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

Vasopressin stimulates mitochondrial Ca^{2+} uptake

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Stimulation of hepatocytes with vasopressin (10nM) in the presence of 1.25mM extracellular Ca^{2+} increased glycogen phosphorylase activity 4-fold within 15s and provoked a rapid efflux of cell-associated Ca^{2+} . Vasopressin also caused a transient increase in the Ca content of a mitochondria-rich fraction separated within seconds of hormone stimulation by a rapid fractionation technique [Shears & Kirk (1984) *Biochem. J.* 219, 375–382]. The Ca content of this fraction was restored to the control value within 2min of hormone addition. These results indicate that mitochondria are not the source of the cell-associated Ca which is mobilized in the cytosol of vasopressin-stimulated hepatocytes. Rather, these organelles buffer the increase in cytosol $[\text{Ca}^{2+}]$ attributable to Ca mobilization from non-mitochondrial sources.

Vasopressin promotes hepatic glycogenolysis by elevating cytosol $[\text{Ca}^{2+}]$, thereby activating glycogen phosphorylase (EC 2.4.1.1). It has been suggested by various workers that this additional Ca^{2+} is mobilized from mitochondria (for reviews, see Exton, 1981; Williamson *et al.*, 1981). Much of the evidence in support of this view has come from experiments in which hepatic mitochondria were isolated by classical differential centrifugation. In such experiments, pretreatment of livers or hepatocytes with vasopressin decreased the Ca^{2+} content of the subsequently isolated organelles (Blackmore *et al.*, 1979; Dehaye *et al.*, 1981; Morgan *et al.*, 1983).

We have three important criticisms of these studies. Firstly, livers were perfused and hepatocytes were incubated with an unphysiologically low concentration of Ca^{2+} (<0.05mM). When extracellular $[\text{Ca}^{2+}]$ is lowered from the physiological range to 0.1mM, then the kinetics of cellular Ca^{2+} fluxes are modified and cell-associated Ca pools are depleted (Barritt *et al.*, 1981). Secondly, it is difficult to exclude the possibility that Ca redistribution occurs during the lengthy procedures employed to prepare mitochondria. This difficulty was acknowledged by Reinhart *et al.* (1982a), who used Percoll gradients to prepare subcellular fractions within 6min of cell disruption.

Abbreviations used: Ca and Ca^{2+} are used to signify total calcium and ionized calcium respectively.

However, we have calculated that a substantial redistribution of mitochondrial Ca may still have taken place in their experiments (see Shears & Kirk, 1984b). Finally, in the studies quoted above (Blackmore *et al.*, 1979; Dehaye *et al.*, 1981; Morgan *et al.*, 1983), mitochondria were isolated after several minutes of hormone stimulation, although Ca mobilization occurs within seconds (Barritt *et al.*, 1981).

We have refined and characterized a technique for rapid cell fractionation, first described by Hoek *et al.* (1980), so that we can now isolate 80% of hepatocyte mitochondria within seconds of cell disruption (Shears & Kirk, 1984a). This technique is particularly appropriate for studies of hormone effects upon mitochondrial Ca content, since mitochondria are largely separated from other Ca-containing organelles (Shears & Kirk, 1984a). Moreover no substantial redistribution of mitochondrial Ca occurs during the isolation procedure (Shears & Kirk, 1984b).

Murphy *et al.* (1980) utilized a similar cell-fractionation technique with Ca-loaded hepatocytes that were subsequently incubated in Ca^{2+} -free medium containing noradrenaline. They observed that stimulation with the hormone for 2.5min decreased the Ca content of a mitochondria-rich fraction. In contrast, when we prepared a mitochondria-rich fraction from hepatocytes that had been stimulated by phenylephrine

for 30s–10min in the presence of 1.25 mM-Ca^{2+} , we found no significant change in mitochondrial Ca content (Shears & Kirk, 1984b). We now report the results of similar investigations with vasopressin.

Materials and methods

Hepatocytes were prepared from male Wistar rats as previously described (Shears & Kirk, 1984a) and incubated at 37°C in a bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 5 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid), 10 mM-glucose , 2% (w/v) bovine serum albumin and 1.25 mM-CaCl_2 . Samples of this suspension were incubated in closed plastic vials which were gassed every 15min with O_2/CO_2 (19:1). Experiments were generally completed within 1.5h. Hepatocytes released $7.7 \pm 0.7\%$ and $12 \pm 0.8\%$ ($n = 7$) of their lactate dehydrogenase activity into the incubation medium after 0.25h and 1.5h respectively.

Preparation of mitochondria-rich fractions

Mitochondria-rich fractions were prepared by a combination of lysis with digitonin and mechanical disruption, followed by rapid centrifugation through a silicone-oil density barrier. The full characteristics of this technique have been described previously (Shears & Kirk, 1984a).

Enzyme assays

Glycogen phosphorylase activity in cell extracts was determined as described by Kirk *et al.* (1979). Results were corrected for the radioactivity of a blank from which cells were omitted. Lactate dehydrogenase (EC 1.1.1.27) and glutamate dehydrogenase (EC 1.4.1.2) activities were determined as described by Shears & Kirk (1984a).

Determination of Ca in intact cells and mitochondria-rich fractions

For the determination of total cell Ca, samples (0.7ml) of intact cells were centrifuged through silicone oil into 0.1 ml of 2 M-HClO_4 , from which 0.08ml samples were removed by microsyringe and assayed for Ca^{2+} (Shears & Kirk, 1984b).

Ca in the mitochondria-rich fractions was determined in samples (0.08ml) taken from the acid layers into which these fractions were centrifuged during rapid cell fractionation (Shears & Kirk, 1984a). The measured Ca contents of the mitochondria-rich fractions were corrected for damaged mitochondria and residual intact cells by assaying the release of glutamate dehydrogenase and lactate dehydrogenase.

Perchlorate-precipitated extracts of intact cells and mitochondria-rich samples were also obtained

from incubations that had been supplemented for at least 30min with $^{45}\text{Ca}^{2+}$ ($0.25\text{ }\mu\text{Ci/ml}$). Radioactivity in these extracts was determined by liquid-scintillation spectrometry. Samples (0.05ml) of the incubation medium, from which the cells had been removed by centrifugation through silicone oil, were also assayed in order to determine the specific radioactivity of extracellular $^{45}\text{Ca}^{2+}$.

The $^{40}\text{Ca}^{2+}$ or $^{45}\text{Ca}^{2+}$ in the extracellular or extramitochondrial medium which contaminated the HClO_4 layers was estimated by using $[\text{U-}^{14}\text{C}]$ -sucrose and $^3\text{H}_2\text{O}$ (Shears & Kirk, 1984a).

Materials

Bovine serum albumin (fraction V), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, oligomycin and vasopressin were obtained from Sigma (London) Chemical Co. The albumin was dialysed before use (Shears & Kirk, 1984a). Radiochemicals were purchased from Amersham International. All other chemicals were of the highest grade commercially available: their sources are given in our previous papers (Shears & Kirk, 1984a,b).

Results and discussion

Effects of vasopressin on phosphorylase activity and the Ca content of intact cells and mitochondria-rich fractions

In agreement with previous reports (Keppens *et al.*, 1977; Creba *et al.*, 1983), vasopressin (10 nM) provoked a rapid activation of hepatocyte glycogen phosphorylase (Fig. 1a). The activity of this enzyme was increased 4-fold within 15s of hormone addition. Since intracellular Ca^{2+} mobilization precedes this activation (see, e.g., Blackmore *et al.*, 1983), these experiments emphasize the importance of establishing the source of hormone-mobilized Ca^{2+} within this time-scale.

Ca^{2+} -mediated activation of glycogen phosphorylase in hormone-stimulated hepatocytes has invariably been associated with a depletion of total cell Ca (Blackmore *et al.*, 1979, 1982; Reinhart *et al.*, 1982a; Morgan *et al.*, 1983; Shears & Kirk, 1984b), which is presumably mediated by a Ca^{2+} -activated ATPase (see, e.g., Chan & Junger, 1983). This phenomenon was also observed after vasopressin treatment in the present study, where total cell Ca was decreased by 0.19 and $0.35\text{ }\mu\text{g-atoms/g dry wt.}$ after 1 and 2min stimulation respectively (Fig. 1b). These changes were statistically significant and represent a release of 13% of total cell Ca within 2min of vasopressin treatment.

The results in Fig. 1(c) demonstrate that $0.85\text{ }\mu\text{g-atoms of Ca/g cell dry wt.}$ was recovered in the mitochondria-rich extract after rapid fractionation of unstimulated hepatocytes. This value represents

Fig. 1. Influence of vasopressin on phosphorylase activity and the Ca content of hepatocytes and mitochondria-rich fractions

Hepatocytes were prepared and incubated as described in the Materials and methods section. Vasopressin (10 nM, ●) or control additions (○) were made at zero time. Glycogen phosphorylase *a* activity (a) and the Ca contents of hepatocytes (b) and mitochondria-rich fractions (c) were measured as described in the Materials and methods section. Phosphorylase determinations were from two separate hepatocyte preparations, and Ca^{2+} was assayed by atomic-absorption spectroscopy in acid extracts from six such preparations. Vertical bars represent S.E.M. Statistical significance was assessed by paired *t* test: * $P < 0.05$; *** $P < 0.01$.

$31 \pm 4\%$ of total cell Ca and is lower than that determined in previous experiments ($41 \pm 4\%$; Shears & Kirk, 1984b). This difference was not associated with any changes in marker-enzyme distribution after rapid cell fractionation (results not shown) and was not statistically significant.

Fig. 2. Influence of vasopressin on ^{45}Ca content in hepatocytes and mitochondria-rich fractions

Hepatocytes were prepared and labelled with $^{45}\text{Ca}^{2+}$ as described in the Materials and methods section. Vasopressin (10 nM, ●) or control additions (○) were made at zero time. [^{45}Ca] in hepatocytes and mitochondria-rich fractions was assayed as described in the Materials and methods section. Results are means $\pm$ S.E.M. from four separate hepatocyte preparations. Statistical significance was assayed by paired *t* test: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

We observed a significant increase in the Ca content of the mitochondria-rich fraction separated 0.5 or 1 min after stimulation of hepatocytes with vasopressin (Fig. 1c). We did not observe any decrease in the Ca content of this fraction at any time during the first 2 min of hormone stimulation. As discussed in our previous paper (Shears & Kirk, 1984b), changes in the Ca content of the mitochondria-rich extract separated by rapid cell fractionation probably reflect changes in mitochondrial Ca content *in situ*. Hence these data indicate that mitochondria are not the source of the cell-associated Ca which is rapidly mobilized in vasopressin-treated hepatocytes. Similar results were obtained when hepatocytes were treated with a higher concentration of vasopressin (0.5 μM ; results not shown).

Effect of vasopressin on ^{45}Ca content in intact cells and mitochondria-rich fractions

When hepatocytes were preincubated with $^{45}\text{Ca}^{2+}$ (0.5 $\mu\text{Ci/ml}$) for 30 min before the addition of vasopressin, the radioactivity of intact cells and mitochondria-rich fractions reached a plateau. Comparison of Figs. 1(c) and 2(b) indicates that, under these conditions, the ^{40}Ca pool of the mitochondria-rich fraction from unstimulated cells was considerably larger than the corresponding ^{45}Ca pool. Thus, only a portion of 'mitochondrial'

Ca was readily exchangeable with ^{45}Ca , but this exchangeable Ca pool appeared to reach a steady state during the 30 min preincubation with $^{45}\text{Ca}^{2+}$. The difference between the sizes of the mitochondria-rich pools of ^{40}Ca and ^{45}Ca ($0.47 \mu\text{g-atoms/g}$ cell dry wt.) was almost identical with the difference between the total cell contents of ^{40}Ca and ^{45}Ca ($0.46 \mu\text{g-atoms/g}$ cell dry wt.). Hence, ^{40}Ca in the mitochondria-rich fraction (which also contains some nuclei; Shears & Kirk, 1984b) accounts for most of the ^{40}Ca that did not exchange with $^{45}\text{Ca}^{2+}$ during our experiments. Barritt (1981) has previously reported that up to half of the Ca of isolated mitochondria is not readily exchangeable with $^{45}\text{Ca}^{2+}$.

Vasopressin (10 nM) provoked a decrease in the ^{45}Ca content of pre-labelled hepatocytes, which achieved statistical significance after 1 and 2 min of treatment with the hormone (Fig. 2a). The pattern of ion efflux was similar to that observed in our studies of total cell ^{40}Ca (Fig. 1b). Also in agreement with the results shown in Fig. 1, vasopressin provoked a transient increase in the ^{45}Ca content of the mitochondria-rich fraction (Fig. 2b). After 0.5 min stimulation with the hormone, this fraction contained almost twice as much $^{45}\text{Ca}^{2+}$ as did that from unstimulated cells. The ^{45}Ca content of the mitochondria-rich fraction returned to the control value after 2 min stimulation. This indicates that the transient rise in radioactivity represents an increase in the size of the readily exchangeable Ca pool of this fraction rather than an effect on the unlabelled, slowly exchangeable, Ca pool. However, the absolute changes in the sizes of the ^{45}Ca pools induced by vasopressin were slightly smaller than those observed with ^{40}Ca , suggesting that the hormone is able to mobilize some slowly exchangeable Ca.

The increase in mitochondrial calcium content that we have observed after vasopressin treatment in hepatocytes (Figs. 1 and 2) probably arises as a result of mitochondrial sequestration of the Ca^{2+} mobilized from another cell-associated store after hormone stimulation. The restoration of 'mitochondrial' Ca content to control values after 2 min stimulation with vasopressin was contemporaneous with the observed diminution in hepatocyte Ca content and may reflect the equilibration of mitochondrial Ca with a diminished $[\text{Ca}^{2+}]$ in the cytosol. The present results do not enable us to identify the cellular locus of the hormone-sensitive Ca store in hepatocytes, but experiments with several tissues have suggested that it may be associated with the endoplasmic reticulum or the plasma membrane (Althaus-Salzmänn *et al.*, 1980; Berthon *et al.*, 1981; Dormer & Williams, 1981; Burgess *et al.*, 1983). It seems likely that this Ca store may have the same identity as that which has

been shown to be mobilized by inositol 1,4,5-trisphosphate in permeabilized pancreatic acinar cells (Streb *et al.*, 1983).

The present study is the first to demonstrate that isolated mitochondria contain increased Ca after stimulation of hepatocytes with vasopressin. Poggioli *et al.* (1980) and Berthon *et al.* (1981) have previously reported that α -adrenergic stimulation in the presence of physiological extracellular $[\text{Ca}^{2+}]$ may provoke a transient increase in the Ca content of hepatocyte mitochondria, even when isolated by classical differential centrifugation. Our previous study using the rapid fractionation technique supported this view, but the effects of α -adrenergic stimulation on mitochondrial $[\text{Ca}]$ were too small and inconsistent to achieve statistical significance (Shears & Kirk, 1984b). Furthermore, Joseph & Williamson (1983) have shown that vasopressin transiently increases the amount of hepatic Ca that may be released by uncouplers of oxidative phosphorylation. This Ca is presumably released from a mitochondrial pool. The failure of others to observe these effects on mitochondrial Ca probably arises from the deficiencies in experimental design discussed in the introduction. In view of the present results and those of Poggioli *et al.* (1980) and Berthon *et al.* (1981), the most important of these deficiencies appear to be the use of unphysiologically low ($<0.05 \text{ mM}$) concentrations of extracellular Ca^{2+} and the failure to measure mitochondrial $[\text{Ca}]$ during the early phase of receptor activation, when Ca^{2+} is first mobilized in the cytosol. A fuller critique of the methodologies adopted by other workers in the field may be found in Shears & Kirk (1984b).

Relationship to other Ca^{2+} -mediated effects in hepatocytes

The idea that changes in mitochondrial $[\text{Ca}^{2+}]$ may reflect, rather than evoke, changes in cytosol $[\text{Ca}^{2+}]$ has been suggested previously by Denton and co-workers (McCormack & Denton, 1980; Denton *et al.*, 1980). These workers reported that intramitochondrial dehydrogenases may be activated by small increases in mitochondrial $[\text{Ca}^{2+}]$, which may themselves be provoked by raising $[\text{Ca}^{2+}]$ in the extramitochondrial compartment.

Several workers have observed a transient increase in respiratory rate after addition of vasopressin to hepatocytes (Reinhart *et al.*, 1982b; Binet & Claret, 1983; Blackmore *et al.*, 1983; Balaban & Blum, 1982). Vasopressin-stimulated uptake of Ca^{2+} into mitochondria may contribute to this effect, either by provoking a transient decrease in $\Delta\psi$ (Nicholls & Crompton, 1980) or by activating intramitochondrial dehydrogenases as described above.

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