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Evidence for β-adrenergic activation of Na^+-dependent efflux of Ca^{2+} from isolated liver mitochondria

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The existence of a Na^+-dependent mechanism for Ca^{2+} efflux from isolated rat liver mitochondria was confirmed. The activity of this system is decreased by 60% in mitochondria isolated from perfused livers. The Na^+-dependent activity is fully restored by infusion of either 1 μM-adrenaline or 1 μM-isoprenaline, but the α-adrenergic agonist phenylephrine is ineffective.

Evidence for the existence of separate carriers for the catalysis of influx and efflux of Ca^{2+} in heart and liver mitochondria was provided by Crompton *et al.* (1976) and Puskin *et al.* (1976). The activity of the efflux system of cardiac mitochondria depends strongly on the presence of Na^+, and subsequent work indicated that the Na^+-dependence reflects the existence of a Na^+/Ca^{2+} antiporter (Crompton *et al.*, 1977), which is highly active in mitochondria from heart, skeletal muscle, brain, brown adipose tissue, parotid gland and adrenal cortex (Crompton *et al.*, 1978; Nicholls, 1978; Al-Shaikhaly *et al.*, 1979). In contrast, the reported lack of a marked effect of Na^+ on Ca^{2+} efflux from liver mitochondria led to their classification as Na^+-insensitive (Nicholls & Crompton, 1980).

More recently, however, the concept that liver mitochondria are Na^+-insensitive has been questioned by two reports of Na^+-induced Ca^{2+} efflux from these mitochondria, albeit of low activity (Haworth *et al.*, 1980; Heffron & Harris, 1981). Clarification of this issue is important in view of the proposals that efflux of mitochondrial Ca^{2+} may be a factor in the gluconeogenic response of liver to α-adrenergic agents (Exton, 1980, and references therein). In the present paper, the existence of a Na^+-induced Ca^{2+} efflux from liver mitochondria is confirmed, and shown to be activated by β-adrenergic, but not α-adrenergic, agonists.

Methods

Mitochondrial preparation

Mitochondria from unperfused rat livers were prepared as described previously (Crompton *et al.*, 1978), except that the homogenization medium contained 210 mM-mannitol, 70 mM-sucrose, 10 mM-Tris/HCl (pH 7.2), 1 mM-EGTA and 0.2% (w/v) bovine serum albumin (fatty acid-free). The rats were killed by decapitation.

Liver perfusions were performed as follows. Rats were injected with heparin (250 international units) and anaesthetized by intraperitoneal injection of 0.25 ml of chloral hydrate [16% (w/v) solution, pH 7]/100 g body wt. The hepatic portal vein was cannulated with a Braunula (no. 0.5) and the liver was perfused with Krebs–Henseleit bicarbonate buffer containing 10 mM-pyruvate (Krebs & Henseleit, 1932) at 37°C and at a flow rate of 40 ml/min; the perfusion medium was gassed continuously with O_2/CO_2 (19:1). The medium was allowed to flow out via an incision in the abdominal vena cava. After perfusion for 30 min, the right lobe was clamped off, removed, homogenized, and mitochondria were prepared as described for unperfused livers. The remaining lobes were perfused for a further 2 min with 1 μM-adrenaline, 1 μM-isoprenaline (isoproterenol) or 10 μM-phenylephrine, which were introduced into the perfusion medium, and then removed and homogenized immediately.

Measurement of Ca^{2+} fluxes

Mitochondria were preloaded with Ca^{2+} as follows. Mitochondria (1.5–3 mg of protein) were incubated at 25°C in 3 ml of medium containing 120 mM-KCl, 4 mM-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH 7.0), 0.17 mM-potassium phosphate, 67 μM-Arsenazo III, 1 μg of rotenone/mg of protein, 4 mM-succinate (Tris salt, pH 7.0), 33 μM-ATP (Tris salt, pH 6.8) and sufficient $CaCl_2$ to yield 15 nmol of Ca^{2+}/mg of protein. Respiration-supported Ca^{2+} uptake was complete after about 1 min, when the extramitochondrial free [Ca^{2+}] was decreased to 0.2–0.3 μM. Ca^{2+} efflux was started by the addition of either 2 nmol of Ruthenium Red/mg of protein, or Rut-

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henium Red plus NaCl. Ca^{2+} efflux was monitored at 675–685 nm with a Perkin–Elmer model 356 dual-wavelength spectrophotometer (Scarpa *et al.*, 1978). The rates of Ca^{2+} release were calculated from calibration curves of $\Delta[Ca^{2+}]$ (total) versus Δabsorbance, which were obtained by adding $CaCl_2$ to the complete incubation media. The extramitochondrial free $[Ca^{2+}]$ (Fig. 1) was calculated from calibration of $\Delta[Ca^{2+}]$ (free) versus Δabsorbance; this was obtained by using Ca^{2+}/*N*-hydroxyethylethylenediaminetriacetic acid buffers as described previously (Crompton *et al.*, 1976). The zero-$[Ca^{2+}]$ point (Fig. 1) was obtained in the presence of 1.8 mM-EGTA.

The data were analysed by Student's unpaired *t* test.

Results and discussion

Fig. 1 reports the effect of Na^+ on the efflux of Ca^{2+} from mitochondria isolated from non-perfused livers; Ruthenium Red was included in the incubation medium to prevent re-uptake of Ca^{2+} by the Ca^{2+} uniporter. In the initial stages, the presence of Na^+ caused a marked increase in the rate of Ca^{2+} efflux, i.e. the Na^+-induced increment in rate (Na^+-dependent efflux) was about 4 times the efflux observed in the absence of Na^+ (Na^+-independent efflux). However, the Na^+-dependent activity decreased as efflux progressed, so that, in the period between 2 and 3 min after the efflux was begun, the Na^+-dependent activity was only 0.7 of the Na^+-independent activity.

This temporal aspect of the Na^+-dependent efflux of Ca^{2+} was observed generally. In addition, the rate of Na^+-dependent efflux from the mitochondria of non-perfused livers varied over an extremely large range, i.e. from 0.55 to 3.7 nmol of Ca^{2+}/per min mg of protein in 17 experiments with 6 mM-Na^+. These two factors may have contributed to earlier failures to detect significant Na^+-induced efflux from liver mitochondria (see the introduction).

The effect of pre-perfusion of the liver on the initial rates of Ca^{2+} efflux from isolated mitochondria is shown in Fig. 2. Perfusion caused a decrease of 61% in the activity of the Na^+-dependent system and a decrease of 50% in the Na^+-independent activity. The decreased activity of the two systems caused by pre-perfusion suggests that both systems may be affected by factors present in the circulation. Potential factors include the concentration of adrenaline, since the rats underwent a variable degree of shock while being killed. This possibility was investigated, as reported in Fig. 2. Infusion of 1 μM-adrenaline for 2 min fully restored the activity of the Na^+-dependent system, i.e. the activity was increased about 3-fold, from 0.50 to 1.46 nmol of Ca^{2+}/min per mg of protein ($P < 0.02$). The capacity of adrenaline to induce activation of the Na^+-dependent system was mimicked by the β-adrenergic agonist isoprenaline ($P < 0.001$), but the α-adrenergic agonist phenylephrine did not cause

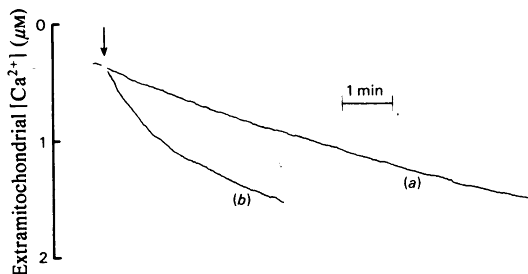

Fig. 1. *Release of Ca^{2+} induced by Ruthenium Red in the presence and absence of Na^+*
Mitochondria (2.1 mg of protein) were preloaded with Ca^{2+} as described in the Methods section. At the arrow, Ca^{2+} efflux was started by the addition of either Ruthenium Red (trace *a*) or Ruthenium Red plus 13 mM-NaCl (trace *b*).

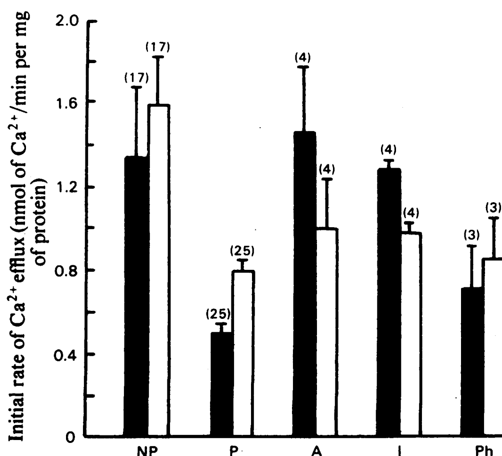

Fig. 2. *Stimulation of the Na^+-dependent and Na^+-independent effluxes of Ca^{2+} by adrenergic agents*
Mitochondria were prepared from rat livers either without perfusion or after a 30 min perfusion period as indicated in the Methods section. The initial rates of Na^+-independent (□) and Na^+-dependent (■) effluxes of Ca^{2+} were measured; Na^+-dependent efflux refers to the increment in the rate of efflux in the presence of Ruthenium Red caused by the addition of 6 mM-Na^+. Bars indicate ± S.E.M. The numbers of mitochondrial preparations tested in each case are given in parentheses. Abbreviations: NP, non-perfused; P, perfused, no agonist; A, perfused, 1 μM-adrenaline for 2 min; I, perfused, 1 μM-isoprenaline for 2 min; Ph, perfused, 10 μM-phenylephrine for 2 min.

a significant activation. In contrast, the activity of the Na^+-independent system in mitochondria from perfused livers was not increased significantly ($P > 0.05$) by infusion of adrenergic agents (Fig. 2). The inability of adrenergic agents to overcome the decreased activity of the Na^+-independent system caused by pre-perfusion suggests that factors other than adrenaline may affect this system.

The selective β-adrenergic activation of the Na^+-dependent system provides additional evidence that the Na^+-dependent and Na^+-independent effluxes of Ca^{2+} are catalysed by separate systems. This concept of two distinct pathways for efflux of mitochondrial Ca^{2+} originates from previous work using heart mitochondria, which demonstrated quite different sensitivities of the Na^+/Ca^{2+} exchange and the Na^+-independent efflux of Ca^{2+} to lanthanides (Crompton *et al.*, 1979). By analogy with heart mitochondria, it seems logical to attribute the Na^+-dependent efflux of Ca^{2+} from liver mitochondria to the presence of a Na^+/Ca^{2+} antiporter. The activity of this system in liver after β-adrenergic activation (1.46 nmol/min per mg) is about 20% of that in heart mitochondria when assayed under similar conditions with 6 mM-NaCl (Crompton *et al.*, 1976).

The reason for the apparent existence of two efflux pathways for Ca^{2+} with comparable activities is not clear, and the possibility must be borne in mind that they belong to different populations of mitochondria. The present data, which indicate that the two efflux systems are subject to independent control, raises the question of their regulatory roles *in vivo*. Many recent investigations (Exton, 1980, and references therein) have attributed the activation of hepatic gluconeogenesis by adrenaline to an α-adrenergic-induced loss of mitochondrial Ca^{2+}. However, this concept is controversial (Denton & McCormack, 1981, and references therein), and it is not supported by the present data. Thus no activation of either the Na^+-dependent or the Na^+-independent efflux systems by α-adrenergic agents was observed in the present work. On the contrary, although β-adrenergic agonists have relatively little effect on hepatic gluconeogenesis (Garrison & Borland, 1979; Kemp & Clark, 1978), isoprenaline induced a selective 2.6-fold activation of Na^+-dependent Ca^{2+} efflux. A similar effect *in vivo* would be predicted to shift the steady-state distribution of Ca^{2+} across the mitochondrial inner membrane (Nicholls & Crompton, 1980) towards lower intramitochondrial and higher cytosolic concentrations of free Ca^{2+}.

In addition, tissue pretreatment with α-adrenergic agonists activates Ca^{2+} influx via the uniporter into mitochondria isolated from both liver and heart (Taylor *et al.*, 1980; Kessar & Crompton, 1981); with liver mitochondria infusion periods greater than 2 min duration were required to develop a significant α-adrenergic activation of Ca^{2+} influx. The puzzling possibility emerges, therefore, that the direction of net Ca^{2+} flux between mitochondria and cytosol may be affected in an opposite manner by α-adrenergic and β-adrenergic agonists, although the development of these effects with time may differ.

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