ROLE OF 3′,5′-CYCLIC AMP IN GLUCAGON-INDUCED STIMULATION OF RUTHENIUM RED-INSENSITIVE CALCIUM TRANSPORT IN AN ENDOPLASMIC RETICULUM-RICH FRACTION OF RAT LIVER

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1. Introduction

A number of studies have employed ruthenium red, a potent inhibitor of mitochondrial Ca^{2+}-transport, to distinguish mitochondrial (i.e., ruthenium red-sensitive) from non-mitochondrial (i.e., ruthenium red-insensitive) Ca^{2+}-transport systems in subcellular fractions of rat liver [1–3].

We have described a subcellular fraction of rat liver having a ruthenium red-insensitive Ca^{2+}-transport system which was responsive to the action of glucagon [3,4]. This fraction was obtained by sedimenting the postmitochondrial supernatant in buffered iso-osmotic sucrose at 34 800 × *g* for 20 min. Glucagon enhanced the Ca^{2+}-transport activity in this fraction when the hormone was administered by intraperitoneal injection to the whole animal or incubated with rat hepatocytes in vitro prior to subcellular fractionation.

In the study employing rat hepatocytes the action of glucagon was mimicked by dibutyryl 3′,5′-cyclic adenosine monophosphate (cAMP) [4]. This suggested that glucagon may stimulate Ca^{2+}-transport in the intermediate fraction by a mechanism involving the action of cAMP, and raised the possibility that Ca^{2+}-transport by the endoplasmic-rich intermediate fraction of rat liver may be controlled by a phosphorylation–dephosphorylation cycle similar to that which regulates Ca^{2+}-transport by the sarcoplasmic reticulum of contractile tissues [5,6]. In view of the potential involvement of such a mechanism in the control of intracellular Ca^{2+} in the liver, these findings were examined in more detail.

This study shows that Ca^{2+}-transport activity in the subcellular fraction described was enhanced by prior incubation of hepatocytes with cAMP analogues or with various agents which elevate the cAMP concentration. Ca^{2+}-transport activity was also stimulated in this fraction following incubation in vitro with 50 μM cAMP, 250 μM ATP and small amounts of a rat liver supernatant fraction.

2. Experimental

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 [7].

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 ice-cold 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 ice-cold 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 a Sorvall RC-2B refrigerated centrifuge (SS-34 rotor) to remove cell debris, nuclei and the bulk of the mitochondria. The supernatant fraction was centrifuged at 34 800 × *g* for 20 min. This fraction has been designated as the 'intermediate fraction'. The pellet obtained was resuspended in an appropriate volume of 250 mM sucrose, 1 mM dithiothreitol plus 5 mM Hepes (pH 6.8).

In experiments where the intermediate fraction

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was prepared from rat liver rather than from hepatocytes the preparative procedure was varied as follows. The livers from rats killed by cervical dislocation were rapidly removed and placed in ice-cold isolation medium containing 250 mM sucrose, 5 mM Hepes buffer (pH 6.8) plus 0.5 mM EGTA. They were minced with scissors and homogenized by 2 passes with a glass–Teflon tissue disintegrator (A. H. Thomas Co., Philadelphia, PA, size C) which was motor-driven at 900 rev./min. The resulting suspension was made up to 80 ml with isolation medium and centrifuged at 1085 × *g* for 5 min in a Sorvall RC-2B centrifuge (SS-34 rotor). The supernatant was centrifuged for a further 5 min at 4340 × *g*. The resultant supernatant fraction was centrifuged for a further 10 min at 7710 × *g* and the supernatant obtained was subjected to a final 20 min period of centrifugation at 34 800 × *g*. The pellet obtained from this final centrifugation step was resuspended in an appropriate volume of 100 mM KCl containing 5 mM sucrose, 5 mM Hepes buffer (pH 6.8) and 1 mM dithiothreitol.

2.3. *Protein determination*

Protein content was measured by the method in [8] with crystalline bovine serum albumin as standard.

2.4. *3′,5′-Cyclic AMP determination*

Following incubation of hepatocytes for 5 min with various agents (see section 3), cells were collected by centrifugation in a Beckman microfuge (5–10 s). The supernatants were rapidly removed and the cell pellets dispersed in 0.5 ml distilled water maintained at 85–95°C. The samples were boiled for a further 10 min with intermittent agitation to aid dispersal of cellular debris. The bulk of the denatured proteins were pelleted by centrifugation (Beckman microfuge, 4 min) and the cAMP content of the supernatant was measured by radioimmunoassay [9]. The procedure was validated in the manner discussed [9]. The specificity of the assay for cAMP was such that purification of samples was not required.

2.5. *Calcium transport*

Uptake of radioactive Ca^{2+} was measured by membrane filtration employing a modification [2] of the method in [10]. 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 preincubation 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 plus 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.

2.6. *Materials*

$^{45}CaCl_2$ was obtained from the Radiochemical Centre, Amersham. Ruthenium red was purchased from Sigma Chemical Co., St Louis, MO and 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 Instr. Co., Ann Arbor, MI. Glucagon (crystalline), theophylline, 3-isobutyl-1-methylxanthine (IBMX) dibutyryl cAMP and 8-bromo-cAMP (crystalline) were acquired from Sigma Chemical Co. Crystalline beef insulin was from Commonwealth Serum Labs. (Parkville, VIC). Roche compound Ro-20-1724 was the kind gift of Dr H. Sheppard, Roche Chemical Co. All other materials were of analytical reagent grade.

3. Results

Ruthenium red-insensitive Ca^{2+}-transport by subcellular fractions of rat liver was determined by initial rate measurements as in [2,3]. In earlier studies, Ca^{2+}-transport activity by the intermediate fraction isolated either from rat liver or from rat hepatocytes was shown to be enhanced by pretreatment of the whole animal or cell suspension with glucagon [3,4]. When hepatocytes were employed, glucagon was reported to be maximally effective following exposure of the cells to the hormone for 20 min. This same exposure time was therefore also used in the present study where the effects of a number of agents on the Ca^{2+}-transport ability of the intermediate fraction were investigated. Data in table 1 show that glucagon

Table 1
Effect of agents on Ca^{2+}-transport by the intermediate fraction and on hepatocyte cAMP concentration

Addition	Relative rate of Ca^{2+}-transport	Relative cAMP concentration
None	100	100
Insulin	125 ± 22 (6)	115 ± 9 (3)
Glucagon	178 ± 9 (8)[a]	375 ± 41 (6)[a]
Glucagon + insulin	115 ± 13 (6)	347 ± 23 (5)[a]
Theophylline	157 ± 12 (6)[a]	146 ± 14 (4)[a]
3-Isobutyl-1-methyl-xanthine	148 ± 16 (4)[a]	204 ± 38 (3)[a]
Ro-20-1724	153 ± 13 (4)[a]	187 ± 18 (4)[a]
8-Bromo-cAMP	163 ± 11 (3)[a]	–
Dibutyryl-cAMP	171 ± 19 (6)[a]	–

[a] $p < 0.01$ compared to no addition

Hepatocytes were incubated at 37°C in Krebs-Henseleit bicarbonate buffer with the additions shown. Incubations were performed for 5 min when the cAMP concentration was determined or for 20 min when Ca^{2+}-transport was determined in the intermediate fraction subsequently isolated. The following agents were added as indicated: glucagon (2×10^{-7} M); insulin (1 mU/ml); theophylline (8×10^{-4} M); 3-isobutyl-1-methylxanthine (5×10^{-4} M); Ro-20-1724 (8×10^{-4} M); 8-bromo-cAMP (5×10^{-4} M); dibutyryl cAMP (5×10^{-4} M). In the absence of further additions the initial rate of Ca^{2+}-transport was 6.4 ± 0.6 nmol . min^{-1} . mg $protein^{-1}$ ($n = 8$) and the cAMP concentration was 0.53 ± 0.04 nmol/g cell wet wt ($n = 10$). Results were calculated relative to these respective control values and expressed as the mean ± SEM for the no. expt indicated in parentheses

enhanced the initial rate of Ca^{2+}-transport in the subsequently isolated intermediate fraction by nearly 80%. Insulin alone was without effect, but completely abolished the stimulatory effect of glucagon when both hormones were added simultaneously. These results essentially confirmed our findings in [4]. Data shown in table 1 also demonstrate that analogues of cAMP (dibutyryl-cAMP and 8-bromo-cAMP) and agents which elevate cAMP concentration by inhibition of cyclic nucleotide phosphodiesterase activity (theophylline, 3-isobutyl-1-methylxanthine (IBM) and Ro-20-1724) all increased, by 40–70%, Ca^{2+}-transport in the intermediate fraction.

The concentration of cAMP was also determined in a parallel series of experiments where cells were exposed to the various agents for 5 min prior to cAMP determination. Hepatocyte cAMP concentrations were increased by almost 300% following treatment with glucagon and by 50–100% after exposure to theophylline, IBMX or Ro-20-1724 (table 1). In contrast, insulin had no significant effect on cAMP concentrations either under control conditions or when levels of the cyclic nucleotide were elevated in the presence of glucagon.

Possible effects of cAMP on Ca^{2+}-transport activity by the intermediate fraction in vitro were also examined and the results shown in fig.1 and 2. Preincubation of the intermediate fraction with 250 μM ATP and 50 μM cAMP for 10 min at 30°C had no significant effect on the initial rate of Ca^{2+}-uptake subsequently determined (fig.1). Inclusion of purified protein kinase (beef heart, 0.1 mg/ml) in the preincubation mixture was also without effect (results not shown) whereas the inclusion of a rat liver supernatant fraction (~3 mg protein/ml) increased the initial rate of Ca^{2+}-transport by ~40% (fig.1). The rat liver supernatant fraction was ineffective in the absence of added cAMP (fig.1,2). The time course of activation of Ca^{2+}-transport following preincubation of the intermediate fraction with ATP, cAMP and supernatant fraction is shown in fig.2. Stimulation of Ca^{2+}-transport occurred between 5–10 min of preincubation and was maximal following a 10 min preincubation period. At later times both basal and stimulated rates of Ca^{2+}-transport declined, presumably reflecting instability of the preparation under these conditions.

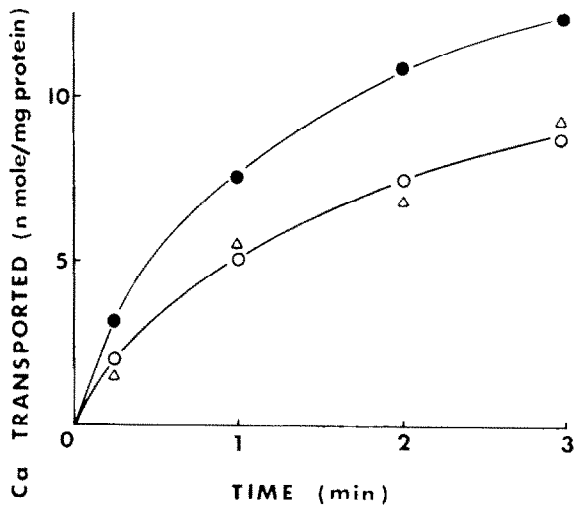

Fig.1. The in vitro effect of dibutyryl cAMP on the rate of ruthenium red-insensitive Ca^{2+}-transport in the intermediate fraction isolated from rat liver. The intermediate fraction was prepared as in section 2 and preincubated with 250 μM ATP at 30°C in the presence of both dibutyryl-cAMP (5×10^{-5} M) and supernatant fraction (~3 mg protein/mg) for 10 min. For some experiments dibutyryl-cAMP was omitted from the preincubation mixture, and for others the supernatant fraction was omitted. Results shown are mean of 3 separate expt. (•) Dibutyryl-cAMP (5×10^{-5} M) and supernatant fraction both present in the preincubation step. (○) Dibutyryl-cAMP omitted from the preincubation mixture. (△) Supernatant fraction omitted from the preincubation mixture.

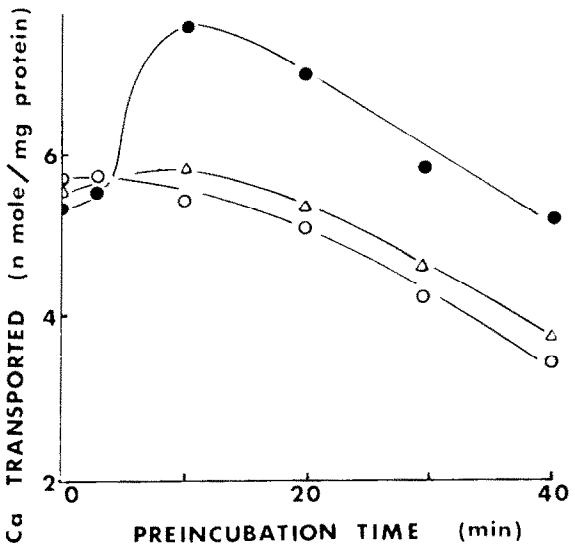

Fig.2. Time course of the in vitro effect of dibutyryl-cAMP on the rate of ruthenium red-insensitive Ca^{2+}-transport in the intermediate fraction from rat liver. The intermediate fraction was prepared as in section 2 and preincubated with ATP, dibutyryl-cAMP and supernatant fraction as in fig.1. The preincubation time was varied as shown. The Ca^{2+}-uptake assay was initiated as in section 2. Results shown are representative of 3 identical expt. (•) Dibutyryl cAMP (5×10^{-5} M) and supernatant fraction both present in the preincubation step. (○) Dibutyryl cAMP omitted from the preincubation mixture. (△) Supernatant fraction omitted from the preincubation mixture.

4. Discussion

The experiments described here confirm our earlier finding that exposure of rat hepatocytes to glucagon enhances ruthenium red-insensitive Ca^{2+}-transport in the intermediate fraction subsequently isolated from the cells [4]. From the data presented it is also evident that a similar stimulation of Ca^{2+}-transport occurs following exposure of hepatocytes to theophylline, IBMX or Ro-20-1724 (table 1). These agents were also shown to elevate hepatocyte cAMP concentrations within 5 min. In addition, analogues of cAMP, dibutyryl cAMP and 8-bromo-cAMP (a potent activator of protein kinase [12]), stimulated Ca^{2+}-transport activity by the intermediate fraction when added to hepatocyte suspensions prior to subcellular fractionation. Moreover, under the conditions in fig.1, the Ca^{2+}-transport ability of the intermediate fraction was stimulated directly by incubation in vitro with cAMP, ATP and the supernatant fraction. Taken together, these data suggest that cAMP may stimulate Ca^{2+}-transport by the intermediate fraction of rat liver.

It is now well established that a cAMP-dependent phosphorylation mechanism operates to control Ca^{2+}-transport in sarcoplasmic reticulum [5,6]. The present data are consistent with the hypothesis that a similar mechanism may operate to control the transport of Ca^{2+} by the intermediate fraction of rat liver. An increase in cAMP may activate a cAMP-dependent protein kinase, which in turn would phosphorylate a protein component of the vesicular membranes in the intermediate fraction, thereby activating the Ca^{2+}-transport system. The protein component may be the 'Ca^{2+}-transporter' itself or may be similar to the phospholamban component known to be involved in the mechanism described for the sarcoplasmic reticulum [5,6].

The data presented in this study indicate that insulin may decrease glucagon-stimulated rates of Ca^{2+}-transport in the intermediate fraction by a mecha-

nism independent of changes in cAMP concentration. Such an effect of insulin may be related to the hormone's ability to promote protein dephosphorylation, presumably through the action of protein phosphatases [13,14].

A further interesting finding in the present study was the observation that cAMP stimulated Ca^{2+}-transport by the intermediate fraction in vitro only when a small amount of rat liver supernatant fraction was also present. This apparent requirement for the rat liver supernatant fraction may simply reflect the distribution of the appropriate protein kinase in the different subcellular fractions with the enzyme presumably being present in the supernatant fraction but virtually absent in the intermediate fraction. In this regard it has been observed that the endogenous cAMP-dependent protein kinase of sarcoplasmic reticulum was rapidly depleted during vesicle preparation [15]. The finding that purified beef heart protein kinase was ineffective when substituted for rat liver supernatant fraction may indicate a high degree of species specificity for the enzyme. Other examples of species-specific protein kinase have been reported [16]. On the other hand, it is possible that the rat liver supernatant fraction contained some other factor required for Ca^{2+}-transport activation such as calmodulin. Maximal stimulation of Ca^{2+}-transport in sarcoplasmic reticulum may only occur following phosphorylation of separate sites on the vesicular membranes catalyzed by cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases, respectively [15]. Finally, recent observations suggest a stimulatory effect of glucagon and cAMP on the uptake and retention of Ca^{2+} by rat liver mitochondria ([17], W. M. T., F. L. B., unpublished). Thus a cAMP-dependent form of regulation of Ca^{2+}-transport by the endoplasmic-rich intermediate fraction of rat liver, may be part of a coordinate alteration of intracellular Ca^{2+}-levels and this in turn may be an integral part of the metabolic effects of glucagon in liver.

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