THE ROLE OF INTRAMITOCHONDRIAL P_i IN STIMULATION OF RESPIRATION BY CALCIUM AND STRONTIUM

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1. Introduction

It was first observed by Chance that calcium ions stimulate respiration reversibly in mitochondria [1]. He realized that respiratory stimulation is probably connected with the active uptake of calcium from the medium. Since that time the study of cation uptake has become an important tool for understanding of energy conservation. The electrophoretic uptake of calcium during respiration strongly supported the chemiosmotic theory according to which electron transport results in charge separation across the mitochondrial membrane [2]. Stimulation of respiration during cation uptake was later found to depend on the presence of certain anions like phosphate, arsenate, acetate or propionate [3–6]. This fact was taken as an indication that respiratory stimulation requires the simultaneous transport of both the cation and the anion. A few years ago Lehninger published convincing evidence that all the anions active in respiratory stimulation acted as proton-donors to the mitochondrial matrix; he further suggested that these anions were primarily transported into mitochondria as a consequence of electron transport while the 'pulling force' for calcium was the anion which entered with its negative charge into the matrix [7].

It follows from these facts that inhibitors of P_i transport were found to interfere seriously with the effect of calcium on respiration if P_i was the only 'permeant' anion added [8,9]. At the time when this was first reported it was also observed that inhibitors of P_i transport abolished respiratory stimulation largely but not entirely [8]. No serious consideration was given to those short-lasting respiratory 'bursts' which followed the first addition of calcium to mitochondria in the presence of P_i transport inhibitors. Although it has been noticed that intramitochondrial P_i might be the cause of the 'burst' the significance of this finding in elucidation of the mechanism by which anions stimulate respiration was overlooked.

The mechanism suggested by Lehninger, the possible importance of anions in genesis of membrane potential and also a series of some very recent reports concerning calcium uptake after inhibition of P_i transport [10–17] prompted us to investigate further the problem. The main problem was whether stimulation of respiration required transport of P_i across the membrane or the presence of P_i within the matrix. We found that when intramitochondrial P_i was varied by various means and P_i transport was inhibited by mersalyl, the stimulation of respiration by either calcium or strontium was, within limits, proportional to the intramitochondrial P_i content. We conclude that it is intramitochondrial P_i which acts as proton donor for the proton pump in calcium or strontium stimulated respiration. The inhibited state of respiration which occurs when divalent cation accumulation takes place in absence of 'permeant' anions (the state 6 of Chance, [18]) sets in when the available proton-donating species of P_i was expended.

2. Methods

Rat liver mitochondria were prepared as in [19]. Respiration was recorded at 25°C using a Clark type

electrode. Mitochondria equivalent to either 6, 9 or 12 mg protein were added to final vol. 2.3 ml medium which contained 241 mM sucrose, 15 mM Tris–HCl, 3 mM $MgCl_2$ and 1 μM rotenone, at pH 7.0. Tris–phosphate (2.1 mM) and Tris–succinate (5.3 mM) were added either before the mitochondria or during the course of the experiment. When added, $CaCl_2$ and $Sr(NO_3)_2$ were added to the respiring suspension. Sr^{2+} was preferably used in this study because it could be given repeatedly and in larger amounts without causing irreversible stimulation of respiration [20].

For determination of the P_i content, a 2 ml aliquot was withdrawn from the thermostated polarographic cell and carefully layered onto 6 ml ice-cold 0.5 M sucrose containing 50 μM mersalyl. The tube was immediately centrifuged for 90 s (following acceleration) at 18 000 × *g* in a Janetzki K 24 refrigerated centrifuge. The supernatant was discarded, the sediment rinsed with cold 0.25 M sucrose and extracted with 1.5 ml 20% trichloroacetic acid at 0°C. The P_i content of the extract was determined colorimetrically using the method in [21].

Table 1
Effect of incubation conditions on the inorganic phosphate content of mitochondria

Sequence of additions	P_i (nmol/mg protein)
Mersalyl, P_i, succinate	17.4
Mersalyl, P_i, succinate, ADP, oligomycin	12.8
P_i, succinate, mersalyl	22.2
P_i, succinate, mersalyl, ADP, oligomycin	15.2
Valinomycin, mersalyl, P_i, succinate	13.3

Mitochondria (5.1 mg protein/ml) were incubated at 25°C in the medium described in section 2. Additions: 2.1 mM Tris–phosphate, 5.3 mM Tris–succinate, 16.7 nmol mersalyl/mg protein, 20.8 nmol ADP/mg protein, 1.25 μg oligomycin/mg protein, and 42 ng valinomycin/mg protein

3. Results

3.1. *Experimentally-induced changes of intramitochondrial P_i content*

The P_i content of freshly isolated mitochondria suspended in the sucrose medium in the presence of mersalyl varied from 13.0–20.5 nmol/mg protein. Under comparable conditions av. 9.8 nmol was found [22] and in single experiments ~20 nmol [23]. If mitochondria were incubated first in the presence of P_i and succinate and mersalyl was added only later, P_i was accumulated (table 1).

Mitochondria were depleted of their P_i content by two procedures. In the first of these P_i movements were inhibited by mersalyl either before or after P_i accumulation, and then ADP was added. This resulted in P_i depletion (table 1): if the starting P_i content was sufficiently high, ADP addition stimulated respiration briefly. Even after repeated ADP additions there was never a total depletion of intramitochondrial P_i, the lowest value observed being 8.4 nmol/mg protein.

In the second procedure mitochondria were suspended in buffered sucrose (containing also rotenone to inhibit endogenous respiration) in the presence of valinomycin. This latter caused a loss of endogenous K^+ (not shown) and P_i. After a brief incubation period mersalyl and succinate were added to inhibit re-uptake of P_i and to start respiration. The depletion under these conditions was similar to that caused by ADP (table 1).

With these manipulations the P_i content of mitochondria could be varied from 8.4–23.2 nmol/mg protein.

3.2. *Stimulation of respiration by calcium or strontium after inhibition of P_i transport at different intramitochondrial P_i levels*

Addition of small amounts of either calcium or strontium to respiring mitochondria which have an intact P_i-transporting system and P_i is present, results in stimulation of respiration (burst). The 'extra' oxygen consumption during the fast phase of respiration is stoichiometrically proportional to the divalent cation added. The calcium:oxygen resp. strontium:oxygen ratio was determined in each preparation and was found to be close to the maximum value of 4, characteristic for succinate oxidation. If P_i transport was inhibited by mersalyl, calcium or strontium caused a much smaller stimulation of respiration as

compared to the control: the 'extra' oxygen consumption was not stoichiometric with the calcium or strontium added but paralleled the P_i content of the mitochondria. After accumulation of P_i the 'extra' oxygen consumption became somewhat larger. Following depletion of P_i by prior addition of ADP the 'extra' oxygen consumption was smaller. When P_i transport was restored by addition of the thiol compound dithiothreitol (DTT) the missing portion of the 'extra' oxygen consumption reappeared immediately. Not only the amount of 'extra' oxygen consumption but also its rate went parallel with the P_i content of mitochondria. It should be noted that added calcium or strontium was still able to stimulate respiration when the added ADP had no more effect on the rate of oxidation.

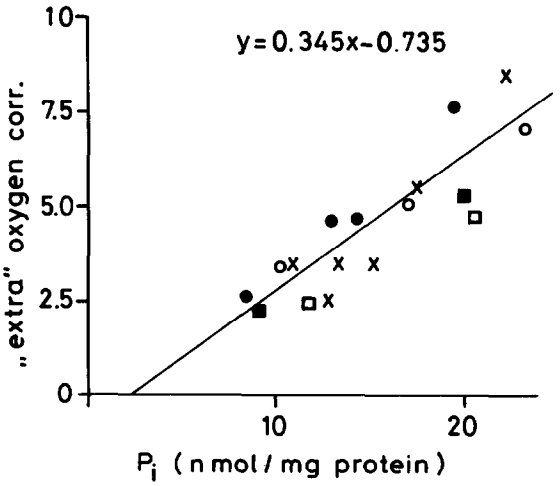

Fig.1. The relationship between 'extra' oxygen consumption and the P_i content of mitochondria. 'Extra' oxygen consumption was initiated by addition of 50 nmol strontium nitrate/mg protein to mitochondria in which P_i transport was inhibited by 16.7 nmol mersalyl/mg protein. The 'extra' oxygen values found in the different experiments were normalized. A correction factor was separately determined in the absence of mersalyl (i.e., with P_i transport intact). The factor for correction was:

$$\frac{Sr^{2+}\text{: 'extra' oxygen consumption found}}{4}$$

The P_i content of the mitochondria was charged as described in section 3.1. The different symbols (□, ○, X, ●, ■) refer to different experimental runs.

In fig.1 the 'extra' oxygen consumption data following strontium addition were plotted against the P_i content of mitochondria measured before the addition of strontium. Because the strontium:oxygen ratios of the different mitochondrial preparations used to construct fig.1 varied slightly, the 'extra' oxygen consumption values were normalized as described in fig.1 legend. There was a linear relation between the P_i content and the corrected 'extra' oxygen consumption. This linear relation was also present if the data were not corrected (data not shown): although the scattering of data was larger, the correlation was still significant. The intercept of the calculated regression line with the X axis indicates that strontium does not increase respiration if the intramitochondrial P_i value is < 2.2 nmol/mg protein.

There is one special condition when no correlation was found between the intramitochondrial P_i content and the 'extra' oxygen consumption. In the presence of P_i transport valinomycin plus K^+ stimulate respiration [5]; mercurials inhibit this latter strongly [8,9]. If valinomycin plus K^+ are given to respiring mitochondria in the presence of mersalyl, respiration is increased temporarily and after this an inhibited state of respiration sets in. This inhibited state resembles 'state 6' of Chance [18]. In this 'state 6'-like condition added strontium did not accelerate respiration although the P_i content of the mitochondria was identical with the respective control. Restoration of P_i transport by DTT immediately started fast respiration.

4. Discussion

Lehninger proposed the following sequence of events in calcium accumulation [7]:

1. H^+ is ejected as part of the charge separation process;
2. P_i is transported into the mitochondria as result of exchange with intramitochondrial OH^-;
3. The negative charge excess of the transported P_i causes electrophoretic movement of calcium into the mitochondria.

It seems probable that the negative charge excess can be generated by intramitochondrial P_i without transport. We suggest that it is intramitochondrial P_i

which can function as H^+ donor for the proton ejection process: the respiration can be stimulated by calcium or strontium only as long as this internal H^+-ion donor is available. Further, if P_i transport is inhibited, it is the deprotonated species of phosphate anions which causes electrophoretic movement of cations. This hypothesis is supported by the fact that under a variety of conditions stimulation of respiration by either calcium or strontium went parallel with the P_i content of mitochondria. The only exception of this parallel was when the transport of K^+ (in the presence of valinomycin) already expended the intramitochondrial $P_i H^+$ donor potential.

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