RUTHENIUM RED AS A PROBE IN ASSESSING THE POTENTIAL OF MITOCHONDRIA TO CONTROL INTRACELLULAR CALCIUM IN LIVER

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1. Introduction

Because of the absolute dependence on Ca^{2+} of a range of cell functions, it is becoming increasingly important to further knowledge on the mechanisms by which intracellular Ca^{2+} is maintained and controlled. Although specific carrier systems for translocating Ca^{2+} are located in at least three cell fractions (mitochondria, microsomes and plasma membrane), little is known of the relative contribution of each to overall control of intracellular Ca^{2+} levels.

Ruthenium Red is widely accepted now as a specific inhibitor of mitochondrial Ca^{2+} transport but its effects on microsome and plasma membrane Ca^{2+} transport are not well established. Experiments in this study show first that concentrations of Ruthenium Red which completely inhibit the initial rate of Ca^{2+} transport by mitochondria, have no significant effect on that by the microsome and plasma membrane fractions. They show too that the Ruthenium Red-sensitive component of Ca^{2+} transport by liver homogenates is very much greater than the Ruthenium Red-insensitive component suggesting that the potential of mitochondrial Ca^{2+} transport to control intracellular Ca^{2+}, greatly exceeds that of the microsome and plasma membranes.

2. Experimental

All membrane fractions were isolated from the livers of male adult Wistar albino rats. Mitochondria [1], plasma membranes [2] and microsomes [3] were prepared as described. Each fraction was resuspended in 250 mM sucrose/5 mM Hepes (pH 7.0).

Mitochondrial Ca^{2+} transport was measured by the procedure of Reed and Bygrave [4]. Transport of Ca^{2+} into the plasma and microsomal membrane vesicles was measured by the procedure of Moore et al. [5] except that initial rates (i.e., those measured over the first minute) and not those of the steady state were determined.

Ruthenium Red was crystallised as previously indicated [6].

3. Results

3.1. *Effect of Ruthenium Red on Ca^{2+} transport in individual cell fractions*

Data in fig.1a shows the effect of increasing concentrations of Ruthenium Red on the initial rate of mitochondrial Ca^{2+} transport as measured at 15°C. The findings are similar to those reported earlier by us [6,7] in that a concentration of only about 100 pmol Ruthenium Red/mg protein produces maximal and near complete inhibition; The K_i is about 30 pmol/mg protein.

The effect of Ruthenium Red on the initial rate of Ca^{2+} transport by the plasma and microsomal membranes on the other hand differs from that of mitochondria. Both systems evidently are much less sensitive to inhibition by Ruthenium Red. Ca^{2+} Transport by the plasma membrane fraction is inhibited by about 10% at a Ruthenium Red concentration of 1 nmol/mg protein; this concentration is approximately ten times greater than that which produces almost complete inhibition of mitochondrial Ca^{2+} transport (see fig.1a). Increasing further the Ruthenium Red concentration produces a steady decline in the initial

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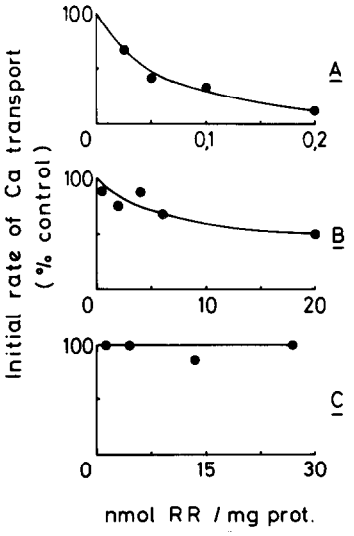

Fig.1. Influence of Ruthenium Red concentration on the initial rate of Ca^{2+} transport by the mitochondria (A), plasma membrane (B) and microsomal membranes (C). The reaction medium for measurement of mitochondrial Ca^{2+} transport contained 250 mM sucrose, 2.5 mM Hepes (pH 7.4), 2 mM succinate and 1 mg mitochondrial protein in final vol. 1 ml. 100 μM $^{45}Ca^{2+}$ was added after a 1 min preincubation and the incubation terminated at 10 s intervals using the EGTA–Ruthenium Red quench technique [4]. The temperature was 15°C. Plasma membrane and microsomal membrane Ca^{2+} transport were measured in a medium consisting of 100 mM KCl, 30 mM imidazole-histidine (pH 6.8), 5 mM ammonium oxalate, 5 mM ATP, 5 mM Mg^{2+} and 0.1 mg protein in final vol. 1 ml. 100 μM $^{45}Ca^{2+}$ was added after a 1 min preincubation and the incubation terminated by millipore filtration at 30 s intervals. The temperature was 37°C.

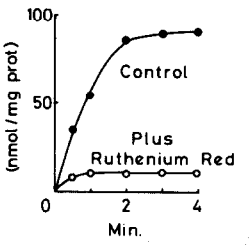

Fig.2. Influence of Ruthenium Red on Ca^{2+} transport by the whole liver homogenate. The reaction medium contained in final vol. 2 ml: 100 mM KCl, 30 mM imidazole-histidine (pH 6.8), 2 mM succinate, 5 mM ATP, 5 mM Mg^{2+}, 5 mM ammonium oxalate and 4 mg liver homogenate. After a 1 min preincubation, the reaction was initiated by addition of 400 nmol $^{45}Ca^{2+}$. Samples (200 μl) were filtered at the indicated times, washed, dried and counted in 10 ml scintillation fluid. The temperature was 37°C and Ruthenium Red was present as indicated at a concentration of 750 pmol/mg protein.

rate of Ca^{2+} transport by the plasma membrane fraction. The data do not exclude the possibility that the small degree of inhibition seen at the low concentrations of Ruthenium Red are due to contaminating mitochondria in the plasma membrane preparation.

Data in fig.1c show the microsomal Ca^{2+} transport is unaffected by Ruthenium Red even when present at a concentration approaching 30 nmol/mg protein.

3.2. *Effect of Ruthenium Red on Ca^{2+} transport by the whole homogenate*

In the experiment shown in fig.2, Ca^{2+} transport by the whole homogenate was measured in a medium supplemented with components reported as being optimal for transport of Ca^{2+} by mitochondria as well as by plasma [8] and microsomal membranes [5]. In the absence of Ruthenium Red, Ca^{2+} transport proceeds very rapidly and is almost complete by 2 min. By contrast, Ca^{2+} transport in the presence of Ruthenium Red (added at 750 pmol/mg protein), proceeds much more slowly and by 4 min has reached only an about one-eight of that of the control.

4. Discussion

Since the discovery by Moore [9] that Ruthenium Red is a potent and specific inhibitor of mitochondrial Ca^{2+} transport, a number of laboratories have utilized this finding to assess the role of mitochondrial Ca^{2+} transport in governing Ca^{2+} movements in the whole cell. As far as we are aware however it has never been shown by direct experimentation whether Ruthenium Red inhibits as well other cell Ca^{2+} transport systems. Some reports have indicated that Ca^{2+}-ATPase activity in the cell (plasma) membrane of erythrocytes [10] and lymphocytes [11] are unaffected by Ruthenium Red (but see ref. [12]).

The present study demonstrates that of the known Ca^{2+} transport systems in liver, only that of mitochondria is inhibited by low concentrations of Ruthenium Red, an observation which has several important implications.

First the findings permit some expression of confidence in studies with whole cells in which Ruthenium Red is used to probe the role of mitochondrial Ca^{2+} transport in the control of cell Ca^{2+} levels (see for example ref. [13,14]). However it must then be assumed that the highly charged molecule has little difficulty in gaining access to the mitochondria through the cell membrane network. Second, sensitivity of Ca^{2+} transport to Ruthenium Red can be used as a 'marker' or index of contamination of, e.g., mitochondria by plasma membrane or vice versa. This provides a more reliable estimate of such contamination than that in which the 'energy source' for transport is blocked; it is now known that mitochondrial Ca^{2+} transport does occur albeit to a limited extent, even when the energy of respiration is inhibited (reviewed in ref. [15]). Finally the findings provide further evidence that the molecular architecture of the Ca^{2+} carrier located in the inner miomitochondrial membrane is different from that of the carrier located in the plasma and microsomal membranes.

For some time mitochondria have been advocated as playing a major role in the control of cell Ca^{2+}. Support for this stems from studies in which the distribution of Ca^{2+} in whole tissue by electron microscopy [16], tracer [17] and direct vision [14] techniques was examined, from studies on kinetic analyses of $^{45}Ca^{2+}$ desaturation curves on cells in tissue culture [18], by analysing the properties of Ca^{2+} accumulation into intact cells [19], from an appreciation of the properties of mitochondrial Ca^{2+} transport as determined in vitro from steady state [20] and initial rate [15] measurements and from knowledge that the 'activity' of mitochondrial Ca^{2+} transport can change according to the development and hormonal status of the tissue of origin [15].

Direct evidence in support of this viewpoint is provided by the data in the present paper (fig.2). In light of the finding that low concentrations of Ruthenium Red fail to inhibit Ca^{2+} transport by microsomal and plasma membranes isolated from the same tissue (fig.1) it is valid to attribute the Ruthenium Red-sensitive component of overall Ca^{2+} transport to that of mitochondria. This component clearly exceeds by several-fold, both in terms of initial rate and capacity, that of the Ruthenium Red-insensitive component which in turn can be attributed to the microsomal and plasma membranes.

Finally, although the present study highlights the great capacity of mitochondria for control of cell Ca^{2+} in liver tissue, the possibility should not be discounted that the activity of microsomal and plasma membrane Ca^{2+} transport is subject to control (for example by specific hormones [21]) in such a manner that they too actively participate in the overall control of cell Ca^{2+}.

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