CALCIUM-MEDIATED ALTERATIONS IN THE OXIDATION–REDUCTION STATE OF PYRIDINE NUCLEOTIDES IN ISOLATED LIVER CELLS

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1. Introduction

In isolated mitochondrial systems calcium ions cause pyridine nucleotide oxidation and increased oxygen consumption, concurrent with energy-dependent calcium accumulation [1,2]. A variety of oxidizable substrates support this process. In the absence of phosphate or other actively permeant anions, calcium causes reduction of cytochrome *b*, described as State 6 [3,4], and, when ATP is present, pyridine nucleotide reduction also occurs [1].

It has recently been observed that the addition of calcium to intact liver cells incubated with NAD-linked substrates causes gradual elevation in the mitochondrial NADH/NAD, most prominent with fatty acid substrate [5,6]. In contrast, when the hepatocytes are incubated with succinate, calcium decreases the mitochondrial NADH/NAD [6].

The redox state of cellular pyridine nucleotides is of major importance in the control of metabolic processes. In view of apparent differences between the action of calcium in isolated mitochondrial systems and that observed in intact liver cells, the influence of calcium on the NAD oxidation-reduction state in hepatocytes was further examined.

As reported herein, pyridine nucleotides, catalase-H_2O_2 and -cyanide complexes and cytochrome oxidase in this isolated hepatocyte preparation were responsive to agents affecting these systems in the perfused liver. Two separate effects of calcium on the oxidative metabolism of liver cells were observed. (a) An immediate effect characterized by oxidation of pyridine nucleotides and increased oxygen consumption. These changes were of greater magnitude in cells incubated with succinate than with palmitate or in the endogenous state. (b) In the presence of fatty acid substrate, an effect of calcium of gradual onset was exhibited by maintenance of the mitochondrial pyridine nucleotides in a more reduced state.

2. Materials and methods

Liver cells were isolated by the procedure of Berry and Friend [7], as previously described [8], without hyaluronidase. About 90% of the cells were intact as measured by trypan blue exclusion. The cells were incubated at 23°C in calcium- and glucose-free Hanks medium [9] containing 10 mM sodium phosphate (pH 7.4) under oxygen with magnetic stirring. Oxygen consumption was measured with a Clark-type electrode [10]. Pyridine nucleotide fluorescence (excitation at 366 nm, emission at 450 nm) together with dual-wavelength absorbance of catalase-H_2O_2 and -cyanide complexes (660–640 nm) and cytochrome oxidase (605–630 nm) were recorded as described previously [11]. Albumin-bound palmitate was prepared [8] after treatment of crystalline bovine serum albumin (Miles

Laboratories) with charcoal [12] for removal of free fatty acids and other impurities. Liver cell cytochrome $a + a_3$ and catalase heme contents were measured as before [13].

3. Results and discussion

The addition of calcium to the hepatocytes caused a moderate but immediate decrease in pyridine nucleotide fluorescence, which reached a steadystate in 1–2 min (fig.1). Subsequent additions of caprylate and ethanol increased fluorescence. Whereas caprylate at 0.6 mM (and in other experiments 200 μM palmitate-0.4% albumin) produced an overshoot in fluorescence, ethanol gradually increased fluorescence without detectable oscillation. Fluorescence changes following the uncoupler pentachlorophenol (PCP) suggest initial oxidation of mitochondrial pyridine nucleotides and secondary gradual oxidation of the cytosolic pyridine nucleotides. The addition of cyanide following antimycin A produced only slightly more fluorescence, indicative of nearly complete reduction of the cellular

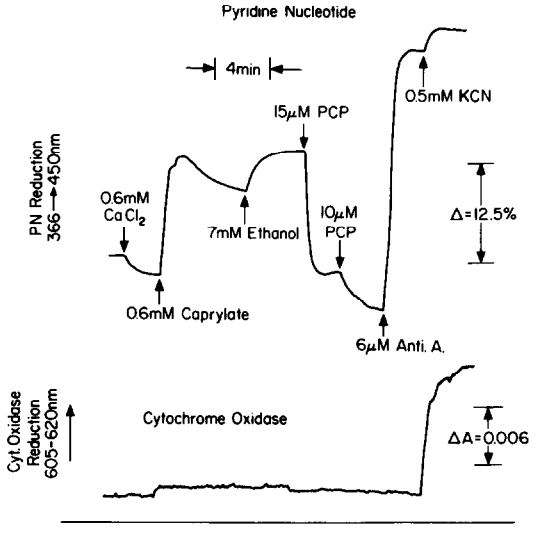

Fig.1. Liver cells (26.1 mg protein) isolated from a rat fasted 24 hr were incubated at room temperature in 3.0 ml of calcium- and glucose-free Hanks solution containing 10 mM sodium phosphate buffer, pH 7.4, in a cuvette with magnetic stirring. Sodium caprylate, pH 7.4, and methanol solutions of pentachlorophenal (PCP) and antimycin A (Anti A) were added where indicated.

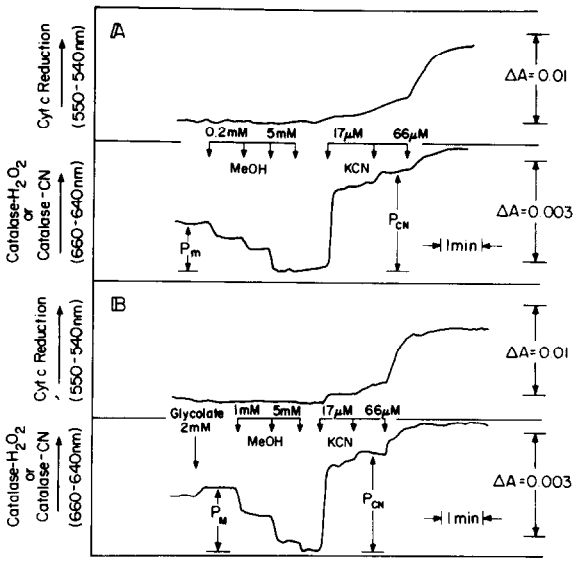

Fig.2. Liver cells (25.2 mg protein) isolated from a fed rat were incubated as described in fig.1 with [A] 10 mM glucose and [B] 10 mM glucose + 2 mM glycolate. P_m = steady state concentration of catalase H_2O_2 without methanol; P_M = maximum concentration of catalase-H_2O_2; P_{CN} = maximum concentration of catalase-CN.

pyridine nucleotides by antimycin A in the presence of the cytosolic reductant ethanol. Cytochrome oxidase was almost completely oxidized in the isolated cells and caprylate caused about 8% reduction.

The steady state concentration of the catalase-H_2O_2 complex, maintained by endogenous H_2O_2 production, was about 80% of its saturation level (fig.2b), as observed in perfused liver [13]. The increased signal following glycolate indicates complete saturation. The marked increase in H_2O_2 production from glycolate is shown by the increased methanol concentration required to produce a steady state catalase-H_2O_2 signal at half maximum; 1 mM methanol ($a_{1/2}$) was required to produce 47% saturation, whereas in the endogenous state (fig.2a) only 0.2 Mm methanol ($a_{1/2}$) was required. The $a_{1/2}$ of 0.2 mM is almost identical to that observed in the perfused liver with endogenous substrate [13,14]. The estimated turnover number of the catalase reaction $[(1/e)(dx_n/dt)(min^{-1})]$ [13] with added glycolate is $(32\ mM^{-1}\ min^{-1}) \times (a_{1/2}) = (32\ mM^{-1}\ min^{-1}) \times (1\ mM) = 32/min$. [15]. The catalase heme content of the liver cells was about 22 nmol/g wet wt. (table 1). The

Table 1
Comparison of cytochrome $a + a_3$ and catalase content of perfused liver and isolated liver cells

	Cytochrome $a + a_3$	Catalase	Ratio
Perfused liver	10.1 ± 0.41 nmol/g*	19.2 ± 0.6 nmol/g*	1.9
Isolated liver cells	46.7 nmol/g protein	102.9 nmol/g protein	2.2

* Mean ± S.E.M.; nmol/g wet weight

H_2O_2 production rate, dx_n/dt, was therefore (22) X (32) = 704 nmol/min/g liver cells. At 0.83 g hepatocytes/g wet wt of liver (16), the converted value is 584 nmol/min/g wet wt at 23°C with 2 mM glycolate as substrate. This can be compared with 720 nmol/min/g wet wt for intact liver with 1 mM glycolate at 30°C [15]. Cyanide (17 μM) (K_D = 10 μM in situ ([13])) markedly affected catalase. Most of the catalase was converted into the cyanide complex by 66 μM KCN (fig.2). Cytochrome *c* was little affected by 17 μM, and largely by 66 μM, cyanide. The absorbance change produced by cyanide was almost twice that produced by excess methanol, as observed in perfused liver and with purified catalase [14]. It is clear that the metabolism of H_2O_2 by the isolated hepatocytes closely resembles that operative in the intact organ.

The oxidation of pyridine nucleotides by calcium in isolated hepatocytes was near maximum at 400 μM $CaCl_2$ (fig.3). This effect of calcium was greater in cells oxidizing succinate than palmitate. Pyridine nucleotide oxidation by calcium in cells incubated with palmitate was suppressed by malonate and therefore appears to be associated with succinate utilization. Oxygen consumption of isolated hepatocytes incubated with 150 μM palmitate – 0.3% albumin and 10 mM succinate was elevated 6% and 30% respectively immediately following the addition of 400 μM calcium. Others have observed that calcium increased oxygen consumption of liver cells incubated with succinate [17,18].

The oxidation of pyridine nucleotides following calcium addition (fig.3) is apparently a response to energy-dependent calcium uptake by the hepatocyte mitochondria. The greater pyridine nucleotide oxidation by calcium in cells oxidizing succinate could be due primarily to facilitation of calcium uptake by succinate or inhibition of reverse electron flow in the respiratory chain by calcium. Elevation of oxygen consumption by calcium in liver cells oxidizing succinate favors the former interpretation. Enhanced calcium uptake would produce a lower phosphorylation state ratio and consequently decrease reverse electron flow. Increased calcium uptake by hepatocytes following succinate addition has recently been reported [18].

The cells were isolated under specified conditions in calcium-free media. Deletion of calcium prior to enzyme treatment improves the cell yield [19]. It is likely that partial depletion of cell calcium provided cells more sensitive to added calcium. During the isolation some cells are slightly damaged; about 10% are stained by trypan blue. These cells may be more permeable to calcium and thereby contribute, to a greater extent

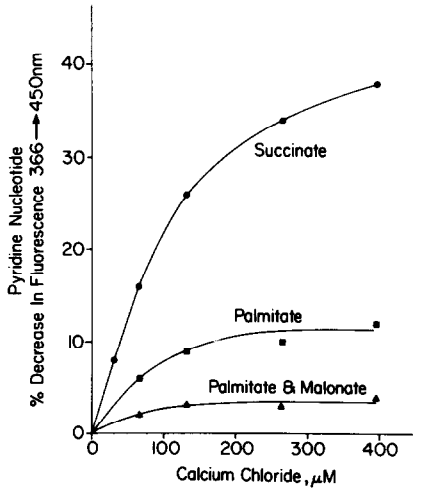

Fig.3. Isolated liver cells (20.9 mg protein) from a rat fasted 24 hr were incubated as described in fig.1 with 10 mM sodium succinate, 200 μM palmitate–0.4% albumin and 1 mM sodium malonate as indicated, following 6 min of preincubation. During the steady fluorescent state after substrate addition, successive increments of $CaCl_2$ were added and the resulting decreases in fluorescence were recorded.

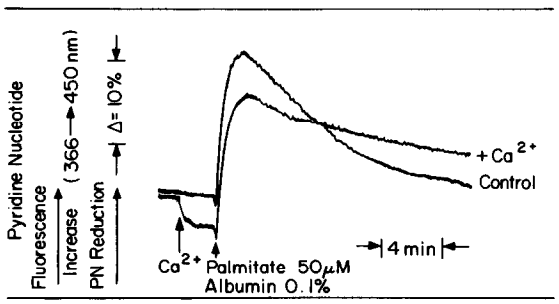

Fig.4. Isolated liver cells (27.0 mg protein) from a rat fasted 24 hr were incubated as described in fig.1. Separate recordings of fluorescence in the presence and absence of 500 μM $CaCl_2$ were superimposed.

than their proportion in the population, to pyridine nucleotide oxidation and enhanced oxygen consumption consequent to calcium addition (fig.1,3).

Although these results are consistent with calcium-dependent pyridine nucleotide oxidation in isolated mitochondrial systems [1,2] and in liver cells incubated with succinate [6], the elevated mitochondrial NADH/NAD caused by calcium in hepatocytes oxidizing NAD-linked substrates for longer periods (8–40 min) [5,6] remained in contrast. However, analysis of the influence of calcium on the gradual change in the oxidation-reduction state of the pyridine nucleotides following reduction by palmitate (fig.4) was supportive of the calcium-induced elevation in the mitochondrial NADH/NAD. Pyridine nucleotide fluorescence in cells incubated with calcium, although initially less, declined at a slower rate following palmitate-induced reduction and eventually exceeded the fluorescence intensity of the control system.

After 30 min of incubation with 750 μM palmitate–1.5% albumin ± 500 μM calcium, when the β-hydroxybutyrate/acetoacetate ration was in a steady state [6], the rate of oxygen consumption of liver cells supplemented with calcium was 13% greater ($P < 0.001$). Cytochrome oxidase was therefore more reduced. In providing a greater oxidation–reduction gradient from the initial segment of the respiratory chain to cytochrome oxidase, this gradual onset effect of calcium may also provide a greater phosphorylation state ratio [20].

These observations with liver cells oxidizing palmitate indicate that calcium interacts with the respiratory chain or the membrane in which the carriers are integrated and, following the initial effects associated with calcium uptake, thereby converts the respiratory carriers into a more reduced state. This transition is slow and it is not yet conclusive whether it is concurrent with greater NADH production or restricted NADH utilization. Although the presence of permeant anions in the cytosol appears to exclude correlation of these results with respiratory State 6 [3,4], this remains equivocal in view of possible compartmentation of such anions. The elevated rate of mitochondrial electron transport following completion of this transition, as measured by oxygen consumption, may be a consequence of greater activity of the metabolic processes which are both calcium- and energy-dependent.

Thus, dual effects of calcium on the oxidation–reduction state of the pyridine nucleotides in liver cells have been observed, an immediate effect characterized by pyridine nucleotide oxidation most prominent with succinate as substrate and a gradual effect exhibited by elevated mitochondrial NADH/NAD in hepatocytes oxidizing fatty acids. The latter effect demonstrates a calcium requirement in liver cells for the maintenance of a physiological oxidation–reduction state of the mitochondrial matrix pyridine nucleotides. This role of calcium may be translated to influences on the function of the respiratory chain in associated phosphorylations and on the rate of reactions responsive to the NADH/NAD ratio in this compartment of liver cells.

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