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Mitochondrial Oscillation and Activation of H^+/Cation Exchange

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Abstract

Mitochondria incubated aerobically in the presence of tetrapropylammonium and weak acids and in the presence of trace amounts of tetraphenylboron undergo a series of damped oscillations reflecting cycles of osmotic swelling and shrinkage. The matrix volume changes are consequent to transport of tetrapropylammonium catalytically stimulated by tetraphenylboron. The amplitude and frequency of the oscillations increase with the concentration of tetrapropylammonium, as required for critical rates and extents of ion influx. Addition of bovine serum albumin abolishes both the uptake of tetrapropylammonium and the oscillations. Volume oscillations are paralleled by cyclic activation and depression of the respiratory rate. Two lines of evidence suggest that the train of damped oscillations depends on the cyclic activation of an electroneutral exchange of H^+ with organic cations rather than on cyclic uncoupling. First, further increase of cation permeability due to a pulse of tetraphenylboron, after initiation of cation efflux, restores cation influx. Second, addition of Mg^{2+}, which abolishes the oscillations, has a much more marked inhibitory effect on the process of cation efflux than on cation influx. Conversely, addition of A23187, which removes membrane-bound Mg^{2+}, promotes cation efflux and thus the oscillations. It is suggested that, in the present system, stretching of the inner membrane and Mg^{2+} depletion result in activation of an electroneutral H^+/organic cation exchange, and that cyclic activation of this reaction results in damped oscillations.

Key Words: Mitochondrial volume oscillations; electroneutral H^+/TPA^+ exchange.

Introduction

Ion fluxes, across the inner membrane of mammalian mitochondria may occur either via endogenous–exogenous carriers or via partition in the lipid phase

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(Azzone and Massari, 1973). After the discovery of the ionophores it was reported that mitochondria may undergo oscillations of the matrix volume, reflecting cycles of influx and efflux of cations (Graven *et al.*, 1966; Hofer and Pressman, 1966; Chance and Yoshioka, 1966; Packer *et al.*, 1966; Utsumi and Packer, 1966; Gooch and Packer, 1974). The cycles of influx and efflux were paralleled by cycles of stimulation and depression of the respiratory rate, and by cyclic changes of the oxidation–reduction state of the endogenous pyridine nucleotides (Utsumi and Packer, 1966). The suggestion was advanced that, consequent to cation uptake, mitochondria undergo a cyclic uncoupling whereby the process of efflux and influx reflects a decrease and an increase of the degree of coupling, respectively (Utsumi and Packer, 1966). However, an efflux of cations from the matrix may occur through either the same or a different pathway from that used for influx. The efflux is consequent to a depression of the thermodynamic force driving cation influx in the former case and driven by a thermodynamic force different from that for influx in the latter case. The distinction between the two mechanisms may be exemplified by the process of Ca^{2+} efflux induced either by uncouplers or by the exogenous ionophore A23187, where the former occurs through a reversal of the endogenous uniporter via a depression of $\Delta\tilde{\mu}_{Ca}$ and the latter through the independent A23187 pathway driven by the H^+ and Ca^{2+} chemical gradients (Reed and Lardy, 1972). In the case of the above-mentioned oscillations it is not clear whether the cyclic cation effluxes occurred through a reversal of the influx or rather through an independent pathway.

The present paper reports a new and well-reproducible oscillatory system in mitochondria. Damped oscillations have been obtained with mitochondria catalyzing the aerobic uptake of organic cations (Bakeeva *et al.*, 1970; Liberman and Skulachev, 1970; Azzone *et al.*, 1976b; 1978b) in the presence of an excess of weak acids and of trace amounts of tetraphenylboron and cytochrome *c*. Two lines of evidence have been obtained favoring the view that, in the present system, the oscillations are due to cyclic activation of a H^+/organic cation exchange rather than to cyclic uncoupling (Azzone *et al.*, 1978b).

Natural carriers catalyzing the electroneutral exchange of H^+ with Na^+ and K^+ have been proposed by Mitchell (1966). However, the rate of operation of these carriers is negligible in the native membrane and becomes appreciable after some modifications of the membrane, among which are membrane stretching or Mg^{2+} depletion (Douglas and Cockrell, 1974; Brierley, 1976; Chavez *et al.*, 1977; Wehrle *et al.*, 1976; Duszynski and Wojtczak, 1977; Azzone *et al.*, 1978a,b; Garlid, 1978, 1979, 1980; Dordick *et al.*, 1980; Shi *et al.*, 1980). Also the carriers catalyzing the exchange of organic cations appear to operate at a negligible rate in native mitochondria and are markedly activated after membrane stretching or Mg^{2+} depletion. The possibility

therefore exists that the molecular nature of the carriers catalyzing the exchange of H^+ with inorganic and organic cations is similar.

Materials and Methods

Rat liver mitochondria were prepared in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EGTA[3] according to Massari *et al.* (1972a). The last washing was carried out in an EGTA-free medium, and mitochondrial protein was assayed with the biuret method, using BSA as a standard.

Since neither isotopically labeled tetrapropylammonium ion (TPA^+) nor a TPA^+-selective electrode were available, TPA^+ transport across the inner mitochondrial membrane was followed indirectly from the changes in light scattering of the mitochondrial suspension at 540 nm. Previous studies by Massari *et al.* (1972b,c) have shown that these absorbance changes are quantitatively related to mitochondrial volume changes accompanying ion transport across the inner membrane. In the present study, however, such a quantitative correlation is seriously hampered by the establishment of a train of damped oscillations. These oscillations begin already at TPA^+ concentrations higher than 1 mM and manifest themselves as a rapid absorbance increase (cf Fig. 3). In the present study, therefore, no attempts were made to relate quantitatively swelling to TPA^+ uptake, and the changes in light scattering are taken as a qualitative reflection of TPA^+ transport. Hence, although the terms "absorbance decrease" and "TPA^+ uptake" have been alternatively used, this limitation must be borne in mind. In control experiments it was, however, ascertained by gravimetric and isotopic measurements that the uptake of TPA^+ was accompanied by an increase of matrix water as predicted by an osmotic swelling (Massari *et al.*, 1972b,c). The optical measurements were performed with an Aminco DW2A dual-wavelength spectrophotometer equipped with magnetic stirring and thermostatic control. The instrument was set in the split-beam mode, with the reference cuvette containing buffer.

H^+ fluxes were monitored with the Aminco DW2A spectrophotometer, following the absorbance changes of phenol red at the wavelength pair 579 minus 595 nm.

Oxygen uptake was measured with a Clark oxygen electrode (Yellow Springs Instruments, Ohio) in a water-jacket thermostated vessel equipped with a magnetic stirrer. The incubation medium is specified in the figure

[3]Abbreviations used: EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*'-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; TPA^+, tetrapropylammonium ion; TPB^-, tetraphenylboron ion; BSA, bovine serum albumin.

legends. Horse heart cytochrome *c* was purchased from Boehringer (Mannheim). Tetrapropylammonium and tetraphenylboron were purchased from Fluka (Buchs). All chemicals were of analytical grade.

Results

In Fig. 1 mitochondria were incubated in the presence of 2 mM TPA^+, in a medium containing 30 mM acetate. Addition of 2 μM TPB^- initiated a phase of rapid decrease of absorbance, corresponding to uptake of TPA acetate (upper trace). After about 90 sec the ion influx levelled off and was followed by a train of damped oscillations. The oscillations had a period of about 90 sec and were roughly symmetrical as for the duration of the phases of efflux and influx. The first cycle was followed by a second, the magnitude of which was about half, and then by a third cycle of still smaller magnitude. The number and dimension of the cycles were slightly variable from preparation to preparation of mitochondria. Figure 1 also shows that the cation influx was accompanied by a H^+ extrusion ending simultaneously to the cation influx (lower trace). The experiment of Fig. 1 indicates that the cycles of cation

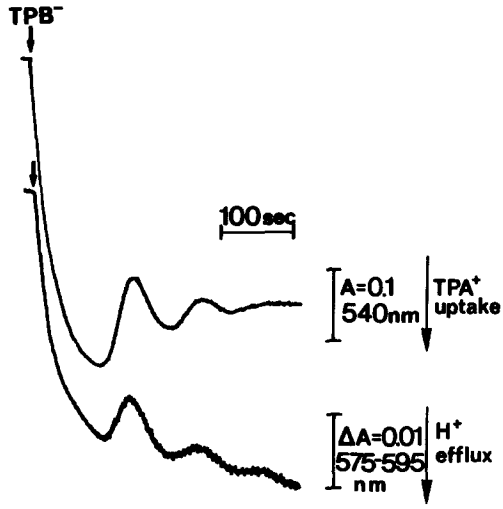

Fig. 1. Correlation between volume changes and polarity of H^+ fluxes during oscillations. The incubation medium contained 0.17 M sucrose, 30 mM choline chloride, 0.5 mM Tris-HCl, pH 7.4, 30 mM Na-acetate, 2 mM Na-succinate, 0.1 mM EGTA-Tris, 5 μM cytochrome *c*, 2 μM rotenone, 50 μM phenol red, and 2.5 mM TPA^+. 1 mg/ml mitochondria, final volume 2 ml, 25°C, Where indicated, 2 μM TPB^- was added.

influx and efflux are paralleled by H^+ fluxes in the opposite direction. Due to the parallel movement of acetic acid, the detected pH changes reflect only a minuscule fraction of the total H^+ fluxes. From the rate of oxygen uptake, which is related with the rate of H^+ extrusion via the stoichiometry of the H^+ pump, it appears that a major part of the H^+ extrusion is masked by the H^+ influx mediated by the transport of acetic acid.

Figure 2 shows the dependence of the oscillations on exogenous cytochrome *c*. When mitochondria were incubated in the absence of cytochrome *c*, the influx phase was followed only by a very slight efflux and no oscillations ensued. Addition of 5 μM cytochrome *c* at this point resulted in immediate and marked enhancement of the influx, followed by the usual train of oscillations. The cytochrome *c* effect is due to the fact that mitochondrial swelling in salt-containing media causes an increase of intermembrane cytochrome *c*. This in turn decreases the respiratory rate and then the activity of the redox H^+ pump (Bernardi and Azzone, 1981). For this reason 5 μM cytochrome *c* was used in all experiments.

Figure 3 shows that the oscillations were strictly dependent on the concentration of TPA^+. Increases of TPA^+ from 0.5 to 2.5 mM resulted in increase of both amplitude and frequency of the oscillations. The experiment indicates that a critical concentration of TPA^+, providing a critical rate of ion

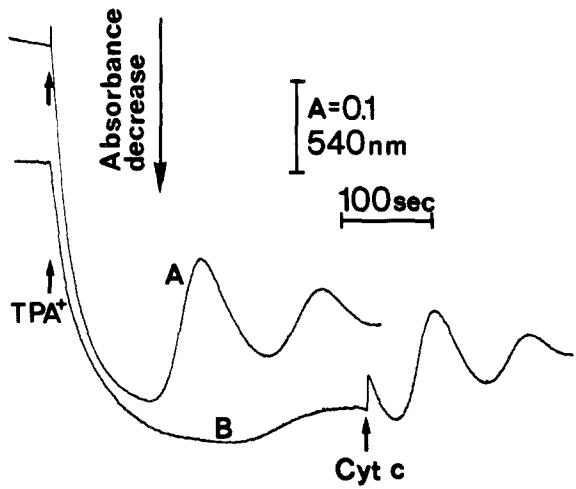

Fig. 2. Dependence of volume oscillations on exogenous cytochrome *c*. The incubation medium contained 0.17 M sucrose, 30 mM choline chloride, 15 mM Tris-Mops, pH 7.4, 30 mM acetate-Tris, 0.1 mM EGTA-Tris, 2 mM succinate-Tris, 2 μM rotenone, and 2 μM TPB^-. In (A) 5 μM cytochrome *c*. 1 mg/ml mitochondria, final volume 2 ml, 25°C. Where indicated, 2.5 mM TPA^+ and 5 μM cytochrome *c* (cyt. *c*) were added.

influx and/or a critical amount of ion uptake, is required for initiation of the oscillations.

Figure 4 shows that a catalytic amount of TPB^- was necessary to obtain a sufficient rate of TPA^+ uptake. TPB^- is known to increase the electric conductance of phospholipid membranes (Grinius *et al.*, 1970), and has been successfully employed to increase the membrane permeability to lipophilic cations in submitochondrial particles (Grinius *et al.*, 1970), in mitochondria (Bakeeva *et al.*, 1970; Azzone *et al.*, 1976b; 1978b), as well as in vesicles from *Escherichia coli* (Hirata *et al.*, 1973; Lombardi *et al.*, 1974; Altendorf *et al.*, 1975; Schuldiner and Kaback, 1975). Although the mechanism of potentiation of cation permeability by TPB^- is not completely understood (see Bakeeva *et al.*, 1970), the concept is widely accepted that TPB^- acts as a catalyst in cation transport, in analogy with the effect of valinomycin on K^+ and Rb^+ transport (Grinius *et al.*, 1970; Bakeeva *et al.*, 1970; Liberman and Skulachev, 1970; Hirata *et al.*, 1973; Lombardi *et al.*, 1974; Altendorf *et al.*, 1975; Schuldiner and Kaback, 1975; Azzone *et al.*, 1976a,b, 1978b).

In fact in the presence of 2.5 mM TPA^+ but in the absence of TPB^-, the rate of ion influx was very slow and no oscillation ensued (Fig. 4A). On the other hand, rapid ion influx and oscillation occurred in the presence of 2 μM TPB^- (Fig. 4C). Addition of BSA resulted in a marked decrease of the rate of TPA^+ influx and abolition of the oscillations (Fig. 4B), suggesting that most of TPB^- is bound by BSA.

This interpretation is supported by the experiments of Fig. 5, showing the

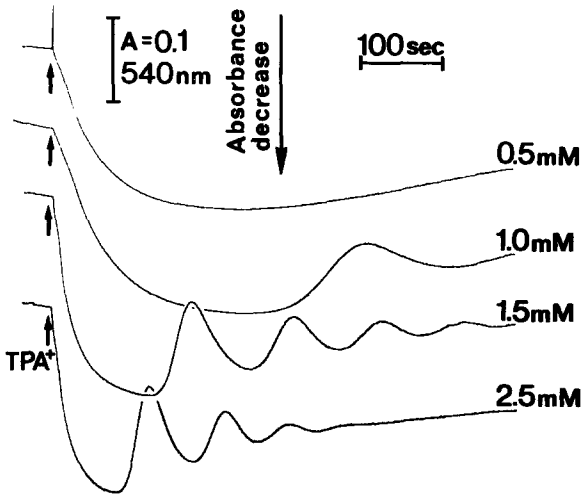

Fig. 3. Dependence of volume oscillations on TPA^+ concentration. Experimental conditions as in Fig. 2A with 0.5 mg/ml mitochondria. TPA^+ addition (arrow) was as indicated on each trace.

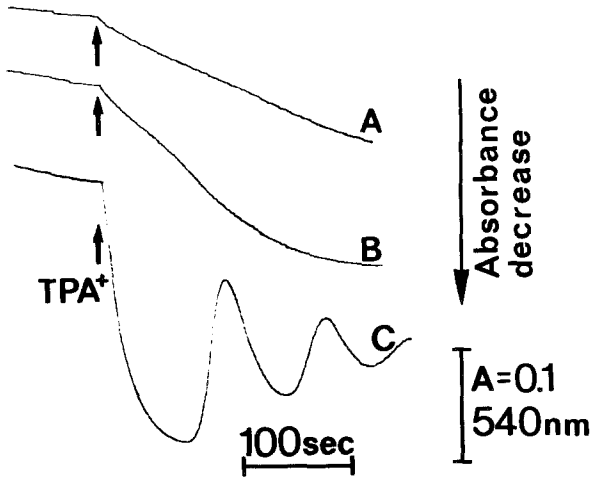

Fig. 4. Effect of BSA and TPB^- on volume oscillations. The incubation medium was as in Fig. 2A, but in trace (A) TPB^- was omitted, whereas in trace (B) 1 mg/ml of BSA was added. 0.5 mg/ml mitochondria, final volume 2 ml, 25°C. Where indicated, 2.5 mM TPA^+ was added.

effect of BSA on TPA^+-stimulated respiration. It is seen that TPA^+ addition did not stimulate the respiration in the absence of TPB^- (dashed upper trace) or in the presence of TPB^- plus BSA (dashed lower trace). Furthermore, addition of BSA after TPA^+ in TPB^--treated mitochondria brought the respiration almost to the basal level preceding TPA^+ addition (solid lower trace). This indicates that TPA^+-stimulated respiration is due to a TPA^+ recycling and not to a TPA^+-induced H^+ recycling.

Figure 6 shows a titration of the inhibitory effect of BSA on either the initial rate of TPA^+ transport or on the steady-state rate of respiration during cycling (cf. Figs. 4–5). Both phenomena were markedly BSA-sensitive although 50% inhibition of the steady-state respiratory rate occurred at slightly higher BSA concentrations. The titrations confirm that the high steady-state respiratory rate involves a cycling of TPA^+ and H^+ and not only of H^+. However, the slight discrepancy in the titration curves between sensitivity of TPA^+ transport and steady-state respiratory rate to BSA suggests that in the latter some H^+ cycling is also involved.

TPA^+ uptake is an electrophoretic process, driven by the transmembrane electrical potential, $\Delta\psi$ (Bakeeva *et al.*, 1970; Liberman and Skulachev, 1970; Azzone *et al.*, 1976b). The cyclic process of TPA^+ efflux, which is responsible for the trains of volume oscillations, could therefore be due either to a cyclic uncoupling, i.e., a cyclic decrease of $\Delta\psi$, as suggested by Utsumi and Packer (1966), or to a cyclic activation of an electroneutral H^+/TPA^+ exchange, with minor changes of $\Delta\psi$ under conditions where the activity of the redox H^+

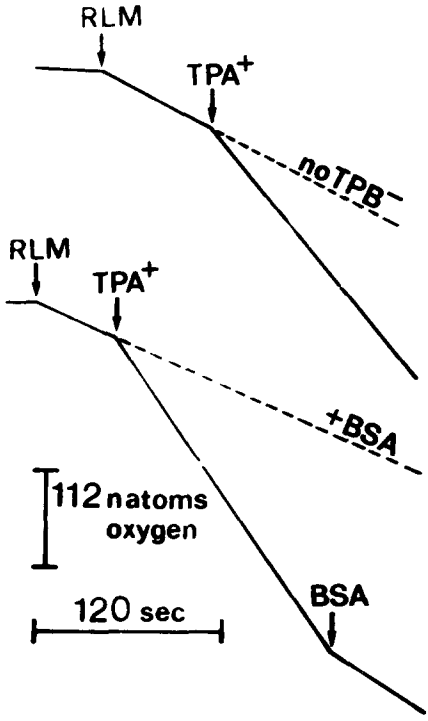

Fig. 5. TPA^+-stimulation of respiration: inhibition by BSA. Incubation medium was as in Fig. 2A. Final volume 2 ml, 25°C. Where indicated, 2 mg of mitochondria (RLM), 2.5 mM TPA^+, and 2 mg bovine serum albumin (BSA) were added. In the dashed line of the upper trace TPB^- was omitted, whereas in the dashed line of the lower trace 2 mg of BSA was added.

pump remains in excess. The experiments reported below were designed to discriminate between these two possibilities.

Figure 7 shows the quantitative dependence on the apparent rate of TPA^+ influx on TPB^- concentration. This titration allows one to assess the role of kinetic factors in determining the cyclic phases of TPA^+ efflux and, therefore, the oscillations. If the phase of TPA^+ efflux reflects a decrease of $\Delta\tilde{\mu}_{TPA}$ due to a decrease of $\Delta\psi$, increasing amounts of TPB^- should increase the rate of TPA^+ efflux along the TPA^+ electrochemical gradient. If, on the other hand, TPA^+ efflux reflects the activation of an endogenous H^+/TPA^+ exchanger, TPA^+ efflux should be largely TPB^--independent, and increasing amounts of TPB^- should decrease or even reverse the phase of TPA^+ efflux. The upper trace of Fig. 8 shows the usual train of oscillations in the presence of 2 μM TPB^-. Addition of a further 6 μM TPB^- pulse, which further increases the TPA^+ permeability and, therefore, the rate of TPA^+ transport (Fig. 7), caused an immediate TPA^+ reuptake (lower trace). This finding confirms that the pathway for TPA^+ efflux is largely TPB^--independent, and that the actual TPA^+ distribution depends on the relative rates of TPA^+ influx and efflux. It is noteworthy that also the rate of active shrinkage of mitochondria swollen in TPA-nitrate is independent of the TPB^- concentration

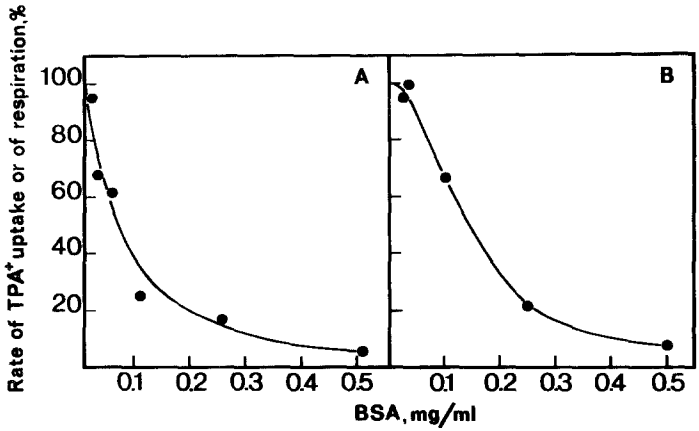

Fig. 6. Inhibitory effect of BSA on TPA^+ uptake and on TPA^+-stimulated respiration. The incubation medium was as in Fig. 2A. In (A) the rate of TPA^+ uptake was followed as the decrease of absorbance at 540 nm following addition of 2.5 mM TPA^+ in the presence of the BSA concentration indicated on the abscissa. In (B), the rate of respiration was followed with the Clark oxygen electrode. The indicated BSA concentration was added 1 min after the addition of 2.5 mM TPA^+. Mitochondrial protein was in both cases 2 mg in a final volume of 2 ml, at 25°C.

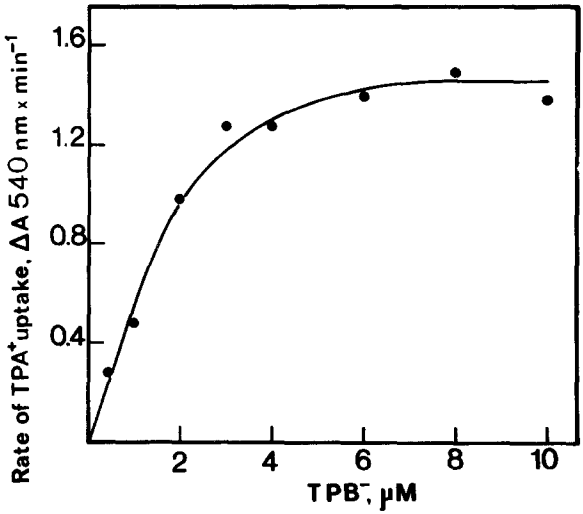

Fig. 7. Relationship between TPB^- concentration and rate of TPA^+ uptake. The incubation medium was as in Fig. 2A, with TPB^- as indicated on the abscissa. 1 mg/ml of mitochondria, final volume 2 ml, 25°C. After 1 min of preincubation, 2.5 mM TPA^+ was added. Values on the ordinate refer to the initial rate of absorbance decrease following TPA^+ addition (chart speed 5 sec/in.).

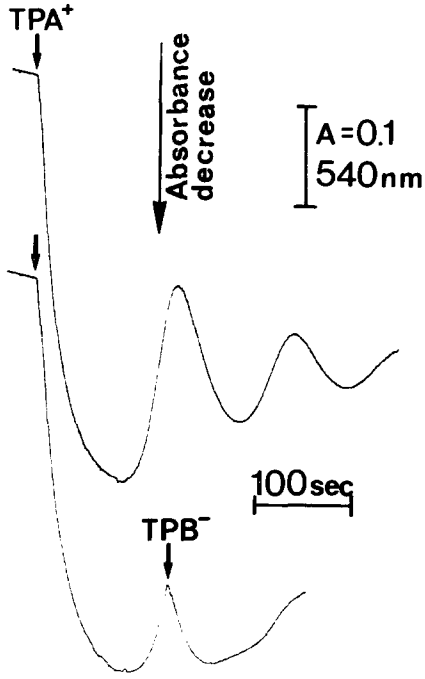

Fig. 8. Asymmetric effect of TPB^- on TPA^+ influx and efflux. Experimental conditions as in Fig. 2A. Where indicated, 2 mM TPA^+ and 6 μM TPB^- were added.

(Azzone *et al.*, 1976b), which is in accord with an asymmetric effect of TPB^- on the processes of TPA^+ influx and efflux, respectively.

Figure 9 analyzes the effect of Mg^{2+} on the oscillatory cycles. Figure 9A shows the usual train of oscillations in the absence of Mg^{2+}. Addition of 3 mM Mg^{2+} almost abolished the oscillations while leaving the rate and extent of TPA^+ uptake practically unaffected (Fig. 9B). Furthermore, addition of 3 mM Mg^{2+} during the phase of TPA^+ efflux led to an immediate TPA^+ reuptake (Fig. 9C), thus supporting the concept that the pathway for TPA^+ efflux is by far more sensitive to Mg^{2+} than the electrical pathway for TPA^+ influx. This is confirmed by the experiments of Fig. 10, where the apparent rate of the first cycle of TPA^+ efflux is plotted against added Mg^{2+}, at two TPA^+ concentrations. The Dixon plot yields an apparent K_i of 1.2 mM Mg^{2+}. Since the electrical pathway for TPA^+ influx is practically unaffected by the same range of Mg^{2+} concentration (4 and 13% inhibition at 1.2 and 3 mM Mg^{2+}, respectively) the experiments further support the concept that the pathway for TPA^+ efflux is different in nature from that for TPA^+ uptake, where only the former is inhibited by Mg^{2+}.

Further support for the view that the mitochondrial oscillations are under Mg^{2+} control, due to an effect of Mg^{2+} on the TPA^+ efflux pathway, is provided by the experiments of Fig. 11. These experiments indicate that the process of TPA^+ efflux is markedly stimulated by the addition of A23187, an

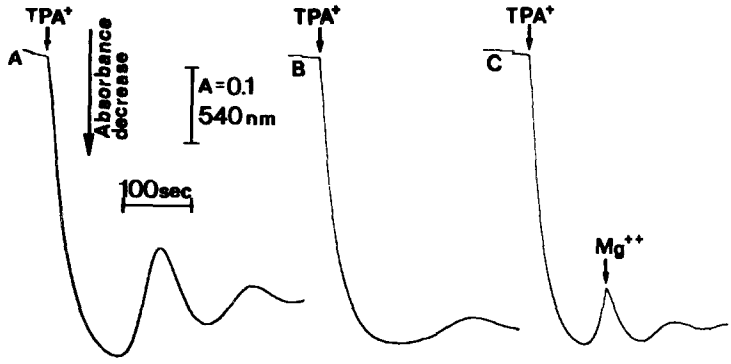

Fig. 9. Effect of $MgCl_2$ on TPA^+ efflux and on volume oscillations. The incubation medium was as in Fig. 2A. In (B) 3 mM $MgCl_2$. 1 mg/ml of mitochondria, final volume 2 ml, 25°C. Where indicated, 3.5 mM TPA^+ and 3 mM $MgCl_2$ were added.

ionophore which is known to cause depletion of membrane-bound Mg^{2+}. Addition of A23187 at the end of the swelling phase initiated a very rapid phase of TPA^+ efflux (trace B). Furthermore, in A23187-supplemented mitochondria, TPA^+ efflux ensued at a much lower degree of swelling, while

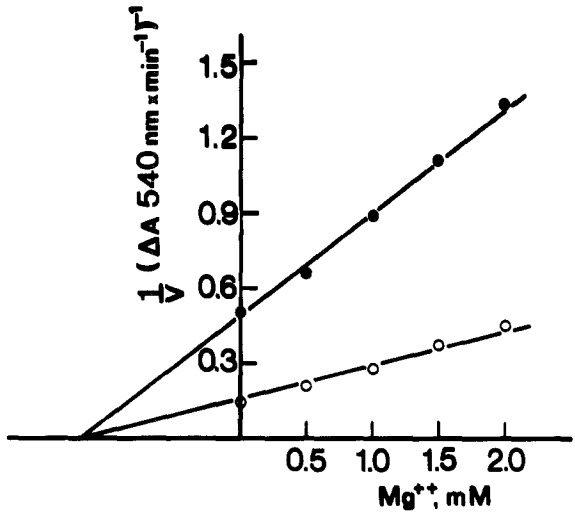

Fig. 10. Kinetic analysis of Mg^{2+} inhibition of TPA^+ efflux. The incubation medium was as in Fig. 2A. 1 mg/ml of mitochondria, final volume 2 ml, 25°C. The experiment was started by the addition of 2 mM (●) or 3 mM (○) TPA^+. The indicated amounts of Mg^{2+} were added 10 sec before the mitochondria reached a maximum degree of swelling, and values on the ordinate refer to the rate of absorbance increase corresponding to the first cycle of TPA^+ efflux.

the frequency of the oscillations was markedly increased (trace A). These effects are presumably also due to A23187-induced activation of the TPA^+ efflux pathway.

Figure 12 shows the relationship between respiratory rate and oscillation cycle. It is seen that the respiratory rate tended to accelerate during the efflux phase and then to be again slightly depressed during the subsequent influx phase. Although the extents of acceleration and depression were of the order of 50%, the results of Fig. 11 clearly indicate a correlation between stimulation of the respiratory rate and cation efflux. This is not different from that observed by Utsumi and Packer (1966). The acceleration of the respiratory rate during the phase of cation efflux was accompanied by a shift of absorbance of the $\Delta\psi$ probe safranine (Åkerman and Wikström, 1975; Zanotti and Azzone, 1980) in a direction indicating $\Delta\psi$ depression (not shown). This is not unexpected in view of the fact that, whatever the mechanism of the oscillation, the phase of cation efflux corresponds to an increased respiratory rate and therefore to an increased energy drain.

Discussion

When mitochondria are incubated aerobically in the presence of a large excess of weak acids, a train of damped oscillations may be established, linked

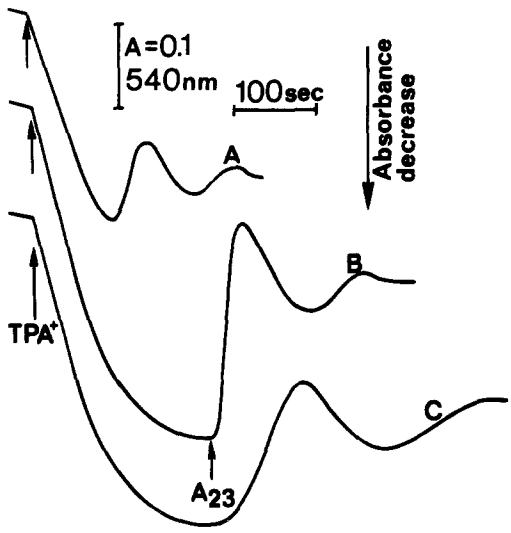

Fig. 11. Effect of A23187 on volume oscillations. Experimental conditions as in Fig. 2A. 1 mg/ml of mitochondria, final volume 2 ml, 25°C. In trace (A) 0.2 μg of A23187. Where indicated, 2 mM TPA^+ and 0.2 μg of A23187 (A 23) were added.

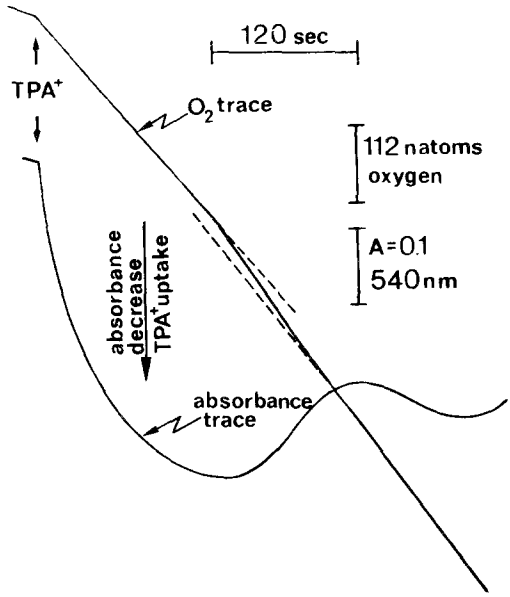

Fig. 12. Phase relationship between volume and respiratory oscillations. The incubation medium was as in Fig. 2A. 1 mg/ml mitochondria, final volume 2 ml, 25°C. Where indicated, 2.5 mM TPA^+ was added.

to the cyclic transport of ions in and out of the matrix (Graven *et al.*, 1966; Hofer and Pressman, 1966; Chance and Yoshioka, 1966; Packer *et al.*, 1966; Utsumi and Packer, 1966). In the present system, initiation of the oscillation depends on critical concentrations of TPA^+ in the presence of cytochrome *c* and of trace amounts of TPB^-. The cytochrome *c* requirement is due to the fact that mitochondrial swelling in salt-containing media is accompanied by decreased binding of cytochrome *c* (Jacobs and Sanadi, 1960; Bernardi and Azzone, 1981). This leads to depression of the rate of e^- transfer and of H^+ pumping, which are restored by the addition of exogenous cytochrome *c* (Jacobs and Sanadi, 1960; Bernardi and Azzone, 1980). The TPB^- requirement indicates that the oscillations require critical rates and/or extent of cation influx, where the rate of cation influx depends on the TPB^--induced increase of cation permeability (Grinius *et al.*, 1970; Bakeeva *et al.*, 1970; Hirata *et al.*, 1973; Lombardi *et al.*, 1974; Altendorf *et al.*, 1975; Schuldiner and Kaback, 1975; Azzone *et al.*, 1976b, 1978b).

The fact that respiring mitochondria undergo a series of damped oscillations is compatible with the view that, consequent to cation influx, modifications are induced in the membrane which enhance cation efflux. Cation efflux is then followed by opposite modifications, leading to novel cation influx. The cycles may be repeated three to four times until the system

reaches a stationary state characterized by a high rate of respiration, steady cation accumulation in the matrix, and swelling. Utsumi and Packer (1966) have already shown that cation influx and efflux are almost synchronous with cyclic changes of the respiratory rate and of the redox state of the mitochondrial pyridine nucleotide, and have suggested that the oscillations are due to some sort of cyclic uncoupling. According to this view, the oscillations can be explained as follows. The phase of cation influx with the accompanying matrix swelling and membrane stretching would lead to increased H^+ leakage and uncoupling. The consequent depression of $\Delta\psi$ would cause depression of $\Delta\tilde{\mu}_{cat}$ and then cation cation efflux. Cation efflux is followed by matrix shrinkage and membrane contraction with decreased H^+ leakage and recoupling. This causes an increase of $\Delta\psi$ and of $\Delta\tilde{\mu}_{cat}$ and then cation influx. This mechanism implies that the oscillations are accompanied by cyclic oscillations of $\Delta\psi$, that both cation influx and efflux occur through the same pathway, and that the polarity of the net ion fluxes is determined only by the value of $\Delta\tilde{\mu}_{cat}$.

After the studies of the sixties, new ionophores have been discovered such as nigericin or A23187 (Reed and Lardy, 1972) which catalyse an electroneutral H^+/K^+ and H^+/Ca^{2+} exchange, respectively. Addition of these ionophores to mitochondria catalyzing an aerobic uptake of K^+ or Ca^{2+} results in net cation efflux, cation cycling, and stimulation of H^+ pumping and e^- transfer. In this case the efflux is not due to changes of the cation electrochemical gradient across the carrier catalyzing the cation influx but to the establishment of a new pathway for cation efflux driven by a different thermodynamic force. Furthermore the stimulation of the respiration is not due to H^+ cycling, as in the case of a H^+ leakage, but rather to H^+/cation cycling. This is shown by the fact that, in the case of A23187, the respiratory stimulation is abolished by Ruthenium Red or EGTA (Heaton and Nicholls, 1976; Bernardi and Pietrobon, 1982). The question now arises as to whether the cation efflux observed in the present study during the oscillation is due to cyclic activation of a H^+ leakage or of a H^+/cation exchanger. An indication favoring the H^+/cation cycling is represented by the effect of BSA, which presumably acts by reducing the amount of free TPB^-. This results in depression of the rate of TPA^+ transport and in a depression of the respiratory rate after initiation of the oscillations (Fig. 5 and 6). The effect of BSA is therefore similar to that of Ruthenium Red or EGTA on Ca^{2+} cycling-linked respiratory stimulation (Reed and Lardy, 1972; Heaton and Nicholls, 1976; Bernardi and Pietrobon, 1982).

Two evidences for an activation of H^+/cation exchange have been obtained in the present study. First, addition of a pulse of TPB^-, after initiation of cation efflux in the course of the oscillations, results in a restoration of the process of cation influx. Garlid (1980) has shown that addition of valinomycin to mitochondria catalyzing K^+ efflux under some

conditions causes an inversion of the polarity of K^+ fluxes, i.e., influx instead of efflux (Garlid, 1980). Since the effect of valinomycin is at the level of the permeability and not of the force, the experiment indicates that, before valinomycin, K^+ efflux proceeds through an independent pathway, namely a H^+/K^+ antiporter (Garlid, 1980). By using a similar argument, if TPA^+ transport occurs only through one pathway, the polarity of the ion flux would be determined only by $\Delta\tilde{\mu}_{TPA}$, and increase of TPA^+ permeability by TPB^- should not cause inversion of efflux into influx. Thus the inversion of polarity due to TPB^- (Fig. 8) indicates that: (1) the polarity of the ion flux depends on the rates of two independent processes of influx and efflux, and (2) increase of TPA^+ permeability renders TPA^+ influx predominant with respect to efflux. Second, addition of Mg^{2+}, which acts as a powerful inhibitor of the oscillations, results in a marked inhibition of the process of TPA^+ efflux, and only in a slight inhibition of the process of TPA^+ influx. The apparent K_i for the inhibitory effect of Mg^{2+} on the efflux is 1.2 mM. Conversely, divalent cation depletion induced by A23187 greatly increases the rate of the efflux over the influx pathway, resulting in rapid TPA^+ efflux and in increased frequency of the oscillations (Fig. 11). These effects of Mg^{2+} over the TPA^+ efflux pathway, and hence on the oscillations, indirectly suggest a parallelism between H^+/TPA^+ and H^+/Na^+ and H^+/K^+ exchange reactions which are also under Mg^{2+} control (Duszynski and Wojtczak, 1977; Azzone *et al.*, 1978a,b; Dordick *et al.*, 1980; Garlid, 1980).

These two lines of evidence hence favor the view that the oscillations during TPA^+ transport reflect the dynamics of a kinetic steady state involving two independent pathways for TPA^+ influx and efflux (see Chay, 1981). The two pathways for cation translocation across the inner membrane are: (1) the electrical TPA^+ diffusion, TPB^- mediated, down the TPA^+ electrochemical gradient, $\Delta\tilde{\mu}_{TPA}$, and (2) the electroneutral H^+/TPA^+ exchange down the two chemical gradients of H^+ and TPA^+, $\Delta\mu_H$ and $\Delta\mu_{TPA}$. The electrical pathway brings TPA^+ to electrochemical equilibrium while the electroneutral pathway brings TPA^+ out of electrochemical equilibrium.

The view that the oscillations depend on a dynamic steady state with two independent pathways for TPA^+ influx and efflux implies that the pathway for TPA^+ efflux, the electroneutral H^+/TPA^+ exchange, becomes cyclically activated during the oscillations. Membrane stretching and lowering of intramitochondrial $[Mg^{2+}]$ during matrix swelling may play a major role in the activation of the H^+/TPA^+ exchange, while deactivation may follow cation efflux, matrix shrinkage, and membrane contraction. The oscillation cycles then depend on the rates of the two transport pathways being out of phase, the electrical pathway being predominant during the influx and the electroneutral pathway during the efflux. This hypothesis explains the restoration of TPA^+ uptake by TPB^-, which stimulates the influx over the efflux

pathway, and the inhibitory effects over the oscillations after addition of either Mg^{2+}, which inhibits the efflux pathway, or of BSA, which inhibits the influx pathway.

As to the molecular nature of the H^+/TPA^+ exchange reaction, a comment is in order here. The H^+/Cat^+ antiporters were postulated by Mitchell (1966) as specific transport reactions for Na^+ and K^+, possessing the physiological role of extruding Na^+ and K^+ against their electrochemical gradients and thus avoiding osmotic burst of the mitochondria *in vivo*.

It appears, however, that the rate of electroneutral exchange of H^+ with K^+ or Na^+ is negligible in intact mitochondria while it is appreciable under conditions leading to modifications of the inner membrane (Azzone *et al.*, 1978a,b). Among these conditions are the decrease of membrane-bound Mg^{2+} and the membrane stretching, induced either by hypotonic media or by isosmotic penetration of ions. Garlid has placed special emphasis on the role of Mg^{2+} as a physiological inhibitor of the H^+/K^+ antiporter by suggesting the "carrier brake" concept, where Mg^{2+} acts as the brake (Garlid, 1980). It is a matter of future investigation to establish whether the exchange reactions observed in the present study occur through a similar pathway as that of the inorganic cations, although the activation by membrane stretching and Mg^{2+} depletion would seem to favor this possibility. If this view proves correct, it follows that the mitochondrial membrane possesses controlled-inducible, unspecific sites responsible for the electroneutral exchange reactions. These sites may be seen as a system of fixed negative charges, interacting electrostatically with H^+ and positively charged species and catalyzing the electroneutral exchange. The accessibility of the fixed negative charge system to the water phase may be controlled by structural parameters, such as the amount of membrane-bound Mg^{2+} or the degree of membrane stretching.

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