DEPENDENCY ON Ca^{++} OF ATP-STIMULATED UNCOUPLED OXIDATION OF SUCCINATE IN RAT LIVER MITOCHONDRIA

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Received November 15, 1971

Rat liver mitochondria depleted of endogenous substrates and high-energy phosphate bonds require exogenous ATP for the maximal uncoupled oxidation of succinate. The present experiments using a mannitol sucrose medium indicate that the effect of ATP is dependent upon the presence of low concentrations of Ca^{++} (less than 5×10^{-6} M). The requirement for Ca^{++} was obvious irrespective of the presence or absence of Mg^{++}, and of the species of anions (Cl^-, HPO_4^{--}, $HAsO_4^{--}$, or acetate) in the buffer. Sr^{++}, but not Mn^{++} could replace Ca^{++}. The Ca^{++}-ATP effect was not obtained in the uncoupled oxidation of NAD-dependent substrates or of ascorbate in the presence of tetramethyl-P-phenylenediamine.

The results of a kinetic analysis of the uncoupled succinate oxidation and of experiments with reagents which interfere with the mitochondrial Ca^{++} translocation suggested that, in the presence of ATP, Ca^{++} after binding on the outer surface of the inner membrane enhanced the mitochondrial succinoxidase activity by lowering the apparent Km for succinate.

In mammalian organs, succinoxidase and one of its major subunits, succinic dehydrogenase, have been assigned a position on the inner mitochondrial membrane where they are thought to serve as an integral part of the membrane structure (1). Accumulated evidence has indicated that the activity of succinic dehydrogenase is under the influence of such various but functionally related factors as substrates (succinate and fumarate), products of the connected reactions (oxalacetate and ATP) and the redox state of pyridine nucleotides and coenzyme Q (2-4). In view of these facts and of the functions performed by this enzyme system in the Krebs' cycle and in the electron transport linked phosphorylation reaction, it is suggested that succinoxidase is one of the crucial regulatory enzymes functioning in the control of aerobic metabolism of mitochondria (2,4).

The present communication describes experimental results indicating that Ca^{++} can be added to the list of the possible factors which may participate in the intracellular control of this regulatory enzyme system. These results are then discussed in relation to the hypothesis that the cellular Ca^{++} flux serves as a mediator in the control of various aspects of cell function.

MATERIALS AND METHODS. Male Wistar rats, weighing 100 to 150 g, were used. Liver mitochondria were prepared by a slight modification of the procedure of Rasmussen and Ogata (5). During the isolation procedure, mitochondria were washed thrice with 0.37 M sucrose + 0.5 mM Tris EDTA (pH 7.0). They were then preincubated at room temperature for 20 min. in an aerobic medium (pH 7.4) consisting of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-Cl, 1 mM $MgCl_2$, 3 mM Tris EDTA, 1 mM Tris AMP, and 0.1 mM 2,4-dinitrophenol (DNP), to deplete endogenous substrates, ATP and Ca (cf. Ref 6), and were finally suspended in 0.37 M sucrose. This mitochondrial preparation, when incubated in a suitable reaction medium, exhibited a satisfactory P : O ratio and respiratory control (P : O, 1.5~1.6, and respiratory control ratio, 5.0~6.0, for 13.3 mM succinate as substrate). The mitochondria thus prepared (hereafter, described as "depleted mitochondria") contained less than 2.5 μ moles of Ca per gram protein. Incubations were performed at 22°. Oxygen consumption was monitored with a Clark type oxygen electrode (Yellow Spring Instrument Co.). Mitochondrial content of elemental calcium was assayed with an atomic absorption spectrophotometer (Hitachi, 207), after extraction with 5% trichloroacetic acid. Mitochondrial protein was estimated by the use of biuret reaction, in which bovine serum albumin served as standard. ATP was generously donated by the Kowa pharmaceutical Co. The other reagents were of the highest purity available commercially.

RESULTS AND DISCUSSION. Rat liver mitochondria that were depleted of considerable portions of their endogenous ATP (6) and Ca^{++} showed a suppressed oxygen consumption with succinate. This was the case even when the respiration was released with an uncoupling concentration (0.1 mM) of DNP (Fig. 1). In a Ca^{++} supplemented medium, the succinate oxidation was markedly enhanced by a later addition of ATP (Fig. 1.A). This finding is in concert with the results of Azzone and Ernster which showed that ATP induced an activation of uncoupled oxidation of succinate in an ATP (but not Ca) depleted rat-liver mitochondria (3). However, this ATP effect did not develop in the absence of Ca^{++} (Fig. 1.B and C). The addition of Ca^{++} before (Fig. 1.A) or after (Fig. 1.B) ATP permitted the ATP-induced uncoupled respiration to proceed at a remarkable rate. These statements hold whether the reaction medium was or was not supplemented with an appropriate concentration (3 mM) of Mg. The ATP-Ca requirement was not observed in the uncoupled oxidation of comparable concentrations of NAD-dependent substrates (β-hydroxybutyrate, malate ± pyruvate, or glutamate ± malate), or of 3 mM ascorbate in the presence of 0.1 mM tetramethyl-P-phenylenediamine.

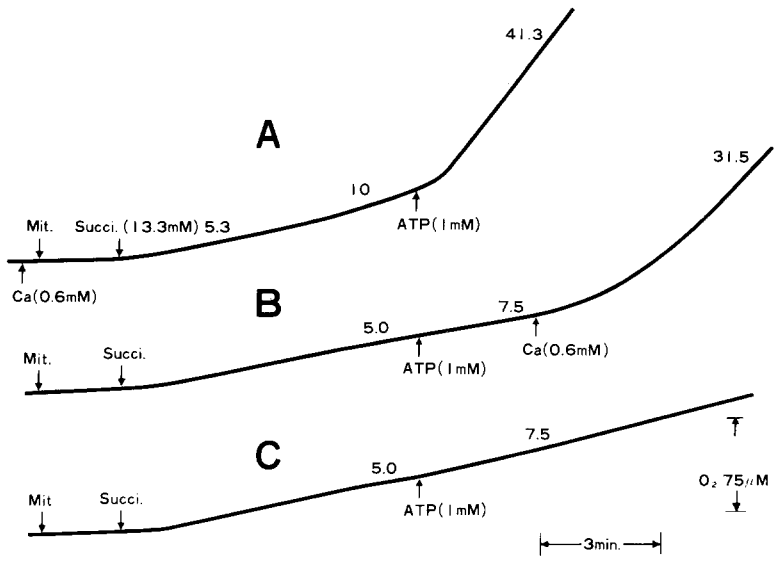

Fig. 1. Effects of Ca^{++} and ATP on uncoupled succinate oxidation by depleted mitochondria.

Incubations were performed with a reaction medium of the following composition in a final volume of 3 ml : 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris Cl (pH 7.4), 3 mM $MgCl_2$, 15 mM Tris HPO_4 and 0.1 mM DNP. The respiratory activity was followed polarographically. The numbers above each line indicate rates of oxygen consumption (mµ moles O_2 per ml per min) at the moments in time indicated.
"Depleted" mitochondria (final concentration, 1.1 mg protein per ml) were prepared as indicated in METHODS and used for three sets of experiments. Succinate (13.3 mM) was added early in each experiment, and ATP (1 mM) was added midway through each experiment. $CaCl_2$ (0.6 mM) was added only to "A" and "B" at the times indicated by the arrows.

Therefore, succinic dehydrogenase is the most likely site of activation by ATP and Ca^{++}. The permissive effect of Ca^{++} in the ATP-induced uncoupled oxidation of succinate (hereafter, described as "Ca effect") was reproduced by comparable concentrations of Sr^{++}, but not by Mn^{++}.
The Ca effect apparently does not depend on the presence of a particular anion. This cation effect was demonstrable whether chloride, acetate, arsenate or phosphate served as the buffer anion.

As shown in Fig. 2, the Ca effect was demonstrable between pH 7.0 and 8.0, and in this pH range, a clear dose-response relationship was indicated. Furthermore, a significant response of considerable reproducibility was observed with concentrations of $CaCl_2$ as low as 5×10^{-6} M. Since the system contained 1 mM ATP, 13.3 mM succinate, and 15 mM phosphate, all of which could avidly trap Ca^{++}, the minimum Ca^{++} activity necessary for the effect should have been lower than 5×10^{-6} M.

Closer examination of the relationship, in the presence or absence of $CaCl_2$, of the rate of the ATP-induced uncoupled oxidation and the succinate

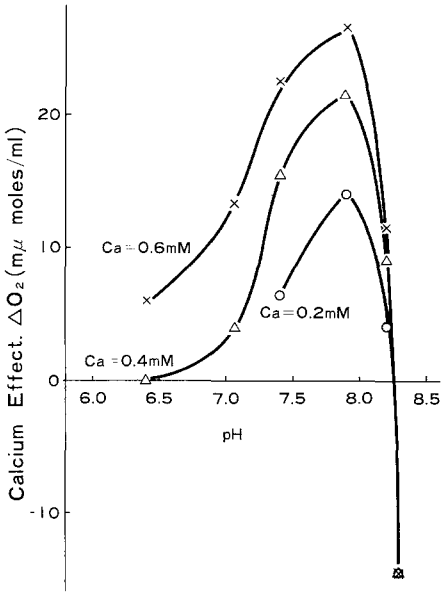

Fig. 2. Medium pH and Ca^{++} stimulation of uncoupled succinate oxidation. The depleted mitochondria were incubated as in Fig. 1. "A" and "C" except that various concentrations of $CaCl_2$ and H^+ were employed. The medium pH was controlled by adding appropriate amounts of Tris base or HCl. As the Ca effect (ordinate) is described the increase in oxygen consumption due to the presence of Ca^{++}, 4 min. after the ATP addition. Mitochondrial concentrations, 0.9 mg protein per ml.

concentration (Fig. 3) revealed that the mechanism of the Ca effect was to lower the apparent Km value of succinoxidase for succinate. In this respect, the Ca effect differs from the enzyme activation induced by the substrates (7). The Ca effect was still demonstrable when the medium was added with rotenone (1 μg/ml) to prevent oxalacetate accumulation.

The ATP-Ca requirement was lost when the mitochondrial preparation was treated with Triton X (0.5 mg/ml). In keeping with Chappell and Crofts (8), the ATP action was prevented by atractyloside which selectively inhibits mitochondrial adenine nucleotide translocase. The Ca effect was abolished when the Ca^{++} binding to the surface of the inner membrane was blocked by a higher concentration (100 mM) of KCl (9). However, the treatment of mitochondria with La^{+++} (200 μM) or Pr^{+++} (64 μM) that should have inhibited the Ca^{++} translocase activity in the inner membrane (10, 11), did not abolish the effect of 5 ~ 400 μM Ca^{++}. Thus, it appears that the ATP-Ca requirement is a membrane structure-dependent property, and that Ca^{++} acts from outside and ATP from inside of the inner membrane.

The cytosol Ca^{++} concentration is considered to range between 10^{-8} and 10^{-6} M (12). If the present effect of Ca^{++} takes place on the outside of mitochondria, the present dose-related Ca effect may have potential physio-

logical significance. A similar observation was made by Carafoli and others

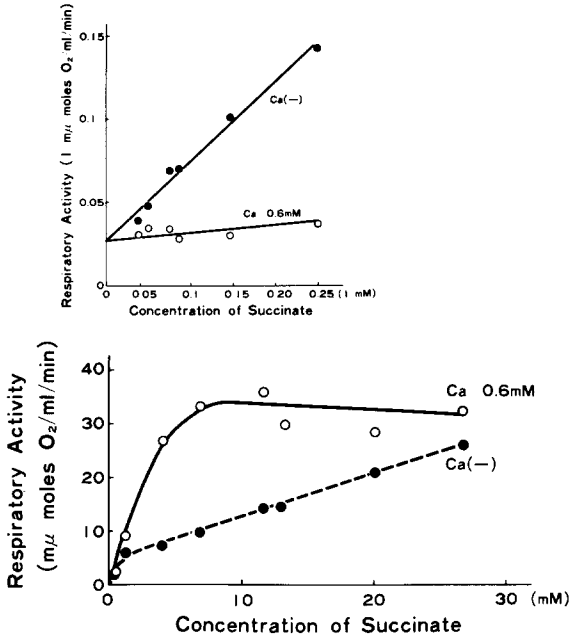

Fig. 3. Effects of substrate concentration and addition of $CaCl_2$ on ATP-induced uncoupled oxidation of succinate.
The depleted mitochondria were incubated as in Fig. 1. "A" and "C", except that KCl in the medium was 5 mM and Tris succinate was added to concentrations as indicated on the abscissa. The "Respiratory Activity" on the ordinate represents the rates of oxygen consumption that were induced by ATP 3 min. after the addition.
Inserted are the Lineweave-Burke plots of the same data.
Mitochondrial concentrations, 1.0 mg protein per ml.

in blow-fly flight muscle mitochondria; Ca^{++} from outside activated another inner membrane-bound enzyme, glycerol-1-phosphate dehydrogenase (e.g., Ref. 13). These facts suggest that alterations in the cytosol concentration of Ca^{++} may regulate a key step in the mitochondrial metabolism of Krebs' cycle intermediates. This view as well as the accumulated evidence that, in response to various stimuli, alterations occur in the Ca^{++} flux across the cell membrane and in the Ca^{++} distribution among subcellular compartments (12, 14), are in keeping with the concept that Ca^{++} is an important factor in the control of intracellular metabolism (14). Further details of the present study will be described in a separate paper.

The authors wish to thank Dr. H. Rasmussen of University of Pennsylvania, U.S.A., for his help in various facets of the present research.

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