Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 173-179

DUAL EFFECTS OF CALCIUM ON THE OXIDATION OF FATTY ACIDS TO KETONE BODIES IN LIVER MITOCHONDRIA

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Received August 23, 1983

The addition of calcium ions (Ca^{2+}) to rat liver mitochondria, under conditions of rapid accumulation of 10-40 nmol Ca^{2+}/mg protein, inhibited the oxidation of long and medium chain fatty acids to ketone bodies, whereas higher quantities of Ca^{2+} activated the process. The mitochondrial NADH:NAD ratio exhibited corresponding depression and elevation. Both inhibitory and stimulatory actions of Ca^{2+} were operative in liver mitochondria from fed and fasted rats and appear to be localized in the mitochondrial inner membrane-matrix region. These observations may signify involvement of Ca^{2+} in the regulation of fatty acid oxidation and ketogenesis.

Earlier reports from this laboratory have described an activating effect of calcium ions (Ca^{2+}) on fatty acid oxidation in isolated rat liver mitochondria (1-3) and in liver cells (1,4,5). In these studies, high concentrations of Ca^{2+} were found to be inhibitory and, in addition, a slight inhibitory effect was always observed at low concentrations of Ca^{2+} in the mitochondrial system (1,2). Under different experimental conditions, it has recently been found that very low concentrations of free Ca^{2+} increase fatty acid oxidation by rat liver mitochondria and higher levels decrease the reaction rate (6). Recent titrations of rat liver mitochondria with Ca^{2+} here have exhibited an inhibitory phase, at low concentrations of total Ca^{2+}, of far greater magnitude than hitherto observed (1,2). These new observations add another dimension to the influence of Ca^{2+} on fatty acid oxidation and ketogenesis at the mitochondrial level and suggest the possibility of dual effects of Ca^{2+} on this oxidative process in the intact hepatocyte.

Materials and Methods

Animals. Adult male Holtzman rats [Crl:CD H(SD)BR], 300-450 g, from Charles River Breeding Laboratories (Wilmington, MA) were given water and Purina Laboratory Chow ad libitum. Food was removed from fasted rats 20 h prior to experiment.

0006-291X/83 $1.50

Isolation of mitochondria. Liver mitochondria were isolated and suspended in 0.25 M sucrose, 3 mM Tris/chloride (pH 7.4) at 0°C, at a concentration of 20-30 mg protein/ml, as previously described (2).

Assays. Protein was assayed by the dye-binding method of Bradford (7), which provides the same results as the biuret procedure (8). Ketone bodies (2) and ATP (2) were analyzed in perchloric acid extracts (4).

Incubation. The mitochondrial suspension (0.2 ml) was incubated with 1.0 ml of 1.5 mM palmitate - 3% bovine serum albumin (fatty acid free), pH 7.4, and 0.8 ml of mixed medium. The following final concentrations were incubated 10 min at 37°C: 0.45 mM ATP, 0.4 mM L-carnitine, 60 mM KCl, 10 mM potassium phosphate, 5 mM $MgCl_2$, 20 µM CoA, 1 mM dithiothreitol, 125 mM sucrose, 1.5 mM Tris/Cl, 0.75 mM palmitate and 1.5% albumin. Fatty acids are oxidized almost exclusively to ketone bodies under these conditions (2). The final volume was 2.0 ml and the pH was 7.2-7.3. This system is the same as that employed previously (1,2) except that the final concentration of sucrose in the prior studies (1,2) was 156 mM and the Tris/Cl was 1.88 mM. Aqueous solutions of $CaCl_2$ were added to achieve the final (zero time) concentrations shown. Octanoate, at a final concentration of 1.5 mM, replaced palmitate in some experiments. The incubation was started by adding 0.2 ml of mitochondrial suspension to the otherwise complete system which was then incubated in a Dubnoff water bath, 37°C, at 90 oscillations/min. The incubation was terminated with perchloric acid (4).

Results and Discussion

The addition of Ca^{2+} to rat liver mitochondria inhibited ketogenesis in the 25-100 µM range of Ca^{2+} (Fig. 1). At the concentration of mitochondria employed (5.3 mg protein in 2 ml total volume), inhibition was observed with approximately 10-40 nmol Ca^{2+}/mg protein. Higher concentrations of Ca^{2+} in the range 300-500 µM, or 110-190 nmol Ca^{2+}/mg protein, caused stimulation as previously reported (1-3). The oxidation of [I-^{14}C]palmitate to total perchloric acid soluble products exhibited the same degrees of inhibition and activation, indicating that ketogenesis (Fig. 1) reflected the rate of β-oxidation.

The inhibition of fatty acid oxidation by Ca^{2+} (25-100 µM) was substantial; only 20% of the control rate was observed at 100 µM Ca^{2+}. Previously, only slight inhibition with this amount of Ca^{2+} was observed (1,2). The greater inhibition in the present study may be related to the higher control rate of fatty acid oxidation, caused by the slightly lower osmolarity in the present system (285 mOsm) than employed before (310 mOsm). Decreased osmolarity is known to enhance the rate of ketogenesis (3).

These data (Fig. 1, curve) were obtained with mitochondria from fasted rats. Mitochondria from fed rats exhibited the same control rate and Ca^{2+}-

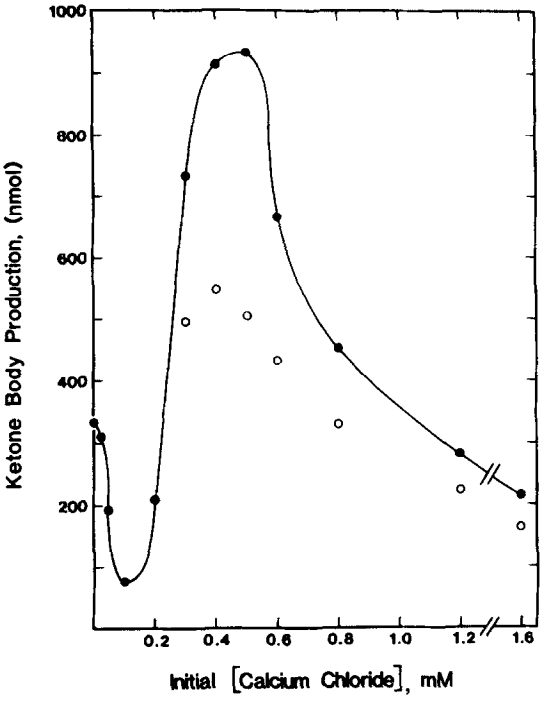

Fig. 1. Influence of calcium ions on fatty acid oxidation by liver mitochondria. Mitochondria were incubated with albumin-bound palmitate 10 min at 37°C. The reaction was terminated with $HClO_4$. Palmitate was oxidized almost exclusively to ketone bodies (acetoacetate + β-hydroxybutyrate) under the conditions employed. The curve illustrates ketogenesis by liver mitochondria (5.3 mg protein) from fasted rats (●). Liver mitochondria from fed and fasted rats produced ketone bodies at similar rates, when incubated with 0-0.2 mM Ca^{2+} (not shown); when incubated with 0.3-0.8 mM Ca^{2+}, the liver mitochondria (5.2 mg protein) from fed rats (○) produced less ketone bodies.

induced inhibition, although activation at higher levels of Ca^{2+} was not as great (Fig. 1, open circles).

The inhibition of fatty acid oxidation by 25-100 μM Ca^{2+} (Fig. 1) was not caused by a deficiency of ATP for fatty acid activation, as a result of energy-dependent Ca^{2+} transport (10,11), since higher amounts of Ca^{2+} (300-500 μM) were not inhibitory. In addition, an energy equivalent of only 100 nmol of ATP is required for the transport of 200 nmol Ca^{2+} (100 μM, 2 ml) and the system contained 900 nmol ATP at zero time. Also, after incubation for 10 min the concentrations of ATP in the mitochondrial systems which contained 0-500 μM Ca^{2+} (Fig. 1) were found to be similar by direct assay (data not shown).

The β-hydroxybutyrate:acetoacetate ratio (Fig. 2) paralleled the rate of fatty acid oxidation (Fig. 1). This relationship is mediated by the produc-

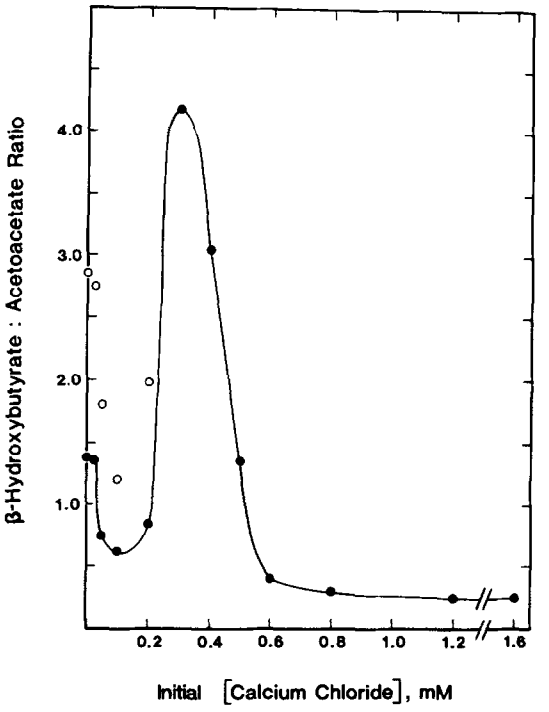

Fig. 2. Influence of calcium ions on the oxidation-reduction state of the mitochondrial pyridine nucleotides. Data are from the experiments described in Fig. 1. The curve illustrates the β-hydroxybutyrate:acetoacetate ratio in mitochondria from fasted rats (●). The mitochondrial NADH:NAD ratio was greater in the mitochondria from fed rats (○), when incubated with 0-0.2 mM Ca^{2+}. The ratio was similiar in mitochondria from fed and fasted rats incubated with 0.3-1.2 mM Ca^{2+}.

tion of NADH by β-oxidation (9). The inhibitory phase of fatty acid oxidation at 25-100 μM Ca^{2+} was accompanied by a corresponding depression in the NADH:NAD ratio. For a given rate of ketogenesis by mitochondria from fed rats (Fig. 1), a higher β-hydroxybutyrate:acetoacetate ratio was produced, throughout the range of inhibition (25-100 μM Ca^{2+}) and stimulation (300-500 μM Ca^{2+}) (Fig. 2). The inhibition of ketogenesis and the collapse of the NADH:NAD at Ca^{2+} concentrations above 800 μM (Fig. 1,2) are caused by Ca^{2+} overloading, structural damage and disruption of mitochondria (10,11).

The uptake of Ca^{2+} by liver mitochondria is very active in the presence of permeant anions such as phosphate (10,11). The rate of Ca^{2+} uptake under the present conditions is approximately 75 nmol/mg protein/min (2). Therefore, throughout the range of inhibition (25-100 μM initial Ca^{2+}) almost complete removal of added Ca^{2+} by the mitochondria probably occurred in the first

minute and, at the highest degree of activation (300 μM initial Ca^{2+}), in 3 minutes. The residual level of extramitochondrial Ca^{2+} throughout the initial concentration range examined (Fig. 1) has not been determined. Since ruthenium red blocks the activation of fatty acid oxidation by Ca^{2+} (2), presumably by blocking Ca^{2+} transport into the mitochondria (12), it is the accumulated Ca^{2+} rather than the extramitochondrial Ca^{2+} which apparently exerts this action. We have observed that ruthenium red also blocks the inhibitory action of 25-100 μM Ca^{2+}. The relation between the inhibition and activation of fatty acid oxidation by Ca^{2+} (1,2, Fig. 1) and those observed by others (6) are not yet clear, in view of the markedly different conditions employed. The presence of EGTA (6) provides low concentrations of free Ca^{2+}, slower rates of Ca^{2+} transport and accordingly less Ca^{2+} in the mitochondrion during a short incubation period.

Activation of fatty acid oxidation by Ca^{2+}, at initial concentrations of 300-500 μM (Fig. 1), and the associated reduction of mitochondrial pyridine nucleotides (Fig. 2) was previously found to be operative with octanoate (1) as well as palmitate substrate (2). Inhibition of fatty acid oxidation to ketone bodies by initial concentrations of 25-100 μM Ca^{2+} (Fig. 1) and the corresponding decrease in the mitochondrial NADH:NAD ratio (Fig. 2) also occurred when octanoate replaced palmitate as the fatty acid substrate (data not shown). This decrease in the reduction of mitochondrial NAD indicates that inhibition of fatty acid oxidation by Ca^{2+} was not caused by inhibition of the utilization of reducing equivalents (generated by β-oxidation) by the respiratory chain, since this would have caused NADH accumulation. Since octanoate oxidation is carnitine-independent, it therefore appears that the site of the inhibitory action of Ca^{2+} (at initial concentrations of 25-100 μM) is the β-oxidation sequence or the conversion of acetyl-CoA, produced by β-oxidation, to ketone bodies.

The enzymes of β-oxidation and ketone body production are located within mitochondrial inner membrane-matrix particles (13,14). The observed actions of Ca^{2+} (Fig. 1,2) are therefore presumeably operative at the inner surface of

the mitochondrial inner membrane or in the matrix compartment. Blockade of these actions of Ca^{2+} by ruthenium red (above) supports this concept.

Perfused livers from fed rats produce about 220 nmol of ketone bodies/g wet wt/min and from fasted rats about 500 nmol/g wet wt/min (15,16). The liver contains 50-60 mg mitochondrial protein/g wet wt (17,18). Mitochondria in liver cells therefore produce 3-10 nmol of ketone bodies/mg mitochondrial protein/min. This corresponds to 150-500 nmol/5 mg mitochondrial protein/10 min, which is the range observed in the current studies (Fig. 1). Therefore, under the conditions employed, the isolated mitochondria produced ketone bodies at rates similiar to those operative under physiological conditions.

Mitochondria in hepatocytes appear to contain 10-20 nmol of Ca^{2+}/mg of mitochondrial protein (19). The mitochondria incubated with up to 50 μM Ca^{2+} (Fig. 1) may therefore contain physiological amounts of Ca^{2+}. Rapid and almost complete uptake of Ca^{2+} occurs under the conditions employed (2). The present observations suggest the possibility that under certain conditions Ca^{2+} may exert two separate and opposite effects on the mitochondrial β-oxidation of fatty acids to ketone bodies in the liver. Inhibition and stimulation of the process may be in part governed by the preexisting mitochondrial Ca^{2+} content and by the nature of the change in Ca^{2+}, i.e. whether characterized by net Ca^{2+} uptake or efflux (20-22). Clearly, further studies are needed to determine the mechanisms and physiological significance of these actions of Ca^{2+} on fatty acid metabolism.

Acknowledgements

This work was supported by Grant HL 23181 from the United States Public Health Service.

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