The activation of Na⁺-dependent efflux of Ca²⁺ from liver mitochondria by glucagon and β -adrenergic agonists

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(Received 8 September 1982/Accepted 20 October 1982)

The Na⁺-induced efflux of Ca²⁺ from liver mitochondria was activated by tissue pretreatment with 1 μ M-adrenaline, 1 μ M-isoprenaline, 10 nM-glucagon and 100 μ M-cyclic AMP when 10 mM-lactate plus 1 mM-pyruvate were present in the perfusion medium. Infusion of the α_1 -adrenergic agonist, phenylephrine (10 μ M), was ineffective. The activation induced by the β -adrenergic agonist, isoprenaline, was maximal after infusion of agonist for 2 min. The isoprenaline-induced activation was very marked (120–220%), with about 7 nmol of intramitochondrial Ca²⁺/mg of protein, but was not evident with >15 nmol of Ca²⁺/mg. Ca²⁺ efflux in the absence of Na⁺ and in the presence of the Ca²⁺ ionophore A23187 was not affected by isoprenaline pretreatment over the range 6–23 nmol of internal Ca²⁺/mg. With 10 mM-lactate plus 1 mM-pyruvate in the perfusion medium, glucagon and isoprenaline infusion increased tissue cyclic AMP content about 8-fold and 3-fold respectively. With 10 mM-pyruvate alone, neither glucagon nor isoprenaline caused a significant increase in cyclic AMP. Omission of lactate also abolished the ability of glucagon, but not of isoprenaline, to activate the Na⁺-induced efflux of Ca²⁺. The data indicate that cyclic AMP may mediate the activation caused by glucagon, but provide no evidence that cyclic AMP is an obligatory link in the β -adrenergic-induced activation.

There is evidence that the actions of α_1 -adrenergic agonists vasopressin and angiotensin in promoting hepatic glycogenolysis involve an increase in cytosolic free [Ca²⁺] (Assimakopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978). The identity of the hormone-sensitive Ca²⁺ pools mobilized are controversial, however, and contributions from various sources including mitochondria (Babcock *et al.*, 1979; Blackmore *et al.*, 1979a,b; Barritt *et al.*, 1981), microsomes (Barritt *et al.*, 1981), plasma-membrane stores (Althaus-Salzmann *et al.*, 1980; Poggioli *et al.*, 1980) and extracellular Ca²⁺ (Michell, 1979; Barritt *et al.*, 1981) have been proposed. In particular, the possible role of mitochondria in supplying the increase in cytosolic [Ca²⁺] is disputed. Mitochondria may contain over 70% of the total Ca²⁺ content in liver (Foden & Randle, 1978). However, there are conflicting reports concerning changes in intramitochondrial [Ca²⁺] after α -adrenergic stimulation. Direct measurements of the Ca²⁺ retained by mitochondria during isolation indicate that α -adrenergic agonists promote a loss of mito-

chondrial Ca²⁺ (Blackmore *et al.*, 1979a,b; Taylor *et al.*, 1980a), whereas tracer studies indicate that the opposite may occur (Foden & Randle, 1978; Althaus-Salzmann *et al.*, 1980; Poggioli *et al.*, 1980).

On the other hand, it is generally considered that the glycogenolytic actions of glucagon and β -adrenergic agonists may be accounted for by cyclic AMP-mediated changes in enzyme activities (Sutherland & Robison, 1966). Nevertheless, recent studies indicate that infusion of liver with β -adrenergic, rather than α -adrenergic, agonists induces a stable activation of Ca²⁺ efflux from subsequently isolated mitochondria (Goldstone & Crompton, 1982). In addition, the finding that several key intramitochondrial enzymes of oxidative metabolism may be regulated by Ca²⁺ (Denton *et al.*, 1980; McCormack & Denton, 1980) has raised the possibility that the prime role of the Ca²⁺-transport systems in the mitochondrial inner membrane may be the regulation of intramitochondrial rather than extramitochondrial [Ca²⁺] (Denton & McCormack, 1980, 1981; Denton *et al.*, 1981). In view of these uncertainties, it appears that investigation of possible hormonal modification of

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

the mitochondrial Ca^{2+} -transport systems may help to clarify the influence of such hormones on changes in net Ca^{2+} flux across the mitochondrial inner membrane.

It is now well established that the distribution of free Ca^{2+} across the mitochondrial inner membrane is determined by steady-state recycling via discrete systems for influx and efflux (Puskin *et al.*, 1976; Crompton *et al.*, 1976; Saris & Åkerman, 1980). The passive influx of Ca^{2+} in response to the negative-inside membrane potential is catalysed by a Ca^{2+} uniporter, and recent work has revealed that this reaction in liver and heart may be stimulated by tissue pretreatment with α_1 -adrenergic agonists (Taylor *et al.*, 1980a; Kessar & Crompton, 1981). The physiological process responsible for active extrusion of Ca^{2+} from liver mitochondria is not settled. The efflux of Ca^{2+} from liver mitochondria that occurs when the uniporter is inhibited by Ruthenium Red in the absence of Na^+ (Puskin *et al.*, 1976) is ill-characterized, and various mechanisms have been suggested; these include $\text{Ca}^{2+}/\text{H}^+$ -antiport (Fiskum & Lehninger, 1979), the involvement of P_i (Wehrle & Pedersen, 1979; Roos *et al.*, 1980), unsaturated fatty acids (Roman *et al.*, 1979) and the redox state of the intramitochondrial nicotinamide nucleotides (Lehninger *et al.*, 1978; Fiskum & Lehninger, 1979). These last two reports have been disputed by others (Nicholls & Brand, 1980; Bardsley & Brand, 1981). Recently, evidence has been obtained that an $\text{Na}^+/\text{Ca}^{2+}$ -antiporter, extensively studied in mitochondria from heart and other tissues (Crompton *et al.*, 1976; Crompton *et al.*, 1978; Nicholls, 1978; Nicholls & Crompton, 1980; Hayat & Crompton, 1982) may also occur in liver mitochondria (Nedergaard & Cannon, 1980; Haworth *et al.*, 1980; Heffron & Harris, 1981; Goldstone & Crompton, 1982). Moreover, the Na^+ -dependent system was activated by pretreatment of the liver with the β -adrenergic agonist isoprenaline (isoproterenol), whereas the Na^+ -independent process was unaffected (Goldstone & Crompton, 1982). The identification of the putative $\text{Na}^+/\text{Ca}^{2+}$ -antiporter as the hormonally-responsive system for Ca^{2+} efflux from liver mitochondria raises the question of its role during adrenergic stimulation of liver, and the mechanism underlying its activation.

The present study examines the response of the Na^+ -dependent system to α_1 -adrenergic stimulation and to treatments that cause large variations in the tissue content of cyclic AMP. It is shown that whereas activation of the Na^+ -dependent system correlates with changes in cyclic AMP induced by glucagon, isoprenaline treatment causes activation under conditions in which no increase in cyclic AMP is observed. The α_1 -adrenergic agonist, phenylephrine, induced no activation under any conditions studied. These findings are discussed.

Methods

Mitochondrial preparation

Liver perfusions were performed as reported previously (Goldstone & Crompton, 1982). Briefly, fed rats were anaesthetized and the hepatic portal vein was cannulated with a Braunula (no. 0.5) and the liver was perfused with Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) containing 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM- MgSO_4 , 1.2 mM- KH_2PO_4 , 1.3 mM- CaCl_2 , 25.3 mM- NaHCO_3 and either 10 mM-Tris/pyruvate or 10 mM-Tris/lactate plus 1 mM-Tris/pyruvate at 37°C at a flow rate of 40 ml/min; the perfusion medium was gassed continuously with O_2/CO_2 (19:1). After perfusion for 30 min, the right lobe was clamped off, removed and homogenized. The remaining lobes were perfused for a further 1–4 min with 1 μM -isoprenaline, 10 μM -phenylephrine, 10 nM-glucagon or 100 μM -Tris/cyclic AMP, which were introduced into the perfusion medium. The treated lobes were then immediately removed and homogenized. Mitochondria from control and treated homogenates were prepared as described previously (Goldstone & Crompton, 1982). The mitochondria were suspended finally in a medium containing 210 mM-mannitol, 70 mM-sucrose and 10 mM-Tris/HCl, pH 7.4. Mitochondrial protein was determined by a modified biuret method (Kröger & Klingenberg, 1966).

Measurement of Ca^{2+} efflux

Mitochondria were preloaded with Ca^{2+} as follows. Mitochondria (containing 3 mg of protein) were incubated for about 10 min at 25°C in 3 ml of medium containing 120 mM-KCl, 4 mM-Tris/Hepes (pH 7.0), 0.17 mM-potassium phosphate, 67 μM -arsenazo III and 3 μg of rotenone, until efflux of endogenous Ca^{2+} was complete (as judged by the fact that further addition of either 0.2 nmol of ionophore A23187/mg of protein or 1% Triton X-100 released no more Ca^{2+}). Sufficient CaCl_2 was then added to yield 7 nmol of Ca^{2+} /mg of protein (except where indicated). Ca^{2+} accumulation was started by the addition of 4 mM-succinate (Tris salt, pH 7.0) plus 33 μM -ATP (Tris salt, pH 6.8), and was complete after about 1 min, when the extramitochondrial free $[\text{Ca}^{2+}]$ was lowered 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 Ruthenium Red plus 6 mM-NaCl, except when the $[\text{Na}^+]$ was varied. Ca^{2+} efflux was monitored at 675–685 nm with a Perkin–Elmer model 356 dual-wavelength spectrophotometer (Scarpa *et al.*, 1978). The system was calibrated by adding CaCl_2 to the complete incubation medium after Ruthenium Red. Each test determination using mitochondria from agonist-treated lobes was accompanied by a control

using mitochondria from untreated lobes of the same liver.

Na^+ -dependent efflux refers to Ca^{2+} efflux in the presence of Ruthenium Red plus Na^+ minus the efflux in the presence of Ruthenium Red alone (Na^+ -independent efflux).

Measurement of cyclic AMP

Samples of perfused liver were quickly excised and freeze-clamped in aluminium tongs pre-cooled in liquid N_2 . The frozen liver was transferred to a mortar pre-cooled with liquid N_2 and ground finely under liquid N_2 . The resulting powder was transferred into a tared vial containing 5 ml of 0.25 M-sucrose, 6% HClO_4 and 20% (v/v) ethanol. The vial was capped and stirred for 10 min in an ice/salt mixture, and weighed again. The precipitated protein was sedimented using a bench centrifuge at 4°C , and the supernatant was adjusted to pH 6–6.5 with successive additions of 10 M-, 2 M- and 0.4 M-KOH. The KClO_4 was sedimented by centrifugation at 4°C . Samples were frozen at -40°C until analysis. The cyclic AMP contents of the extracts were determined as described by Gilman (1970)

Results

The effects of isoprenaline on Ca^{2+} efflux from liver mitochondria

It has been reported previously that infusion of liver with the β -adrenergic agonist isoprenaline leads to an increased rate of Na^+ -induced efflux of Ca^{2+} from isolated mitochondria (Goldstone & Crompton, 1982). Fig. 1 shows representative time courses of the efflux of Ca^{2+} from mitochondria isolated from liver perfused in the presence and absence of isoprenaline. The rate of Ca^{2+} efflux induced by addition of Ruthenium Red alone (Na^+ -independent efflux) was not changed detectably by isoprenaline pretreatment. The further addition of Na^+ stimulated Ca^{2+} efflux from both control and isoprenaline-treated mitochondria. However, the Na^+ -induced increment in the rate of Ca^{2+} efflux (i.e. Na^+ -dependent efflux) was markedly greater with mitochondria from isoprenaline-treated tissue.

Further experiments were carried out to establish the assay conditions under which the activation of the Na^+ -dependent efflux was maximal. Fig. 2 reports the $[\text{Na}^+]$ -dependence of the initial rates of Na^+ -induced efflux of Ca^{2+} from mitochondria isolated from control and isoprenaline-pretreated liver. Saturation kinetics were observed in both cases and half-maximal velocities attained with 7–11 mM- Na^+ , which is similar to the $[\text{Na}^+]$ required for half-maximal activity of the $\text{Na}^+/\text{Ca}^{2+}$ -antiporter of heart and other mitochondria (8–10 mM; Crompton *et al.*, 1978). Isoprenaline caused activation at all

Fig. 1. The effect of tissue pretreatment with $1\text{ }\mu\text{M}$ -isoprenaline on the Na^+ -dependent and Na^+ -independent Ca^{2+} effluxes

Mitochondria (containing 3 mg of protein) were isolated from lobes of the same liver perfused with buffer containing 10 mM-pyruvate and in (a) the absence and (b) the presence of isoprenaline ($1\text{ }\mu\text{M}$, 2 min). Ca^{2+} efflux from Ca^{2+} -preloaded mitochondria was monitored as described in the Methods section on the addition (at the arrow) of Ruthenium Red in the presence and absence of 6 mM- Na^+ as indicated.

Fig. 2. The dependence of the rate of Na^+ -induced efflux of Ca^{2+} from control and isoprenaline-treated mitochondria on the extramitochondrial $[\text{Na}^+]$

Mitochondria were isolated from lobes of the same liver perfused with buffer containing 10 mM-pyruvate and in the presence (●) and absence (○) of isoprenaline ($1\text{ }\mu\text{M}$, 2 min). The initial rates (v) of Na^+ -dependent efflux of Ca^{2+} were determined with 7 nmol of intramitochondrial Ca^{2+} /mg of protein as described in the Methods section.

$[\text{Na}^+]$ studied, but the degree of activation increased as the $[\text{Na}^+]$ decreased, e.g. the percentage activation at 6 mM and 30 mM were 230% and 45% respectively.

Fig. 3. The dependence of the rates of Ca^{2+} efflux from control and isoprenaline-treated mitochondria on the intramitochondrial content of Ca^{2+}

Mitochondria were prepared from lobes of the same liver perfused with buffer containing 10 mM-pyruvate and with (●, ▲ and ■) and without (○, △ and □) isoprenaline (1 μM , 2 min). The mitochondria were preloaded with 4–23 nmol of Ca^{2+} /mg of protein as indicated, and the initial rates of efflux (v) were determined over the range 0.4–0.6 μM extramitochondrial Ca^{2+} . (a), Na^+ (6 mM)-dependent efflux; (b), Na^+ -independent efflux; (c), efflux in the presence of Ruthenium Red plus 0.2 nmol of A23187/mg of protein.

Figs. 3(a) and 3(b) show the dependence of the rates of the Na^+ -dependent and Na^+ -independent effluxes of Ca^{2+} on the intramitochondrial Ca^{2+} content. The activation of the Na^+ -dependent Ca^{2+} efflux by isoprenaline pretreatment was very marked with Ca^{2+} contents less than about 10 nmol of Ca^{2+} /mg of protein; above about 15 nmol of Ca^{2+} /mg of protein, pretreated and control mitochondria exhibited similar behaviour (Fig. 3a). In three separate experiments, the isoprenaline-induced stimulations with 6 nmol of Ca^{2+} and 23 nmol of Ca^{2+} /mg were $+174 \pm 18\%$ and $-1 \pm 6\%$ respectively (means $\pm$ S.E.M.). In contrast, the response of the Na^+ -independent process to changes in intramitochondrial content of Ca^{2+} was not modified by isoprenaline pretreatment (Fig. 3b). The acute sensitivity of the isoprenaline-induced stimulation of the Na^+ -dependent activity on the Ca^{2+} load raises the possibility that the affinity of the Na^+ -dependent system for internal Ca^{2+} may be changed by hormone pretreatment.

It is likely, however, that only a small fraction of the accumulated Ca^{2+} is free. Studies with liver and heart mitochondria indicate that up to 95% of the total intramitochondrial Ca^{2+} may be bound (Puskin *et al.*, 1976; Crompton & Heid, 1978). Thus an unambiguous analysis of the effects of isoprenaline pretreatment on the affinity of the Na^+ -dependent system for internal Ca^{2+} requires knowledge of the intramitochondrial buffering power for Ca^{2+} and, in particular, whether this is changed in hormone-pretreated mitochondria. The lack of response of the

Na^+ -independent system to isoprenaline pretreatment at non-saturating contents of internal Ca^{2+} suggests that the intramitochondrial buffering power was not changed. Nevertheless, to clarify this point, the effect of hormone pretreatment on the rates of Ca^{2+} efflux induced by the Ca^{2+} ionophore A23187 in the presence of Ruthenium Red was investigated.

Fig. 3(c) shows that isoprenaline pretreatment induces no change in the rates of A23187-catalysed efflux of Ca^{2+} over the range 6–23 nmol of Ca^{2+} /mg of protein. Since the rates of ionophore-induced Ca^{2+} efflux increased as the Ca^{2+} load was increased, the ionophore was evidently not saturated by internal Ca^{2+} , and it would be predicted that any change in intramitochondrial buffering power would be manifested as a changed rate of Ca^{2+} efflux. The data of Fig. 3, therefore, are consistent with an isoprenaline-induced change in the apparent affinity of the Na^+ -dependent system for intramitochondrial Ca^{2+} . In all subsequent experiments, the conditions adopted include the use of 7 nmol of Ca^{2+} /mg of protein and 6 mM external Na^+ to maximize the observed activation. The relation of these to physiological conditions is considered in the Discussion section.

The effects of the infusion period on the activation of Ca^{2+} efflux by adrenergic agonists

Fig. 4 reports the time course of the activation of the Na^+ -dependent release of Ca^{2+} by adrenergic agonists. The activation of the Na^+ -induced release by isoprenaline (Fig. 4a) was evident after 1 min

Fig. 4. The effects of tissue pretreatment with adrenergic agonists on the rates of Na⁺-dependent and Na⁺-independent efflux of Ca²⁺

Livers were perfused with buffer containing 10 mM-pyruvate as described in the Methods section. Mitochondria were isolated from control lobes and from lobes treated with agonist for the time periods indicated. Data are reported as the percentage increase in the rate of Ca²⁺ efflux from mitochondria of hormone-pretreated lobes with respect to control mitochondria from the same liver. The internal Ca²⁺ contents were 7 nmol/mg, and 6 mM-Na⁺ was added where indicated. Bars indicate $\pm$ S.E.M. The number of separate experiments is given in parentheses. (a), Isoprenaline (1 μ M) was infused; (b), 1 μ M-adrenaline was infused; (c), 10 μ M-phenylephrine was infused; (d), no agonist was infused. Closed symbols ($\blacktriangle$, $\bullet$, $\blacksquare$ and $\blacklozenge$) refer to Na⁺-dependent Ca²⁺ efflux; open symbols ($\triangle$, $\circ$, $\square$ and $\lozenge$) refer to Na⁺-independent Ca²⁺ efflux.

infusion, maximal after 2 min, and was maintained for at least 4 min. The time course of the isoprenaline-induced activation mimicks that caused by adrenaline (Fig. 4b). In contrast, pretreatment with phenylephrine, an α_1 -adrenergic agonist, yielded no significant activation (Fig. 4c). These data support the conclusion drawn previously (Goldstone & Crompton, 1982) that the adrenergic activation of the Na⁺-dependent system of liver mitochondria is mediated via a β -adrenergic mechanism.

The data of Fig. 4 also show an apparent small activation of the Na⁺-independent efflux. However, it is essential to consider possible complications arising from the experimental procedure involved in preparing control and treated mitochondria from lobes of the same liver. This procedure, in which the control lobes were removed, and then homogenized while hormone was infused into the treated lobes, necessarily involved retention of the control homogenate (on ice) until the treated homogenate was ready, after which the two homogenates were dealt with together. A valid comparison of data from control and treated mitochondria, therefore, requires that any time-dependent changes during the retention period of the control homogenate (1–4 min) be accounted for. This aspect was investigated as reported in Fig. 4(d) and Table 1.

Table 1. The stabilities of the Na⁺-independent and Na⁺-dependent efflux systems after tissue homogenization

Control livers were perfused with buffer containing 10 mM-pyruvate as described in the Methods section, and then homogenized and placed on ice. Portions of the homogenate were then centrifuged (and treated according to the standard mitochondrial isolation procedure; see the Methods section) at 4 min, 16 min and 30 min after homogenization. Ca²⁺ efflux was measured in the presence and absence of 6 mM-Na⁺ and with 7 nmol of Ca²⁺/mg of protein. The rates of Ca²⁺ efflux from mitochondria of extracts retained on ice are expressed as a percentage of the activities with mitochondria from the same extracts centrifuged 4 min after homogenization.

Time (min)	Activity (%)			
	Na ⁺ -independent		Na ⁺ -dependent	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
16	53	75	98	100
30	—	44	—	96

In the experiments of Fig. 4(d), the control lobes were removed and homogenized at periods of 1 min and 2 min before removal and homogenization of the

remaining lobes (hormone untreated); both homogenates were then centrifuged together, i.e. the standard procedure was adopted except that the 'test' lobes (shams) received no infusion of hormone. It is evident that the Na^+ -independent activity of mitochondria from the sham-test lobes was significantly greater than the activity of mitochondria from the control lobes. This suggests that storage of the control homogenate on ice led to a time-dependent loss of Na^+ -independent activity. This conclusion was tested as shown in Table 1. In this case, single control homogenates were divided and the portions were retained on ice for various periods before centrifugation. Mitochondria from the retained homogenates lost up to about half of the Na^+ -independent activity with respect to the 4 min controls. It is concluded that the apparent increases in Na^+ -independent activity (e.g. Fig. 4b) are within the limits predicted from the time-dependent loss of activity during retention of the control homogenates and do not reflect hormonally-induced changes. On the other hand, the Na^+ -dependent process was remarkably stable. No significant change in Na^+ -dependent activity was observed when homogenates were retained as long as 30 min on ice (Table 1). This stability is reflected by the fact that the Na^+ -dependent activity of mitochondria from sham-test lobes was no greater than in controls (Fig. 4d).

The influence of tissue redox state on the capacity of isoprenaline, glucagon and cyclic AMP to activate Ca^{2+} efflux

It is considered that β -adrenergic mechanisms in general involve cyclic AMP as mediator (Sutherland

& Robison, 1966). Accordingly the response of the Na^+ -dependent process for Ca^{2+} efflux to treatments that cause changes in tissue levels of cyclic AMP were investigated. Fig. 5(a) compares the capacity of tissue pretreatment with isoprenaline, glucagon and cyclic AMP to induce an activation of the Na^+ -dependent system when the perfusion medium contained 10 mM-lactate plus 1 mM-pyruvate. All three treatments caused a comparable stimulation of the Na^+ -dependent efflux of Ca^{2+} in isolated mitochondria (120–165%). In the presence of lactate plus pyruvate, infusion of isoprenaline and glucagon increased the cyclic AMP content from 0.65 nmol/g to 1.61 and 5.4 nmol/g respectively (Table 2); these

Table 2. *The effects of isoprenaline and glucagon on the tissue content of cyclic AMP*

Livers were perfused with buffer containing either (a) 10 mM-pyruvate or (b) 10 mM-lactate plus 1 mM-pyruvate, in the presence and absence of isoprenaline (1 μM , 2 min) and glucagon (10 nM, 2 min). Cyclic AMP contents were determined as described in the Methods section. Results are means $\pm$ S.E.M.; where two separate experiments were performed, both values are reported. The numbers of separate experiments is given in parentheses.

Agonist	Cyclic AMP content (nmol/g)	
	(a) Pyruvate	(b) Lactate plus pyruvate
None	0.42, 0.60 (2)	0.65 $\pm$ 0.21 (3)
Isoprenaline	0.61 $\pm$ 0.16 (3)	1.61 $\pm$ 0.25 (3)
Glucagon	0.62, 0.74 (2)	5.40 $\pm$ 0.78 (3)

Fig. 5. *Effect of tissue pretreatment with isoprenaline, glucagon and cyclic AMP on the Na^+ -dependent and Na^+ -independent release of Ca^{2+} from isolated mitochondria*

Livers were perfused with buffer containing either 10 mM-lactate plus 1 mM-pyruvate (a) or 10 mM-pyruvate (b). Mitochondria from control and treated liver lobes were isolated as described in the Methods section. Assays of the initial rates of Ca^{2+} efflux were performed with 7 nmol of internal Ca^{2+} /mg of protein and in the presence and absence of 6 mM- Na^+ . Data are expressed as the percentage change in the rates of Ca^{2+} efflux from mitochondria of treated lobes with respect to control mitochondria isolated from the same liver. Bars indicate $\pm$ S.E.M. The numbers of separate experiments are given in parentheses. Open columns denote Na^+ -dependent activity and filled columns Na^+ -independent activity. Abbreviations: I, isoprenaline (1 μM , 2 min); G, glucagon (10 nM, 2 min); C, cyclic AMP (100 μM , 2 min).

values are similar to those reported by others (Exton *et al.*, 1970, 1971). The degree to which exogenous administration of 100 μM -cyclic AMP increases cytosolic cyclic AMP is not known, but the effectiveness of this treatment in activating cyclic AMP-dependent phenomena is well documented (Exton *et al.*, 1971). Thus, under these conditions an apparent correlation may be drawn between activation of Na^+ -dependent Ca^{2+} transport and the tissue content of cyclic AMP. On the other hand, changes in the tissue content of cyclic AMP were not associated with significant changes in the activity of the Na^+ -independent process for Ca^{2+} efflux (Fig. 5a; Table 2).

The studies by Clark & Jarrett (1978) have shown that the addition of glucagon to hepatocytes causes a marked increase in cyclic AMP levels only when sufficient lactate is provided in the incubation medium. Incubation with oxidized substrate alone (e.g. pyruvate) drastically diminishes the cyclic AMP response to glucagon. This observation provides a means to test further the correlation between the activity of the Na^+ -dependent system and the tissue content of cyclic AMP.

Experiments were conducted to investigate whether the activities of the Na^+ -dependent and Na^+ -independent systems and the tissue content of cyclic AMP were affected by the lactate and pyruvate composition of the perfusion medium. Table 3 shows that the inclusion of 10 mM-pyruvate in the perfusion medium in place of 1 mM-pyruvate plus 10 mM-lactate caused no significant changes in the activity of the Na^+ -dependent system for Ca^{2+} efflux in the absence of hormone infusion. Replacement of lactate with pyruvate caused some increase in Na^+ -independent activity, but this was small (about 24%; Table 3). In addition, with no hormone present, substitution of pyruvate plus lactate by

pyruvate alone did not change significantly the tissue level of cyclic AMP (0.65 ± 0.21 and $0.42 - 0.60$ nmol/g respectively; Table 2).

The use of pyruvate alone, however, prevented the ability of glucagon to increase the tissue content of cyclic AMP (Table 2), in agreement with the data of Clark & Jarrett (1978). Moreover, with pyruvate alone present, glucagon administration did not lead to stimulation of the Na^+ -induced efflux of Ca^{2+} , although exogenous supply of cyclic AMP under these conditions did cause an activation (100%) of the Na^+ -dependent system (Fig. 5b). It may be tentatively concluded therefore that the lack of glucagon-induced activation of the Na^+ -dependent system in the absence of lactate was due to the fact that tissue cyclic AMP was not increased under these conditions.

Table 2 shows that the ability of isoprenaline to increase cyclic AMP was also abolished when lactate was replaced by pyruvate in the perfusion medium. However, unlike glucagon, the capacity of isoprenaline to activate the Na^+ -dependent system was not removed by replacement of lactate by pyruvate (Fig. 5b). Thus, with isoprenaline, there is no apparent correlation between activation of the Na^+ -dependent system and cyclic AMP content. These data suggest that the β -adrenergic-induced stimulation of the putative $\text{Na}^+/\text{Ca}^{2+}$ -antiporter of liver mitochondria may not be mediated by cyclic AMP.

Discussion

The contribution of changes in cytoplasmic $[\text{Ca}^{2+}]$ to hormone-induced glycogenolysis in liver has been the subject of extensive investigation (for review, see Exton, 1980; Williamson *et al.*, 1981). These studies have provided evidence that the activation of glycogen phosphorylase *b* kinase by Ca^{2+} (Khoo & Steinberg, 1975; Keppens *et al.*, 1977; Vandenheede *et al.*, 1977) may be a critical factor in the actions of hormones that do not cause changes in cyclic AMP, i.e. α_1 -adrenergic agonists, vasopressin and angiotensin (Sherline *et al.*, 1972; Exton, 1980). On the other hand, no role has been assigned to Ca^{2+} movements during the action of glucagon and β -adrenergic agonists, for which cyclic AMP has been established as a second messenger (Sutherland & Robison, 1966; Williamson *et al.*, 1981). The object of the present work was to assess the contribution of mitochondrially-derived Ca^{2+} to hormone-induced glycogenolysis as judged by the capacity of various hormone pretreatments to cause stable changes in mitochondrial Ca^{2+} efflux.

The study requires some understanding of the physiological process(es) responsible for Ca^{2+} extrusion from liver mitochondria. Four groups of workers have provided evidence that liver mito-

Table 3. The effects of the pyruvate and lactate contents of the perfusion medium on the initial rates of Na^+ -independent and Na^+ -dependent Ca^{2+} efflux

Mitochondria were isolated from control livers perfused with buffer containing either (a) 10 mM-pyruvate, or (b) 10 mM-lactate plus 1 mM-pyruvate. The rates of Ca^{2+} efflux were determined with 7 nmol of Ca^{2+} /mg of protein and in the presence and absence of 6 mM- Na^+ . Results are given as means $\pm$ S.E.M. The numbers of separate experiments are given in parentheses.

Substrate	Ca^{2+} efflux (nmol of Ca^{2+} /mg per min)	
	Na^+ -independent	Na^+ -dependent
(a) Pyruvate	0.72 ± 0.04 (49)	0.48 ± 0.03 (49)
(b) Lactate plus pyruvate	0.58 ± 0.03 (14)	0.44 ± 0.04 (14)

chondria possess an Na^+ -dependent system for Ca^{2+} efflux (Nedergaard & Cannon, 1980; Haworth *et al.*, 1980; Heffron & Harris, 1981; Goldstone & Crompton, 1982). The mechanism of this process in liver has not been investigated, but extensive studies of the Na^+ -induced efflux of Ca^{2+} from heart mitochondria are most logically interpreted in terms of an $\text{Na}^+/\text{Ca}^{2+}$ exchange (Crompton *et al.*, 1977; Crompton, 1980; Hayat & Crompton, 1982) and a similar mechanism may be provisionally assigned to the liver system.

Liver mitochondria also exhibit an Na^+ -independent release of Ca^{2+} (Puskin *et al.*, 1976; Nicholls, 1978), which has a comparable activity to the Na^+ -dependent system (Goldstone & Crompton, 1982). This raises the question of whether the Na^+ -independent activity reflects a residual capacity of the Na^+ -dependent system to function in the absence of Na^+ , or whether two distinct systems are present. Available evidence, although far from conclusive, favours the latter interpretation. Thus, studies of the Na^+ -dependent and Na^+ -independent systems of heart mitochondria revealed their quite different sensitivities to inhibition by the lanthanide series (Crompton *et al.*, 1979). The present studies have shown that pretreatment of liver with glucagon and β -adrenergic agonists selectively activates the Na^+ -induced rate of Ca^{2+} efflux (Figs. 2, 3a, 4 and 5). In particular, the finding that hormone infusion changes the apparent affinity of Na^+ -induced efflux for internal Ca^{2+} (Fig. 3a) is difficult to interpret in terms of a single carrier for Ca^{2+} efflux since, if this were the case, a similar response of Na^+ -induced and Na^+ -independent effluxes of Ca^{2+} would be predicted.

The pronounced dependence of the isoprenaline-induced activation of the Na^+ -dependent system on the intramitochondrial Ca^{2+} load may be related to the recent work of Reinhart *et al.* (1982), who, by using a rapid procedure for the purification of rat liver mitochondria in the presence of inhibitors of mitochondrial Ca^{2+} fluxes, obtained mitochondrial contents of 5–7 nmol of Ca^{2+} /mg of mitochondrial protein. This suggests that the physiological level of intramitochondrial Ca^{2+} may be within the range of maximal sensitivity to β -adrenergic-induced changes in efflux rate.

The ability of glucagon pretreatment to activate the Na^+ -dependent system for Ca^{2+} efflux and to increase tissue cyclic AMP are both critically dependent on the presence of lactate in the perfusion medium (Fig. 5; Table 2). Presumably, this reflects the control of cyclic AMP levels by the redox state of the cytoplasmic nicotinamide nucleotides. Ethanol infusion has been shown to alleviate the requirement of a low pyruvate/lactate ratio (Sugano *et al.*, 1980) for glucagon-induced glucose release in liver, and Clark & Jarrett (1978) have reported that the

enzymes responsible for cyclic AMP production and breakdown are sensitive to changes in the NAD^+/NADH ratio. In addition, infusion of cyclic AMP was equally effective in activating the Na^+ -dependent system (Fig. 5). These data indicate a role for cyclic AMP in the mediation of the glucagon-induced activation of the Na^+ -dependent system. The point at which cyclic AMP may be involved remains to be elucidated. Studies in this laboratory (T. P. Goldstone & M. Crompton, unpublished work) have failed to produce any activation of the Na^+ -dependent system by the addition of cyclic AMP or the catalytic subunit of protein kinase to isolated liver mitochondria, in agreement with previous reports (Scarpa *et al.*, 1976; Borle, 1976).

The glucagon-induced activation of the Na^+ -dependent efflux system for Ca^{2+} is in accordance with the reports that administration of glucagon to liver and hepatocytes induces a decrease in mitochondrial Ca^{2+} content (Foden & Randle, 1978; Blackmore *et al.*, 1979a; Taylor *et al.*, 1980a) and an inhibition of net $^{45}\text{Ca}^{2+}$ uptake into the mitochondrial fraction (Foden & Randle, 1978), and provides a possible biochemical basis for these phenomena. Taken together, these observations suggest that intracellular redistribution of Ca^{2+} may occur during glucagon action, but the physiological significance of this cannot be assessed until the compartment to which Ca^{2+} is relocated is identified. Ultimate relocation to the cytoplasm is not borne out by the studies of Williamson and co-workers (Murphy *et al.*, 1980), who detected no change in the cytoplasmic $[\text{Ca}^{2+}]$ of hepatocytes after glucagon administration. Ca^{2+} may be mobilized to another compartment; stimulation of liver microsomal Ca^{2+} uptake by cyclic AMP (Taylor *et al.*, 1980b) and by tissue pretreatment with glucagon (Waltenbaugh & Friedmann, 1978; Taylor *et al.*, 1979, 1980b) have been reported.

There is now extensive evidence that an increase in cytosolic $[\text{Ca}^{2+}]$ may activate phosphorylase *b* kinase in liver during α_1 -adrenergic stimulation, but, as outlined in the introduction section, there is no agreement as to the nature of the compartment that may provide this Ca^{2+} . The concept that mitochondrial Ca^{2+} may be involved, based on observed losses of mitochondrial Ca^{2+} after α_1 -adrenergic stimulation (Blackmore *et al.*, 1979a,b; Taylor *et al.*, 1980a), must also take into account possible consequences of mitochondrial oxidative metabolism. Denton & McCormack (1980) have reported that several intramitochondrial dehydrogenases of liver (pyruvate dehydrogenase, NAD^+ -isocitrate dehydrogenase, oxoglutarate dehydrogenase) are activated by increases in $[\text{Ca}^{2+}]$, so that a loss of mitochondrial Ca^{2+} would be expected to depress oxidative metabolism. However, in the fed animal, the promotion of glycogenolysis more than accounts

for the increased glucose output during α_1 -adrenergic activation and is accompanied by increases in glycolysis and in oxidative metabolism (Williamson *et al.*, 1980; Hue *et al.*, 1981a,b). In addition, tissue pretreatment with vasopressin increases the proportion of pyruvate dehydrogenase in the active form (Hems *et al.*, 1978). The present work revealed no support for the concept that α_1 -adrenergic stimulation causes a release of mitochondrial $[\text{Ca}^{2+}]$. No stable changes in the activities of either the Na^+ -dependent or the Na^+ -independent efflux systems were induced by phenylephrine pretreatment of livers perfused either with pyruvate (Fig. 4c) or with 10 mM-lactate plus 1 mM-pyruvate (results not shown). However, these results do not exclude the possibility that either activation of efflux or inhibition of influx of mitochondrial Ca^{2+} occurs in a manner that does not survive the mitochondrial isolation procedure.

The present data indicate that the capacity of adrenaline to induce stable activation of the Na^+ -dependent system (Fig. 4b) may be attributed to a β -adrenergic mechanism since isoprenaline pretreatment was equally effective (Fig. 4a). It is generally assumed (Hue *et al.*, 1981a) that β -adrenergic actions on liver are mediated in the same manner, via cyclic AMP, as those after stimulation by glucagon. However, in contrast with the effects exerted by glucagon, the capacity of isoprenaline to activate the Na^+ -dependent system was not related to changes in tissue content of cyclic AMP. This observation, together with reports of β -adrenergic effects on reticulocyte ghosts, guinea-pig atria, S49 lymphoma cells and adipocytes that occur independently of increases in cyclic AMP (Hirata *et al.*, 1979; Nawrath *et al.*, 1980; Maguire & Erdos, 1980; Cheng *et al.*, 1981) suggest that, in some instances, the assumption that cyclic AMP is an obligatory messenger in β -adrenergic responses may not hold. An alternative explanation is that a cascade may operate between cyclic AMP and the $\text{Na}^+/\text{Ca}^{2+}$ -antiporter so that very small changes in [cyclic AMP], perhaps localized, may be amplified into a measurable activation of the antiporter. The loss of components of such a cascade during mitochondrial isolation would account for the observations, reported here, that administration of cyclic AMP to the whole tissue causes activation of the $\text{Na}^+/\text{Ca}^{2+}$ -antiporter, whereas addition of cyclic AMP or protein kinase to isolated mitochondria does not. However, this hypothesis does not easily account for the fact that the presence of lactate profoundly influences the capacity of both isoprenaline and glucagon to increase cyclic AMP, but affects only the ability of glucagon to cause antiporter activation.

This work was supported by the Science Research Council (GR/B 22581) and a grant to T. P. G. from the

Medical Research Council for training in research methods.

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