results presented herein demonstrate that atractyloside which also inhibit adenine nucleotide translocase does not inhibit dinitrophenol-induced Ca^{2+} efflux. This finding tends to support the rationalization that bongkrekic acid could be an inhibitor of a specific Ca^{2+} efflux carrier. Furthermore, Ionophore A23187 also induces Ca^{2+} efflux but this efflux is not inhibited by bongkrekic acid. The bongkrekic acid sensitive pathway bypassed by A23187 suggests the presence of a Ca^{2+} efflux carrier on the mitochondrial membrane.

If the efflux of H^+ accompanied by Ca^{2+} uptake in Fig. 1a indicates the establishment of a pH gradient across the mitochondrial membrane, then the subsequent reuptake of H^+ on the addition of dinitrophenol would be expected if dinitrophenol is only functioning as a proton carrier. However, bongkrekic acid prevents the reuptake of protons on addition of dinitrophenol while the uncoupling effect on oxygen consumption is unaffected as can be seen in Fig. 1b. Such a mechanism implies that there is an effect of dinitrophenol on energy-linked Ca^{2+}-H^+ exchange process in the mitochondrial membrane that is separate from its action as a proton carrier.

Several physiological functions have been proposed for the mitochondrial Ca^{2+} transport process. Mitochondria may play a role in skeletal calcification [23, 24], in cardiac muscle contraction and relaxation where sarcoplasmic reticulum is poorly

developed [25, 26], and in regulating cellular Ca^{2+} concentration [27, 28]. However, these physiological roles of mitochondria are not proven at the present time, although the ability of mitochondria to relax myofibrils has been demonstrated [29]. We have previously demonstrated that phosphoenolpyruvate, an intermediate of glycolytic and gluconeogenic pathways, is able to induce Ca^{2+} efflux from rat liver mitochondria [10]. Recently, Asimakis and Sordahl have also shown that palmitoyl coenzyme A causes Ca^{2+} efflux from cardiac mitochondria [30]. These results indicate that these physiological metabolites may play an important role in regulating cytosolyic Ca^{2+} concentration by stimulating Ca^{2+} efflux from mitochondria. Although dinitrophenol-induced Ca^{2+} efflux from heart mitochondria is not an in vivo phenomenon, this study suggests that the efflux of Ca^{2+} from mitochondria is mediated by a bongkrekic acid sensitive component which is apparently not identical to Ca^{2+} influx carrier.

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