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INHIBITION BY Sr^{2+} OF SPECIFIC MITOCHONDRIAL Ca^{2+}-EFFLUX PATHWAYS

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The effect of Sr^{2+} on the set point for external Ca^{2+} was studied in rat heart and liver mitochondria with the aid of a Ca^{2+}-sensitive electrode. In respiring mitochondria the set point is determined by the rates of Ca^{2+} influx on the Ca^{2+} uniporter and efflux by various mechanisms. We studied the Ca^{2+}-Na^{+} exchange pathway in heart mitochondria and the $\Delta\psi$-modulated efflux pathway in liver mitochondria. Prior accumulation of Sr^{2+} was found to shift the set points towards lower external Ca^{2+} both in heart mitochondria under conditions of Ca^{2+}-Na^{+} exchange and in liver mitochondria under conditions that should promote opening of the $\Delta\psi$-modulated pathway. The effect on the set point was found to be due to inhibition of Ca^{2+} efflux by Sr^{2+} taken up by the mitochondria, while Sr^{2+} efflux was too slow to be measurable.

Introduction

The distribution of Ca^{2+} between the mitochondrial matrix space and the external medium (cytosol) is influenced by factors affecting the relative rates of influx and efflux. Influx by the so-called uniporter is driven by the membrane potential, negative on the matrix side, formed on 'energisation' by respiration or ATP hydrolysis according to the chemiosmotic theory. This mechanism has high capacity, but the influx rate is low in the range of Ca^{2+} concentrations of physiological interest, e.g., about 1 μM. The set point of external Ca^{2+} is here mainly influenced by changes in the rates of specific efflux mechanisms which may be modulated (for recent reviews on mitochondrial Ca^{2+} transport, see Refs. 1–3). The uniporter has rather low specificity and is able to transport many other divalent cations as well, while nothing is known about the specificity of efflux mechanisms [1].

The best described efflux mechanism is the Ca^{2+}-Na^{+} exchange present mainly in mitochondria fom heart and other excitable tissues [1–4]. The efflux mechanisms in liver mitochondria are much less known, but it appears likely that a Ca^{2+}-H^{+} exchange mechanism is involved [1–3,5]. Recently, an increase of the set point of external Ca^{2+} concentration was found when $\Delta\psi$ was raised above 130 mV. These data were interpreted as evidence in favour of a $\Delta\psi$-modulated efflux pathway which is activated at high membrane potential [6,7].

In this study we show that Sr^{2+}, an analogue of Ca^{2+}, that is readily transported by the Ca^{2+} uniporter, is not appreciably transported by the Ca^{2+}-Na^{+} exchange mechanism in heart mitochondria, nor by the membrane potential-modulated pathway in rat liver mitochondria, and that Sr^{2+} taken

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, membrane potential; $TPMP^{+}$, triphenylmethylphosphonium ion; $pCa_o = -\log[Ca^{2+}]$ outside the mitochondrial compartment; Mops, 4-morpholinepropanesulphonic acid.

up by the mitochondria inhibits the efflux of Ca^{2+} by these pathways.

Materials and Methods

The experimental procedures and the preparation of rat liver mitochondria have been described in detail elsewhere [8]. Rat heart mitochondria were prepared in a similar way to liver mitochondria, but the homogenisation of the minced tissues was carried out with the aid of a Polytron PT 10 homogenizer for 15 s with the rheostat in position 4. ^{45}Ca and ^{89}Sr were obtained from the Radiochemical Centre, Amersham, U.K., and used in efflux studies by filtration through 0.45 μm pore-size Millipore filters.

Results and Discussion

Inhibition by Sr^{2+} of efflux of Ca^{2+} in heart mitochondria

Rat heart mitochondria were suspended in a sucrose-based medium in the presence of acetate as the permeant anion, succinate as substrate, and bovine serum albumin as a protecting agent. Fig. 1 shows that rat heart mitochondria were able to lower the $[Ca^{2+}]$ in the medium to a set point of pCa_o equal to 6.2. Addition of Na^+ (6 mM) caused an immediate shift of the signal towards a lower pCa_0, due presumably to an artefactual effect upon the electrode. This was followed by a slower decrease in pCa_o due to the efflux of Ca^{2+}. When a new set point was approached, 10 μM Sr^{2+} (broken line) or Ca^{2+} (continuous line) was added. Both additions caused an immediate signal which shows that Sr^{2+} is also sensed by the electrode. In the case of addition of Sr^{2+}, the new set point approached as the external concentration of Ca^{2+} + Sr^{2+} was lowered by the activity of the Ca^{2+} uniporter was substantially below that before addition of Sr^{2+}.

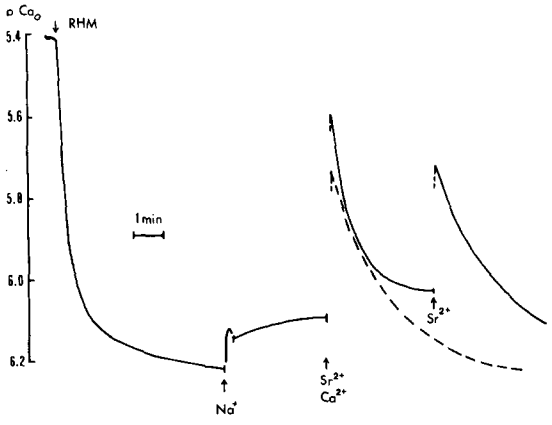

Fig. 1. Effect of Sr^{2+} on the set point for external Ca^{2+} in rat heart mitochondria. Medium: 140 mM sucrose, 40 mM choline chloride, 10 mM Tris-Mops buffer, 5 mM succinate, 10 mM potassium acetate, 2 μM rotenone, 5 μM cytochrome *c*, 1 mg bovine serum albumin/ml, pH 7.0, 30°C. Additions: rat heart mitochondria (RHM), 0.75 mg protein/ml; Na^+, 6 mM; Ca^{2+} or Sr^{2+}, 10 μM as chloride. Solid line: Ca^{2+} added as indicated. Broken line: Sr^{2+} added as indicated.

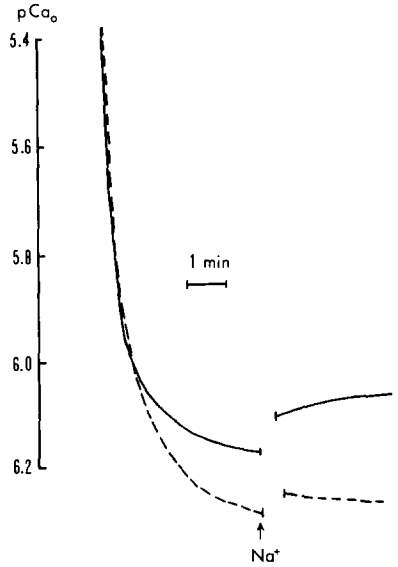

Fig. 2. Inhibition of Ca^{2+}-Na^+ exchange in rat heart mitochondria by Sr^{2+}. The experimental conditions were as in Fig. 1. Solid line: 10 μM Ca^{2+} was added before rat heart mitochondria. Broken line: 10 μM Sr^{2+} was added before rat heart mitochondria.

Addition of 10 μM Ca^{2+} (Fig. 1, continuous trace), on the other hand, caused the new set point to be shifted towards lower pCa_o, which was to be expected since the activity of Ca^{2+} in the matrix would be increased in the presence of acetate, and efflux therefore stimulated. Addition of an aliquot of Sr^{2+} at this point again led to higher pCa_o.

Fig. 2 shows a similar experiment in which 10 μM Ca^{2+} (continuous line) or Sr^{2+} (broken line) was added before rat heart mitochondria. The

effect of Sr^{2+} on the set point was as in Fig. 1 in the presence of added Ca^{2+}. In the presence of Sr^{2+} no efflux of divalent cations was seen, only the artefactual shift caused by Na^+.

It is well known that the mitochondrial Ca^{2+} uniporter has a rather low specificity and is able to transport Sr^{2+} readily [1–3]. Ca^{2+} acts as a competitive inhibitor of Sr^{2+} influx by this system, and Sr^{2+} also inhibits Ca^{2+} uptake though it could not be shown with certainty that the latter inhibition was competitive [9]. Inhibition of the uniporter would result in a set point at higher external $[Ca^{2+}]$. Since the opposite was found with Sr^{2+} present, it is obvious that the inhibition of Ca^{2+} efflux via the Ca^{2+}-Na^+ exchanger is more strongly inhibited by Sr^{2+} than the influx via the uniporter.

Fig. 2 (broken line) demonstrates that addition of 6 mM Na^+ was unable to effect efflux of accumulated Sr^{2+}, while in the absence of Sr^{2+}, Na^+ clearly caused efflux of Ca^{2+} (Figs. 1 and 2). At least under these experimental conditions Sr^{2+} was not able to be transported by the Ca^{2+}-Na^+ exchanger. If such a transport could indeed occur, it was not able to compensate for the inhibition of Ca^{2+} efflux by Sr^{2+} as shown by the set points at lower concentration of divalent cations in the presence of Sr^{2+} (Figs. 1 and 2). It thus seems evident that the Ca^{2+}-Na^+ exchanger exhibits a greater discrimination between divalent cations than does the Ca^{2+} uniporter.

Inhibition by Sr^{2+} of efflux of Ca^{2+} in liver mitochondria

Rat liver mitochondria are able to reduce the external $[Ca^{2+}]$ to approx. 1–2 μM (pCa_o 6) as shown in Fig. 3. In this experiment a sucrose-based medium without acetate was used; in addition to bovine serum albumin, Mg^{2+} was present in order to prevent an artefactual release of accumulated Ca^{2+} which may be brought about by P_i [1–3]. Such a release is seen only with much higher amounts of added Ca^{2+} than used in this study. In the control experiment (trace 1), addition of P_i caused the set point to be shifted towards lower pCa_o. Addition of a small amount of an uncoupling agent, FCCP, sufficient to lower $\Delta\psi$ slightly but not to cause complete uncoupling, then caused the set point to be shifted towards higher pCa_o. This experiment has been described before and

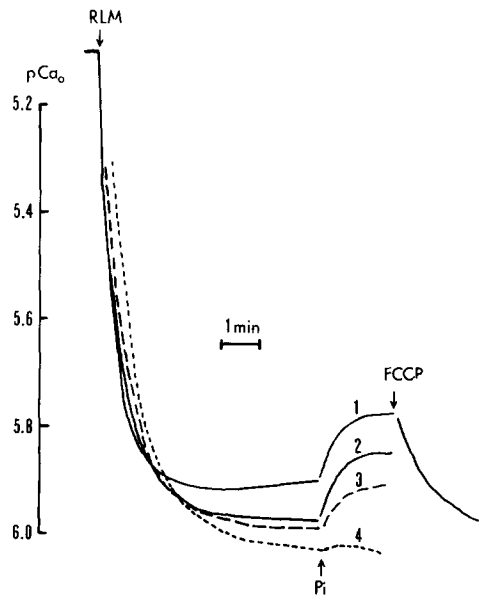

Fig. 3. Effect of Sr^{2+} on the set point for external Ca^{2+} in rat liver mitochondria. The experimental conditions were as in Fig. 1, but the acetate was omitted, and 1.5 mM $MgCl_2$ and 1 μg oligomycin/ml were present. Additions: rat liver mitochondria (RLM), 1 mg/ml; P_i, 0.5 mM; FCCP 300 nM. Sr^{2+} was present in the medium in a concentration of: (trace 1) 0, (trace 2) 5 μM, (trace 3) 15 μM, and (trace 4) 30 μM.

has been interpreted as indicating the presence of a $\Delta\psi$-modulated efflux pathway [6]. According to this interpretation, the effect of P_i is to raise $\Delta\psi$ and thereby open the pathway, while FCCP would lower $\Delta\psi$ and close it. The effect of P_i in increasing $\Delta\psi$ would be due to its effect of decreasing the transmembrane ΔpH; since the proton-motive force that can be formed on respiration stays constant, $\Delta\psi$ must rise. Other means of altering $\Delta\psi$, e.g., K^+ influx mediated by valinomycin, partial inhibition of respiration, and H^+ current through F_1-ATPase. modulate pCa_o in an analogous manner [7].

Traces 2–4 in Fig. 3 show the effect of the presence of progressively higher concentrations of added Sr^{2+}. The efflux of divalent cations following the addition of P_i was then clearly inhibited. This could be due to a direct inhibition of the pathway by Sr^{2+} as was shown for the efflux via the Ca^{2+}-Na^+ exchanger (Figs. 1 and 2). However, the effect could also be due to an effect of Sr^{2+}

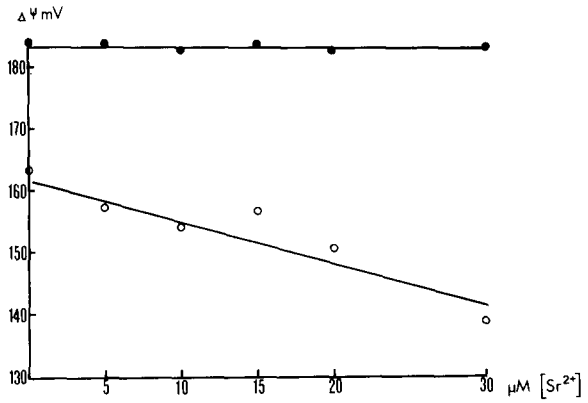

Fig. 4. Effect of Sr^{2+} on $\Delta\psi$ before and after addition of P_i. The experimental conditions corresponded to those in Fig. 3. Open circles without P_i; filled circles with P_i.

upon $\Delta\psi$. In parallel experiments $\Delta\psi$ was measured by the method of distribution of [^{14}C]TPMP$^+$ and estimation of the sucrose-impermeable space (for experimental details, see Ref. 8). Fig. 4 shows that after the addition of P_i, the $\Delta\psi$ was virtually constant at 183 mV in the presence of Sr^{2+}, while in the absence of P_i $\Delta\psi$ was progressively lowered in the presence of increasing amounts of Sr^{2+}. (The lowering of $\Delta\psi$ is likely to be due to increased alkalinisation of the matrix due to accumulation of the added Sr^{2+}. The increased ΔpH would then result in lowering of the $\Delta\psi$ component of the constant proton-motive force.) It is thus evident that the inhibition of efflux by Sr^{2+} is not due to an effect upon $\Delta\psi$. If indeed the pathway is opened to Ca^{2+} by increased $\Delta\psi$, then the inhibition of efflux likely is due to an inhibition by Sr^{2+} on the level of efflux.

Fig. 3. also shows that the set point for external $[Ca^{2+}] + [Sr^{2+}]$ prior to the addition of P_i is lowered (apparent pCa_o increased) in the presence of Sr^{2+}. This could be explained by two mechanisms. As was argued in the case of the Ca^{2+}-Na^+ exchanger and its inhibition by Sr^{2+}, this could be due to more extensive inhibition of the Ca^{2+}-efflux pathway by Sr^{2+} than of the uniporter. The other mechanism would be that as $\Delta\psi$ was progressively lowered in the presence of increasing amounts of Sr^{2+} (Fig. 4), the $\Delta\psi$-modulated pathway would be closed. Both mechanisms might contribute to the observed effect.

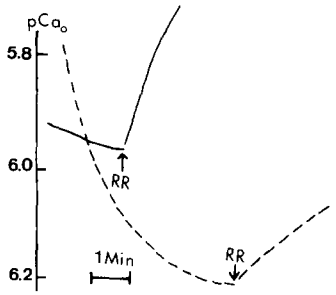

Fig. 5. Inhibition by Sr^{2+} of ruthenium red-insensitive Ca^{2+} efflux. The experimental conditions were as in Fig. 3 with P_i present. Continuous trace without, broken trace with 20 μM Sr^{2+} present. RR, ruthenium red addition 250 pmol/mg protein.

The experiments in Fig. 3 demonstrate that Sr^{2+} efflux does not occur in the presence of the highest concentration used, 30 μM Sr^{2+}. Trace 4 showed hardly any increase in the signal on addition of P_i though the electrode is responsive to Sr^{2+}. It is therefore likely that the pathway is able to discriminate between Ca^{2+} and Sr^{2+} to a considerable extent.

The inhibition by Sr^{2+} of Ca^{2+} efflux can be seen not only as an effect upon the set point of pCa_o but also as a decreased rate of efflux following the inhibition of the Ca^{2+} uniporter with ruthenium red as shown in Fig. 5. Data from

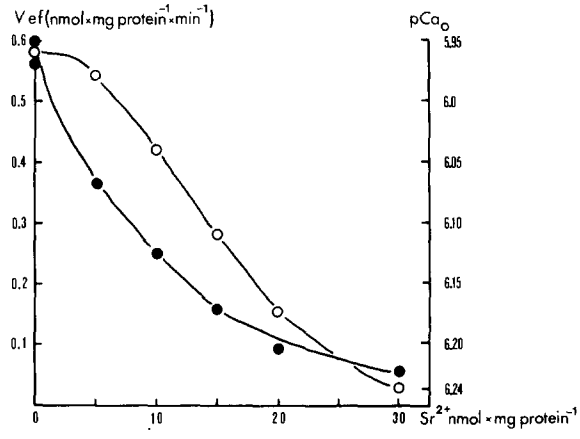

Fig. 6. The rates of Ca^{2+} efflux and pCa_0 as a function of added Sr^{2+}. The experimental conditions were as in Fig. 5. Filled circles, rate of efflux after addition of ruthenium red as nmol/mg protein per min; open circles, pCa_o.

experiments of this design with varying Sr^{2+} concentrations are plotted in Fig. 6. The rate of ruthenium red-insensitive M^{2+} efflux was drastically reduced as the amount of added Sr^{2+} was inreased. The M^{2+} efflux was due exclusively to Ca^{2+} efflux. In experiments using ^{89}Sr-labelled Sr^{2+} (5 nCi/mg protein) and a 2 min sampling after addition of ruthenium red, no efflux of Sr^{2+} could be demonstrated even after accumulation of the highest amount of Sr^{2+} used, while this amount of Sr^{2+} (30 nmol/mg protein) inhibited ^{45}Ca-labelled Ca^{2+} efflux by 84%.

Modulation of specific Ca^{2+}-efflux pathways

The physiological significance, if any, of the Na^+-stimulated efflux of Ca^{2+} in heart and other excitable tissue mitochondria still remains debatable [3]. The $\Delta\psi$-modulated Ca^{2+}-efflux pathway that only recently was described [6] seems to be absent from heart mitochondria (Bernardi, P., unpublished result). It may have a role in keeping the matrix Ca^{2+} concentration high enough for the activation of citric acid enzymes in states of active respiration [10], e.g., during phosphorylation of ADP when the $\Delta\psi$ tends to be slightly lowered. An effect of addition of ADP on the set point has been observed [7,11].

It was not clear from the original data [6] whether $\Delta\psi$ influences the Ca^{2+}-efflux pathway directly or indirectly. The experiments were carried out without oligomycin and therefore changes in $\Delta\psi$ are likely to change the phosphorylation potential. Especially the phosphorylation state of the adenine nucleotides and P_i might have special roles in controlling the Ca^{2+} fluxes (for reviews, see Refs. 1–3). However, the presence of oligomycin alone or with added ADP did not change the behaviour of the system in experiments analogous to the one in Fig. 3, trace 1 (Saris and Bernardi, unpublished resuls). It appears likely that the effect of $\Delta\psi$ is a direct one.

The finding that a Ca^{2+}-efflux pathway seems to be activated when $\Delta\psi$ rises above 130 mV [6,7] is at variance with earlier data [12] indicating a constant set point above 130 mV. This may have been due to different experimental conditions. In the study 160 mV was the highest $\Delta\psi$ measured and the last point may be interpreted as indicating a higher set point (Fig. 6 in Ref. 12).

P_i may have other effects than increasing $\Delta\psi$. It may stimulate Ca^{2+} efflux directly and may promote a spontaneous release of accumulated Ca^{2+} under experimental conditions where substantial amounts of Ca^{2+} have been added in the absence of Mg^{2+} (see Refs. 1–3). In this study the latter conditions were carefully avoided. The spontaneous release of Ca^{2+} may be due to activation of a mitochondrial phospholipase A_2 with accumulation of fatty acids and lysophosphatides which increase the permeability of the inner membrane or to activation of efflux pathways. Thus, the $\Delta\psi$-modulated pathway may be involved in the early events leading to the catastrophic increase in permeability. It is of interest that uncoupling agents at low concentrations may postpone the spontaneous release of Ca^{2+} [13] and that Sr^{2+} does not undergo such release after having been accumulated by the uniport pathway [14], but may even prevent spontaneous Ca^{2+} release (Saris, unpublished result). It is thus possible that P_i and matrix Ca^{2+} may stimulate, and Mg^{2+} and Sr^{2+} inhibit the activity of the $\Delta\psi$-modulated efflux pathway.

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