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Stimulation of Calcium Efflux from Rat Liver Mitochondria by Adenosine 3′5′ Cyclic Monophosphate

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Summary. The uptake or release of Ca^{2+} from rat liver mitochondria was studied by means of a sensitive Ca-electrode. It was found that using palmitoyl coenzyme A together with carnitine and ATP as substrates that Ca^{2+} was released gradually from mitochondria by adenosine 3′5′ cyclic monophosphate. The effect was obtained with either mitochondria preloaded with Ca^{2+} or with their physiological content of Ca^{2+}. No such release was obtained with the usual substrates used to provide energy for Ca^{2+} uptake by mitochondria.

Little is known about the mechanism of release of Ca^{2+} from rat liver mitochondria under physiological conditions. There is evidence that the mechanism of efflux of Ca^{2+} differs from the influx mechanism [5, 9, 18, 19]. In heart mitochondria Na^+ may be the physiological stimulus for release of Ca^{2+} [8]. In the small intestine, where massive translocation of Ca^{2+} takes place, it was shown that Ca-binding protein causes release of Ca^{2+} from mitochondria [13]. It has been suggested that phosphoenolpyruvate, PEP [17], or palmitoyl CoA [1] might cause release of Ca^{2+} in liver mitochondria. In the preceding paper it was shown that PEP in the presence of pyruvate kinase in fact aids retention of Ca^{2+} and that the effects of palmitoyl CoA are overcome by carnitine and ATP, both of which would be present in the cytosol of liver cells [14]. A recent paper has described the role of the oxidation-reduction status of the pyridine nucleotides in controlling the movement of Ca^{2+} into or out of mitochondria of heart muscle, liver, and of Erlich tumor cells [15]. The redox state of NADH/NAD was manipulated by adding β-hydroxybutyrate or acetoacetate [15]. It has been shown that glucagon or dibutyryl *c*AMP lowered the ratio of β-hydroxybutyrate/acetoacetate of isolated rat hepatocytes when palmitate was the substrate [6]. Since we have shown that palmitoyl CoA, in the presence of carnitine and ATP or palmitoyl carnitine, can be used as an energy substrate for mitochondria [14], it was decided to reinvestigate the effect of *c*AMP on Ca^{2+} efflux from mitochondria with palmitoyl CoA or palmitoyl carnitine as substrates.

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Materials and Methods

The preparation of rat liver mitochondria and the study of their uptake or release of Ca^{2+} by the Ca-electrode or the ^{45}Ca radioactive technique and the analytic methods used were described in the previous paper [14]. The characteristics of the sensitive and stable amplifier for the Ca-ion electrode are given in the accompanying paper [14]. To study the low concentrations of Ca associated with mitochondria with only their endogenous Ca, the output of the amplifier connected to the Ca-electrode was connected to a second amplifier containing an antilog stage. With this amplifier a linear output to the recorder could be obtained in the range 0.5×10^{-6} M to 10^{-5} M Ca^{2+}.

Estimation of Adenosine and Pyridine Nucleotides

At different time intervals 0.5-ml samples of the incubation mixtures were layered on silicone oil (Silicone Fluid M.S. 704, Midland Silicone Ltd., Barry, Glamorgan) and centrifuged at $12{,}000 \times g$ for 5 min through the oil into a layer of 1 N $HClO_4$ in 12.5% wt/vol sucrose. The lower perchloric acid layer was removed, neutralized with KOH, and examined for adenosine nucleotides as previously described [14]. This acid extract was also used to determine the concentrations of NAD and NADP by an enzymic and fluorimetric assay [22]. For the estimation of reduced pyridine nucleotide, the 0.5 ml of incubation mixture was pipetted immediately into 0.5 ml, of 0.5 N KOH in 50% vol/vol ethanol. After neutralization, NADH and NADPH were measured fluorimetrically [22].

Results

Uptake and Release of Ca^{2+} by Alteration of the Redox State of Mitochondria

The uptake and release of Ca^{2+} was monitored by a Ca-sensitive electrode [14], and we were able to confirm the observations of Lehninger, Vercesi and

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Table 1. Calcium release and re-uptake in rat liver mitochondria as monitored by the redox state of mitochondrial pyridine nucleotides and the corresponding change in mitochondrial adenine nucleotides

	Ca^{2+} (nmol/mg mitochondrial protein)	ATP (nmol/mg mitochondrial protein)	ADP	AMP
Uptake	20	6.8	10.0	10.0
1st release by oxaloacetate	15.5	9	10.3	6.0
Re-uptake by β-hydroxybutyrate	20	7	10.6	10.6
2nd release by oxaloacetate	14.8	8	10.2	6.3
Re-uptake by β-hydroxybutyrate	20	6.7	9.8	9.8
3rd release by oxaloacetate	16	10	10.3	12.0

Calcium uptake by rat liver mitochondria using a Ca-sensitive electrode: rat liver mitochondria, 20 mg, was added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 10 mM Na acetate at pH 7.2, 1 mM $MgCl_2$, 72 mM KCl, 2 mM Na succinate, 10^{-5} M rotenone, and 400 nmol Ca^{2+}. Calcium release from mitochondria was induced by 0.5 mM oxaloacetate and calcium re-uptake by adding 4 mM β-hydroxybutyrate. 0.5-ml samples at various points during the successive cycles of Ca^{2+} uptake and release were estimated for adenine nucleotides. The mitochondrial pellets were obtained by silicone centrifugation. Maximum possible calcium accumulated in mitochondria = 20 nmol/mg mitochondrial protein.

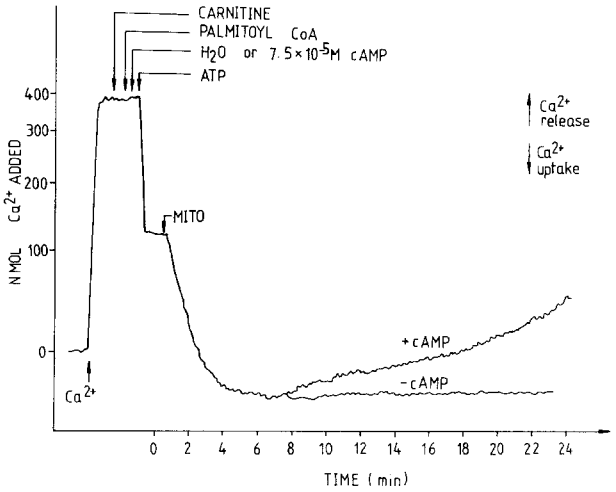

Fig. 1. Effect of 7.5×10^{-5} M *c*AMP on Ca^{2+} release from mitochondria with substrates palmitoyl CoA, carnitine and ATP. The Ca-electrode was used to monitor Ca^{2+} movement. Rat liver mitochondria, 6 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 400 nmol Ca^{2+}, 2 mM DL carnitine HCl, 20 μM palmitoyl CoA and 1 mM ATP. When present, the concentration of *c*AMP = 7.5×10^{-5} M. The Ca^{2+} added is shown on a log scale

Bababunmi [15], in that oxaloacetate caused release of Ca^{2+} from preloaded mitochondria and β-hydroxybutyrate caused reuptake and the cycle could be repeated at least three times. A new observation was made that, after release of Ca^{2+} caused by oxaloacetate, the addition 30 μM of bovine serum albumin (BSA) to the incubation mixture caused reuptake of Ca^{2+} and further cycles could continue. Table 1 shows that during three cycles with β-hydroxybutyrate and oxaloacetate, the concentrations of adenosine nucleotides in mitochondria remained steady, although Ca^{2+} was lost or gained during the cycles.

The Effect of cAMP on Ca^{2+} Release from Mitochondria

The movement of Ca^{2+} into mitochondria or back into the medium was monitored with a Ca-sensitive electrode, which allows the study of changes of ionic Ca^{2+} in the medium. Figure 1 shows that when 1 mM ATP was added to 5 ml medium containing 1 mM Mg and 400 nmol Ca^{2+} then 275 nmol of Ca^{2+} are chelated by the ATP (log scale). Addition of mitochondria resulted in a rapid uptake of Ca^{2+} to a level of 0.5×10^{-6} M in the medium, which is at the limit of detection of this particular electrode. When 7.5×10^{-5} M *c*AMP was present in the medium, the uptake of Ca^{2+} by mitochondria was not affected but Ca^{2+} began to leave the mitochondria at approx. 7 min after uptake and was steadily released thereafter. The control mitochondria retained their Ca^{2+} through the duration of the experiment. By addition of known amounts of Ca^{2+} to the incubation medium and calibration of the system with Ca buffers [14], it was calculated that of the 66 nmol Ca^{2+}/mg protein accumulated by mitochondria, 8–10 nmol were released in 20 min. The effects of *c*AMP were duplicated by dibutyryl *c*AMP but *c*GMP did not cause the release of Ca^{2+}.

Since ATP would chelate Ca^{2+} released from mitochondria, the extent of Ca^{2+} release is not immediately apparent from Fig. 1. By using palmitoyl carnitine as substrate, the concentration of ATP could be reduced to 0.1 mM and Fig. 2 shows that *c*AMP caused a significant release of Ca^{2+} commencing approx. 6 min after the uptake of Ca^{2+}. The results of Figs. 1 and 2 are for individual experiments. Individual experiments differed slightly in the time at which release of Ca^{2+} was first detected. In 18 experiments significant release of Ca^{2+} was obtained by *c*AMP. The condition of the mitochondria was important in obtaining this effect of *c*AMP. No effects

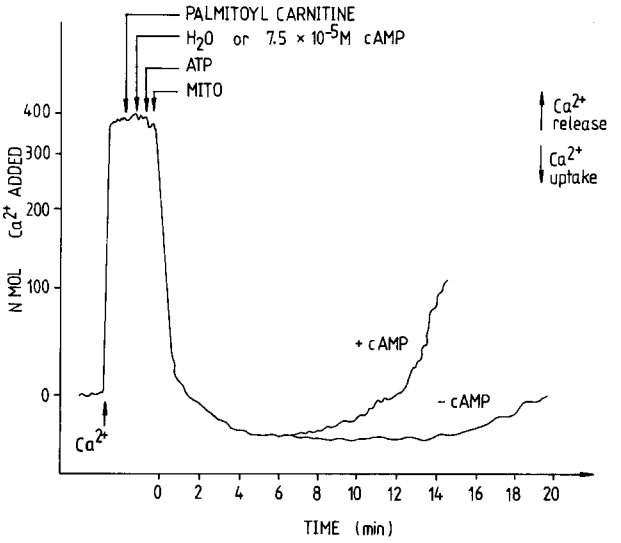

Fig. 2. Effect of 7.5×10^{-5} M *c*AMP on Ca^{2+} release from mitochondria with substrates palmitoyl carnitine and ATP. The Ca-electrode was used to monitor Ca^{2+} movement. Rat liver mitochondria, 7.5 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 400 nmol Ca^{2+}, 50 μM palmitoyl carnitine, and 0.1 mM ATP. When present, the concentration of *c*AMP $= 7.5 \times 10^{-5}$ M. The Ca^{2+} added is shown on a log scale

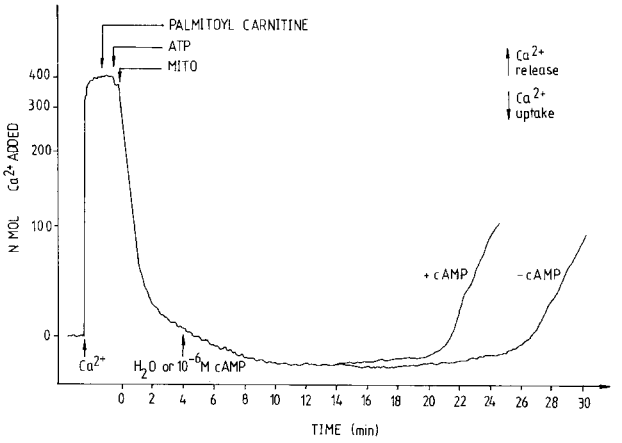

Fig. 3. Effect of 10^{-6} M *c*AMP on Ca^{2+} release from mitochondria. The Ca-electrode was used to monitor Ca^{2+} movement. Rat liver mitochondria, 8.8 mg, were added to 5 ml of 250 mM sucrose. 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 400 nmol Ca^{2+}, 50 μM palmitoyl carnitine, and 0.1 mM ATP. Addition of 10^{-6} M *c*AMP was at 4 min after mitochondrial Ca^{2+} uptake. The Ca^{2+} added is shown on a log scale.

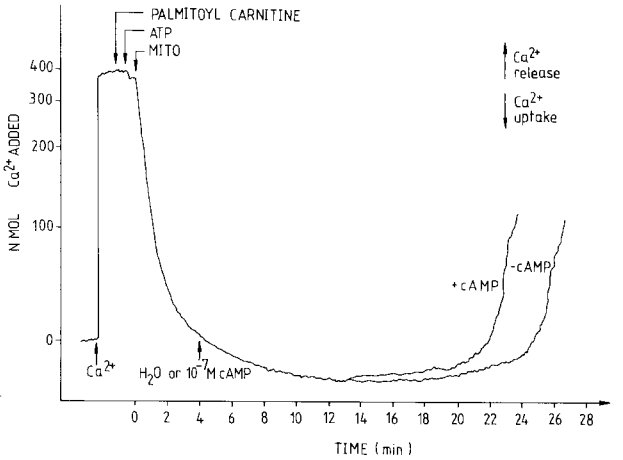

Fig. 4. Effect of 10^{-7} M *c*AMP on Ca^{2+} release from mitochondria. The Ca-electrode was used to monitor Ca^{2+} movement. Rat liver mitochondria, 8.5 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 400 nmol Ca^{2+}, 50 μM palmitoyl carnitine, and 0.1 mM ATP. Addition of 10^{-7} M *c*AMP was at 4 min after mitochondrial Ca^{2+} uptake. The Ca^{2+} added is shown on a log scale

were obtained in 4 experiments in which the mitochondria were kept on ice for longer than 1 hr after isolation, although these mitochondria still showed a P/O ratio 1.6–1.8 with succinate and would undergo cyclic uptake and release of Ca^{2+} with the β-hydroxybutyrate/oxaloacetate couple. The inclusion of 10^{-5} M rotenone in the system diminished the releasing effect of *c*AMP. When succinate plus ATP were used as substrates, with or without rotenone, the effect of *c*AMP could not be demonstrated (8 experiments, data not shown).

Significant effects of *c*AMP on Ca^{2+} release could be observed at 10^{-6} M and 10^{-7} M as shown in Figs. 3 and 4. In these experiments *c*AMP was added at 4 min when uptake of Ca^{2+} was completed.

For the study of mitochondria containing a more physiological level of Ca, the mitochondria were isolated as usual but without EGTA in the homogenizing medium [14]. These mitochondria contained 20 nmol Ca^{2+}/mg protein. When examined with the Ca-electrode connected to the anti-log amplifier to give a linear response these mitochondria showed a release of Ca^{2+} into the medium when 7.5×10^{-5} M *c*AMP was present. The concentration of Ca^{2+} in the medium at 4 min was 0.5×10^{-6} M for mitochondria with without *c*AMP, but 8 min later a significant release of Ca^{2+} began in the presence of *c*AMP. The release was greater than appears in Fig. 5 since Ca^{2+} was chelated by the 1 mM ATP present in the medium.

By calibration with additions of known quantities of Ca^{2+} it was estimated that 3 nmol Ca^{2+}/mg protein were released after 20 min incubation. The results were reproducible with mitochondria kept not longer than 1 hr after isolation.

Uptake and Release of Ca^{2+} by Mitochondria from Fed or Starved Rats

Mitochondria were prepared from normal stock fed rats and from their litter mates that were starved

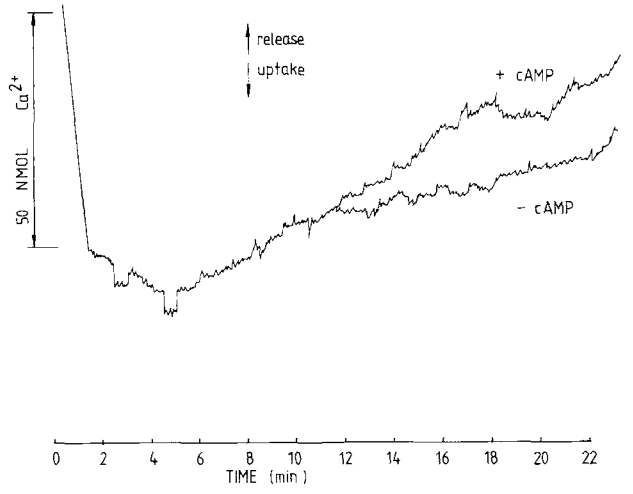

Fig. 5. Effect of 7.5×10^{-5} M cAMP on Ca^{2+} release from mitochondria not preloaded with Ca^{2+}. Ca^{2+} movement was studied by means of the Ca-electrode which was connected to a second amplifier containing an antilog stage. Rat liver mitochondria, 12 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 nM KCl, 2 mM DL carnitine HCl, 20 μM palmitoyl CoA, and 1 mM ATP. 7.5×10^{-5} M cAMP was added before the mitochondria

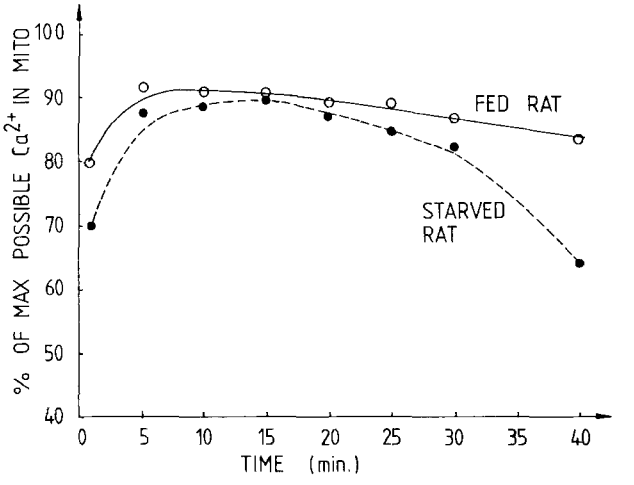

Fig. 6. Effect of starvation on Ca^{2+} release from mitochondria. Ca^{2+} movement studied by the radioassay technique. Starved rat liver mitochondria, 7.6 mg, or fed rat liver mitochondria, 8.1 mg, were added to 5 ml of 250 mM sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 2 mM DL carnitine HCl, 20 μM palmitoyl CoA, 1 mM ATP, and 400 nmol $^{45}CaCl_2$

for 40 hr in a cage with adequate water but which prevented coprophagy. Mitochondria from both groups were prepared and used simultaneously. Since with the Ca-electrode only one experiment at a time could be performed for the uptake and release of Ca^{2+} for these experiments of fed and starved mitochondria, $^{45}Ca^{2+}$ was used in the radioactive quench technique [14]. In eight separate experiments significant differences could be detected in the uptake or

Table 2. Concentrations of calcium and pyridine nucleotides in starved and fed rat liver mitochondria

	(nmol/mg mitochondrial protein)	
	Fed	Starved
Calcium	30	24
NAD^+	2.6	3.3
NADH	0.4	0.2
$NADP^+$	2.6	4.5
NADPH	Not detectable	Not detectable
NADH/NAD^+ ratio	0.15	0.06

Calcium uptake by rat liver mitochondria was by the radioassay technique: 10 mg of mitochondrial protein from starved (40 hr starvation) or fed rats were added to 5 ml of 0.25 M sucrose, 2.5 mM HEPES at pH 7.4, 2 mM P_i at pH 7.4, 1 mM $MgCl_2$, 72 mM KCl, 20 μM palmitoyl CoA, 2 mM carnitine, 1 mM ATP, and 400 nmol Ca^{2+}. Calcium and pyridine nucleotides in mitochondria were estimated at 1 min after adding mitochondria in the incubation medium.

release of $^{45}Ca^{2+}$ by fed or starved mitochondria, when 20 μM palmitoyl CoA, 2 mM carnitine, and 1 mM ATP were the substrates as shown in Fig. 6. Uptake of $^{45}Ca^{2+}$ by mitochondria from starved rats was slower than when fed rats were used. Also, mitochondria from starved rats began to lose their $^{45}Ca^{2+}$ earlier than mitochondria from fed rats. The analysis of the different pyridine nucleotides was determined at 1 min and Table 2 shows that the fed rat liver mitochondria had a higher ratio NADH/NAD than those from the starved rats. The total free fatty acid content of the liver mitochondria was slightly higher in the starved rats, 46 nmoles/mg mitochondrial protein, than the fed rats, 43 nmol/mg.

Discussion

Cyclic AMP has long been favored as the factor that modulates Ca^{2+} concentration in mitochondria or cytosol [20] and cAMP was reported to release Ca^{2+} from preloaded kidney, heart, and liver mitochondria [2, 16]. However, many workers, using a variety of substrates and different regimes for the rats [21], were unable to repeat these observations. The claims for the effect of cAMP were withdrawn by the original author pending further evidence [3]. Dramatic changes could be produced of the uptake or release of Ca^{2+} by rat liver mitochondria *in vitro* by manipulation of the oxidation-reduction status [15]. Therefore it was of interest to look for some physiological way of causing such a redox change. It was reported that glucagon or dibutyryl cAMP, acting on isolated rat hepatocytes metabolizing palmitate, cause a lower-

ing of the ratio β-hydroxybutyrate/acetoacetate [6]. Since we had previously shown that palmitoyl CoA in the presence of carnitine plus ATP or palmitoyl carnitine was a suitable substrate for studying mitochondrial Ca^{2+} uptake [14], we reinvestigated the effects of *c*AMP using this substrate. With mitochondria preloaded with Ca^{2+}, a substantial release of Ca^{2+} was obtained with 7.5×10^{-5} M *c*AMP or by dibutyryl *c*AMP, and significant release of Ca^{2+} was obtained at 10^{-6} M and 10^{-7} M *c*AMP. No effects were observed with cyclic GMP. The release of Ca^{2+} from mitochondria by *c*AMP could not be observed in a succinate plus ATP medium either with or without rotenone confirming [21]. Rotenone partially obscured the effect of *c*AMP when added to the palmitoyl CoA system. By the use of the sensitive Ca-electrode, it was possible to detect the release of Ca^{2+} from mitochondria that were not preloaded with Ca^{2+} and therefore presumably contained close to physiological levels of Ca^{2+}. A large fraction of the Ca released into the medium would be buffered; i.e., complex, with the 1 mM ATP present in the medium. This topic was discussed fully in the previous paper [14]. For this reason the changes in ionic Ca^{2+} shown in Fig. 5 do not reveal the full extent of Ca^{2+} released from the mitochondria. Estimates of the total release were made by calibration of the electrode in the medium used, including 1 mM ATP, and adding known amounts of Ca^{2+}. These estimates lead to a figure of approx. 3 nmol Ca^{2+} released from the original 20 nmol Ca^{2+}/mg mitochondrial protein in 20 min. The phenomenon was reproducible and the only failures to obtain release of Ca^{2+} by addition of *c*AMP were when mitochondria were used that had been isolated more than 1 hr before performing the experiment. Mitochondria older than 1 hr were still capable of Ca^{2+} uptake and retention for long periods with media reported previously [14].

There are several reasons why the work reported in this paper found significant effects of *c*AMP on Ca^{2+} release, when other authors were unsuccessful. The differences in methods that might account for this are: (i) a sensitive and stable amplifier coupled to a Ca-ion-sensitive electrode was used for Ca^{2+} measurement; (ii) mitochondria prepared from fed rats were used within 1 hr after isolation; (iii) the external medium always contained ATP which we have shown allows Ca^{2+} to be retained by mitochondria for long periods; (iv) the use of palmitoyl CoA plus carnitine or palmitoyl carnitine as the substrate for oxidation by mitochondria; (v) no inhibitors such as rotenone were present in the system. Previous authors had used mitochondria preloaded with Ca^{2+}, and it is shown in this paper that *c*AMP will release Ca^{2+} from mitochondria that were not preloaded but that contained close to their physiological level of Ca.

An attempt was made to alter the redox status of mitochondria by using starved rats. Starvation increases the levels of glucagon in blood [12] which would be expected to increase the *c*AMP levels in liver [11]. Glucagon was postulated to have a "push-pull" effect on fatty acid oxidation in liver by making more acyl-CoA and acyl-carnitine available for β-oxidation and also facilitating this oxidation by lowering the NADH/NAD ratio [4]. The experiments reported in this paper found a lower NADH/NAD ratio in starved rat mitochondria and the mitochondria accumulated Ca^{2+} more slowly and lost this Ca^{2+} more rapidly than mitochondria from fed rats.

The question arises which occurs first: does the hormone, glucagon, by increasing the concentration of *c*AMP cause the release of Ca^{2+} or does *c*AMP alter the activity of certain enzymes, promoting a more oxidized state in the mitochondria which would cause release of Ca^{2+}? Using perfused rat hepatocytes, it was shown that either glucagon or dibutyryl cyclic AMP caused stimulation of Ca efflux from hepatocytes and glucagon inhibited the uptake of ^{45}Ca into the mitochondria of hepatocytes [10]. A further complication has arisen in that glucagon has been implicated in the control of the redox state of hepatocytes and it was suggested the NADH inhibits phosphodiesterase thus maintaining the concentration of *c*AMP [7]. If it is true that only small shifts of Ca^{2+} in the μM range are required to alter the activities of Ca-sensitive enzymes, then it seems possible from the data in this paper for *c*AMP to bring about these small changes.

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