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Accumulation of Calcium and Phosphate Stimulated by Carboxylic Antibiotics into Mitochondria*

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Abstract

Carboxylic ionophores such as nigericin, dianemycin, the monensins and compounds Lilly A 217 or X-537 A, stimulate an electron-transport dependent accumulation of Ca^{2+} and phosphate into mitochondria. Ion accumulation is stimulated under conditions of limited Ca^{2+} loading imposed by phosphate in the presence of β-hydroxybutyrate. Carboxylic ionophores do not affect divalent ion uptake when β-hydroxybutyrate is replaced for by succinate. They block Ca^{2+} and phosphate accumulation when energy is provided from the hydrolysis of ATP, or from the oxidation of glutamate, α-ketoglutarate, pyruvate or glutamate + malate. Nigericin-like antibiotics also transform the indefinite prolongation of state 3 respiration induced by Ca^{2+} and phosphate on β-hydroxybutyrate oxidation, into tightly coupled state 3 to 4 transitions. Evidence suggests that electrophoretic Ca^{2+} transport occurs in parallel with proton or K^+ carriers. The anion movements associated to Ca^{2+} uptake are most probably driven by the existent ΔpH across the mitochondrial membrane.

It is known that the translocation of Ca^{2+} energized by substrate oxidation or by ATP hydrolysis, is linked to the efflux of protons in intact mitochondrial membranes [1-6].

It has also been reported that an oligomycin-insensitive efflux of K^+ induced by valinomycin in non-respiring mitochondria stimulates the translocation of Ca^{2+} into these organelles [7-9].

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The evidence hereby presented indicate that several carboxylic antibiotics from the nigericin family stimulate an electron transport-dependent accumulation of Ca^{2+} and phosphate into mitochondria. It is suggested that the membrane potential changes generated upon the induced efflux of protons and K^+ from mitochondria, parallel to the simultaneous translocation of some oxidizable substrates and phosphate, facilitate the electrophoretic flux of Ca^{2+} into mitochondria. The possibility that nigericin-like compounds operate as mobile carriers for Ca^{2+} transport is also considered.

Materials and Methods

Mitochondria were prepared from livers of male rats weighing 125-150 g as described by Johnson and Lardy [10]. The initial homogenization step was carried out in the presence of 1 mM EDTA.

Measurement of K^+ and H^+ Movements and Oxygen Uptake

A continuous recording of oxygen consumption and variations in the extramitochondrial concentration of K^+ and H^+ was carried out using an apparatus designed, developed and constructed by Chance, Mayer, Pressman and Graham [11-13].

Measurement of the Accumulation of Ca^{2+} and Inorganic Phosphate in Liver Mitochondria

Rat liver mitochondria were incubated and rapidly isolated as previously described [14-16]. The accumulated orthophosphate was extracted by the procedure of Falcone and Witonsky [17] and measured in the organic solvent phase by determination of the radioactivity when (^{32}P) Pi was used, or chemically by the method of Martin and Doty [18] as modified by Lindberg and Ernster [19]. The accumulated (^{45}Ca) Ca^{2+} was measured in the aqueous phase of mitochondrial extracts obtained after rapid centrifugation of the incubation vessel contents.

Other Analytical Procedures

Radioactivity measurements were made with a Nuclear Chicago gas-flow low background counter. ATPase activity was measured as described by Lardy and Wellman [20]. Inorganic phosphate from ATP hydrolysis was determined as described [21]. Mitochondrial protein was estimated by the Biuret method. (^{32}P) Pi was obtained from Tracerlab (Waltham, Mass.); (^{45}Ca)-Ca^{2+} was obtained from Amersham Searle Co. The antibiotics nigericin, dianemycin, monensin A, X-537 A, Lilly A-217

and nonactin used in these experiments were a generous gift from Dr. Henry A. Lardy, The Institute for Enzyme Research, University of Wisconsin, and Dr. Marvin Gorman, The Lilly Research Laboratories.

Results

In the presence of β-hydroxybutyrate and inorganic phosphate, increasing concentrations of nigericin stimulate the aerobic accumulation of Ca^{2+} into mitochondria (Fig. 1). Such phenomenon is prevented when either β-hydroxybutyrate or phosphate are omitted (Fig. 2) or else when 5×10^{-7} M antimycin or 2×10^{-7} M rotenone are added to the medium. It is apparent that very low concentrations of nigericin (1×10^{-7} M), are required for the antibiotic to stimulate maximal accumulation of Ca^{2+} into mitochondria.

Figure 2 (panel A) shows a plot of the magnitude of Ca^{2+} uptake induced by 3×10^{-7} M nigericin versus concentrations of Ca^{2+} added.

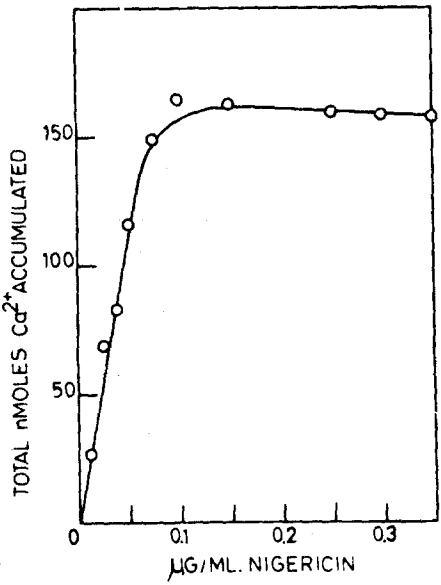

Figure 1. The effect of the concentration of nigericin on the accumulation of Ca^{2+} in liver mitochondria in the presence of β-hydroxybutyrate and phosphate. The reaction mixture contained: 10 mM triethanolamine (Cl^-) pH 7.4, 15 mM KCl, 2.5 mM phosphate (TEA) 200 μM $CaCl_2$, 20,000 c.p.m. (^{45}Ca)-Ca^{2+}, 10 mM β-hydroxybutyrate, 200 mM sucrose, the indicated concentrations of nigericin and approximately 9 mg protein N of mitochondria in 2.0 ml volume at 28°. After 10 min incubation, the mitochondria were rapidly isolated and the radioactivity of (^{45}Ca)-Ca^{2+} determined in the pellets.

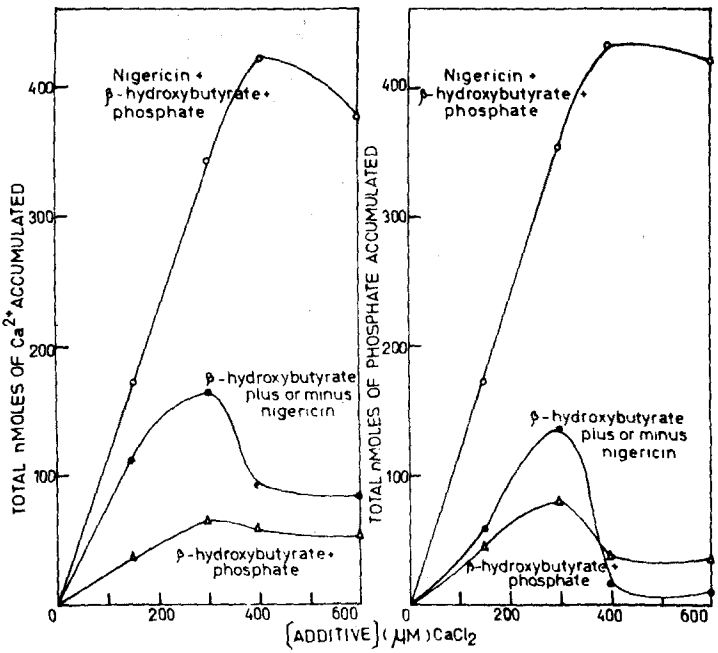

Figure 2. The effect of calcium concentration on the accumulation of Ca^{2+} and inorganic phosphate stimulated by nigericin in liver mitochondria. The experimental conditions were essentially those of Fig. 1 except for the indicated concentrations of Ca^{2+} and that of 0.1 μg/ml nigericin. *Panel A* shows a plot of the accumulation of (^{45}Ca)-Ca^{2+} against Ca^{2+} concentration in the medium. *Panel B* indicates the dependence of the accumulation of inorganic phosphate on Ca^{2+} concentration.

The antibiotic not only prevents the inhibitory effect of orthophosphate on Ca^{2+} retention [22] but also clearly stimulates Ca^{2+} accumulation above the levels energized by β-hydroxybutyrate alone or with added phosphate. Panel B of Fig. 2 indicates that nigericin also stimulates the net accumulation of orthophosphate from the medium, when mitochondria are supplemented with β-hydroxybutyrate and increasing concentrations of Ca^{2+}. Maximal Ca^{2+} or phosphate accumulation is induced by nigericin at an external concentration of 400 μM Ca^{2+}; higher concentrations of Ca^{2+} tend to inhibit net Ca^{2+} and phosphate uptake in a parallel form.

Nigericin does not affect Ca^{2+} or phosphate translocation in the absence of added inorganic phosphate regardless of the presence of oxidizable substrates or ATP. Apparently, phosphate must inhibit Ca^{2+} loading [22] for the antibiotic to stimulate net Ca^{2+} uptake into mitochondria. The translocation of Ca^{2+} stimulated by nigericin is negligible when β-hydroxybutyrate is replaced for by succinate in the

presence of orthophosphate. Moreover, the carboxylic ionophore almost completely blocks the accumulation of Ca^{2+} and phosphate linked to glutamate, α-ketoglutarate or malate oxidation. Thus, except where indicated, most of the experiments to be described were carried out with β-hydroxybutyrate as energy source for ion transport.

As shown in Fig. 3, the accumulation of Ca^{2+} stimulated by nigericin is sensitive to the concentration of protons in the medium. An increase of Ca^{2+} uptake is induced by the carboxylic antibiotic from pH 6.0 to 6.5, whereas a progressive decrease is observed above pH 7.0. Identical pH values are found for the accumulation of phosphate stimulated by nigericin. The antibiotic does not stimulate Ca^{2+} or phosphate uptake into mitochondria above pH 8.0.

The ability of carboxylic ionophores to stimulate Ca^{2+} uptake is also sensitive to the concentration of mitochondrial protein nitrogen in the medium. It is apparent that the protein/antibiotic ratio is implicated with the extent of divalent ion accumulation (Table I). Parallel results to

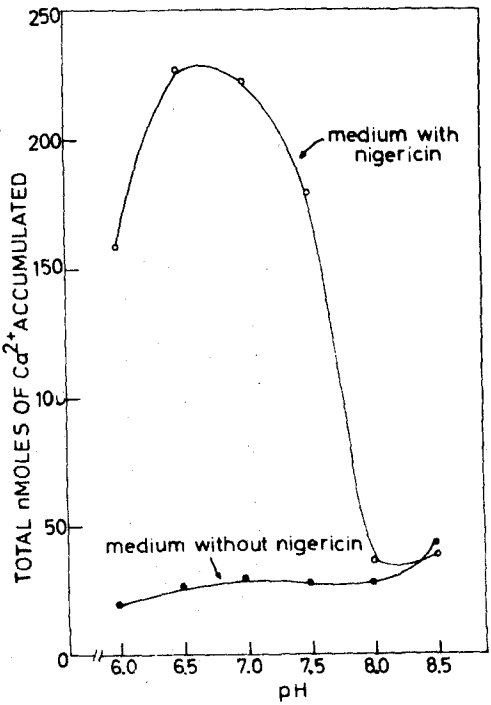

Figure 3. The effect of pH change on the accumulation of Ca^{2+} which occurs in the presence of nigericin, β-hydroxybutyrate and phosphate in liver mitochondria. The reaction mixture for these experiments was similar to that of Fig. 1 except for the addition of 6 mM histidine and variable concentrations of HCl to obtain the indicated pH values. The concentration of nigericin used was 0.1 μg/ml.

TABLE I. The effect of the concentration of mitochondria protein N on the extent of calcium accumulation mediated by nigericin

Total concentration of mitochondrial protein nitrogen	Ca^{2+} accumulated without nigericin	Ca^{2+} accumulated in the presence of nigericin
	Total nmoles of Ca^{2+} accumulated	
1.65	15	23
3.3	23	44
4.95	33	43
6.6	37	98
9.9	55	143
13.2	79	188
19.8	160	188
23.1	165	185

The reaction mixture contained 10 mM triethanolamine (Cl) pH 7.4, 15 mM KCl, 2.5 mM phosphate (TEA), 150 μM $CaCl_2$, 20,000 cpm (^{45}Ca)-Ca^{2+}, 10 mM β-hydroxybutyrate, 200 mM sucrose, 0.25 μg/ml nigericin and the indicated concentrations of mitochondrial protein added in 2.0 ml volume at 25°. A zero time value (Ca^{2+} accumulated in mitochondria incubated with 1 mg/ml antimicin and rotenone plus 5×10^{-5} M, 2.4 dinitrophenol), was subtracted from each experimental at the different alternatives reported. Time of incubation 10 min. The isolation of mitochondria after the incubation, as well as the measurement of the radioactivity of (^{45}Ca)-Ca^{2+} in the mitochondrial pellets was carried out as indicated in methods.

those obtained for Ca^{2+} transport have also been found for inorganic phosphate movements in the above parameter.

Different carboxylic antibiotics replace for nigericin to stimulate Ca^{2+} and phosphate translocation linked to β-hydroxybutyrate oxidation in mitochondria. This is shown in Table II. It indicates that nigericin, dianemycin, compound Lilly A-217 (almost identical to HLR-206) monensin A and X-537 A substitute for nigericin in the above effect.

Previous observations by Lardy, Johnson and McMurray [23] showed that low concentrations of nigericin block the ATPase stimulated by Ca^{2+} in liver mitochondria. As illustrated in Fig. 5, the inhibition by both monensin A or X-537 A of the hydrolysis of ATP stimulated by Ca^{2+} (Panel A of Fig. 4) is associated to a parallel inhibition by the antibiotics of the accumulation of Ca^{2+} energized by ATP (Panel B of Fig. 4). Thus carboxylic antibiotics stimulate the accumulation of Ca^{2+} when mitochondria are energized with β-hydroxybutyrate in the presence of phosphate, while inhibiting that promoted by ATP.

Table III indicates that electron transport inhibitors as well as proton or cation conductors block the uptake of Ca^{2+} and phosphate stimulated by nigericin into mitochondria. Antimycin and rotenone as well as 2,4-dinitrophenol or FCCP inhibit such phenomena. Moreover, in the presence of increasing concentrations of KCl, nonactin completely

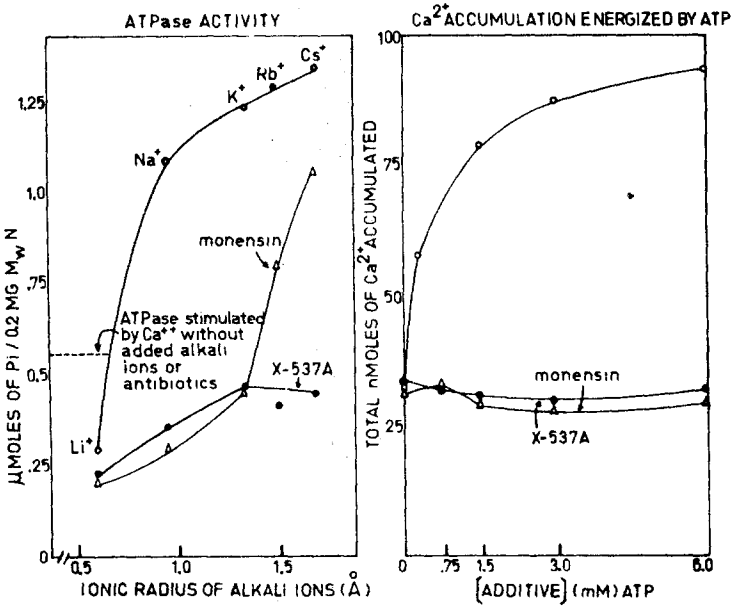

Figure 4. The effect of monensin A and X-537 A on the ATPase activity stimulated by Ca^{2+} (Panel A) and the uptake of Ca^{2+} energized by ATP into mitochondria (Panel B). The reaction mixture from *Panel A* contained: 6 mM ATP (Tris) pH 7.4, 50 mM of the chloride salts of either Li^+, Na^+, K^+, Rb^+ or Cs^+, 400 μM $CaCl_2$, 180 mM sucrose, 1 μg/ml of either HLR-537 or monensin A and 2.6 mg of mitochondrial protein in 1.0 ml volume at 30°. The time of incubation was 10 min. The reaction mixture from *Panel B* was that of Fig. 1 except for the indicated concentrations of ATP instead of β-hydroxybutyrate as well as the addition of 400 μM Ca^{2+} and 4.6 mg mitochondrial protein/ml.

TABLE II. Effect of different carboxylic antibiotics on the accumulation of Ca^{2+} and phosphate into mitochondria

Additions	Total nmoles of Ca^{2+} accumulated	Total nmoles of Pi accumulated
None	50.93	69.00
Nigericin	221.90	275.00
Monensin	192.80	280.00
Dianemycin	217.00	275.00
X-537	219.00	279.00
Lilly A-217	188.00	280.00

The reaction mixture was essentially that from Table I, except for the addition of 0.6 μg/ml of the indicated carboxylic antibiotics, 200 μM CaCl and mg of mitochondrial protein nitrogen.

blocks the uptake of Ca^{2+} and phosphate stimulated by nigericin (Fig. 5A B). Valinomycin and macrocyclic polyether XXXIV [25] replace for nonactin in such inhibitory action (Table III). From the above data it may be concluded that an energized state of the mitochondrial membrane is necessary for nigericin-like antibiotics to stimulate Ca^{2+} and phosphate accumulation into mitochondria.

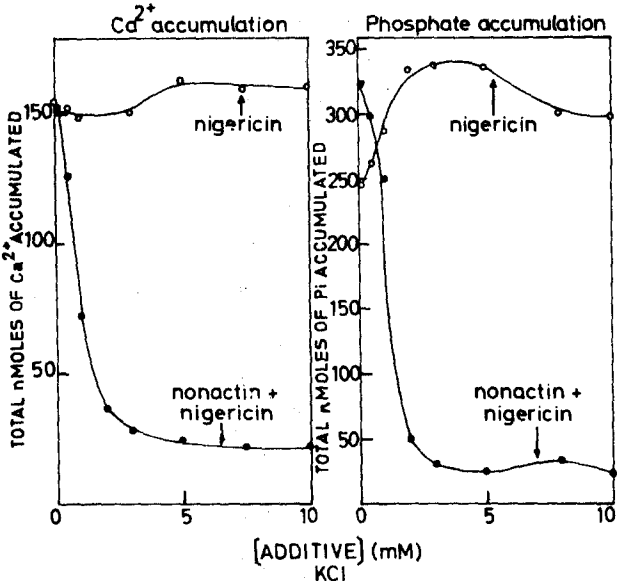

Figure 5. The effect of the concentration of KCl on the inhibition by nonactin of the accumulation of Ca^{2+} (Panel A) or phosphate (Panel B) stimulated by nigericin into mitochondria. The reaction mixture for these experiments was similar to that of Fig. 1, except for the addition of 2×10^{-7} M nonactin, and the indicated concentrations of KCl.

Rossi, Carafoli, Drahota and Lehninger [22] have shown that orthophosphate causes an indefinite prolongation of the state 3 respiration in the presence of Ca^{2+} and β-hydroxybutyrate. When the medium is supplemented with nigericin, Ca^{2+} is capable of inducing state 3 to 4 respiration transitions, even in the presence of 10 mM phosphate (Fig. 6A B). The addition of ADP after Ca^{2+} and phosphate, causes tightly coupled bursts of oxygen uptake only in the presence of nigericin. Thus, parallel to their ability to stimulate Ca^{2+} and phosphate uptake in the presence of β-hydroxybutyrate, carboxylic ionophores prevent the uncoupling effect of phosphate plus Ca^{2+} on β-hydroxybutyrate oxidation.

The possible relationship existent between K^+ and H^+ movements and the antibiotic induced-Ca^{2+} accumulation in mitochondria is shown in

TABLE III. The effect of inhibitors of electron transfer and energy conservation as well as cation or proton conductors on the accumulation of Ca^{2+} stimulated by nigericin into mitochondria

Nigericin plus	Total nmoles of Ca^{2+} accumulated into mitochondria
(Control) Without nigericin added	48.2
Nigericin with no addition	225.4
2,4-dinitrophenol	32.2
Antimycin	31.1
Rutamycin	216.8
Rotenone	67.4
Valinomycin	28.6
Macrocyclic polyether XXXIV	38.2

The reaction mixture was identical to that from Fig. 1. The concentration of nigericin was 0.25 μg/ml. Other additions were as follows: 2,4-dinitrophenol 5×10^{-6} M; Antimycin 3×10^{-7} M; Rutamycin 1×10^{-6} M; rotenone 5×10^{-7} M; Valinomycin 1×10^{-6} M; Macrocyclic polyether XXXIV 5×10^{-5} M.

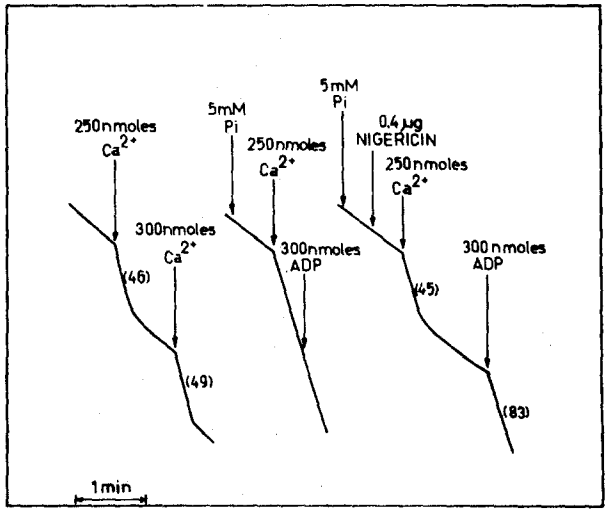

Figure 6. The effect of nigericin and Ca^{2+} on the respiration of mitochondria oxidizing β-hydroxybutyrate in the presence of phosphate. The reaction mixture contained: 10 mM triethanolamine (Cl^-) pH 7.2, 30 mM KCl, 3 mM phosphate (TEA), 5 mM β-hydroxybutyrate (TEA), 180 mM sucrose, 1.7 mg mitochondrial N, and where indicated, 0.1 μgl ml nigericin, 100 μM $CaCl_2$ and 80 μM ADP (Na^+).

Figs. 7 and 8. Figure 7 shows independent experiments on the effect of the order of addition of Ca^{2+} and nigericin on K^+ and H^+ movements as well as mitochondrial oxygen uptake. Such media was supplemented with phosphate and two different oxidizable substrates. In the presence

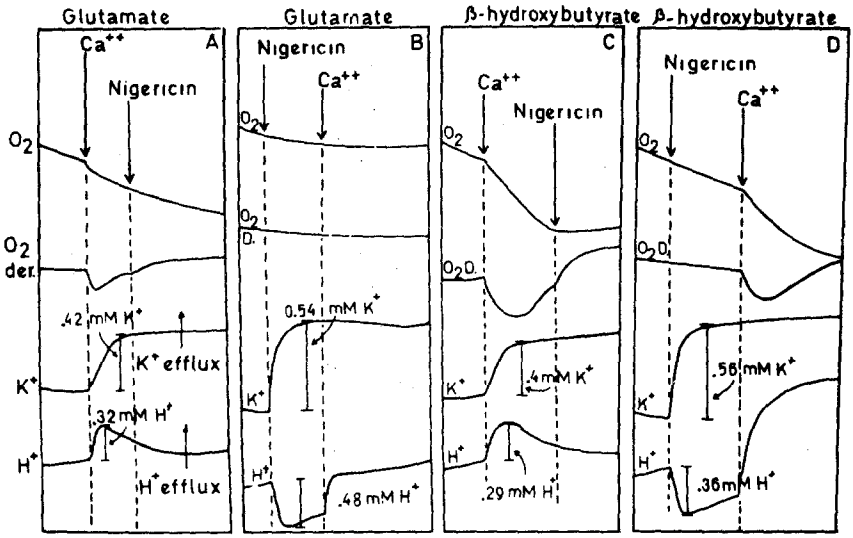

Figure 7. The effect of the order of addition of Ca^{2+} and nigericin on K^+ and H^+ movements in mitochondria oxidizing glutamate or β-hydroxybutyrate. An upward deflection of K^+ or H^+ tracings represent an increase in the concentration of these ions in the medium or efflux from the mitochondria. The reaction mixture contained: 2 mM triethanolamine (Cl^-) pH 7.4, 3 mM either glutamate or β hydroxybutyrate (TEA), 2 mM phosphate (TEA), 4 mM KCl, 180 mM sucrose, and 1.4 mg mitochondrial N, in 5 ml volume at 28°. Where indicated, Ca^{2+} and nigericin were added at a concentration of 200 μM and 0.4 μg/ml respectively.

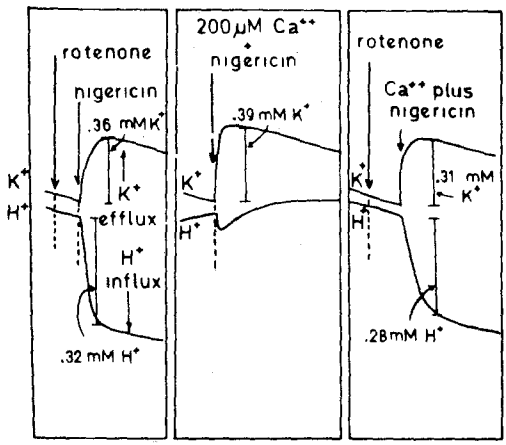

Figure 8. The effect of the simultaneous addition of Ca^{2+} and nigericin on the movements of K^+ and protons in aerobically incubated or respiratory-inhibited mitochondria. Experimental conditions were similar to those of Fig. 7 except for the addition of 1 μg/ml of rotenone where indicated.

of glutamate, 200 μM Ca^{2+} cause a short burst of oxygen uptake, a cyclic efflux of protons and a slow release of mitochondrial K^+ (Panel A, Fig. 7). The subsequent addition of nigericin does not affect the K^+ or H^+ tracings. Essentially similar results in K^+ and H^+ movements, except for a faster oxygen uptake rate, are found when Ca^{2+} is added prior to nigericin in the presence of β-hydroxybutyrate (Panel C of Fig. 7). The oxidation of this latter substrate is not inhibited by carboxylic ionophores [13, 23]. When nigericin is added prior to Ca^{2+} in the presence of glutamate (Panel B of Fig. 7), it causes a rapid release of K^+ in exchange for external protons. It also inhibits glutamate oxidation. The subsequent addition of Ca^{2+} only results in a reversal to the base line of the nigericin-mediated H^+ influx, without affecting the K^+ tracing. On the other hand, when this experiment is carried out in the presence of β-hydroxybutyrate (Panel D of Fig. 7), the addition of Ca^{2+} after nigericin causes a large and very slowly reversible proton efflux subsequent to the antibiotic-induced K^+ release. It is only with this latter oxidizable substrate that nigericin stimulates Ca^{2+} accumulation in the presence of phosphate. Under no circumstance glutamate supports Ca^{2+} accumulation in the presence of carboxylic antibiotics, nor does it facilitate H^+ efflux by Ca^{2+}, after nigericin addition (Panel B, Fig. 7). Figure 8 gives additional evidence for the possible participation of K^+ and H^+ movements on the mechanism of Ca^{2+} accumulation promoted by carboxylic antibiotics in a medium which contains phosphate. It shows that nigericin catalyzes a 1 : 1 K^+/H^+ exchange in mitochondria suspended in a medium containing β-hydroxybutyrate plus rotenone without added Ca^{2+} (Panel A, Fig. 8). In the absence of an added respiratory inhibitor, and with β-hydroxybutyrate as substrate (Panel B of Fig. 8) the simultaneous addition of Ca^{2+} and nigericin induces K^+ release from mitochondria, while Ca^{2+} completely prevents the passive net H^+ influx catalyzed by nigericin. Finally, Panel C of Fig. 8 indicates that Ca^{2+} does not affect the H^+ movements catalyzed by nigericin in the presence of β-hydroxybutyrate plus rotenone.

Discussion

Carboxylic antibiotics stimulate Ca^{2+} and phosphate uptake into mitochondria only under conditions of limited Ca^{2+} loading imposed by phosphate in media supplemented with β-hydroxybutyrate (Figs. 1-3, Tables I-II). When the latter substrate is replaced for by ATP or else by succinate, glutamate, malate, α-ketoglutarate or pyruvate as energy source for transport, the antibiotics block Ca^{2+} or phosphate accumulation. The inhibition by nigericin-like compounds of the accumulation and oxidation of substrates such as glutamate, pyruvate, malate or α-ketoglutarate, secondary or parallel to an induced-block in the penetration of phosphate [13, 16, 23, 24, 27-29] may account for

the inability of such substrates to energize Ca^{2+} accumulation by nigericin. An inhibitory effect by carboxylic antibiotics of the accumulation of ATP into mitochondria, secondary to the proton movements catalysed by the antibiotics, may also be related to their induced block of both the hydrolysis of ATP stimulated by Ca^{2+} (cf. ref. [23] Fig. 4A) and the accumulation of Ca^{2+} supported by ATP hydrolysis (Fig. 4B). On the other hand, it is likely that the ability of β-hydroxybutyrate but not succinate to support Ca^{2+} accumulation by nigericin, primarily reflects a coupling existent between the movements of the former substrate, but not succinate, with the translocation of Ca^{2+} across the membrane. Nigericin does not affect the uptake or the oxidative phosphorylation of saturating concentrations of either substrate [23, 26]. However, Kimmich and Rasmussen [30] have found that when rat liver mitochondria accumulate Ca^{2+}, only β-hydroxybutyrate, glutamate, HCO_3^- and lactate, but not all of the Krebs cycle intermediates, including succinate, served as co-ions for Ca^{2+} transport. With respect to the above-mentioned penetrant anions for Ca^{2+} movements, it is worth noting that nigericin inhibits glutamate but not β-hydroxybutyrate uptake and oxidation. Moreover, in contrast to β-hydroxybutyrate, neither lactate nor HCO_3^- could possibly serve as an energy source for ion transport in mitochondria. Thus, β-hydroxybutyrate emerges as the only possible oxidizable substrate which can fulfil the dual role of being an adequate energy source and an efficient mobile anion for facilitating the transport of Ca^{2+} stimulated by nigericin.

Previous observations by Rossi *et al.* [22] showed that, similar to nigericin-like ionophores (Fig. 6), oligomycin also prevents the uncoupling effect of concentrations of phosphate higher than 2 mM on the Ca^2 induced respiration. Furthermore, data to be described elsewhere indicate that in the presence of phosphate and β-hydroxybutyrate, and similar also to nigericin, oligomycin stimulates Ca^{2+} accumulation into mitochondria, although to a lesser extent that nigericin. The central question from this analogy is: what is the mechanism responsible for the apparent respiratory control induced in intact mitochondria by nigericin-like compounds and oligomycin? Mitchell and Moyle [31] suggested that oligomycin caused a decrease in the permeability of the mitochondrial membranes to protons. The prevention by oligomycin of the H^+ influx phase, which discretely follows the proton extrusion linked to substrate oxidation, has been invoked [31] as an important reason for its ability to couple phosphorylating respiration in submitochondrial particles [32]. In fact, it has been shown that oligomycin decreases the proton conductance of membranes from mitochondria [33], chloroplasts [34, 35] and chromatophores from photosynthetic bacteria [36]. Data from Figs. 7

and 8 of the present manuscript indicate not only that Ca^{2+} prevents the passive net proton accumulation caused by nigericin in mitochondria (Panel B of Fig. 8), but also that the combination of Ca^{2+} plus nigericin induces a marked and very slowly reversible proton outflux, higher in magnitude to the rapid cyclic H^+ release mediated by Ca^{2+} alone. It is likely that the increase in the availability of protons mediated by nigericin in the interior of the mitochondria, electrically balanced by the efflux of K^+, allows Ca^{2+} to catalyse a quantitatively more significant H^+ efflux phase. In line with this explanation is the ability of Ca^{2+} to prevent the passive internal acidification caused by nigericin in mitochondria (Fig. 8). The combined efflux of H^+ and K^+ mediated by Ca^{2+} and nigericin (Fig. 7) would also secondarily enhance the extent of the inner negative membrane potential. Thus, by a different mechanism, both nigericin and oligomycin could further stimulate the maintenance of a negative membrane potential in the presence of phosphate, Ca^{2+} and β-hydroxybutyrate. This could explain the parallel ability of both antibiotics to couple the phosphorylation of ADP in media containing Ca^{2+} and phosphate (cf. Rossi *et al.* [22] and Fig. 6). The above hypothesis could also explain the mechanism by which Ca^{2+} transport is stimulated in mitochondria by carboxylic antibiotics or oligomycin. By enhancing the extent of an internal negative membrane potential the antibiotics, and particularly nigericin-like compounds, could stimulate an electrophoretic influx of Ca^{2+} down the electric gradient. In line with this suggestion, it is possible that Ca^{2+} movements occur in parallel with the translocation of K^+ and protons catalysed by carboxylic ionophores. However, it is also very attractive to consider the alternative of the antibiotics functioning as Ca^{2+} mobile carriers across the lipid phase of the mitochondrial membrane.

The accumulation of phosphate which accompanies Ca^{2+} movements may also be accounted for by the associated internal alkalinization consecutive to the combined effects of Ca^{2+} and nigericin in proton translocation. The existence of a ΔpH across the mitochondrial membrane, parallel to the generated negative membrane potential, would be the reason for the translocation of phosphate as a proton-anion symport [5, 6] into the organelle. Evidence which supports this explanation has been given by Palmieri and Quagliariello [37], who found that in respiratory inhibited mitochondria, the prevention of anion uptake and efflux of endogenous anions were dependent upon the uptake of H^+ by the mitochondria and vice versa, H^+ ejection was responsible for stimulation of anion uptake. These findings are essentially the reverse from the suggestions by Harris and Pressman [38] and Van Dam and Tsou [39] who proposed that energization leads to the generation of a positive membrane potential in the mitochondrial interior which, in turn drives anion uptake.

References

1. F. D. Vasington and J. V. Murphy, *J. Biol. Chem.*, 237 (1962) 2670.
2. L. Saris, *Acta Chem. Scan.*, 17 (1963) 882.
3. C. S. Rossi and A. L. Lehninger, *J. Biol. Chem.*, 239 (1964a) 3971.
4. B. Chance, *J. Biol. Chem.*, 240 (1965) 2729.
5. P. Mitchell, *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, Cornwall, 1968.
6. P. Mitchell, *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin, Cornwall, 1966.
7. G. F. Azzone and A. Azzi, in: *Regulation of Metabolic Processes in Mitochondria*, Vol. 7, J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater (eds.), Elsevier, Amsterdam, 1966, p. 332.
8. C. S. Rossi, A. Azzi and G. F. Azzone, *J. Biol. Chem.*, 242 (1967) 951.
9. E. Scarpa and G. F. Azzone, *European J. Biochem.*, 12 (1970) 328.
10. D. Johnson and H. A. Lardy, in: *Methods in Enzymology*, Vol. 10, S. P. Colowick and N. O. Kaplan (eds.), Academic Press, New York, 1967, p. 95.
11. B. C. Pressman, *Proc. Natl. Acad. Sci., U.S.*, 53 (1965) 1076.
12. B. C. Pressman, in: *Methods in Enzymology*, Vol. 10, S. P. Collowick and N. O. Kaplan (eds.), Academic Press, New York, 1967, p. 714.
13. S. N. Graven, S. Estrada-O. and H. A. Lardy, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 654.
14. B. C. Pressman, *J. Biol. Chem.*, 232 (1958) 967.
15. G. Brierley, E. Murer, E. Bachman and D. E. Green, *J. Biol. Chem.*, 238 (1963) 3482.
16. S. Estrada-O. and E. Calderón, *Biochemistry (Wash.)*, 9 (1970) 2092.
17. A. B. Falcone and P. Witonsky, *J. Biol. Chem.*, 239 (1964) 1964.
18. J. B. Martin and D. M. Doty, *Anal. Chem.*, 21 (1947) 946.
19. O. Lindberg and L. Ernster, *Methods Biochem. Anal.*, 3 (1956) 61.
20. H. A. Lardy and H. Wellman, *J. Biol. Chem.*, 201 (1953) 537.
21. J. B. Sumner, *Science*, 100 (1944) 413.
22. C. S. Rossi, E. Carafoli, Z. Drahota and A. L. Lehninger, in: *Regulation of Metabolic Processes in Mitochondria*, Vol. 7, J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater (eds.), Elsevier, Amsterdam, 1966, p. 317.
23. H. A. Lardy, D. Johnson and W. McMurray, *Arch. Biochem. Biophys.*, 78 (1958) 587.
24. S. M. F. Ferguson, S. Estrada-O. and H. A. Lardy, *J. Biol. Chem.* (1971), in press.
25. C. J. Pedersen, *Fed. Proc.*, 27 (1968) 1305.
26. P. F. J. Henderson and J. B. Chapell, *Biochem. J.*, 105 (1967) 16.
27. E. J. Harris, K. Van Dam and B. C. Pressman, *Nature, London*, 213 (1967) 1126.
28. S. Estrada-O., S. N. Graven and H. A. Lardy, *Fed. Proc.*, 26 (1967) 610.
29. P. J. F. Henderson, J. O. McGivan and B. Chappell, *Biochem. J.*, 111 (1969) 521.
30. G. Kimmich and H. Rasmussen, *Fed. Proc.*, 27 (1968) 1748.
31. P. Mitchell and J. Moyle, *Nature*, 208 (1965) 1208.
32. C. P. Lee and L. Ernster, in: *Regulation of Metabolic Processes in Mitochondria*, Vol. 7, J. M. Tager, S. Papa, F. Quagliariello and E. C. Slater (eds.), Elservier, Amsterdam, 1966, p. 218.
33. M. Montal, B. Chance and C. P. Lee, *J. Membrane Biol.*, 2 (1970) 201.
34. S. J. D. Karlish and M. Avron, *Biochim. Biophys. Acta*, 153 (1968) 153.
35. L. V. Von Stedingk and H. Baltschefsky, *Arch. Biochem. Biophys.*, 117 (1966) 400.

36. M. Nishimura, K. Kadota and B. Chance, *Arch. Biochem. Biophys.*, **125** (1968) 308.
37. F. Palmieri and E. Quagliariello, *European J. Biochem.*, **8** (1969) 473.
38. E. J. Harris and B. C. Pressman, *Biochim. Biophys. Acta*, **172** (1969) 66.
39. K. Van Dam and C. S. Tsou, *Biochim. Biophys. Acta*, **162** (1968) 301.