STABLE ENHANCEMENT OF RUTHENIUM RED-INSENSITIVE CALCIUM TRANSPORT IN AN ENDOPLASMIC RETICULUM-RICH FRACTION FOLLOWING THE EXPOSURE OF ISOLATED RAT LIVER CELLS TO GLUCAGON

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1. Introduction

Studies [1–3] employing the potent inhibitor of mitochondrial Ca^{2+} transport, ruthenium red, have provided a rational basis for discriminating mitochondrial from non-mitochondrial Ca^{2+} transport by subcellular fractions from rat liver. These have been designated ruthenium red-sensitive and ruthenium red-insensitive Ca^{2+} transport components, respectively [4].

In extending initial studies [5,6], we have employed initial rat measurements to investigate the properties of ruthenium red-insensitive Ca^{2+} transport in fractions isolated from rat liver [2,3]. One important property we have described relevant to the present considerations, is that intraperitoneal administration of glucagon to rats stimulates by up to 2-fold, the initial rate of MgATP-dependent, ruthenium red-insensitive Ca^{2+} transport [3]. A stimulation of microsomal Ca^{2+} transport has also been recently reported following the exposure of perfused rat liver [7] to glucagon. In our studies, the subcellular liver fraction most susceptible to the action of the hormone was enriched with the glucose 6-phosphatase activity and was therefore presumably derived from the endoplasmic reticulum. It was obtained by sedimenting the post-mitochondrial supernatant in buffered isoosmotic sucrose at 24 800 × *g* for 20 min [3].

Because these observations have a direct bearing on the mechanism(s) of hormonal control of intracellular Ca^{2+} (see also [8]), they have been investigated further. The present study shows that incubation of hepatocytes with glucagon leads to a stable enhancement of ruthenium red-insensitive Ca^{2+} transport.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis (-amino-ethyl ether) *N,N'*-tetraacetic acid; butyl PBD, 2-(4'-*t*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxidazole

2. Experimental procedures

2.1. *Preparation of hepatocytes*

Male Wistar rats (200–250 g) fed ad libitum were used in all experiments. The methods of preparation and incubation of hepatocytes were those detailed in [9].

2.2. *Preparation of membrane fractions*

At the conclusion of the incubation, hepatocytes were collected by brief centrifugation in a bench centrifuge (500 × *g* for 1 min), resuspended in 5 mM Hepes buffer (pH 6.8) and homogenized by hand using a 40 ml glass Dounce homogenizer (20 strokes).

The homogenate was resuspended in 250 mM sucrose, 5 mM Hepes buffer (pH 6.8) plus 0.5 mM EGTA and centrifuged at 12 000 × *g* for 10 min in an RC-2B Sorvall centrifuge to remove cell debris, nuclei and the bulk of the mitochondria. The supernatant fraction was centrifuged at 24 800 × *g* for 20 min. This fraction has been designated as the 'intermediate fraction'. The resulting supernatant

fraction was then centrifuged at 105 000 × *g* for 60 min in a Beckman Model L-2 Ultracentrifuge to yield the 'microsomal fraction'.

The pellet obtained from each fractionation step was resuspended in an appropriate volume of 250 mM sucrose plus 5 mM Hepes (pH 6.8).

2.3. *Protein determination*

Protein content was measured by the method in [10]. Crystalline bovine serum albumin was used as a standard.

2.4. *Calcium transport*

Uptake of radioactive Ca^{2+} was measured by membrane filtration employing a modification [2] of the method in [6]. Transport assays were performed at 37°C in the following incubation medium: 100 mM KCl, 20 mM Hepes buffer (pH 6.8), 5 mM $MgCl_2$, 3.3 mM ammonium oxalate, 2.5 μM ruthenium red and 0.3–0.5 mg protein in 1.5 ml final reaction vol. After a pre-incubation time of 2 min, 5 mM ATP (pH 6.8) was added and the assay was initiated by the addition of 5 μl 10 mM $^{45}Ca^{2+}$. At specified time intervals, a 100 μl sample of the reaction medium was removed and filtered through a membrane filter (0.45 μm porosity) prewashed with 250 mM sucrose plus 5 mM Hepes. The membrane filters were washed again to remove non-sequestered $^{45}Ca^{2+}$, then dissolved in 10 ml scintillant (6 g butyl PBD in 400 ml 2-methoxyethanol and 600 ml toluene). Radioactivity retained by the filters was measured by liquid scintillation spectrometry. The specific activity of the radioisotope in each incubation was determined by counting an unfiltered 100 μl sample.

2.5. *Materials*

$^{45}Ca^{2+}$ was obtained from the Radiochemical Centre, Amersham, Bucks. Ruthenium red purchased from Sigma Chemical Co., St Louis, MO was recrystallized by the method in [11].

Nucleotides were from Boehringer, Mannheim GmbH. Collagenase (type B) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Membrane filters (0.45 μm porosity) were obtained from the Gelman Instrument Company, Ann Arbor, MI.

Glucagon (crystalline), dibutyryl cAMP and puromycin dihydrochloride (crystalline) were acquired from Sigma Chemical Co. Crystalline beef insulin was from Commonwealth Serum Labs. (Parkville, Victoria). All other materials were of analytical reagent grade.

3. Results

Two phases of activity characterize ruthenium red-insensitive Ca^{2+} transport by subcellular fractions isolated from rat liver [2,3]. The first phase, which is rapid and complete by 2–5 min, merges into the second which is slower and remains linear for ⩾ 40 min [2]. Because the second phase is one in which considerable cycling of Ca^{2+} most likely occurs, it does not adequately reflect the true ability of the vesicles to transport Ca^{2+}.

Data in fig.1A show the initial phase of Ca^{2+} transport activity by the intermediate fraction isolated from rat hepatocytes incubated for 20 min with and without glucagon. Exposure of the cells to the hormone clearly enhances by ~ 80% the

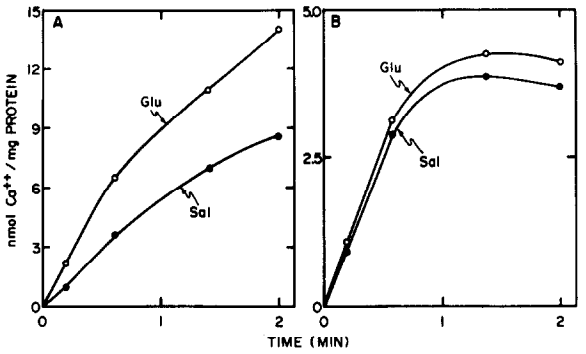

Fig.1A,1B. Ruthenium red-insensitive Ca^{2+} transport by the intermediate and microsomal fractions isolated from rat hepatocytes. The incubation medium used in the Ca^{2+} transport studies contained in 1.5 ml final vol.: 100 mM KCl, 20 mM Hepes buffer (pH 6.8), 3.3 mM ammonium oxalate, 5 mM ATP, 5 mM $MgCl_2$, 2.5 μM ruthenium red and 0.3–0.5 mg protein. The reaction was initiated by the addition of 50 nmol Ca^{2+} containing 0.5 μCi $^{45}Ca^{2+}$. The reaction was at 37°C. For further details see section 2. Hepatocytes were incubated at 37°C in Krebs-Henseleit buffer gassed with O_2, CO_2 (95:5) for 20 min in the presence of 5×10^{-7} M glucagon (Glu) (○) and in the absence of hormone (Sal) (●), prior to preparation of the subcellular fractions. Results shown are from a representative experiment.

initial rate of ruthenium red-insensitive Ca^{2+} transport by this fraction.

In contrast, glucagon treatment of hepatocytes did not significantly increase the initial phase of ruthenium red-insensitive Ca^{2+} transport activity by the microsomal fraction (fig.1B). A lack of effect of glucagon on the Ca^{2+} transport activity of the microsomal fraction was reported in an earlier study where the hormone was administered in vivo [3]. In these earlier studies, total Ca^{2+} transport activity was greater in the microsomal fraction than in the intermediate fraction. In this study, Ca^{2+} transport activity was higher in the intermediate fraction, possibly as a result of the general instability of the microsomal fraction isolated from hepatocytes (W.M.T., F.L.B., unpublished).

Data in fig.2 show that the extent to which glucagon promotes an increase in ruthenium red-insensitive Ca^{2+} transport by the intermediate fraction, is dependent on the concentration of glucagon added to the cell suspension. Half-maximal and maximal effects occur at $\sim 5 \times 10^{-9}$ M glucagon and 10^{-7} M glucagon, respectively.

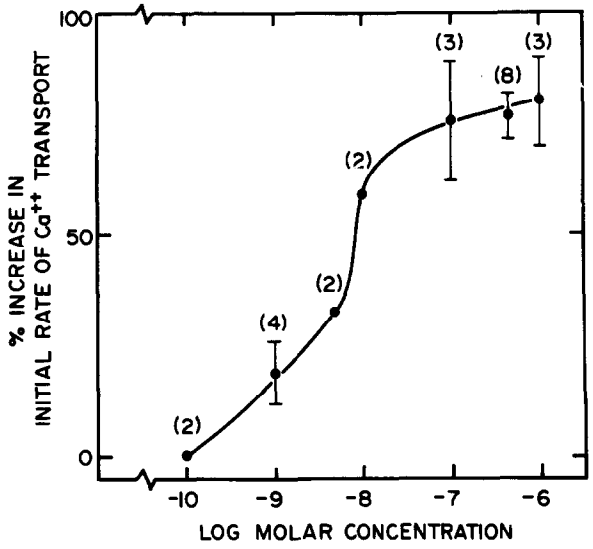

Fig.2. Ruthenium red-insensitive Ca^{2+} transport by the intermediate fraction isolated from rat hepatocytes pre-exposed to varying glucagon concentrations. Ca^{2+} transport was measured as in fig.1. Hepatocytes were incubated for 20 min at 37°C with glucagon present at the concentrations indicated before preparation of the intermediate fraction. Results are expressed as the mean ± SEM for the no. expts indicated in parentheses.

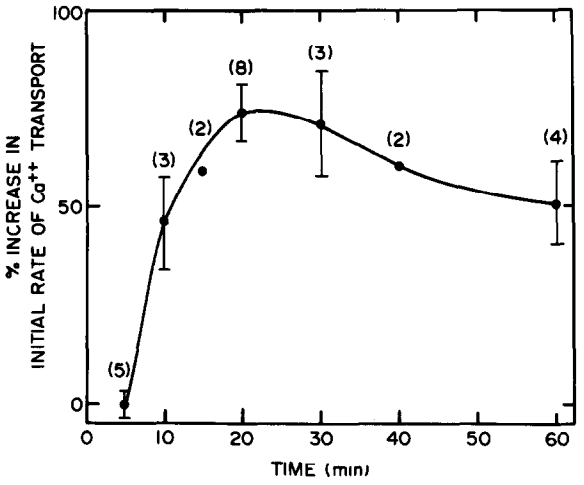

Fig.3. Ruthenium red-insensitive Ca^{2+} transport by the intermediate fraction isolated from rat hepatocytes pre-exposed to glucagon for varying time periods. Ca^{2+} transport was measured as in fig.1. Hepatocytes were incubated in the presence of 5×10^{-7} M glucagon for the times indicated. Results are expressed as the mean ± SEM for the no. expts shown in parentheses.

The effect of time of incubation of the cells with a fixed concentration of glucagon (5×10^{-7} M) on the stimulation of Ca^{2+} transport is shown in fig.3. No response is seen until after 5 min exposure to the hormone. By 10 min, the initial rate of Ca^{2+} transport is stimulated > 50% and by 20 min it has reached maximal stimulation. Thereafter the degree of stimulation gradually falls away.

A comparison of the effects of incubation of the cells with glucagon, insulin or dibutyryl cAMP on the Ca^{2+} transport activity of the intermediate fraction is shown in table 1. Although insulin treatment alone had no significant effect on the ruthenium red-insensitive Ca^{2+} transport activity subsequently determined in the intermediate fraction, co-administration of the hormone with glucagon completely abolished the stimulatory effect of glucagon described above. Treatment of hepatocytes with dibutyryl cAMP led to a stimulation of Ca^{2+} transport activity in the intermediate fraction similar to that observed after treatment of cells with glucagon.

4. Discussion

The experiments described here clearly establish

Table 1
Effects of glucagon, insulin, glucagon plus insulin and dibutyryl cAMP on Ca^{2+} transport in intermediate fraction

Addition	Stimulation of initial rate of Ca^{2+} uptake (% increase over control)
None	0
Glucagon	69 ± 8[a]
Insulin	25 ± 22
Glucagon + insulin	15 ± 13
Dibutyryl cAMP	71 ± 19[a]

[a] $p < 0.01$ compared to control

For details of hepatocyte incubation and intermediate fraction preparation see fig. 1A. Where indicated, additions were made at the following final conc.: glucagon 5×10^{-7} M; insulin 3×10^{-9} M (1 mU/ml); dibutyryl cAMP 5×10^{-4} M. Results are expressed as mean ± SEM for 7 separate expts, each assayed in duplicate

that the exposure of rat hepatocytes to low concentrations of glucagon induces a considerable enhancement of ruthenium red-insensitive Ca^{2+} transport in the intermediate (endoplasmic reticulum-rich) fraction isolated from the cells. The in vitro effects reported here are remarkably similar to those seen following administration of glucagon to adult and prenatal rats in vivo [3]. This enhances the likelihood that glucagon itself is the hormone primarily responsible for inducing the changes in ruthenium red-insensitive Ca^{2+} transport in the in vivo situation [3].

The observations that glucagon acts relatively rapidly, is effective at low concentrations, and that its effect is inhibited by insulin, suggest that these findings may be of physiological consequence. The effect probably also represents a mechanism by which glucagon stimulates the short-term redistribution of intracellular Ca^{2+}, which may be linked to the effect of the hormone on gluconeogenesis [12–14].

A further observation of note is the stimulation of ruthenium red-insensitive Ca^{2+} transport activity by dibutyryl cAMP. This result indicates a possible involvement of cAMP in the stimulation of Ca^{2+} transport activity and is the subject of further investigation.

Finally, as observed (F.L.B., P. A. Jacomb, unpublished) [3], it is important to appreciate that the glucagon-sensitive Ca^{2+} transport system resides in the intermediate fraction and not in the fraction obtained by high-speed centrifugation, even though both membrane fractions appear to be derived from the endoplasmic reticulum (F. L. B., P. A. Jacomb, unpublished) [3]. These properties are of interest in that they are analogous to those of the ruthenium red-sensitive Ca^{2+} transport system, which is also modified by glucagon action [3], and has a heterogeneous distribution among mitochondrial populations in rat liver [15].

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