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Stable Changes to Calcium Fluxes in Mitochondria Isolated from Rat Livers Perfused with α-Adrenergic Agonists and with Glucagon

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Mitochondria isolated from rat liver after a short-term perfusion with the α-adrenergic agonist phenylephrine or with glucagon exhibited enhanced rates of uptake of Ca^{2+} and prolonged retention of Ca^{2+} in the presence of 4 mM-P_i. The effect of Ca^{2+} retention was apparent after perfusion with phenylephrine for only 1 min and was maximal after 7 min of treatment. The changes induced by glucagon, although similar, were less rapid. Adrenaline caused similar changes to phenylephrine and its effects were blocked by the α-adrenergic antagonist phenoxybenzamine, but not by the β-antagonist propranolol. The Ca^{2+} content of the isolated mitochondria decreased by 30% 1 min after the onset of perfusion with phenylephrine; by 6 min it had begun to return to the original value which was reached at 10 min. A similar loss in calcium content was induced by glucagon but the changes were not as great and occurred more slowly. Mitochondria from phenylephrine-treated livers exhibited decreased rates of Ca^{2+} efflux induced by addition of 2 mM-EGTA, a 50% increase in the contents of ADP and total adenine nucleotides, a small increase in the transmembrane pH gradient, and a reduced rate of oxaloacetate-induced NADPH oxidation. This study thus shows that stimulation of liver by α-adrenergic agonists, like that by glucagon, induces within minutes a stable modification of mitochondria leading to alterations in the Ca^{2+}-translocation cycle (increased Ca^{2+} uptake and retention) and alterations in mitochondrial energy-linked reactions.

It is becoming increasingly evident that many hormones exert large effects on liver mitochondrial Ca^{2+} fluxes and that these are probably important in some of the metabolic effects of these hormones (Rasmussen & Goodman, 1977; Bygrave, 1978). Although work has been done on the changes in Ca^{2+} fluxes in mitochondria isolated from livers of rats treated with glucagon *in vivo* (Yamazaki, 1975; Hughes & Barritt, 1978; Prpić *et al.*, 1978) or from livers perfused with glucagon *in vitro* (Hughes & Barritt, 1978), there have been no reports of the actions of catecholamines, even though these agents produce larger changes in mitochondrial Ca^{2+} content than does glucagon (Blackmore *et al.*, 1979*a*,*b*).

The present work was designed to examine the effects of α-adrenergic stimulation on liver mitochondrial Ca^{2+} fluxes. Attention was focused on α-adrenergic action since the effects of catecholamines on liver cell Ca^{2+} fluxes (Assimacopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978) and on liver mitochondrial Ca^{2+} content (Blackmore *et al.*, 1979*a*,*b*) are mediated almost exclusively through α-receptors. We show that perfusion of rat livers with α-adrenergic agonists induces rapid and large changes in many liver mitochondrial energy-linked functions. These include components of the Ca^{2+}-translocation cycle (Ca^{2+} uptake, efflux and retention), oxidation of NADPH, and the transmembrane pH gradient.

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Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Experimental

Details of animals and perfusion technique

Male Wistar strain albino rats (200–300 g body wt.) were obtained from the John Curtin School of Medical Research. They were allowed free access to food, with a cycle of alternating period of 12 h each of light and dark.

Rats were anaesthetized with intraperitoneal sodium pentobarbitone (50 mg/kg) just prior to dissection. Perfusion details were essentially as described by Hutson *et al.* (1976) with the following changes. The perfusion medium was Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932)

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and a Silastic tubing oxygenator (Berry *et al.*, 1973) was included in the perfusion system. Rat livers were perfused at a flow rate of 5 ml/min per g wet wt. of liver for 15 min *in situ*. The right main liver lobe then was tied off and taken for the preparation of control mitochondria. Perfusion of the remaining liver lobes was continued for up to 20 min during which time hormones or other agents were continuously infused into the portal cannula using a syringe driven by an infusion pump at a flow rate of 0.5 ml/min.

At the conclusion of the perfusion, mitochondria were prepared from the caudate and papilliform lobes. In control experiments in which saline was infused, it was shown that mitochondria obtained from the right main lobe or at varying times later from the caudate and papilliform lobes exhibited essentially identical rates of Ca^{2+} uptake and retention.

Isolation of mitochondria

Liver lobes were rapidly excised and placed in ice-cold isolation medium containing 250 mM-sucrose, 5 mM-Hepes and 0.5 mM-EGTA adjusted to pH 7.4 with KOH. They were minced with scissors and homogenized by two passes with a glass/Teflon tissue disintegrator (A. H. Thomas Co., Philadelphia, PA, U.S.A.; size C) which was motor driven at 900 rev./min. The resulting suspension was made up to 80 ml with isolation medium and centrifuged at 900 ***g*** for 5 min in a Sorvall RC-2B refrigerated centrifuge fitted with an SS-34 rotor. The pellets were resuspended in the above medium and centrifuged at 800 ***g*** for 5 min. The combined supernatants were centrifuged at 4500 ***g*** for 5 min. The mitochondrial pellets were washed with EGTA-free isolation medium by centrifuging at 4500 ***g*** for 5 min and resuspended in this medium at a protein concentration of 70–100 mg/ml. The mitochondrial fraction so prepared is enriched in 'heavy' mitochondria that have an enhanced ability to transport Ca^{2+} (Bygrave *et al.*, 1978).

Measurement of mitochondrial protein

Mitochondrial protein concentration was assayed by the biuret method (Layne, 1957) after solubilization with deoxycholate (Jacobs *et al.*, 1956). Corrections were made for non-biuret colour and turbidity by subsequent cyanide treatment (Szarkowska & Klingenberg, 1963).

Measurement of Ca^{2+} transport

Ca^{2+} transport was measured by using techniques described by Reed & Bygrave (1974, 1975). For studies on Ca^{2+} retention, the medium contained in a final volume of 2 ml: 230 mM-sucrose, 10 mM-KCl, 5 mM-Hepes, 5 mM-sodium succinate, 1 μM-rotenone and 4 mM-potassium phosphate. The final pH was 7.4 and the temperature 25°C. Mitochondria (2 mg of protein) were added and the mixture was allowed to incubate for 1 min. $CaCl_2$ (100 μM, containing 0.5 μCi of $^{45}Ca^{2+}$ was added to initiate the reaction and Ca^{2+} transport was terminated by transferring 100 μl of the incubation mixture at appropriate times into 100 μl of ice-cold quench medium (150 mM-KCl/0.5 mM-EGTA/2 μM-Ruthenium Red) contained in Eppendorf centrifuge tubes. These were centrifuged to pellet the mitochondria and 100 μl of the supernatant was transferred to vials containing 10 ml of scintillation fluid (Dorman *et al.*, 1975). The ^{14}C channel of a Beckman scintillation counter was used for counting the sample for radioactivity.

Initial rates of Ca^{2+} transport were measured by the same technique, but with the temperature adjusted to 4°C. EGTA-induced Ca^{2+} efflux (Reed & Bygrave, 1974) was determined essentially under the conditions described for Ca^{2+} transport but with the following changes. The reaction medium contained in a total volume of 2 ml: 230 mM-sucrose, 0.5 mM-KCl, 5 mM-Hepes and 2 mg of mitochondrial protein. The pH was 7.4 and the temperature 25°C. $CaCl_2$ (100 μM, containing 0.5 μCi of $^{45}CaCl_2$) was added and 3 min later duplicate samples of the suspension (100 μl) were taken to determine the initial Ca^{2+} concentration in the medium. EGTA (final concentration 2 mM) was added to induce Ca^{2+} efflux and samples of suspension were taken at appropriate times thereafter and mixed with quench medium as described above to determine Ca^{2+} efflux.

Protonmotive force

The protonmotive force (Δp) was determined by using the ion distribution technique of Nicholls (1974). The incubation medium contained 150 mM-LiCl, 0.5 mM-KCl, 3 mM-Hepes (pH adjusted to 7.4 with Tris), 5 mM-sodium succinate, 10 μM-^{86}RbCl (0.12 μCi/ml), 50 μM-[^{14}C]methylamine (0.3 μCi/ml), 50 μM-sodium [^{3}H]acetate (1.2 μCi/ml), 1 μM-rotenone and 0.5 μM-valinomycin. The scintillation fluid contained 6 g of butyl-PBD [5-(biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole]/litre in toluene/2-methoxyethanol (3:2, v/v) containing 10% Biosolv BBS-3 (Beckman Instruments, Fullerton, CA, U.S.A.). Radioactivity was counted by using the three channels of a Packard Tri-Carb scintillation counter. Corrections were made for cross-over and background. The three discriminator control settings were such that crossovers of 21 and 3.6% were allowed from the ^{32}P channel into the ^{14}C and ^{3}H channels respectively, and 26.8% allowed from the ^{14}C channel into the ^{3}H channel. The components of Δp were calculated exactly as described by Nicholls (1974). A limiting matrix volume of 0.4 μl/mg of mitochondrial protein was used for the calculations, as assumed by Nicholls (1974) and Mitchell & Moyle (1969).

Redox state of nicotinamide adenine nucleotides

The wavelength pair 340–370nm was used to monitor spectrophotometrically (Lehninger *et al.*, 1978) the concentration of nicotinamide adenine nucleotides in a reaction medium containing in a total volume of 5ml: 130mM-sucrose, 65mM-KCl, 3mM-Hepes, 5mM-$MgCl_2$, 0.2mM-P_i, 4μM-rotenone, 1mM-sodium succinate and 7mg of mitochondrial protein. The temperature was 25°C and the pH 7.2. $CaCl_2$ (600nmol) was added and 1min later oxidation of nicotinamide adenine nucleotides was initiated by the addition of 2.5μmol of sodium oxaloacetate. Nicotinamide adenine nucleotide oxidation was monitored spectrophotometrically for up to 6min.

Measurement of calcium content of mitochondria

Aliquots (0.5ml) of mitochondrial suspension were mixed with 0.1ml of 11M-$HClO_4$ and denatured protein was removed by centrifugation (1500*g* for 5min). The Ca^{2+} content of the supernatant fluid was determined using a Perkin-Elmer atomic absorption spectrophotometer (model 603).

Other methods

Adenine nucleotides were determined enzymatically in freshly-isolated mitochondria to which only oligomycin (1μg/mg of protein) was added. ATP was determined by the method of Lamprecht & Trautschold (1974) and ADP and AMP were determined by the method of Jaworek *et al.* (1974). Mitochondrial respiration and acceptor control ratios were determined in media identical to that used for Ca^{2+} transport, and oxygen uptake was measured polarographically using a Clark-type oxygen electrode.

Chemicals

All radiochemicals used were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Glucagon, adrenaline and phenylephrine hydrochlorides, propranolol and phenoxybenzamine were all from Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals used were of analytical grade.

Results

Ca^{2+} retention in mitochondria isolated from rat liver perfused with phenylephrine or glucagon

Recent work from this laboratory has provided evidence that mitochondria isolated from rat liver display considerable heterogeneity in their ability to accumulate and retain Ca^{2+} (Bygrave *et al.*, 1978). Since Ruthenium-Red-sensitive Ca^{2+} transport is enriched in mitochondria that sediment in buffered iso-osmotic sucrose at relatively low centrifugation forces (Bygrave *et al.*, 1978), all the experiments reported in the present study were carried out with mitochondria that had been isolated in a manner that permitted enrichment of these relatively 'heavy' mitochondria.

Fig. 1 shows how the ability of mitochondria to retain accumulated Ca^{2+} changed after perfusion of rat liver for 7min with a maximally effective concentration of either glucagon or the α-adrenergic agonist phenylephrine. In the absence of hormone, mitochondria accumulated 85–90nmol of Ca^{2+}/mg of protein within 10s at 25°C in the presence of 4mM-P_i and retained this Ca^{2+} for approximately 9min. Mitochondria isolated from glucagon-perfused (Fig. 1*a*) or phenylephrine-perfused (Fig. 1*b*) livers, on the other hand, consistently accumulated greater than 90nmol of Ca^{2+}/mg of protein and Ca^{2+} retention times were extended to about 17 and 21min, respectively. The data may be compared with earlier findings that glucagon administration to the rat *in vivo* for 60min (Prpić *et al.*, 1978; Hughes & Barritt, 1978) or to the perfused liver *in vitro* for up to 60min (Hughes & Barritt, 1978) induces liver mitochondria to retain Ca^{2+}.

Fig. 2 shows that the glucagon- or phenylephrine-induced changes in mitochondrial Ca^{2+} retention were relatively rapid in onset. Mitochondria isolated from rat livers perfused with phenylephrine for 1min exhibited an increased ability to retain Ca^{2+} and maximal effects were observed after 7min. The effect of glucagon on mitochondrial Ca^{2+} retention was less rapid in onset and initially of lower magnitude than the effect of phenylephrine. A significant increase in Ca^{2+} retention was observed after perfusion with glucagon for 4min. The effect of glucagon increased progressively as the perfusion time was increased up to 20min. The addition of cycloheximide (2μM) or puromycin (100μM) to the perfusate did not alter the ability of either glucagon or phenylephrine to exert their effects on mitochondrial Ca^{2+} retention (data not shown).

Fig. 3 indicates how the mitochondrial Ca^{2+} concentration varied after perfusion of rat liver for increasing periods of time with either phenylephrine or glucagon. Both elicited a rapid transient decrease, similar to the findings of Blackmore *et al.* (1979*a*,*b*). Phenylephrine significantly lowered the Ca^{2+} content after only 1min with a maximal effect (decrease of 40%) observed after 4min. The response to glucagon was slightly slower and of smaller magnitude; the Ca^{2+} content was decreased after 4min and was maximally effective at 7min (decrease of approximately 30%). By 10min the Ca^{2+} content of both phenylephrine- or glucagon-treated mitochondria had returned to the original pretreatment level.

The ability of mitochondria to retain Ca^{2+} following treatment of the perfused liver for 7min with varying doses of phenylephrine was also investigated. A significant increase in mitochondrial

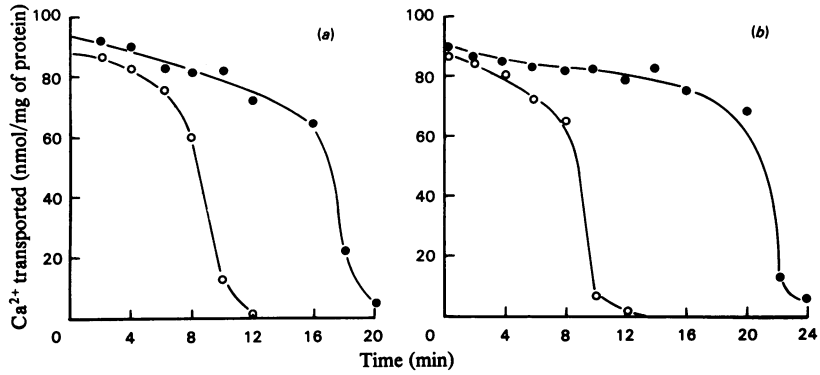

Fig. 1. *Ability of liver mitochondria to retain Ca^{2+} after perfusion of liver with glucagon or phenylephrine*
Rat livers were perfused *in situ* for 15 min, at which time the right main lobe was tied off and taken for the preparation of control mitochondria. The remaining liver was perfused for a further 7 min in the presence of 10^{-7} M-glucagon (Fig. 1*a*) or 10^{-5} M phenylephrine (Fig. 1*b*); the caudate and papilliform lobes were then taken for the preparation of hormone-treated mitochondria. The ability of control and hormone-treated mitochondria to retain accumulated Ca^{2+} was then compared. Ca^{2+} retention was measured as described in the Experimental section. The reaction medium contained, in a total volume of 2.0 ml: 230 mM-sucrose, 10 mM-KCl, 5 mM-Hepes, 5 mM-sodium succinate, 1 μM-rotenone, 4 mM-P_i, 200 nmol of $^{45}Ca^{2+}$ and 2 mg of mitochondrial protein. The pH was 7.4 and the temperature 25°C. ○, Control; ●, hormone-treated.

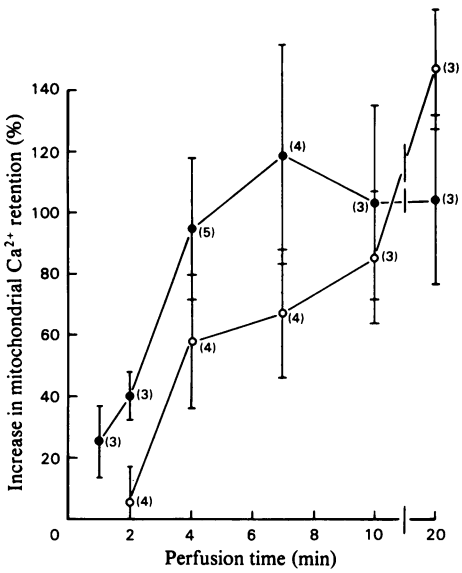

Fig. 2. *Time-course effect of phenylephrine and glucagon on retention of Ca^{2+} by rat-liver mitochondria*
Experimental details were as described in the legend to Fig. 1 except that the time of perfusion with glucagon (10^{-7} M; ○) or phenylephrine (10^{-5} M; ●) was varied as detailed above. Results were expressed relative to their respective control value and are reported as the means ± S.E.M. for the number of experiments indicated in parentheses.

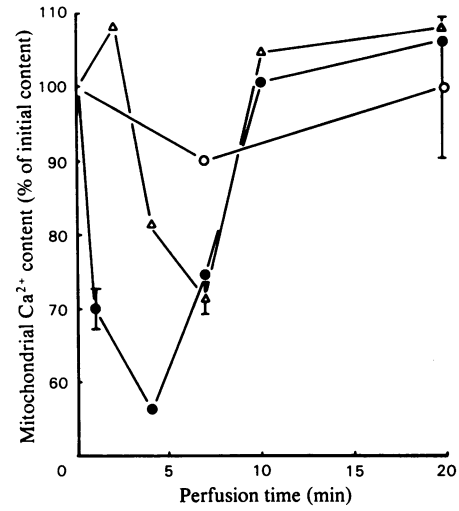

Fig. 3. *Ca^{2+} content of mitochondria following perfusion of rat liver with phenylephrine or with glucagon*
The Ca^{2+} content of the mitochondria was measured as indicated in the Experimental section after the exposure of the liver to the hormones for the indicated times. The livers were perfused with phenylephrine (●), glucagon (△) or without hormones (○). In the absence of hormone, the Ca^{2+} content of the control lobe at zero time was 8.7 ± 0.5 nmol/mg of protein (n = 13). Results are reported as means ± S.E.M. for three separate experiments or as mean of two separate experiments where no S.E.M. values are shown.

Ca^{2+} retention was observed (data not shown) at a concentration of 10^{-7}M-phenylephrine and the ability to retain Ca^{2+} increased further with increasing agonist concentrations up to the maximum dose employed (10^{-5}M).

Mitochondria isolated from rat livers perfused for 7min with 10^{-5}M phenylephrine also exhibited increased initial rates of Ca^{2+} uptake. Phenylephrine enhanced the initial rate of Ca^{2+} uptake (0–10s period) by nearly 60% under these conditions (Fig. 4).

Fig. 5 shows that perfusion of rat livers with adrenaline affected the flux of Ca^{2+} in subsequently isolated mitochondria in a manner similar to that described for phenylephrine. Treatment with 10^{-6}M-adrenaline increased Ca^{2+} retention by more than two-fold (Fig. 5*a*), and the initial rate of Ca^{2+} uptake (Fig. 5*d*) was increased by almost 80% following perfusion of livers with the hormone. When the α-adrenergic antagonist phenoxybenzamine was also present during the perfusion (Fig. 5*b*,*e*), no effect of adrenaline on Ca^{2+} retention or uptake was observed. In contrast, the β-adrenergic antagonist propranolol (Figs. 5*c*,*f*) did not significantly diminish the increase of Ca^{2+} retention and uptake induced by adrenaline.

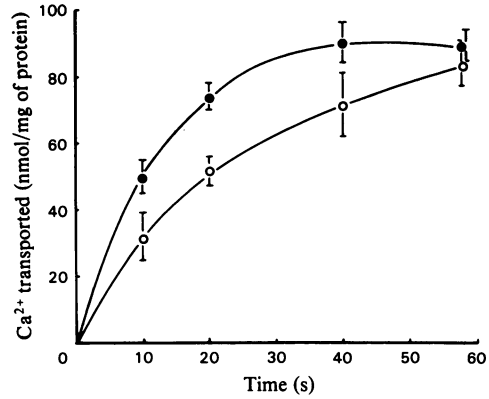

Fig. 4. *Effect of phenylephrine on mitochondrial Ca^{2+} uptake*

Perfusion details were as described in the legend to Fig. 1. The ability of control (○) or phenylephrine (10^{-5}M; ●)-treated mitochondria to take up Ca^{2+} was compared. Ca^{2+} uptake assays were performed as described in the Experimental section. The reaction medium and assay conditions were as described for Ca^{2+} retention determinations (Fig. 1) with the exceptions that rotenone was omitted from the reaction mixture and the temperature was 4°C. Results are reported as the means ± S.E.M. for three separate experiments.

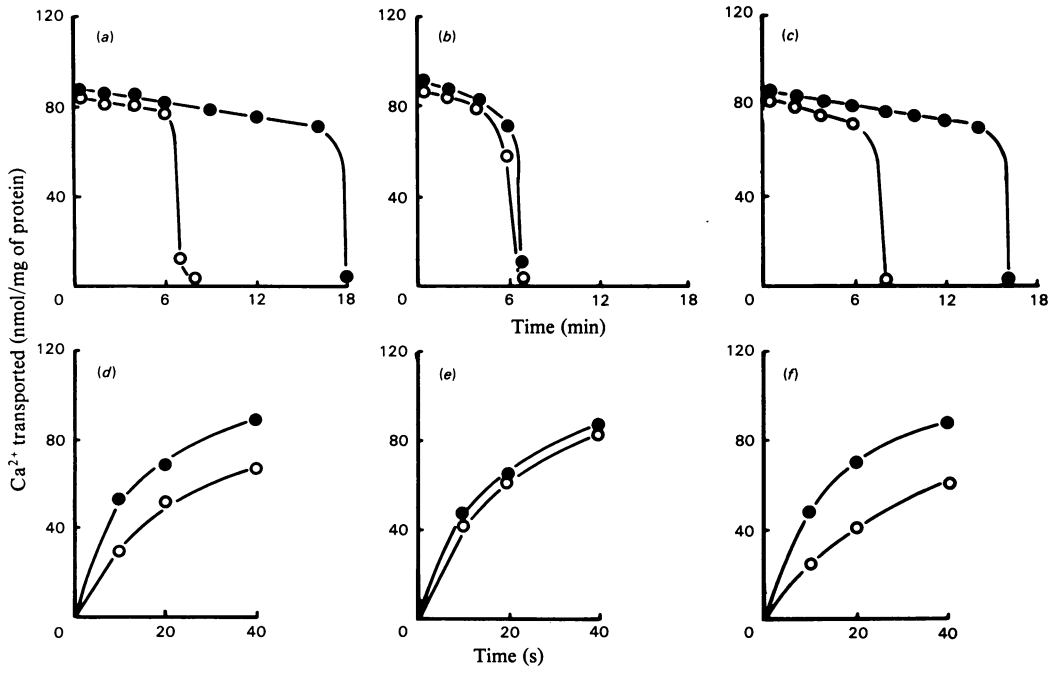

Fig. 5. *Effect of α- and β-adrenergic antagonists on alteration of mitochondrial Ca^{2+} fluxes induced by adrenaline*

For experimental details refer to Figs. 1 and 4. The ability of control and treated mitochondria to take up and retain added Ca^{2+} was compared. Symbols indicate treatments employed as follows: *a*, *d*: ○, control; ●, 10^{-6}M-adrenaline; *b*, *e*: ○, 10^{-5}M-phenoxybenzamine; ●, 10^{-6}M-adrenaline plus 10^{-5}M-phenoxybenzamine; *c*,*f*: ○, 10^{-5}M-propranolol; ●, 10^{-5}M-propranolol plus 10^{-6}M-adrenaline.

Effect of perfusion with phenylephrine or glucagon on EGTA-induced mitochondrial Ca^{2+} efflux

As discussed by Bygrave (1978) a Ca^{2+}-translocation cycle may operate at the inner mitochondrial membrane to regulate Ca^{2+} fluxes. Data presented above suggest that α-adrenergic agonists stimulate one component of such a cycle, namely Ca^{2+} uptake. The experiments described in Table 1 were designed to investigate possible effects of phenylephrine or glucagon on mitochondrial Ca^{2+} efflux, the other component of the cycle. The results indicate that phenylephrine and glucagon both inhibited by approximately 50% the initial rate of mitochondrial Ca^{2+} efflux induced by EGTA.

Effect of phenylephrine treatment on mitochondrial adenine nucleotide content, nicotinamide adenine nucleotide oxidation and components of protonmotive force

The potential involvement of adenine nucleotides, nicotinamide adenine nucleotide oxidation and components of protonmotive force in Ca^{2+} retention by mitochondria has been described (Prpić *et al.*, 1978; V. Prpić & F. L. Bygrave, unpublished work). Possible effects of phenylephrine on these mitochondrial parameters were therefore investigated.

Table 2 shows that the ADP and total adenine nucleotide contents of mitochondria obtained from rat livers perfused with phenylephrine were increased over their respective controls by approximately 50%. Phenylephrine treatment also resulted in small changes in components of the protonmotive force and in the acceptor control ratio. There was a very small increase in the membrane potential, but significant increases of about 6 and 10 mV occurred in the transmembrane pH gradient and protonmotive force, respectively. Small, but significant, increases in the mitochondrial acceptor control ratio and ADP-stimulated respiration were observed fol-

Table 1. *Effect of glucagon and phenylephrine treatment on mitochondrial Ca^{2+} efflux induced by EGTA*

Control or hormone-treated mitochondria were prepared as described in the legend to Fig. 1. EGTA-induced Ca^{2+} efflux was determined as described in the Experimental section. Results are reported as the means ± S.E.M. for three separate experiments.

Pretreatment of mitochondria	Initial rate of Ca^{2+} efflux (nmol Ca^{2+}/min per mg of protein)
None	28 ± 2.0
Glucagon (10^{-7}M)	12.6 ± 2.7
None	28 ± 4.1
Phenylephrine (10^{-5}M)	13 ± 2.5

Table 2. *Effect of phenylephrine treatment on adenine nucleotide content and on the components of the protonmotive force in rat-liver mitochondria*

Control and phenylephrine-treated mitochondria were prepared as described in the legend to Fig. 1. Adenine nucleotides, oxygen uptake and the components of the protonmotive force were determined as described in the Experimental section. The data are given as means ± S.D.; numbers in parentheses indicate the number of individual experiments. Acceptor control ratio is (rate of respiration in the presence of ADP)/(rate of respiration in the absence of ADP). Data were analysed by Student's unpaired *t* test: $*P < 0.1$; $**P < 0.01$; $***P < 0.005$; $\dagger P < 0.0025$; N.S. not significant.

Liver	Content (μmol/g of protein) of				Membrane potential (mV)	Transmembrane pH gradient (mV)	Protonmotive force (mV)	Respiration (ng-atoms of O/min per mg of protein) in		Acceptor control ratio
	ATP	ADP	AMP	Total adenine nucleotides				State 3	State 4	
Control	2.00 ± 0.86 (6)	8.10 ± 1.86 (6)	3.00 ± 0.57 (6)	13.60 ± 3.06 (6)	149.5 ± 0.5 (3)	80.6 ± 1.3 (3)	230.3 ± 1.1 (3)	128 ± 27 (4)	41.3 ± 9.5 (4)	3.11 ± 0.26
Phenylephrine-treated	2.60 ± 0.76 (6)*	13.10 ± 1.51 (6)†	3.70 ± 0.97 (6)*	19.50 ± 1.11 (6)†	153.1 ± 1.0 (3)**	87.6 ± 1.7 (3)†	240.6 ± 1.6 (3)†	158 ± 25 (4)*	38.5 ± 7.1 (4) N.S.	4.12 ± 0.34 ***

lowing phenylephrine treatment. Similar changes in adenine nucleotide content, acceptor control ratio and the components of proton-motive force were reported in a previous study of the effect of glucagon on these parameters and Ca^{2+} retention (Prpić *et al.*, 1978).

Mitochondrial NADH and NADPH oxidation induced by addition of oxaloacetate was also determined by measuring the difference in the absorption spectrum at 340–370nm (V. Prpić & F. L. Bygrave, unpublished work, see also Lehninger *et al.*, 1978). Two kinetically distinguishable phases of oxaloacetate-induced oxidation of pyridine nucleotides were observed. Fluorimetric analyses of the pyridine nucleotides made over the course of the oxidation trace revealed that essentially all NADH oxidation occurred during the initial rapid portion of the trace and that the bulk of the NADPH was oxidized more slowly over the later slower portion of the oxidation profile (V. Prpić & F. L. Bygrave, unpublished work).

Phenylephrine treatment did not significantly alter mitochondrial NADH oxidation in the system described, but like glucagon, caused a marked decline in the rate and extent of NADPH oxidation as indicated by the much slower second portion of the oxidation trace (data not shown).

Discussion

The experiments reported here indicate that α-adrenergic stimulation of the isolated perfused liver induces changes in many mitochondrial energy-linked reactions which are similar to those exerted by glucagon. This finding, together with the lack of any inhibitory effect of puromycin or cycloheximide on the hormone-induced changes to the mitochondria in this system, are among the items of new information provided by the work described in this paper.

Hughes & Barritt (1978) already have demonstrated that perfusion of rat liver with glucagon leads to enhanced mitochondrial Ca^{2+} retention. The majority of their data was obtained however following the perfusion of the organ with the hormone for up to 60min. In these circumstances, the hormone-induced changes were completely prevented by co-perfusion with cycloheximide.

Data in the present study agree with those of Hughes & Barritt (1978) to the extent that perfusion of rat liver with glucagon leads to an enhancement of mitochondrial Ca^{2+} retention (Figs. 1 and 2). They differ on the other hand in several important respects that bear directly on the elucidation of the mechanism by which the hormones induce *in situ* the changes in the mitochondria. The present study employed 'heavy' mitochondria, now known to be enriched in their ability to carry out Ruthenium-Red-sensitive Ca^{2+} transport (Bygrave *et al.*, 1978), and showed that exposure of livers to glucagon for times as short as 4min produces significant prolongation of mitochondrial Ca^{2+} retention. The α-adrenergic agonist, phenylephrine, exerted its effects even more rapidly, being evident at 2min and maximal at approx. 7min. Where tested, the effects of phenylephrine were mimicked by the α-adrenergic component of the natural agonist, adrenaline. Within the time period examined (7min), no β-adrenergic effects of this catecholamine on mitochondrial Ca^{2+} uptake or retention were detected. The rapidity with which glucagon exerted its effects on mitochondrial energy-linked reactions in the present study is consistent with data presented elsewhere (Yamazaki, 1975; Yamazaki *et al.*, 1977; Bryla *et al.*, 1977).

The second important finding in this work was that the short-term exposure of rat liver to hormones leading to alterations in mitochondrial Ca^{2+} fluxes was completely unaffected by cycloheximide or puromycin, thus rendering unlikely any mechanism of hormone action that purportedly implicates the involvement of protein synthesis (Cf. Dorman *et al.*, 1975; Hughes & Barritt, 1978; Prpić *et al.*, 1978).

The findings made here that α-adrenergic agonists on the one hand, and glucagon, on the other, induce similar changes in liver mitochondria *in situ* is of especial interest particularly since, by contrast with glucagon, α-adrenergic activation of glycogenolysis and gluconeogenesis in hepatocytes occurs by mechanisms not involving an increase in total cellular cyclic AMP or activation of the cyclic AMP-dependent protein kinase (Cherrington *et al.*, 1976).

Clearly both hormones promote Ca^{2+} retention by liver mitochondria as a consequence of both an enhanced Ca^{2+} uptake and an inhibited Ca^{2+} release; i.e. by alteration of both components of the mitochondrial Ca^{2+}-translocation cycle (Bygrave, 1978). Presumably these changes are associated with the same change(s) that induce the increases in adenine nucleotide content, protonmotive force and ADP-stimulated respiration and a decreased ability to carry out oxaloacetate-induced NADPH oxidation. As discussed elsewhere (Prpić *et al.*, 1978; V. Prpić & F. L. Bygrave, unpublished work), these changes in mitochondria could have a significant influence on mitochondrial Ca^{2+} fluxes.

Halestrap (1978) has evinced data indicating that electron flow between cytochromes c_1 and c is stimulated as a result of glucagon action, and has suggested that this is due to phosphorylation of cytochrome c (which is located on the outer surface of the inner mitochondrial membrane) by cyclic AMP-dependent protein kinase. In the case of α-adrenergic agonists, evidence is accumulating that

Ca^{2+}-stimulated phosphorylation may be involved in their action (Garrison, 1978) and in view of the similar modes of action of α-adrenergic agonists and glucagon on mitochondrial Ca^{2+} fluxes, a similar mechanism may operate here.

Another question raised in the present study is how the findings relate to observations in intact liver cells (Chen *et al.*, 1978) and perfused livers (Blackmore *et al.*, 1978, 1979*a*,*b*) showing that the initial effect of α-adrenergic stimulation is to cause a large release of Ca^{2+} from intracellular organelles, including mitochondria. The early phase of mitochondrial Ca^{2+} loss (Fig. 3) that would lead initially to a rise in cytosolic Ca^{2+} may be due to a transient non-stable change in the mitochondria, i.e. the action of a natural ionophore, which does not result in alterations that can be detected after mitochondria have undergone the several steps involved in their isolation. The stable alterations seen here would then relate to the 'recovery phase' of hormone action in the liver, especially when the mitochondrial Ca^{2+} content is near-maximally depleted and when Ca^{2+} re-accumulation by the mitochondria becomes apparent.

Although much additional work is required to determine the nature of the changes in liver mitochondrial function induced by α-adrenergic and by glucagon stimulation, the present findings provide another example of the remarkable similarity of the effects of these two hormones in the liver and introduce the possibility that their respective mechanisms of action may have a number of common features. Appropriate deployment of the experimental system described here should provide further information both on these points and on the mechanism and role of the mitochondrial Ca^{2+}-translocation cycle in cell metabolism (Bygrave, 1978).

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