Effect of the α -agonist noradrenaline on total and $^{45}\text{Ca}^{2+}$ movements in mitochondria of rat liver cells

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Ca^{2+} movements triggered by noradrenaline were determined in isolated cells and mitochondria from rat livers. It has been shown that these depend on experimental conditions. In cells incubated in 1.8 mM-Ca^{2+} , results suggest that noradrenaline mobilizes Ca^{2+} from reticulum before releasing Ca^{2+} from mitochondria.

It is widely accepted that the various physiological responses of mammalian hepatocytes to α -receptor activation (glycogenolysis, loss of Ca^{2+} and loss or gain of K^{+}) result from a single signal, namely cytosolic Ca^{2+} (Jenkinson *et al.*, 1978; Exton, 1979). According to this hypothesis, α -agonists mobilize Ca^{2+} from an internal pool leading to an increase in intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$), which stimulates the intracellular system sensitive to Ca^{2+} ; phosphorylase *b* is converted into its active form, phosphorylase *a*, and the Ca^{2+} -pump and Ca^{2+} -sensitive K^{+} channels (in the livers of species that show this permeability) are stimulated. It has been proposed that in rat liver, mitochondria are the main source of the Ca^{2+} mobilized by α -agonist on the basis of the observation that the activation of α -receptors is associated with a large net release of Ca^{2+} from mitochondria (Chen *et al.*, 1978; Blackmore *et al.*, 1979; Murphy *et al.*, 1980). In contrast, α -agonists have been shown to promote a transient uptake of $^{45}\text{Ca}^{2+}$ by mitochondria (Althaus-Salzmann *et al.*, 1980; Poggioli *et al.*, 1980). Poggioli *et al.* (1980) suggested therefore that mitochondria were not the primary source of the Ca^{2+} mobilized and that other Ca^{2+} pools were involved in the α -response.

In the present work, we have shown that these discrepancies between the results and their interpretation may be due to the differences between experimental protocols (time of incubation with the hormone, Ca^{2+} concentration of the medium incubating cells, method for measuring Ca^{2+}). Our results suggest that in cells incubated in 1.8 M-Ca^{2+} , the α -adrenergically mediated $[\text{Ca}^{2+}]_i$ increase is the sum of the sequential release of Ca^{2+} first from endoplasmic reticulum and then from mitochondria.

Abbreviation used: FCCP, carbonyl cyanide *p*-tri-fluoromethoxyphenylhydrazine.

Experimental

Cell isolation and incubation

Liver cells were prepared from 220–250 g white female Wistar rats as reported previously (Poggioli *et al.*, 1980). Routinely, cells were suspended at 37°C for 30–60 min in Eagle's medium, containing (mM): NaCl, 116; KCl, 5.4; CaCl_2 , 1.8; MgSO_4 , 0.81; NaH_2PO_4 , 0.96; NaHCO_3 , 25; as well as (mg/litre): amino acids, 805; vitamins, 8.1; glucose, 2000; L-glutamine, 292; Phenol Red, 10). This medium was supplemented with 2% albumin (fraction V; Sigma). The pH was maintained at 7.4 by circulating CO_2/O_2 (1:19) above the cell suspension. Cells were then resuspended in $^{45}\text{Ca}^{2+}$ Eagle's media (1 mCi/litre) containing propranolol ($5\text{ }\mu\text{M}$) in the presence or in the absence of albumin. $^{45}\text{Ca}^{2+}$ contents, dry weights and viability of cells were determined as described previously (Burgess *et al.*, 1979).

Isolation of mitochondria from cell suspension or from perfused liver

Mitochondria were isolated from cell suspensions or from perfused liver equilibrated in the presence or in the absence of $^{45}\text{Ca}^{2+}$ as described previously (Claret-Berthon *et al.*, 1977; Poggioli *et al.*, 1980). All operations were carried out at 0°C . Cell or liver-lobe samples were homogenized in 10 ml of ice-cold medium containing (mM): sucrose, 250; EGTA, 1.3; tetracaine, 2; Tris/HCl, 10 (pH 7.4), with a Potter Teflon homogenizer. The resulting homogenates were centrifuged at 800 g for 10 min in an MSE refrigerated centrifuge. The supernatant fractions were centrifuged at 8500 g for 10 min and the mitochondrial pellet was washed twice in the same buffer. Low temperature (0°C) and Ca^{2+} -chelators have been shown to be adequate both to prevent uptake and loss of Ca^{2+} by mitochondria

during their isolation from perfused livers (Claret-Berthon *et al.*, 1977). It was assumed that this also held for mitochondria isolated from hepatocytes. This is justified by the following observations. (1) Total Ca^{2+} and exchangeable $^{45}\text{Ca}^{2+}$ contents were similar when measured in mitochondria isolated from dispersed liver cells or from perfused livers (Foden & Randle, 1978; Murphy *et al.*, 1980; B. Berthon, J. Poggioli, T. Capniod & M. Claret, unpublished work). (2) The net uptake of $^{45}\text{Ca}^{2+}$ occurring within 30 s after α -agonist addition was observed in both preparations (Althaus-Salzmann *et al.*, 1980; B. Berthon, J. Poggioli, T. Capniod & M. Claret, unpublished work).

Other methods

$^{45}\text{Ca}^{2+}$, $^{40}\text{Ca}^{2+}$ and protein contents of mitochondria were determined as indicated previously (Claret-Berthon *et al.*, 1977; Poggioli *et al.*, 1980).

Chemicals

Noradrenaline and adrenergic blockers were obtained from Sigma and $^{45}\text{Ca}^{2+}$ from Amersham.

Results and discussion

Cell and mitochondrial $^{45}\text{Ca}^{2+}$ movements generated by noradrenaline

Noradrenaline causes a large efflux of $^{45}\text{Ca}^{2+}$ from isolated hepatocytes due to mobilization of an intracellular Ca^{2+} compartment. The $^{45}\text{Ca}^{2+}$ release, which represents up to 20–30% of the cell $^{45}\text{Ca}^{2+}$, was dose-dependent with a dissociation constant of about 50 nM and was substantially blocked by the α -blocker phenoxybenzamine (50 μM). In a previous study (Poggioli *et al.*, 1980), we showed that the Ca^{2+} loss was complete by 3 to 4 min but that the Ca^{2+} -pump flux was maximal 30 s after the hormone addition, indicating that the internal pool responsible for the increase in $[\text{Ca}^{2+}]_i$ had been released by that time. Fig. 1 shows that mitochondria isolated from cells after 30 s of incubation with noradrenaline exhibited a net uptake of $^{45}\text{Ca}^{2+}$ (up to about 0.4 nmol/mg of mitochondrial protein). The uptake was inhibited by 10 μM -FCCP, an uncoupling agent that causes the electrochemical proton gradient to collapse. These results are in agreement with those obtained previously (Poggioli *et al.*, 1980). They show that an uptake of $^{45}\text{Ca}^{2+}$ by mitochondria is occurring at the time when the Ca^{2+} -pump located in plasma membranes is fully activated by noradrenaline.

Effect of short-term incubation in Ca^{2+} -free media

Cells were first equilibrated with 1.8 mM- $^{45}\text{Ca}^{2+}$ for 30 min then rapidly washed and resuspended in Eagle's medium containing either 1.8 mM- $^{45}\text{Ca}^{2+}$ or

Fig. 1. Effect of FCCP (10 μM) and of a decrease in external Ca^{2+} on the Ca^{2+} uptake by mitochondria provoked by the addition of noradrenaline (5 μM) on cell suspension in the presence (a) or in the absence (b) of 2% albumin

Cells were incubated with $^{45}\text{Ca}^{2+}$ for 30–60 min. Mitochondria were isolated from cell suspension just before and 30 s after hormone addition. Samples (2 ml) of cell suspension were added to 10 ml of ice-cold buffer and immediately homogenized (see the Experimental section). When used, FCCP was added to the suspension 5 min before noradrenaline. For experiments in Ca^{2+} -free media, cells pre-equilibrated with 1.8 mM- $^{45}\text{Ca}^{2+}$ were rapidly centrifuged and washed twice (50 g, 90 s) in washing solution containing (mM): NaCl, 154; EGTA, 2; pH 7.4 at 0°C. The cell pellets were then resuspended in Ca^{2+} -free Eagle's media (supplemented with 100 μM -EGTA). The $^{45}\text{Ca}^{2+}$ content of mitochondria was: (a) 2.00 ± 0.20 nmol/mg ($n = 10$); (b) 1.90 ± 0.14 nmol/mg ($n = 6$). Note the difference in ordinate scales between (a) and (b).

in Ca^{2+} -free medium supplemented with 100 μM -EGTA. Noradrenaline was added 30 s later; mitochondria were isolated 30 s after the hormone. Parallel experiments were carried out with cells suspended in the absence of albumin, a condition that has been shown to increase the α -mediated $^{45}\text{Ca}^{2+}$ uptake by mitochondria (Poggioli *et al.*, 1980). Fig. 1 shows that the reduction of extracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_o$) completely blocked the response. When cells were incubated in the absence of albumin, the $^{45}\text{Ca}^{2+}$ uptake was also blocked by FCCP and Ca^{2+} -free solutions. From these results it is not possible to conclude whether the unknown mechanism by which noradrenaline releases Ca^{2+} from an internal Ca^{2+} pool is dependent on Ca^{2+} influx or not. The inhibition observed in Ca^{2+} -free media may result from depletion of Ca^{2+} from this pool if it is exchanging Ca^{2+} rapidly. In this respect, it is obvious that long-term incubations of cells in

media containing lower concentrations than 1.8 mM may explain the apparent absence of Ca^{2+} uptake by mitochondria reported by others (Chen *et al.*, 1978; Blackmore *et al.*, 1979; Murphy *et al.*, 1980; Taylor *et al.*, 1980).

Time course of the effect of noradrenaline on mitochondrial $^{40}\text{Ca}^{2+}$ content

Mitochondria were isolated from livers perfused with normal Ca^{2+} concentration just before (control) and 30 s, 1 min and 4 min after noradrenaline perfusion ($5\text{ }\mu\text{M}$). They were also isolated from livers pre-perfused with FCCP ($10\text{ }\mu\text{M}$) for 5 min. The mitochondrial $^{40}\text{Ca}^{2+}$ was decreased by about 45% in livers treated with the inhibitor (Fig. 2) in agreement with the fact that uncoupling agents abolish the ability of mitochondria to accumulate Ca^{2+} . In control cells, noradrenaline triggered a rapid uptake of $^{40}\text{Ca}^{2+}$, which is completely blocked by the uncoupler FCCP. This is followed by a large release that could be detected 1 min after addition of the hormone and that was significant after 4 min. These results demonstrate that the uptake of Ca^{2+} is not due to differences in the method for measuring Ca^{2+} . In contrast, the release of Ca^{2+} was not detected when using labelled Ca^{2+} (Poggioli *et al.*, 1980). The observed uptake of Ca^{2+} by mitochondria suggests that the Ca^{2+} first mobilized by noradrenaline is not coming from mitochondria but from another intracellular compartment.

Effect of caffeine on cellular $^{45}\text{Ca}^{2+}$ movements caused by noradrenaline

Besides mitochondria, the most likely source of Ca^{2+} is endoplasmic reticulum, which has been shown to have a high capacity for concentrating Ca^{2+} in liver (Moore *et al.*, 1975). To test this hypothesis, hepatocytes were loaded with 1.8 mM- $^{45}\text{Ca}^{2+}$ for 30 min and the effect of noradrenaline was determined in the presence of 2 mM-caffeine, a drug that potentiates Ca^{2+} release in reticulum of muscle cells (Endo, 1977). Caffeine added 5 min before noradrenaline treatment in 14 coupled experiments had no depleting effect on the $^{45}\text{Ca}^{2+}$ content: $2.52 \pm 0.14\text{ nmol/mg}$ dry wt. in control cells and $2.42 \pm 0.12\text{ nmol/mg}$ in treated cells. Instead, it increased the net Ca^{2+} loss by the cells normally seen after noradrenaline treatment by 38%. This effect of caffeine supports the suggestion that the potentiating effect of methylxanthines on α -agonist-induced carbohydrate metabolism is mediated by Ca^{2+} (Chan & Exton, 1978). It also suggests that the endoplasmic reticulum is involved as the primary pool of Ca^{2+} released by α -adrenergic activation.

The present work shows that net Ca^{2+} movements triggered by noradrenaline in mitochondria of rat liver cells may depend on experimental conditions, including the time of incubation with the

Fig. 2. Effect of noradrenaline ($5\text{ }\mu\text{M}$) on the total Ca^{2+} content of mitochondria *in situ* isolated from livers perfused with non-recirculating physiological solutions in the absence (a) or in the presence (b) of $10\text{ }\mu\text{M}$ -FCCP. The media contained $5\text{ }\mu\text{M}$ propranolol. Noradrenaline was added at 30 min and FCCP 5 min before the hormone. Ordinate values are expressed as percentages of the Ca^{2+} content before noradrenaline addition. [Controls, $9.39 \pm 0.81\text{ nmol/mg}$ ($n = 12$); FCCP-treated mitochondria, $5.2 \pm 0.53\text{ nmol/mg}$ ($n = 5$)].

hormone or the presence of Ca^{2+} in the media. In cells equilibrated in 1.8 mM- Ca^{2+} , mitochondria exhibit a biphasic response to the hormone with a transient uptake at a moment when the intracellular Ca^{2+} -sensitive systems (phosphorylase, K^{+} channels, Ca^{2+} -pump) are maximally activated (Burgess *et al.*, 1979; Aggerbeck *et al.*, 1980; Poggioli *et al.*, 1980). A plausible hypothesis is that at least part of the Ca^{2+} mobilized originates from endoplasmic reticulum. The Ca^{2+} stored by reticulum is large

enough to account for a release that represents 20–30% of the cell calcium (Claret-Berthon *et al.*, 1977). Caffeine, which has been shown to potentiate the so-called Ca^{2+} -induced Ca^{2+} release from sarcoplasmic reticulum (Endo, 1977) increases the Ca^{2+} release mediated by α -adrenergic hormones. If this model is valid, the increase in $[\text{Ca}^{2+}]_i$ responsible for the glycogenolysis and for cation movements should be due to the sequential release of Ca^{2+} from endoplasmic reticulum and then from mitochondria.

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