Determination of mitochondrial calcium content in hepatocytes by a rapid cellular-fractionation technique

α -Adrenergic agonists do not mobilize mitochondrial Ca^{2+}

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A rapid cellular fractionation technique [the preceding paper, Shears & Kirk (1984) *Biochem. J.*, **219**, 375–382] was employed to separate a mitochondria-rich fraction from hepatocytes within seconds. Mitochondrial Ca was estimated to be no more than 41% of total cell Ca. At least half of the mitochondrial Ca was present in an energy-dependent pool; 20% of total cell Ca was accessible to EGTA within 10s. The α -adrenergic agonist phenylephrine stimulated glycogen phosphorylase activity by 100% within 0.5 min and induced a loss of 20% of total cell Ca after 10 min from the EGTA-inaccessible pool. However, between 0.5 and 10 min after the addition of phenylephrine to hepatocytes there was no significant change in the Ca content of the mitochondria-rich fraction. Hepatocytes that were preloaded with Ca^{2+} during 90 min incubation at 0–4°C expelled this cation during 20 min incubation at 37°C. After this time, phenylephrine failed to alter the Ca content of a mitochondria-rich fraction. It is concluded that α -adrenergic agonists do not mobilize Ca^{2+} from hepatocyte mitochondria.

The α -adrenergic agonists evoke an increase in cytosolic $[\text{Ca}^{2+}]$, thereby provoking an activation of glycogen phosphorylase (EC 2.4.1.1.) (for a review, see Exton, 1981). Investigations of the source of the extra Ca^{2+} involved in this process have been carried out in several laboratories. Some experiments utilizing $^{45}\text{Ca}^{2+}$ have been interpreted to provide evidence that α -agonists cause liver cells to accumulate Ca^{2+} from the external environment (Keppens *et al.*, 1977; Foden & Randle, 1978; Poggioli *et al.*, 1980). However, it is unclear to what extent the latter phenomenon contributes to the total rise in cytosolic Ca^{2+} concentration (Blackmore *et al.*, 1978, 1982).

It has been suggested that α -agonists mobilize Ca^{2+} from one or more intracellular sources, but many studies have led to the conclusion that the major pool of mobilized Ca^{2+} is mitochondrial in origin. The techniques which have been employed in these investigations include chlortetracycline fluorescence (Babcock *et al.*, 1979), compartmental

analyses of hepatocyte $^{45}\text{Ca}^{2+}$ fluxes (Barritt *et al.*, 1981) and measurements of Ca content in mitochondria after classical differential centrifugation in order to isolate these organelles from hepatocytes or perfused livers (Blackmore *et al.*, 1979a,b; Dehaye *et al.*, 1980, 1981; Taylor *et al.*, 1980). In the latter experiments it is difficult to exclude the possibility that a redistribution of Ca occurs during the lengthy isolation procedures. In an effort to decrease the isolation time, Reinhart *et al.* (1982a,b) employed Percoll gradients to separate a mitochondrial fraction from liver homogenates within 6 min of cell disruption. These workers also reported that pretreatment of perfused liver with α -agonists resulted in a decreased Ca content of the mitochondrial fraction.

Murphy *et al.* (1980) measured Ca in a mitochondrial fraction obtained from hepatocytes within seconds. A crucial advantage of these experiments was the apparent absence of significant Ca transfer between mitochondria and other cell constituents during the fractionation procedures. Murphy *et al.* (1980) found that, in Ca^{2+} -loaded hepatocytes, the Ca content of the mitochondrial fraction decreased by 40% after 2.5 min of treatment with α -agonists. In view of the importance of these data, we have attempted to

Abbreviations used: CCFP, carbonyl cyanide *p*-tri-fluoromethoxyphenylhydrazine; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid. Ca and Ca^{2+} are used to signify total calcium and ionized calcium respectively.

repeat and extend these experiments. In doing so, we sought to improve the experimental design adopted in this earlier study.

Murphy *et al.* (1980) incubated their hepatocytes in the absence of extracellular Ca^{2+} , after first subjecting them to a Ca^{2+} -loading procedure by incubation for up to 90 min on ice. We wished to avoid the intervention of these aphysiological loading procedures. Moreover, when the extracellular Ca^{2+} concentration is decreased from the physiological range to ≤ 0.1 mM, then the kinetics of cellular Ca^{2+} fluxes are modified (Barritt *et al.*, 1981; Berthon *et al.*, 1981; Kleineke & Söling, 1983). Consequently, we have included 1.25 mM- Ca^{2+} in our incubation media during rapid fractionation experiments carried out as described in the preceding paper (Shears & Kirk, 1984). Finally, since α -agonists may double phosphorylase activity within 0.5–1 min (Keppens *et al.*, 1977; Dehay *et al.*, 1980; Murphy *et al.*, 1980), we have looked for changes in mitochondrial Ca content within this time. The characterization of the time course of Ca^{2+} movements is essential in determining whether changes in mitochondrial Ca have an initiating role in phosphorylase activation or are a secondary manifestation of earlier events. A preliminary account of some of our findings has been published (Shears & Kirk, 1983).

Experimental

Methods

Preparation and incubation of hepatocytes. Hepatocytes were prepared from male Wistar rats as described in the preceding paper (Shears & Kirk, 1984). Unless otherwise stated, hepatocytes were incubated at 37°C in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 5 mM-Tes, 10 mM-glucose, 2% (w/v) bovine serum albumin and 1.25 mM- CaCl_2 . Cells were incubated at 37°C as 2 ml portions in stoppered plastic vials and gassed every 15 min with O_2/CO_2 (19:1).

Determination of Ca in intact cells and mitochondria-rich fractions. For the determination of total cell Ca, portions (0.7 ml) of intact cells were centrifuged through silicone oil into 0.1 ml of 2M- HClO_4 [the preceding paper (Shears & Kirk, 1984)], and 0.08 ml samples of this lower phase were removed by microsyringe for Ca^{2+} determination by atomic-absorption spectrometry (see below). The Ca^{2+} in the extracellular medium that contaminated the HClO_4 layers was estimated with $[\text{U-}^{14}\text{C}]\text{sucrose}$ (Shears & Kirk, 1984).

Mitochondrial Ca was determined in samples (0.08 ml) taken from the acid layers into which mitochondria-rich samples were centrifuged during rapid cell fractionation (Shears & Kirk, 1984). In these experiments, Ruthenium Red and EGTA

were included in the fractionation medium (Shears & Kirk, 1984) in order to inhibit mitochondrial Ca^{2+} uptake (Reed & Bygrave, 1975). The efficacy of this inhibition was determined by the addition of 1 μl of $^{45}\text{Ca}^{2+}$ (0.01 μCi) to 0.9 ml of cell suspension, simultaneously with the addition of the cells to the fractionation medium. The radioactivity in the mitochondria-rich fraction was assessed as described in the preceding paper (Shears & Kirk, 1984). Ca^{2+} uptake into the mitochondria-rich fraction during its separation from other cellular material was estimated from the known specific radioactivity of the $^{45}\text{Ca}^{2+}$. Contamination of the HClO_4 layers with extramitochondrial Ca^{2+} was estimated from the known concentration of this ion in the extramitochondrial volume, which was itself measured with $[\text{U-}^{14}\text{C}]\text{sucrose}$ (Shears & Kirk, 1984). The difference between Ca^{2+} uptake by the mitochondria-rich fraction and contaminating Ca^{2+} in the extramitochondrial medium, was found to be 0.017 ng-atom/mg cell dry wt. (from two preparations of hepatocytes). This value, which was unaffected by phenylephrine treatment, is about 1% of total Ca in the mitochondria-rich fraction (see the Results section). These experiments confirm that mitochondrial Ca^{2+} uptake is almost entirely inhibited during the fractionation procedures.

For the determinations of Ca^{2+} in acid-quenched extracts (see above), these samples were first adjusted to 0.2 ml with 2M- HClO_4 . In determining mitochondrial Ca, the extracts from each pair of centrifuge tubes were first combined. Then 2.8 ml of 33 mM- LaCl_3 was added and Ca^{2+} was assayed with a Pye-Unicam SP.9 atomic-absorption spectrometer. Standards (2.5–20 μM) contained equivalent concentrations of LaCl_3 and HClO_4 . The Ca^{2+} in the extracellular or extramitochondrial medium which contaminated the HClO_4 layers was estimated with $[\text{U-}^{14}\text{C}]\text{sucrose}$ (Shears & Kirk, 1984). Mitochondria-rich Ca was corrected for damaged mitochondria and residual intact cells by measuring release of glutamate dehydrogenase and lactate dehydrogenase (see below). Statistical significance was assessed by Student's paired *t* test.

Enzyme assays. Glycogen phosphorylase (EC 2.4.1.1) was determined as described by Kirk *et al.* (1979). Glutamate dehydrogenase (EC 1.4.1.2) and lactate dehydrogenase (EC 1.1.1.27) were determined as described by Shears & Kirk (1984).

Materials

Bovine serum albumin (fraction V), CCFP, oligomycin and phenylephrine were purchased from Sigma. The albumin was dialysed before use (Shears & Kirk, 1984). Pindolol was supplied by Sandoz, Feltham, Middx., U.K.

Results

Ca content of intact cells and mitochondria-rich fractions

A rapid fractionation technique was employed to isolate 80% of hepatocyte mitochondria within seconds of cell disruption (Shears & Kirk, 1984). The Ca in the mitochondria-rich fraction represented 41% of total cell Ca (Table 1). The addition of EGTA to hepatocytes resulted in an immediate 20% decrease in cell Ca (Table 1), presumably due to the removal of this cation from the outer face of the plasma membrane (Blackmore *et al.*, 1978, 1982). A slower efflux of intracellular Ca was not evident for several minutes (Blackmore *et al.*, 1982; S. B. Shears & C. J. Kirk, unpublished work). After subtracting the apparent extracellular pool, the proportion of the total cell Ca in the mitochondria-rich fraction was about half.

Oligomycin did not affect the Ca content of the mitochondria-rich fraction (Table 1). However, there was a significant release of Ca^{2+} from non-mitochondrial pools, in agreement with the results of other workers (Blackmore *et al.*, 1982).

The mitochondria-rich fraction was contaminated with 53% of cell nuclei (Shears & Kirk, 1984), which have been reported to contain Ca (Bygrave, 1978). Other subcellular fractions that contain Ca were largely separated from the mitochondria (Shears & Kirk, 1984).

We attempted to distinguish between nuclear and mitochondrial Ca on the basis that there is no known mechanism for an efflux of nuclear Ca in the presence of CCFP plus oligomycin. This is in contrast with the effects of these compounds on mitochondrial Ca (Nicholls, 1978). In the present study, when the mitochondrial electrical potential was substantially decreased by CCFP plus oligomycin (Shears & Kirk, 1984), the Ca content of the mitochondria-rich fraction was halved (Table 1).

These data suggest that 0.65 ng-atom of Ca/mg dry wt. is to be found in an energy-dependent mitochondrial pool. Mitochondria will also retain some Ca in the presence of CCFP plus EGTA, and this is sufficient to account for the Ca in the fraction obtained from cells treated with oligomycin plus CCFP (Reed & Bygrave, 1974; S. B. Shears & C. J. Kirk, unpublished work). We therefore conclude that hepatocyte mitochondrial Ca is in the range 0.65–1.35 ng-atoms/mg cell dry wt. (Table 1).

Effect of phenylephrine on cell Ca content and phosphorylase activity

Phenylephrine was added to hepatocytes at a concentration (10 μM) reported to maximize the stimulation of phosphorylase activity (Blackmore *et al.*, 1978; Aggerbeck *et al.*, 1980). Both control and agonist-treated hepatocytes were incubated with 0.5 μM -pindolol to block β -adrenergic receptors (Aellig, 1976). It is clear from Fig. 1 that glycogen phosphorylase activity was enhanced by about 100% within 0.5 min of the addition of phenylephrine. Phosphorylase activity then declined, so that by 5 min it was identical with the control values.

Phenylephrine induced a significant loss of 0.37 ng-atom of cell Ca/mg cell dry wt. within 1 min. By 10 min this loss was 0.63 ng-atom of Ca/mg cell dry wt. or 20% of total cell Ca (Fig. 1). However, it is clear that a substantial proportion of Ca^{2+} efflux from cells was detected only after the peak of phosphorylase activation. It should be noted that Ca^{2+} efflux under the influence of α -agonists has also been observed when extracellular Ca^{2+} was removed by EGTA (Blackmore *et al.*, 1978, 1982; Barritt *et al.*, 1981). This suggests that phenylephrine mobilized Ca^{2+} from a cell-associated pool. We confirmed this observation by adding EGTA (12 mM final concn.) to cells 10 s before their centrifugation through silicone oil. At

Table 1. *Ca content of intact cells and mitochondria-rich fractions: the effects of oligomycin, CCFP and EGTA*
Hepatocytes were preincubated for 30 min as described under 'Methods'. Oligomycin and CCFP were added in 0.01 ml of ethanol, and EGTA was added in 0.01 ml of water. Data are means $\pm$ s.e.m. for the numbers of hepatocyte preparations given in parentheses. Significance: * $P < 0.05$ and ** $P < 0.01$ for a comparison with paired controls. Abbreviation used: n.d., not determined.

Addition	Period of incubation	Ca content (ng-atoms of Ca^{2+} /mg cell dry wt.)		$100 \times \frac{M}{C} (\%)$
		Cell (C)	Mitochondria-rich fraction (M)	
Ethanol	10 min	3.28 ± 0.38 (5)	1.35 ± 0.16 (8)	41
Oligomycin (5 mg/ml)	10 min	2.24 ± 0.36 (5)**	1.40 ± 0.24 (5)	58
Oligomycin (5 mg/ml) + 50 μM -CCFP	10 min	2.50 ± 0.32 (5)*	0.70 ± 0.11 (8)**	28
Water	10 s	3.16 ± 0.30 (6)	n.d.	
12 mM-EGTA	10 s	2.56 ± 0.25 (6)**	n.d.

Fig. 1. Effect of phenylephrine on (a) glycogen phosphorylase activity and (b) cell Ca content

Hepatocytes were preincubated for 30 min as described under 'Methods' with the addition of $0.5 \mu\text{M}$ -pindolol, a β -adrenergic antagonist. At zero time, 0.02 ml of phenylephrine (●, final concn. $10 \mu\text{M}$) or incubation medium (○) was added. Samples were taken from two preparations of hepatocytes for the assay of glycogen phosphorylase, and from six hepatocyte preparations for determinations of cell Ca content. Vertical bars represent the S.E.M. Significance: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$ (for difference between paired treated and control samples).

1 and 2 min after the addition of phenylephrine, cell Ca^{2+} was then decreased by, respectively, $0.55 \pm 0.11 \text{ ng-atom/mg dry wt.}$ ($n = 4$, $P < 0.02$) and $0.42 \pm 0.1 \text{ ng-atom/mg dry wt.}$ ($n = 3$, $P < 0.05$).

Effect of phenylephrine upon mitochondrial Ca

The Ca content of the mitochondria-rich fraction was determined in normal hepatocytes and in paired samples treated with $10 \mu\text{M}$ -phenylephrine. The agonist did not affect the pattern of enzyme release during fractionation (results not shown). Between 0.5 and 10 min after the addition of the agonist, no significant change in mitochondrial Ca content was observed (Fig. 2). These results are in

Fig. 2. Effect of phenylephrine on Ca content of a mitochondria-rich fraction obtained by rapid cell fractionation

Hepatocytes were preincubated for 30 min as described under 'Methods', with the addition of $0.5 \mu\text{M}$ -pindolol, a β -adrenergic antagonist. At zero time, 0.02 ml of phenylephrine (●, final concn. $10 \mu\text{M}$) or incubation medium (○) was added. Results are means $\pm$ S.E.M. for six hepatocyte preparations.

contradiction to those of Murphy *et al.* (1980), who reported that α -adrenergic agonists decrease mitochondrial Ca content by 40%. We therefore studied whether phenylephrine treatment would have any effect on mitochondrial Ca if, as in the experiments of Murphy *et al.* (1980), hepatocytes were preloaded with Ca^{2+} by incubation at $0-4^\circ\text{C}$ for 90 min in the presence of 5 mM-Ca^{2+} . Although this procedure increased both total cell and mitochondrial Ca (Fig. 3), much of this Ca was lost after 20 min incubation at 37°C . However it was noted that, under these conditions, mitochondrial Ca was 67% of total cell Ca. This is within the range reported by Murphy *et al.* (1980) and greater than that for cells incubated with 1.25 mM-Ca^{2+} (Table 2).

Murphy *et al.* (1980) added α -agonists to cells after 10 min incubation at 37°C following the Ca^{2+} -loading procedures. Our data (Fig. 3) indicate that such cells only achieve steady-state Ca distribution after 20 min of such incubation, at which time we conducted our experiments. Table 2 shows that, in agreement with Murphy *et al.* (1980), $10 \mu\text{M}$ -phenylephrine did not induce a significant Ca^{2+} efflux from cells under these conditions. On the other hand, we were unable to confirm that phenylephrine significantly altered mitochondrial Ca content as reported by Murphy *et al.* (1980).

Fig. 3. Ca content of (a) hepatocytes and (b) mitochondria-rich fractions after Ca^{2+} loading of the cells

Hepatocytes were incubated for 90 min at $0-4^{\circ}\text{C}$ as 30 ml portions in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 5 mM-Tes and 5 mM- CaCl_2 . Cells were washed (see under 'Methods') three times with Ca^{2+} -free, ice-cold buffer (Krebs & Henseleit, 1932); 2 ml portions were gassed with O_2/CO_2 (19:1) and samples were taken for Ca^{2+} analysis at the times indicated. Data are individual determinations from six hepatocyte preparations.

Table 2. Effect of phenylephrine on calcium content of cells and mitochondria-rich fractions obtained from cells pre-loaded with Ca^{2+}

Hepatocytes were loaded with Ca^{2+} as described in the legend to Fig. 5. After 20 min incubation at 37°C , either 0.01 ml of buffer or 0.01 ml of phenylephrine was added. Control samples were assayed for Ca content immediately and treated samples were taken 2.5 min later. Data are means $\pm$ S.E.M. from the number of hepatocyte preparations in parentheses. The values obtained after phenylephrine treatment are not significantly different from the controls.

	Ca content (ng-atoms/mg cell dry wt.)	
	Cell	Mitochondria-rich fraction
Control	1.76 ± 0.24 (6)	1.02 ± 0.11 (5)
+ Phenylephrine ($10 \mu\text{M}$)	1.77 ± 0.20 (6)	1.15 ± 0.20 (5)

Discussion

Our estimates of the Ca content of the mitochondria-rich fraction may be considered reliable

provided that mitochondrial Ca content does not change markedly during the fractionation procedure. In this respect we were able to inhibit mitochondrial Ca^{2+} uptake by EGTA and Ruthenium Red (see under 'Methods'). Ca^{2+} efflux from mitochondria can occur by two independent mechanisms (Nicholls, 1978). Firstly, Ca^{2+} will be lost through the Ruthenium Red-insensitive $\text{Ca}^{2+}/2\text{H}^{+}$ exchange carrier. Even if mitochondrial Ca^{2+} content were ten times that of our mitochondria-rich extract, the activity of this Ca^{2+} -transport system at $30-37^{\circ}\text{C}$ would be within the range of 1–5 ng-atoms of Ca^{2+} /min per mg of mitochondrial protein (Nicholls, 1978; Dawson & Fulton, 1980; Barritt, 1981; Shears & Bronk, 1981; Coll *et al.*, 1982). This range of values was employed to estimate the efflux of Ca^{2+} during fractionation. The loss of mitochondrial Ca^{2+} after extrusion of the cell extract was computed from the integrated area under a curve depicting the time course for the retention of intact mitochondria above the silicone-oil barrier during 0–20 s centrifugation (assuming 0.24 mg of mitochondrial protein/mg cell dry wt.; Hoek *et al.*, 1980). This value was added to the estimated mitochondrial Ca^{2+} efflux during the 5 s exposure of cells to digitonin immediately before extrusion. In this way it was calculated that, during fractionation, between 0.03 and 0.17 ng-atom of Ca^{2+} /mg cell dry wt. may be lost from the mitochondria. Therefore, the apparent mitochondrial Ca^{2+} content of control hepatocytes (0.65–1.35 ng-atom/mg cell dry wt.; see the Results section) may be a slight underestimate.

Ca^{2+} can also be lost from mitochondria by a Ruthenium Red-insensitive process, which occurs independently of the $\text{Ca}^{2+}/2\text{H}^{+}$ -exchange carrier, but only when the electrical potential across the mitochondrial membrane falls below 130 mV (Nicholls, 1978). Our data indicate that this did not occur after the fractionation of either control hepatocytes (Shears & Kirk, 1984) or those treated with phenylephrine (results not shown). Moreover, immediately after lysis of the plasma membrane, the electrical potential across the mitochondrial membrane will be that between the mitochondrial matrix and the incubation medium (202 mV; Shears & Kirk, 1984). This phenomenon contributes to maintaining the potential above 130 mV. We conclude that our determinations of mitochondrial Ca^{2+} content represent reliable estimates.

We found that $10 \mu\text{M}$ -phenylephrine increased glycogen phosphorylase activity by 100% and induced a loss of up to 20% of cell Ca (Fig. 1). Very similar effects have been reported elsewhere (Blackmore *et al.*, 1982). Fig. 1 also shows that the peak of phosphorylase stimulation preceded the

onset of a significant loss of cellular Ca by 0.5 min. However, Reinhart *et al.* (1982b) employed a Ca^{2+} -sensitive electrode to detect a significant loss of Ca^{2+} from perfused liver within 7 s of the addition of phenylephrine. Moreover, when α -agonists are added to cells previously labelled with $^{45}\text{Ca}^{2+}$, an efflux of the radioactive label can be observed within 10 s (Barritt *et al.*, 1981; Blackmore *et al.*, 1982). It appears that the use of a Ca^{2+} electrode or $^{45}\text{Ca}^{2+}$ may be more sensitive in detecting early changes in Ca^{2+} fluxes than our determinations of total cell Ca.

If the phenylephrine-mediated loss of cell Ca (Fig. 1) had arisen from the mitochondria, it would have represented a substantial proportion of the mitochondrial Ca content (see Table 1). Such a phenomenon was not detected in our experiments (Fig. 2); no significant phenylephrine-mediated change in mitochondrial Ca was observed for up to 10 min after addition of the α -agonist. Indeed, at 0.5 min, by which time glycogen phosphorylase activation was maximal (Fig. 1), mitochondria from phenylephrine-treated cells contained slightly higher levels of Ca, although this effect was not statistically significant. Berthon *et al.* (1981) have also indicated that, after 0.5 min treatment of hepatocytes with α -agonists, mitochondrial Ca content might increase.

Our observation that α -agonists do not mobilize mitochondrial Ca is in conflict with similar studies with Ca^{2+} -loaded mitochondria (Murphy *et al.*, 1980), longer-term isolations of subcellular fractions (Blackmore *et al.*, 1979a,b; Dehaye *et al.*, 1980, 1981; Taylor *et al.*, 1980; Reinhart *et al.*, 1982b), compartmental analyses of $^{45}\text{Ca}^{2+}$ fluxes (Barritt *et al.*, 1981) and experiments with chlorotetracycline fluorescence (Babcock *et al.*, 1979), all of which have been taken to suggest that α -adrenergic agonists mobilize mitochondrial Ca.

Considering first the experiments of Murphy *et al.* (1980), it appears from our data (Fig. 3) that, after the Ca^{2+} -loading procedures employed by these workers, neither total cell nor mitochondrial Ca would be at a steady state before 20 min incubation at 37°C. This is due to expulsion of Ca^{2+} from the cells. Indeed, some of Murphy's co-workers noted this Ca^{2+} efflux in a later publication (Joseph *et al.*, 1983). Murphy *et al.* (1980) studied the effects of α -agonists on mitochondrial Ca after 12.5 min incubation of hepatocytes at 37°C, at which time our data (Fig. 3) indicate mitochondrial Ca content would still be decreasing in control cells. Furthermore, when we employed the non-physiological Ca^{2+} -loading procedures of Murphy *et al.* (1980), the relative mitochondrial Ca content of hepatocytes was increased (compare Table 1 with Table 2), and we were unable to detect

α -adrenergic-mediated Ca^{2+} efflux from cells (compare Fig. 1 with Table 2 and see Murphy *et al.*, 1980). We must, therefore, express reservations concerning the observation by Murphy *et al.* (1980) that α -agonists mobilize mitochondrial Ca.

During long-term mitochondrial isolation studies it is difficult to exclude the likelihood of substantial loss of mitochondrial Ca due both to operation of $\text{Ca}^{2+}/2\text{H}^{+}$ exchange and to de-energization-induced Ca^{2+} efflux (see Nicholls, 1978). Therefore, although it is not immediately apparent why mitochondria isolated in this manner from α -agonist treated liver generally contain less Ca than controls (see above), our data indicates that this phenomenon is not the cause of Ca^{2+} mobilization in intact cells.

Substantial loss of Ca^{2+} from mitochondria will occur even when the fractionation time is decreased to 6 min (Reinhart *et al.*, 1982a,b) in the presence of nupercaine, which reduces $\text{Ca}^{2+}/2\text{H}^{+}$ exchange to 0.5 nmol/min per mg of mitochondrial protein (Dawson & Fulton, 1980). Under these conditions the Ca^{2+} lost would be expected to equal 0.72 ng-atom of Ca^{2+} /mg cell dry wt. (assuming 0.24 mg of mitochondrial protein/mg cell dry wt.; Hoek *et al.*, 1980), which represents 50–110% of our range of values for mitochondrial Ca content. This calculation does not take into account any loss of Ca^{2+} due to mitochondrial de-energization (which was not investigated by Reinhart *et al.*, 1982b), but it is sufficient to indicate the benefits of the rapid fractionation procedures described here. It should also be noted that Reinhart *et al.* (1982b) carried out most of their experiments on livers which had been perfused for 5 min with an aphysiologically low concentration of Ca^{2+} (10 μM). Their data indicate that the perfusion of livers with 1.3 mM- Ca^{2+} markedly decreased the statistical significance of the phenylephrine-induced changes in mitochondrial Ca content.

In considering the experiments of Barritt *et al.* (1981), there are a number of difficulties with compartmental analyses of $^{45}\text{Ca}^{2+}$ fluxes in liver cells which may have contributed to the conclusion that α -agonists mobilize mitochondrial Ca. Firstly, $^{45}\text{Ca}^{2+}$ may exchange with less than 50% of total cell and mitochondrial Ca during the time of the experiments (Foden & Randle, 1978; Barritt, 1981; Barritt *et al.*, 1981), so that $^{45}\text{Ca}^{2+}$ movements may not accurately assess $^{40}\text{Ca}^{2+}$ fluxes. Secondly, as emphasized by Barritt (1981), the technique may not be sufficiently sensitive to resolve all the cellular compartments of exchangeable Ca. Therefore, the Ca compartment assigned as being mitochondrial by Barritt *et al.* (1981) may, in fact, include several non-mitochondrial pools of Ca.

Babcock *et al.* (1979) found that α -agonists decreased cellular chlortetracycline fluorescence; they regarded this as indicative of a decrease in the Ca content of mitochondrial membranes. However, chlortetracycline fluorescence will detect Ca bound to a variety of membranes (Caswell & Hutchinson, 1971). The data of Babcock *et al.* (1979) do not unequivocally characterize a mobilization of mitochondrial Ca.

Our data indicate that, in hepatocytes incubated with near-physiological concentrations of Ca^{2+} , α -adrenergic activation of glycogen phosphorylase is not a consequence of a mobilization of mitochondrial Ca. Our work does not give an indication of the true source of the Ca^{2+} mobilized by hormonal stimulation, although it has been proposed by other workers to arise largely from endoplasmic reticulum or plasma membranes (Poggioli *et al.*, 1980; Berthon *et al.*, 1981; Capiod *et al.*, 1982).

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