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Phosphate Transport, Membrane Potential, and Movements of Calcium in Rat Liver Mitochondria

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Abstract

The membrane potential and calcium accumulation of mitochondria were followed by ion-specific electrodes in the presence of the proton-donor anions phosphate, acetate, glutamate, and beta-hydroxybutyrate. Phosphate was the only anion which allowed rapid and complete restoration of both the membrane potential and the steady-state extramitochondrial calcium concentration after the uptake of 100–200 nmol calcium per mg protein. If there was no influx of any proton-donor anion, the extent of calcium uptake depended on the intramitochondrial phosphate content. Both the fall of the membrane potential and the increase of the external calcium concentration brought about by a given amount of uncoupler were counteracted by phosphate transported into the mitochondria.

Key Words: Mitochondria; liver; transport; calcium; phosphate; membrane potential; uncoupler.

Introduction

According to Mitchell's chemiosmotic hypothesis, the charge separation systems of the respiratory chain build up chemical (ΔpH) and electric ($\Delta\Psi$) potential[2] differences across the inner mitochondrial membrane (Mitchell, 1968). The values of ΔpH and $\Delta\Psi$ may vary in a wide range, but the sum of them, the proton electrochemical potential difference ($\Delta\mu_{H^+}$), is kept constant by the changing activity of the respiratory chain. Under physiological

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[2]Abbreviations: $\Delta\mu_{H^+}$, proton electrochemical potential difference across the inner mitochondrial membrane; $\Delta\Psi$, membrane potential across the inner mitochondrial membrane; ΔpH, pH difference across the inner mitochondrial membrane; pCa, negative logarithm of the extramitochondrial free calcium concentration; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; P_i, inorganic phosphate; TPP^+, tetraphenylphosphonium; TMPD, *N,N,N',N'*-tetramethylparaphenylenediamine; BOH, DL-beta-hydroxybutyrate.

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conditions the membrane potential dominates, representing 150–180 mV out of a total $\Delta\mu_{H^+}$ of 220–230 mV (Nicholls, 1982).

It is the inside negative membrane potential which can move various cations (Ca^{2+}, Sr^{2+}, or K^+ in the presence of valinomycin) down the electric gradient but against their concentration gradient into the matrix space of mitochondria. Movements and distribution of the monovalent cations are determined exclusively by $\Delta\Psi$, whereas the distribution of the divalent cations does not achieve electrochemical equilibrium (for a review see Nicholls and Akerman, 1982). Instead, a steady state is reached between the influx through the electrogenic uniporter and efflux via a separate carrier which exchanges in liver mitochondria internal Ca^{2+} for external H^+. The activity of the uniporter sharply depends on the external calcium concentration, whereas the rate of the exchange carrier is determined by the internal calcium concentration. Thus, the extramitochondrial free calcium concentration (generally expressed as pCa) is controlled mainly by these kinetic factors, and thermodynamic regulation takes place only if $\Delta\Psi$ is considerably decreased (by approximately 30–40 mV).

The electric capacity of the inner mitochondrial membrane is so low that the transfer of just 1 nmol calcium per mg mitochondrial protein can discharge $\Delta\Psi$ and, by this, diminish $\Delta\mu_{H^+}$. The decrease of the proton electrochemical potential gradient stimulates the respiratory chain, and the enhanced rate of charge separation partially restores $\Delta\Psi$. By this process the transmembrane pH difference reaches its maximum value. Permeating weak acids, i.e., anions which are transported in an electroneutral process in symport with proton(s)—such as phosphate, acetate, beta-hydroxybutyrate—are able to dissipate the pH gradient and therefore allow the continuous H^+ ejection and restoration of $\Delta\Psi$. In the presence of these weak acids, large amounts (300–400 nmol per mg) of the cation and the anion in question can be accumulated, the process being accompanied by enhanced respiration and H^+ ejection.

In most of the earlier studies, H^+-transporting anions were omitted from the incubation mixture, and in this case mitochondrial calcium uptake was limited to 40–60 nmol per mg, a process termed "membrane loading" (Chance, 1965). It was not realized that calcium was also transported under these conditions into the matrix space. The major part of this calcium accumulation was later explained by the finding that mitochondrial preparations contain various amounts of phosphate as a consequence of leakage during preparation and storage (Reynafarje *et al.*, 1976; Nicholls, 1978). However, even when P_i transport was completely inhibited, mitochondria were still able to take up approximately 20 nmol calcium per mg protein (Nicholls 1978). It was postulated that in this case the internal buffering capacity (amounting to 30–40 nmol per mg according to Mitchell and Moyle, 1969)

could provide the protons necessary for the compensatory activity of the respiratory chain, but the nature of the intramitochondrial buffer remains open.

Recently a new suggestion has also been published on the role of phosphate in the course of calcium movements (Bernardi and Azzone, 1982). In these experiments the authors report that addition of phosphate results in calcium release in perfectly coupled mitochondria. The explanation is that addition of phosphate increases the membrane potential, and by this action it activates an outwardly directed calcium pump with the final result of a decrease of steady-state pCa. On the other hand, Bernardi and Azzone claimed that depressing $\Delta\Psi$ by uncoupling agents should inhibit this pump and shift the steady-state pCa to higher values.

The contradictions of these experimental findings and explanations prompted us to reinvestigate the role of phosphate and other proton-donor anions in mitochondrial calcium transport and the underlying membrane potential changes.

Methods

Rat liver mitochondria were prepared according to Johnson and Lardy (1967). Protein content was measured by the biuret method with bovine serum albumin as standard. The amount of transported substances is referred to mg mitochondrial protein.

Membrane potential was calculated on the basis of the movements of the lipid-soluble cation tetraphenylphosphonium (TPP^+). An electrode specific for TPP^+ was prepared according to Kamo *et al.* (1979). In the range of 0.2–10 μM the electrode response depended linearly on the logarithm of the TPP^+ concentration. The quantity $\Delta\Psi$ was calculated as described by Lötscher *et al.* (1980). Calcium movements were followed by a Ca^{2+}-selective electrode as described previously (Ligeti *et al.*, 1981). Both electrodes were attached to a Radelkis OP-205 pH-meter and a potentiometric recorder (OH-814).

Mitochondrial phosphate content was manipulated as described by Fonyó and Ligeti (1978). Phosphate determination was carried out according to Wahler and Wollenberger (1958) after centrifugation of the suspension and acid extraction of the pellet.

The uptake of ^{45}Ca was measured in a medium in which the free calcium concentration was kept constant by nitrilotriacetate buffer. Mitochondria (2.4 mg protein per ml) were incubated in the presence of 100 mM sucrose, 75 mM KCl, 7.5 mM $MgCl_2$, 5 mM Tris-HCl, 1 μM rotenone, 5.7 mM *n*-butylmalonate, 1.9 mM Tris-ascorbate, 53 μM TMPD, 3 mM Tris-phosphate, 2 μg

oligomycin per ml, 0.1 mM ADP, 57 μM mersalyl, 0.6 mM $CaCl_2$, 10 mM NTA, and 7 kBq $^{45}CaCl_2$ per ml, the final pH being 7.0. The free ionic concentrations, calculated according to Perrin *et al.* (1967), were the following: Ca^{2+}, 24 μM; Mg^{2+}, 2.1 mM; phosphate, 1.8 mM; and ADP, 20 μM. After 2 min of incubation a 0.1-ml aliquot of the suspension was withdrawn and filtered through 0.45 μm pore size Sartorius nitrocellulose membrane filter. The filters were then washed with 0.5 ml chilled medium (^{45}Ca omitted) and placed into 5-ml scintillation cocktail. Radioactivity was counted in a Beckman LS-200 liquid scintillation spectrometer. Only those experiments were evaluated where the ^{45}Ca content of mitochondria remained constant for at least 4 min. The obtained values were corrected for ruthenium-red-insensitive binding and for ^{45}Ca–^{40}Ca exchange. All the experiments were carried out at 25°C. Further details are given in the legends to the figures.

Beta-hydroxybutyrate and acetazolamid (Diamox) were obtained from Serva, CCCP from Calbiochem, TMPD from Merck, TPP^+ from Fluorochrom Ltd. (Derbyshire, U.K.), and nitrilotriacetate from Fluka. All the other chemicals were analytical grade reagents.

Results

Effect of Proton-Donor Anions on the Membrane Potential

Respiring mitochondria build up a membrane potential of 180–190 mV, which is further increased by 15–20 mV upon addition of 0.4–2.0 mM inorganic phosphate (Fig. 1; see also Lötscher et al., 1980, and Akerman, 1978). This effect of phosphate was independent of the type of the applied respiratory substrate (beta-hydroxybutyrate, succinate or ascorbate plus TMPD), or the presence of ruthenium red or oligomycin, and of the sequence of additions. However, phosphate became ineffective if the P_i carrier was inhibited by mersalyl (not shown). Thus, the intact function of the phosphate carrier is an essential requirement of the observed phenomenon.

Other proton-donor anions changed $\Delta\Psi$ in the same direction, but there was a great difference in their effectiveness. As shown in Fig. 1B and C, acetate applied in 20 mM concentration brings about only half of the effect of 2 mM phosphate.

Calcium Uptake in the Presence of Various Proton-Donor Anions

In earlier experiments, phosphate, acetate, beta-hydroxybutyrate, glutamate, lactate, and bicarbonate were shown to support energy-dependent accumulation of calcium (Lehninger, 1974; Harris, 1978). In these experiments stimulation of respiration and ^{45}Ca-uptake were measured. On the basis

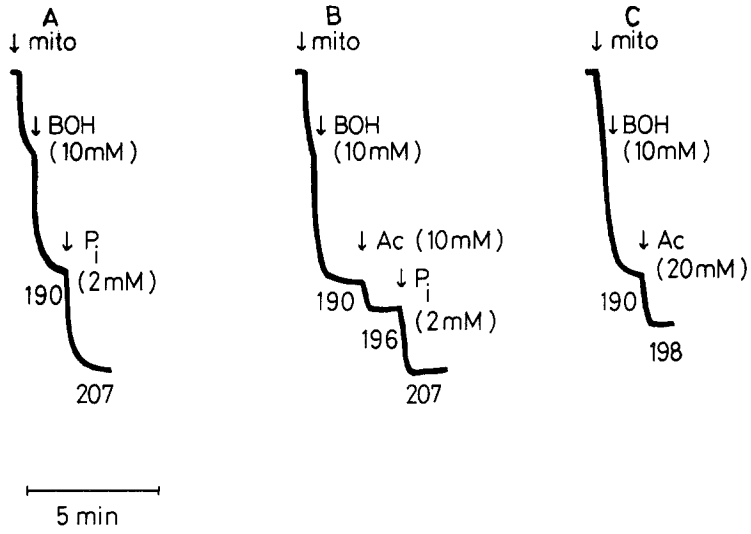

Fig. 1. Comparison of the effect of phosphate and acetate on $\Delta\Psi$. Original trace of the TPP^+ electrode. Composition of the medium: 239 mM sucrose, 15 mM Tris-HCl, pH 7, 3 mM $MgCl_2$, 1 mM KCl, 2.4 μM TPP^+, and 1.2 mg mitochondrial protein per ml. 10 mM beta-hydroxybutyrate served as substrate. Further additions: 2 mM Tris-phosphate and 10 mM Tris-acetate. The numbers refer to $\Delta\Psi$ (mV).

of these publications, all the proton-donor anions were regarded as equally effective (Nicholls and Akerman, 1982), although they were never thoroughly compared. Recording the membrane potential changes during calcium uptake, we found substantial differences between these anions.

In the presence of phosphate, initiation of calcium uptake into respiring mitochondria causes a temporary fall of $\Delta\Psi$ followed by a rapid restoration of the membrane potential (Lötscher *et al.*, 1980). This cycle can be repeated several times. In contrast to this, in the absence of any proton-donor anion, a significant pulse of calcium permanently lowers the membrane potential. In the experimental reproduced in Fig. 2A, 80 nmol calcium per mg protein decreased $\Delta\Psi$ from 202 to 140 mV. Subsequent addition of phosphate completely restored the membrane potential. All the other anions added were only partially effective (Fig. 2B–D), although they were applied at a concentration an order of magnitude higher than that of phosphate.

In another type of experiment, small pulses of calcium were repeatedly added in the presence of either phosphate or beta-hydroxybutyrate (Fig. 3). If phosphate was the accompanying anion, even a load of 160 nmol calcium per mg protein lowered $\Delta\Psi$ by only 10 mV, whereas in the presence of beta-hydroxybutyrate already the first pulse (15 nmol calcium per mg protein) effected a definite fall (18 mV) with a further decrease of $\Delta\Psi$ upon the

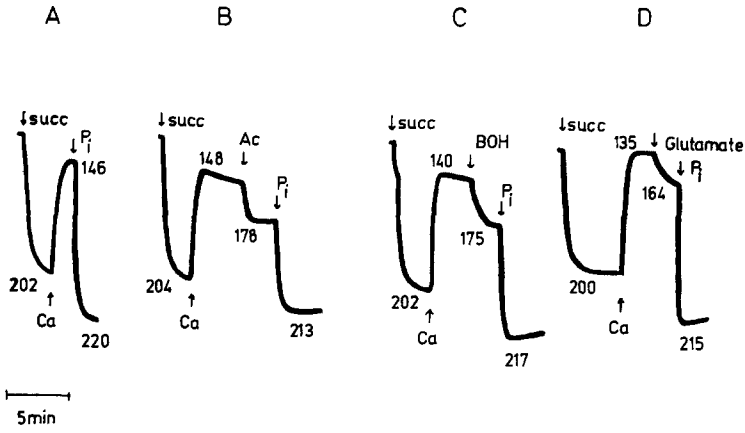

Fig. 2. Changes of $\Delta\Psi$ during calcium accumulation. Effect of various proton-donor anions. Original trace of the TPP^+ electrode. Composition of the medium: 240 mM sucrose, 10 mM tris-HCl, pH 7, 3 mM $MgCl_2$, 3.2 μM TPP^+, 1 μM rotenone, 2 mM Tris-succinate, and 1.2 mg mitochondrial protein per ml. Further additions: 80 nmol $CaCl_2$ per mg protein, 0.4 mM Tris-phosphate, 5 mM Tris-acetate, 5 mM beta-hydroxybutyrate, and 5 mM Tris-glutamate. The numbers refer to $\Delta\Psi$ (mV).

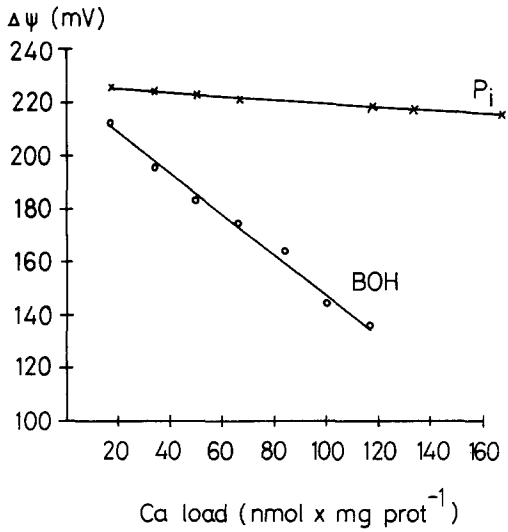

Fig. 3. $\Delta\Psi$ as a function of the amount of calcium added to mitochondria. Effect of phosphate and beta-hydroxybutyrate. Experimental conditions are the same as in Fig. 2. 0.5 mM Tris-phosphate (×) or 5 mM beta-hydroxybutyrate (O) were initially present.

successive calcium additions. We obtained the same results when beta-hydroxybutyrate was replaced by glutamate or acetate (not shown). Thus, phosphate was the only anion capable of keeping $\Delta\Psi$ unaltered during calcium accumulation.

Differences between phosphate and all the other proton-donor anions were also found if we recorded the steady-state pCa value (Fig. 4). Again, small pulses of the divalent cation were added. In the presence of phosphate, mitochondria behaved as powerful calcium buffers: pCa decreased only by 0.3 unit upon a load of 250 nmol per mg. Steady-state pCa values were consequently lower if beta-hydroxybutyrate or glutamate served as accompanying anions, and these plots exhibited two clearly distinguishable phases: up to 80 nmol calcium per mg protein, pCa declined slightly (0.29 and 0.15 unit, respectively), whereas further calcium loads induced a steep fall of the plot. In the first phase, $\Delta\Psi$ was still high enough (see Fig. 3) not to exert thermodynamic control of calcium movements. The differences of pCa value observed under these conditions point to differences of the kinetic control. Formation of insoluble calcium phosphate precipitate reduces the intramitochondrial free calcium concentration and lowers the activity of the efflux pathway (Zoccarato and Nicholls, 1982). We suggest that the other proton-donor anions allow much higher internal calcium concentrations, and the more active efflux carrier explains the lower pCa value in the steady state. The steep decline of pCa in the absence of phosphate upon the addition of larger calcium pulses (above 80–100 nmol per mg) is in accordance with the low membrane potential observed under these conditions (see Fig. 3).

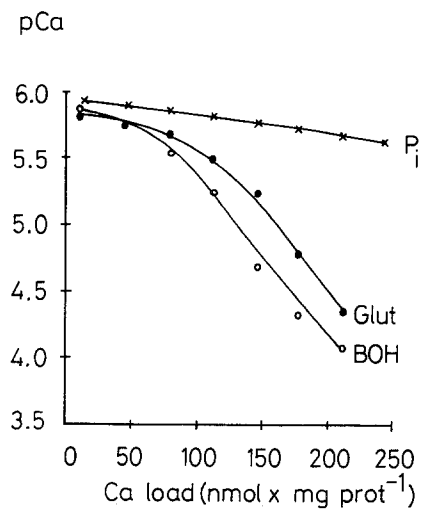

Fig. 4. Steady-state pCa as a function of the amount of calcium added to the mitochondria. Effect of various proton-donor anions. Experimental conditions are the same as in Fig. 2, but TPP^+ was omitted. 0.5 mM Tris-phosphate (×), 5 mM beta-hydroxybutyrate (○), or 5 mM tris-glutamate (●) were initially present.

Effect of Low Concentration of Uncouplers on Calcium Distribution

The protonophore uncouplers, like CCCP, are weak acids so highly lipophilic that the deprotonated base also remains lipid soluble. They shuttle within the membrane, and the cyclic movement of the acidic form to the matrix surface and the backflow of the deprotonated form to the outer surface results in the increase of H^+ permeability and collapse of ΔpH and $\Delta\Psi$. If the fall of $\Delta\Psi$ exceeds 30–40 mV in calcium-loaded mitochondria, then the reversed electrochemical potential gradient of Ca^{2+} causes net calcium efflux through the uniporter with a consequent decrease of pCa. Recently, however, just the opposite effect of uncoupling has been observed by Bernardi and Azzone (1982): pCa was shifted to higher values.

To find the basis of this unexpected result, we measured parallelly the effect of uncoupling on membrane potential and steady-state pCa value (Fig. 5). In both experiments mitochondria were allowed to accumulate a low dose of calcium and to reach steady state. Following this, very small amounts of CCCP were added consecutively. The membrane potential decreased in small incremental steps. Phosphate augmented $\Delta\Psi$ throughout the whole range of CCCP concentrations examined ($\Delta\Psi$ values below 110 mV are not measurable by the TPP^+ electrode). Thus, in the presence of phosphate, higher amounts of uncoupler were needed to obtain the same fall of $\Delta\Psi$.

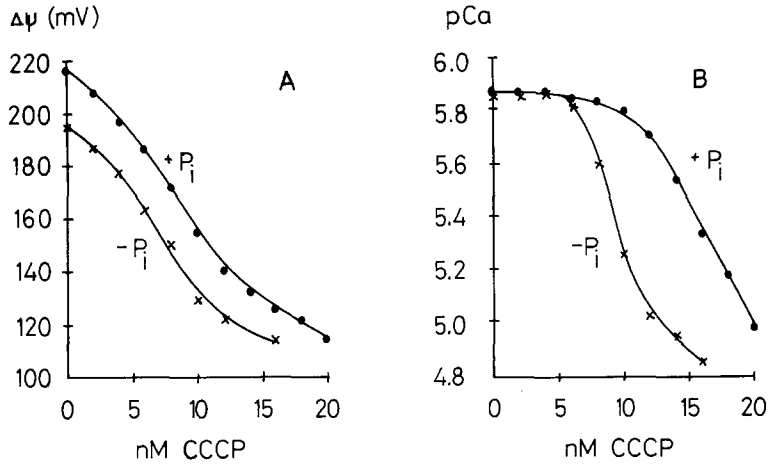

Fig. 5. Effect of CCCP on $\Delta\Psi$ (A) and on steady-state pCa (B). Composition of the medium: 240 mM sucrose, 10 mM Tris-HCl, pH 7.0, 3 mM $MgCl_2$, 2 mM beta-hydroxybutyrate, and 1.2 mg mitochondrial protein per ml. When measuring $\Delta\Psi$, 3.2 μM TPP^+ was also present. In both experiments mitochondria have accumulated 13 nmol calcium per mg protein before CCCP was added. Where indicated, the medium was supplemented with 1 mM Tris-phosphate.

Phosphate renders the intramitochondrial milieu more acidic, and this change would slow down the dissociation of the protonated form of the uncoupler at the internal surface. As a result, the same amount of CCCP would carry less protons to the matrix space.

The effect of CCCP on calcium movements was recorded first in the absence of phosphate. Addition of low concentrations (up to 6 nM) of CCCP did not alter pCa although $\Delta\Psi$ was gradually decreased. Application of more than 6 nM CCCP induced prompt calcium efflux, and within 1 min a new steady-state pCa was established at a lower level. The presence of phosphate shifted the whole plot to the right, i.e., the same pCa value was reached by significantly higher concentration of CCCP. As in the absence of phosphate, in which more CCCP was needed to decrease $\Delta\Psi$ to a level where calcium distribution was determined thermodynamically, it is fully comprehensible that also more CCCP was needed to decrease steady-state pCa (compare Fig. 5A and 5B). In similar experiments phosphate also counteracted the effect of CCCP both on $\Delta\Psi$ and on pCa if the anion was added after the uncoupler (Fig. 6). Thus, in contrast to the findings of Bernardi and Azzone (1982), in our hands CCCP was the agent which decreased pCa, and phosphate was that which increased it.

Calcium Uptake in the Absence of Permeable Anions

Mitochondria are able to accumulate 15–20 nmol calcium per mg protein even if no proton-donor anion is added and phosphate transport is blocked. Under these conditions both the oxygen consumption and the H^+ ejection accompanying the cation movement were found to be dependent on the phosphate content of mitochondria (Fonyó and Ligeti, 1978). Calcium uptake was not measured in these early studies, and therefore we followed the uptake of ^{45}Ca after the manipulation of the intramitochondrial phosphate content. Endogenous phosphate was increased by incubating mitochondria in a medium containing 2 mM phosphate, and it was decreased by using up internal phosphate for ATP synthesis. Further movements of phosphate were then stopped by mersalyl plus butylmalonate, and ATPase was inhibited by oligomycin. Calcium uptake was measured at different internal phosphate contents. The extent of accumulated calcium changed in parallel with the intramitochondrial phosphate content, and a linear relationship between these two parameters was found (Fig. 7A). Harris (1978) reported that inhibition of carbonic anhydrase by Diamox reduced the amount of calcium taken up. In our experiments Diamox did not decrease calcium uptake at any given level of intramitochondrial phosphate (Fig. 7B); thus, bicarbonate formed intramitochondrially from CO_2 does not seem to play a significant role.

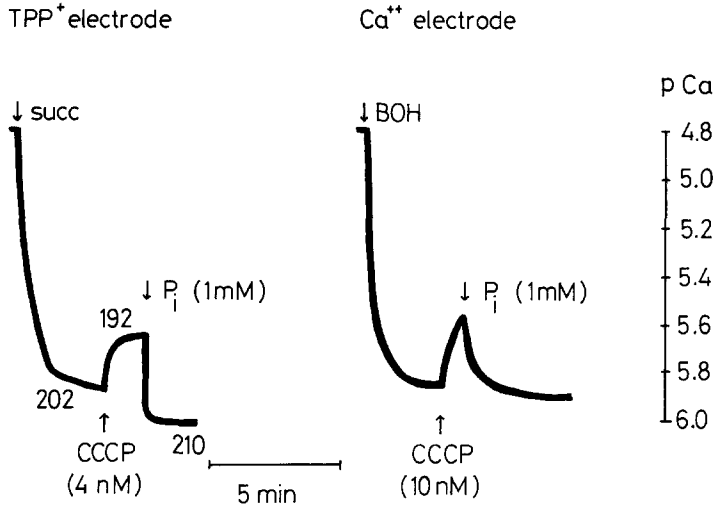

Fig. 6. The opposing effect of CCCP and P_i on the membrane potential (TPP^+ electrode trace) and on steady-state pCa (Ca^{2+} electrode trace). The experimental conditions are the same as in Fig. 5. Further additions: 4 and 10 nM CCCP, 1 mM Tris-phosphate, 2 mM Tris-succinate, and 2 mM beta-hydroxybutyrate. The numbers refer to $\Delta\Psi$ (mV).

Discussion

Our experiments revealed a distinctive role of phosphate ions in the course of calcium uptake:

1. It was the intramitochondrial phosphate content that determined the extent of calcium uptake if the influx of proton-donor anions was prevented.
2. Phosphate brought about the complete restoration of $\Delta\Psi$ during calcium accumulation.
3. In the presence of a functional phosphate transport system the change of steady state pCa was minimal even in the case of massive calcium loading.

In order to explain these observations, we have to consider the chemical properties distinguishing phosphate from the rest of the proton-donor anions: it is a trivalent anion with the second pK around 7, and it has a low stability constant with calcium.

It follows from the second pK value that both ionic species of phosphate in the internal space of respiring mitochondria (the pH being approximately 7.5) are able to dissociate protons. By the formation of the calcium phosphate precipitate, additional protons are liberated. Thus, intramitochondrial phos-

phate content can be regarded as a possible source of protons for the respiratory chain. The fact that an increase of the amount of internal phosphate enhances the capacity of mitochondria to take up cations (Fig. 7), i.e., to restore $\Delta\Psi$ by H^+ ejection, proves that phosphate indeed serves as an intramitochondrial buffer.

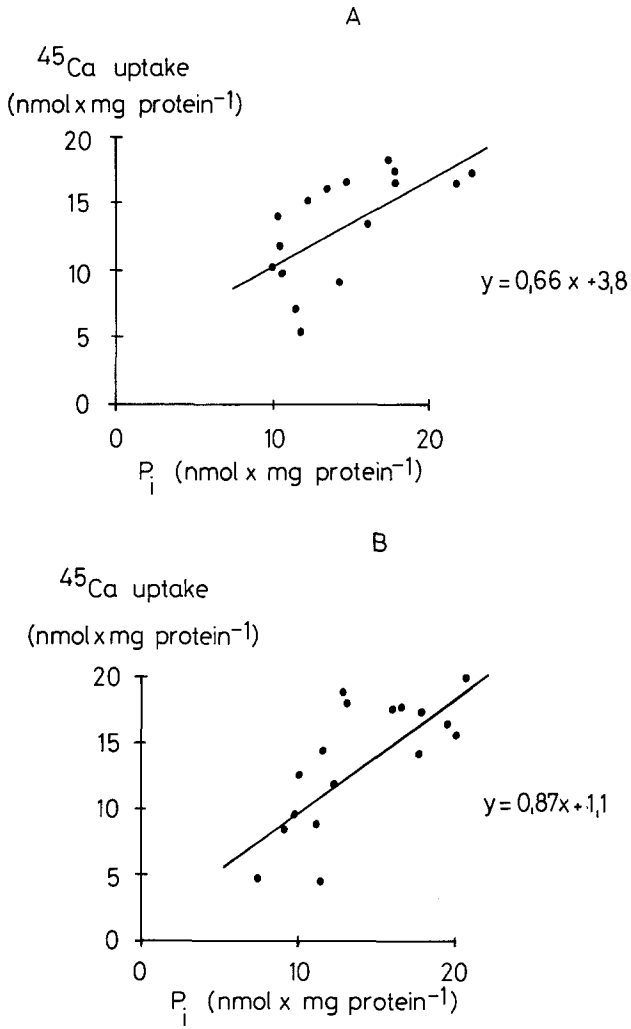

Fig. 7. Dependence of ^{45}Ca uptake on the intramitochondrial phosphate content in the absence (A) and in the presence (B) of Diamox. All details of the experiment are given in Methods. Data of three independent experiments are represented. In plot B 10 mM Diamox was present initially.

The difference in the effect of phosphate and the other H^+-donor anions on $\Delta\Psi$ during calcium accumulation might also be the result of the different pK values. At neutral pH the transported form of acetate (the undissociated acid) represents only a minor fraction of the total concentration, whereas approximately 50% of phosphate is present as $H_2PO_4^-$, the true substrate of the phosphate carrier (Fonyó *et al.*, 1982). As a result of these differences, phosphate is more effective in dissipation of the pH gradient than any of the other H^+-donor anions, allowing the interconversion of ΔpH into $\Delta\Psi$. However, speculations on some kind of direct interaction between phosphate and the H^+-pumping proteins of the respiratory chain might also be considered.

The low stability constant of the calcium phosphate salts results in the precipitation of calcium and phosphate ions in the matrix space. Thus, intramitochondrial calcium concentration and therefore the rate of efflux through the exchange carrier remain unchanged during calcium accumulation, explaining the constancy of external pCa during this process.

Summing up, all the effects of phosphate—increasing the internal buffer capacity, keeping $\Delta\Psi$ high and lowering the rate of calcium efflux—favor prompt uptake of calcium into mitochondria in cases of acute rise of the extramitochondrial calcium concentration.

The v_{max} of the P_i carrier is at least ten times higher than that of the adenine nucleotide carrier (Vignais, 1976). Consequently only a small fraction of the P_i transport activity is utilized during oxidative phosphorylation. On the other hand, the apparent v_{max} of calcium uptake is in the range of that of the P_i carrier (Nicholls and Akerman, 1982). The role of phosphate in rapid calcium accumulation might give an explanation for the ample reserve of P_i transport capacity.

Finally, our results concerning the effect of uncouplers on calcium movements disagree with the data of Bernardi and Azzone (1982). Very low concentrations of CCCP decreased $\Delta\Psi$ in small steps but, in agreement with earlier data relating $\Delta\Psi$ to calcium distribution (Nicholls, 1978), did not affect pCa. If the fall of $\Delta\Psi$ exceeded 30 mV, pCa started to decrease. The presence of phosphate increased the concentration of CCCP required for inducing calcium efflux, but it did not influence pCa in the absence of uncoupler. On the basis of our results, we do not feel that the suggestion about an energy-dependent calcium-efflux pathway is justified.

Acknowledgments

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References

Akerman, K. E. O. (1978). *Biochim. Biophys. Acta* **502,** 359–366.
Bernardi, P., and Azzone, G. F. (1982). *FEBS Lett.* **139,** 13–16.
Chance, B. (1965). *J. Biol. Chem.* **240,** 2729–2748.
Fonyó, A., and Ligeti, E. (1978). *FEBS Lett.* **96,** 343–345.
Fonyó, A., Ligeti, E., and Lukács, G. (1982). In *Cell Function and Differentiation* (Akoyunoglou, G., Evangelopoulos, A. E., Georgatsos, J., Palaiologos, G., Trakatellis, A., and Tsiganos, C. P., eds.), FEBS Vol. 65, Alan R. Liss, Inc., New York, pp. 409–422.
Harris, E. J. (1978). *Biochem. J.* **176,** 983–991.
Johnson, D., and Lardy, H. (1967). *Methods Enzymol.* **10,** 94–96.
Kamo, N., Muratsugu, M., Hongoh, R., and Kobatake, Y. (1979). *J. Membr. Biol.* **49,** 105–121.
Lehninger, A. L. (1974). *Proc. Natl. Acad. Sci.* **71,** 1520–1524.
Ligeti, E., Bodnár, J., Károly, É., and Lindner, E. (1981). *Biochim. Biophys. Acta* **656,** 177–182.
Lötscher, H. R., Winterhalter, K., Carafoli, E., and Richter, C. (1980). *Eur. J. Biochem.* **110,** 211–216.
Mitchell, P. (1968). Chemiosmotic Coupling and Energy Transduction. Glynn Res. Ltd., Bodmin.
Mitchell, P., and Moyle, J. (1969). *Eur. J. Biochem.* **7,** 471–484.
Nicholls, D. G. (1978). *Biochem. J.* **176,** 463–474.
Nicholls, D. G. (1982). *Bioenergetics,* Academic Press, London and New York.
Nicholls, D. G., and Akerman, K. (1982). *Biochim. Biophys. Acta* **683,** 57–88.
Perrin, D. D., and Sayce, I. G. (1967). *Talanta* **14,** 833–842.
Reynafarje, B., Brand, M. D., and Lehninger, A. L. (1976). *J. Biol. Chem.* **251,** 7442–7451.
Vignais, P. V. (1976). *Biochim. Biophys. Acta* **456,** 1–39.
Wahler, B. E., and Wollenberger, A. (1958). *Biochem. Z.* **329,** 508–520.
Zoccarato, F., and Nicholls, D. G. (1982). *Eur. J. Biochem.* **127,** 333–338.