CALCIUM MOVEMENTS IN IN SITU MITOCHONDRIA FOLLOWING ACTIVATION OF α-ADRENERGIC RECEPTORS IN RAT LIVER CELLS

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1. Introduction

Several groups have shown that activation of α-, and not only β-adrenergic receptors, increases phosphorylase activity and glucose release in the liver of several species (reviewed [1,2]). The α-adrenergic response, in contrast to the β-one, is not associated with an increase in cyclic AMP or with the activation of adenylate cyclase [3,4]. It is thought instead to be mediated by a rise in intracellular Ca which may play a role similar to that of cAMP in the β-response [5]. According to this scheme, the activation of α-receptors would cause Ca to be released from internal stores and the resulting rise in cytosolic Ca would be responsible for the conversion of phosphorylase *b* to its active form phosphorylase *a*. It has been proposed [6–8] that mitochondria are the source of the Ca release, being in some way 'permeabilized' by an intracellular messenger which becomes active following the binding of catecholamines to α-receptors.

These observations prompted us to determine the time course of the ^{45}Ca movements elicited by α-adrenergic activation both in isolated cells and in in situ mitochondria. The results confirm that noradrenaline causes an increase in cytosolic calcium. The increase is provided partly by the Ca influx through the plasma membrane of cells and partly by the release of Ca from internal stores which are not however mitochondria. Rather, the mitochondria exhibit a rapid and transient uptake of Ca (30–60 s) which correlates closely with extrusion of Ca from the cells (30–60 s) presumably as a consequence of activation of the Ca pump. Of the Ca mobilized, 25–85% is taken up by mitochondria. It is suggested that mitochondria behave as a buffer for internal ionized calcium and that they are not the source of the Ca released in the α-response.

Abbreviation: FCCP, carbonyl-cyanide *p*-trifluoromethoxyphenyl hydrazone

2. Materials and methods

Hepatocytes were isolated from the liver of female Wistar rats as in [9,10]. The cells were equilibrated at 37°C for 30–60 min in Eagle's medium (Wellcome) containing (mM): NaCl, 116; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 0.81; NaH_2PO_4, 0.96; $NaHCO_3$, 25 and (mg/l): amino acids 805; vitamins, 8.1; glucose, 1000; L-glutamine, 292; phenol red, 10. This medium was supplemented with 2% albumin (fraction V, Sigma). The pH was maintained at 7.40 by circulating 5% CO_2 in O_2 above the cell suspension which was shaken at 120 strokes/min. Cells were then centrifuged at 50 × *g* for 2 min and the pellet resuspended in ^{45}Ca Eagle's media in the presence or in the absence of albumin. ^{45}Ca content were determined by counting the ^{45}Ca content of the pellet after centrifuging the cells at 12 000 × *g* for 30 s through an oil phase as in [11]. The viability of the cells was checked before and after each experiment by their ability to exclude Trypan Blue 0.4%. The dry weight of the cell suspension was estimated by desiccation at 105°C of the pellets obtained by centrifugation for 3 min at 5000 × *g*.

For isolating mitochondria, 2 ml cell suspension samples were mixed with 10 ml medium containing 0.25 M sucrose, 1.3 mM EGTA, 1 mM tetracaine, and 10 mM Tris–HCl (pH 7.40) and homogeneised in a Potter teflon homogeneiser. All operations were carried out at 0°C. Low temperature, calcium-chelators

(EGTA) and inhibitors of Ca transport (tetracaine) were used to prevent any redistribution of mitochondrial ^{45}Ca during homogenization [12,13]. Homogenates were centrifuged at 800 × *g* for 10 min. The supernatant fractions were then centrifuged at 8500 × *g* for 10 min and the pellet washed and centrifuged twice at 8500 × *g* for 10 min in the same buffer. The resulting mitochondrial pellet was resuspended in deionised water. The protein content of the pellet was determined by the method of Lowry using bovine serum albumin (Sigma, fraction V) as standard. Citrate synthase was assayed according to [14]. The ^{45}Ca content of cells and mitochondria were determined by counting pellet homogenates with a liquid scintillation spectrometer.

3. Results and discussion

Fig.1a shows the effect of noradrenaline (5.10^{-6} M) on the ^{45}Ca content of rat liver cells equilibrated with the tracer for 30 min, a period long enough to allow most of the cytoplasmic calcium including that in the mitochondria to exchange [12]. As extracellularly bound ^{45}Ca was removed by a large excess of EGTA during the centrifugation period, the values given in this study refer to the internal ^{45}Ca content of the hepatocytes. In the presence of albumin, noradrenaline caused an immediate net extrusion of Ca from the cells. Most of the Ca was pumped out within 90 s and the response was effectively complete by 3 min. The net loss of Ca was 25% of the initial content (1.73 nmol/mg ± 0.11) which represents a quantity of 0.430 nmol/mg. This phase of Ca extrusion triggered by activation of α-receptors has been already reported for rat liver cells and has been attributed to Ca released from mitochondria [6–8]. Fig.1c shows that the net Ca flux calculated from the time course of the adrenergic-sensitive Ca response was maximal at 30 s. Since there is no desensitization phenomenon of α-receptors in isolated cells [15] at that time, the internal stores may have been already depleted. This observation is in excellent agreement with the fact that maximal activation of phosphorylase or of the Ca-sensitive K permeability were observed within the 30 s following addition of noradrenaline in isolated liver cells [11,15]. In perfused liver the Ca fluxes elicited by activation of α-receptors appears to be delayed since the maximal response is generally observed 2–3 min after addition of the hormone [7,16,17]. This suggests that diffusion barriers may hinder free diffusions of hormones and ions in the extracellular and interstitial spaces in this preparation and as a consequence may mask the sequence of intracellular events occurring under such conditions.

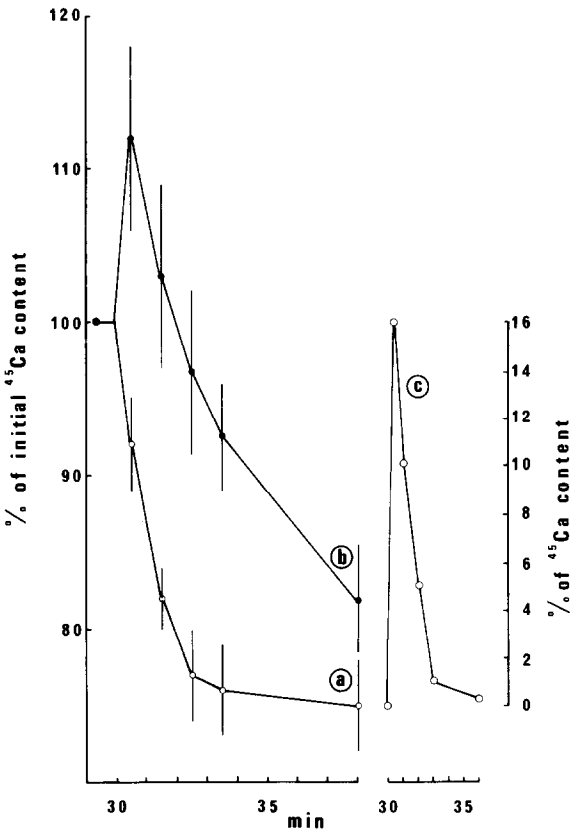

Fig.1. Effect of noradrenaline (5.10^{-6} M) on the ^{45}Ca content of rat liver cells equilibrated in the presence (a) or in the absence (b) of 2% albumin. ^{45}Ca was included in the suspension fluid from zero time onwards. Propanolol (5.10^{-6} M) was added at zero time and noradrenaline at 30 min. Ordinate values have been expressed as percentages of the content before noradrenaline (mean value = 1.80 ± 0.12 nmol Ca/mg dry wt, *n* = 27). The vertical bars indicate the SEM of 14 obs. The curve (c) represents the net Ca flux calculated from curve (a) (% ^{45}Ca exchanged/min). Abscissa, incubation time in ^{45}Ca (min).

The dose response of noradrenaline on ^{45}Ca extrusion was tested in cells equilibrated with the hormone for 8 min. The maximal effect was seen at 10^{-6} M and was equivalent to a net loss of 23% of the initial Ca content. The concentration for half-maximal effect was ~5.10^{-8} M. The response was observed in

the presence of the β-blocking agent propanolol and was completely inhibited by 5.10^{-5} M phenoxybenzamine.

The time course of the Ca response was altered in the absence of albumin. Fig.1b illustrates this observation. The activation of α-receptors now caused an initial increase in the ^{45}Ca content of the cells. This occurred during the first 30 s and was then followed by a period of Ca extrusion during which the ^{45}Ca content fell to below the pre-hormone level just as in cells in the presence of albumin. The mechanism of the first phase is unclear. The Ca content is determined by the balance between the passive diffusional influx and the active pump efflux of Ca^{2+}. It has been shown that noradrenaline increases the unidirectional Ca influx [5,18]. Results showed in fig.1a suggest that in the presence of albumin this increase is masked by the sudden activation of the Ca pump. In the absence of albumin, the α-mediated Ca influx may be drastically enhanced and/or the Ca pump partially inhibited. As a consequence the increase in cytosolic Ca concentration may saturate the Ca pump which will become unable to extrude the accumulated calcium sufficiently rapidly. The apparent Ca mobilized by the activation of α-receptors in the absence of albumin was larger than in control cells. It came to 0.54 nmol/mg dry wt.

Fig.2 shows the ^{45}Ca movements triggered by noradrenaline (5.10^{-6} M) in in situ mitochondria. Mitochondria were isolated from samples of cells suspended in labelled Eagle's medium for 30-60 min. At that time the mean ^{45}Ca content of in situ mitochondria was 1.41 ± 0.20 nmol/mg. This amount represented the exchangeable Ca pool, i.e., the soluble fraction of internal Ca [19]. In the presence of albumin (fig.2a), a rapid phase of Ca uptake occurred (30 s) which was followed by a slow release of the Ca accumulated. During this first phase mitochondria increased their exchangeable Ca content by 44%, i.e., 0.62 nmol/mg. The time course of the Ca uptake by in situ mitochondria correlated very closely with that of the net extrusion of Ca by the cells. It was maximal within 15–30 s following hormone application to the cell suspension, confirming the supposition that internal Ca stores were rapidly mobilized following the binding of hormone to the receptor site.

As might be expected from in fig.1b, the effect of noradrenaline on ^{45}Ca uptake by mitochondria was much more pronounced in albumin-free than in control solutions (fig.2b). The mitochondria increased their Ca content by 240% which corresponds to a net uptake of 2.49 nmol/mg. These Ca uptakes were substantially inhibited by 10^{-5} M FCCP an uncoupler of oxidative phosphorylation indicating that the

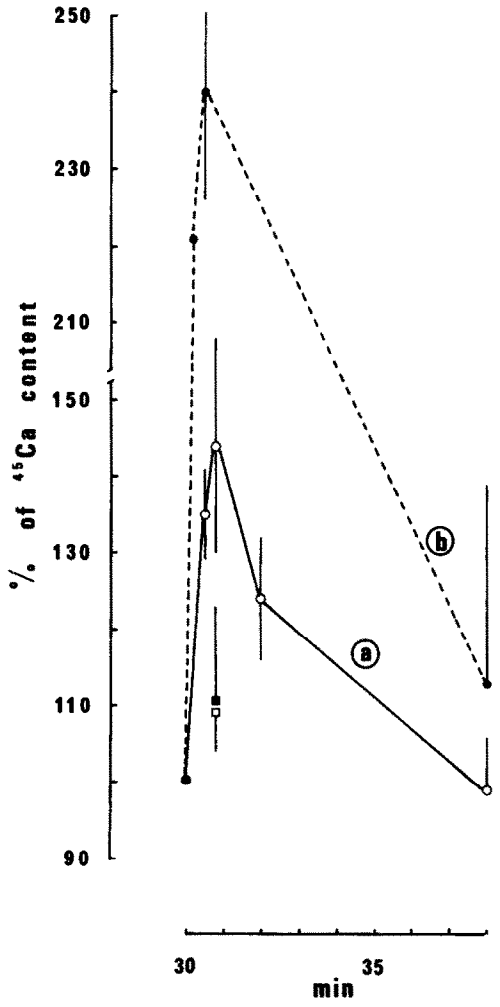

Fig.2. Effect of noradrenaline (5.10^{-6} M) on the ^{45}Ca content of in situ mitochondria isolated from cells equilibrated for 30 min in the presence (a) or in the absence (b) of albumin. Same experimental protocol as in fig.1. The exchangeable ^{45}Ca content was: (a) 1.41 ± 0.20 (n = 22) nmol/mg mitochondrial protein in control; (b) 1.78 ± 0.13 (n = 18) nmol/mg in the absence of albumin. The activity of citrate synthase found in mitochondrial pellets was used as an index of pellet enrichment. It was equal to 0.046 ± 0.004 (n = 5) IU/mg protein. FCCP (10^{-5} M) inhibited 80% and 88% of the mitochondrial ^{45}Ca uptake triggered by noradrenaline in the presence (□) and in the absence (■) of albumin.

uptake of calcium cannot be referrable to other subcellular organelles or to Ca binding proteins.

These results show that mitochondria are not the source of the Ca released by α-receptor activation as proposed [6–8] and that instead these organelles behave as a buffer system for internal ionized Ca. This interpretation agrees with the observation [16] that epinephrine caused an increase in mitochondrial Ca content in perfused liver. Probably other cellular pools are involved in the α-adrenergic response and are linked in some way to Ca influx to produce a sudden large and transient rise in the level of cytosolic calcium. During this rise in $[Ca^{2+}]_i$ phosphorylase *b* would be converted to its active form phosphorylase *a*, hence stimulating glycogenolysis before the calcium is removed from the cytosol by the mitochondria and by the Ca pump located in plasma membrane. This buffering capacity of in situ mitochondria can be illustrated by the following calculation. In the presence of albumin the maximal net uptake of Ca by mitochondria is 0.62 nmol/mg which corresponds on the basis of 0.185 mg mitochondrial protein/mg isolated cell dry wt [20] to 0.11 nmol/mg cell dry wt. Since 0.430 nmol Ca/mg dry wt were extruded by the Ca pump it may be estimated that under normal conditions, ~26% of the Ca mobilized by noradrenaline is temporarily buffered by in situ mitochondria. A similar calculation may be made for cells equilibrated in the absence of albumin. It indicates that as the pump failed to extrude rapidly Ca from the cells, in situ mitochondria then buffered ~85% of the Ca mobilized in the adrenergic response.

The source of the internal Ca pool which is associated with the influx through the plasma membrane to increase cytosolic calcium concentration is not known. However, endoplasmic reticulum is probably involved in the mobilization of intracellular Ca by α-agonists. Preliminary experiments show that caffeine enhances the net Ca extrusion which is in keeping with the fact that the drug potentializes the Ca-induced Ca release in muscle sarcoplasmic reticulum [21]. If so, there would be no feed for a new messenger to explain the mechanism by which Ca is released. A small increase in $[Ca]_i$ due to Ca influx could trigger the Ca-dependent Ca release from hepatocyte endoplasmic reticulum. This hypothesis is under study.

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