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HORMONAL REGULATION OF MITOCHONDRIAL FUNCTION

DESCRIPTION OF A SYSTEM CAPABLE OF MIMICKING SEVERAL EFFECTS OF GLUCAGON

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Isolated rat liver mitochondria were incubated at 0°C in a medium consisting of 225 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4 (buffer 1) for 10 min, centrifuged and resuspended in 0.3 M sucrose. This treatment resulted in a stimulation of mitochondrial functions, mimicking several of the effects that follow glucagon treatment of the intact rat or isolated hepatocytes. Both phosphate and potassium are required for this effect; the addition of magnesium serves to enhance it. Mitochondrial respiration is essential for the development of the activated state as the stimulation is blocked by increasing concentrations of rotenone in the incubation. The intramitochondrial ATP/ADP ratio is increased, but when this increase was prevented by including low levels of rotenone or oligomycin in buffer 1, the stimulation of mitochondrial function was not diminished, thus demonstrating that an increased ATP/ADP ratio is not essential for activation. The rate of citrulline formation was unaffected by buffer 1 treatment unless glutamate was also included in the medium, indicating that control of this mitochondrial function differs from other functions studied.

Introduction

In isolated rat hepatocytes or in the liver of intact rats, glucagon treatment results in activation of several enzymes and a stimulation of a variety of mitochondrial functions. These include pyruvate carboxylation [1], uncoupler-dependent ATPase [2,3], succinate dehydrogenase [4–7], citrulline formation [8–11], glutaminase [12], oxidative phosphorylation and uncoupled respiration [13], P_i-ATP exchange [3], rate of swelling in the presence of K^+, ATP and valinomycin [14], membrane potential [15] and proton gradient [15,16]. The 'energization' of intact hepatic mitochondria is also retained in isolated submitochondrial particles, as evidenced by a stimulation of reverse electron flow, energy-dependent transhydrogenase, and State 3 and uncoupled respiration when succinate or NADH are substrates [17]. The primary mitochondrial modification that results in this broad stimulation of mitochondrial activities is not known, although proposals have been forwarded implicating changes in substrate transport [3,18–20], metabolite compartments [3,11,21], pH gradient [16] and respiratory chain components [13,15]. A central problem in the elucidation of the mechanism of glucagon's action at the level of the mitochondrion has been the inability to duplicate the effect of the hormones in broken cell preparations. Traditionally, the

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

hormone has been administered to the intact rat or hepatocyte and interpretation of subsequent events is hampered by the multiplicity of glucagon's effects on the intact cell. In an earlier study [21] we demonstrated that rat liver mitochondria incubated at 0°C in a buffered, isotonic medium containing K^+, P_i and Mg^{2+} showed a stimulation of several mitochondrial functions similar to that observed after glucagon treatment of the intact animal. This included a stimulation of respiration, both State 3 and uncoupled, succinic dehydrogenase activity, pyruvate carboxylation and the rate of uncoupler-dependent ATPase. In the following report, we further investigate this phenomenon, with the goal of establishing it as a model system capable of mimicking a number of the in vivo effects of glucagon on rat liver mitochondria. Such a system may be useful in the investigation of the mechanism by which glucagon activates hepatic mitochondria.

Materials and Methods

FCCP was obtained from Pierce Chemical Co.; ATP, dinitrophenol, oligomycin, digitonin, glucagon, bovine serum albumin, rotenone, *N*-acetylglutamate, trichloracetic acid and pyruvate kinase were purchased from Sigma. Triethanolamine, lactic dehydrogenase and hexokinase were obtained from Boehringer-Mannheim and Triton X-100 from Beckman Instruments. [^{14}C]Bicarbonate was purchased from New England Nuclear.

Fed, male Wistar strain rats weighing between 200 and 350 g were used. The animals were anesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg) and were immediately treated subcutaneously with 100 μg glucagon or its injection vehicle, saline/0.05% bovine serum albumin. After 20 min, the livers were removed and mitochondria prepared by differential centrifugation in 0.3 M sucrose with the mitochondrial fraction washed once. For activation of functions, mitochondria were incubated in 225 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM KH_2PO_4, 5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4 (buffer 1), at 0°C for 10 min. Mitochondrial protein was varied from 1.5 to 15 mg/ml. After incubation, mitochondria were centrifuged and resuspended to 5 mg/ml in 0.3 M sucrose.

Mitochondrial ATPase activity was measured by the release of P_i at 30°C over a period of 8 min in the absence and presence of 0.18 mM 2,4-dinitrophenol as described by Weiner and Lardy [22]. Mitochondrial pyruvate carboxylation was measured as previously described [1]. Mitochondrial citrulline formation was assayed by the incorporation of [^{14}C]bicarbonate into citrulline using succinate as the energy source, as described by Yamazaki and Graetz [8]. *N*-Acetylglutamate content was determined in heat-treated extracts of the mitochondria, as previously described [11]. Inorganic ions were measured in trichloracetic acid extracts of mitochondria; Mg^{2+} with an Instrument Laboratory atomic absorption dual-beam spectrophotometer, model 751, K^+ with an Instrument Laboratory flame photometer, model 143, and P_i as described by Fiske and Subbarow [23]. Protein was determined by the method of Lowry et al. [24] using crystalline bovine serum albumin as standard. Adenine nucleotides were determined using the fluorometric assays described by Lowry et al. [25].

Submitochondrial particles were prepared as previously described [17], except the last wash was omitted. The energy-linked pyridine nucleotide transhydrogenase reaction and the energy-dependent reduction of NAD were measured according to the method of Ernster and Lee [26].

Results

Effect of buffer 1 treatment of mitochondria on the uncoupler-dependent ATPase

Titheradge and Haynes [3] have shown that glucagon treatment of the intact animal results in an increased resistance of the uncoupler-dependent ATPase of hepatic mitochondria to the inhibitory effects of high concentrations of uncouplers. As can be seen in Fig. 1, we confirm this and also show that incubation at 0°C in a buffered isotonic medium containing K^+, P_i and Mg^{2+} (buffer 1), as described in Materials and Methods, produces a resistance to inhibition by high concentrations of FCCP, resembling that caused by glucagon. This simple treatment of the mitochondria stimulated the rate of the uncoupler-dependent ATPase in both control (Fig. 1A) and glucagon-treated mitochondria (Fig. 1B).

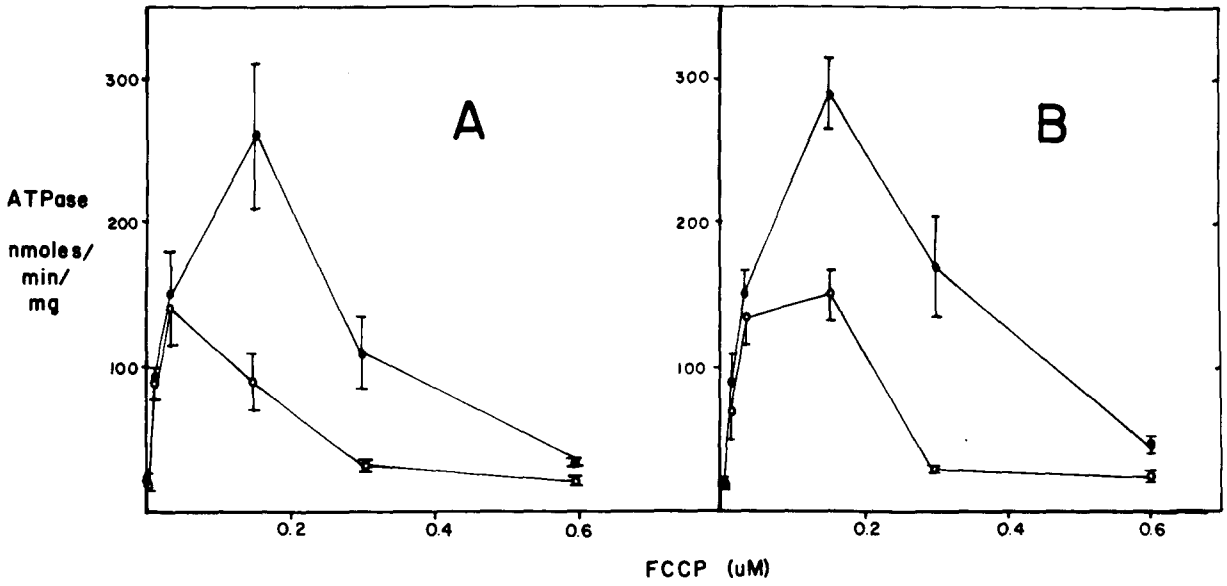

Fig. 1. Inhibition of the uncoupler-dependent ATPase by increasing concentrations of the uncoupler, FCCP. Mitochondria from control rats (A) or from glucagon-treated rats (B) were incubated in buffer 1 (closed circles). The uncoupler-dependent ATPase was assayed as described in Materials and Methods except FCCP replaced dinitrophenol in the assay medium. Values are the mean ± S.E. of four experiments.

It can be seen that maximal stimulation of the ATPase occurred at 0.15 μM FCCP in buffer 1-treated as well as glucagon-treated mitochondria, in contrast to the peak occurring at 0.075 μM in control mitochondria. The mechanism by which high concentrations of uncouplers inhibit the ATPase is not clear [27,28], but it is apparent that the incubation in buffer 1 and glucagon treatment resulted in a similar response. These data resemble those of Bertina and Slater [29], who showed that incubation with increasing concentrations of potassium phosphate displaced the optimum of the FCCP effect curve to higher FCCP concentrations and increased the rate of the uncoupler-dependent ATPase. It was suggested by these investigators that endogenous phosphate may be a regulator of the turnover of the ATPase in intact mitochondria.

Effect of buffer 1 treatment of mitochondria on submitochondrial particles prepared subsequently

A previous report [17] from this laboratory noted that effects of glucagon treatment on isolated rat mitochondria persist in submitochondrial particles produced by sonication. The stimulation of the energy-dependent transhydrogranse, reverse electron flow, oxidative phosphorylation and 8-anilino-1-napththalenesulfonic acid fluorescence indicated an increased degree of energization in submitochondrial particles from glucagon-treated rats. If exposure to buffer 1 does indeed mimic in vivo glucagon stimulation, the stimulation should persist in submitochondrial particles prepared from these mitochondria. Table I shows that after this treatment, rates of both the energy-dependent transhydrogenase and reverse electron flow did not differ significantly from those of submitochondrial particles of glucagon-treated rats. It can also be seen that incubation of mitochondria from glucagon-treated mitochondria in buffer 1 did not further stimulate these two functions.

Energy dependence of activation procedure

A series of experiments was performed to determine if the buffer 1-induced stimulation was energy dependent, i.e., if respiration was required. Table II shows the effect of such incubations supplemented with increasing concentrations of

TABLE I

EFFECT OF INCUBATION OF MITOCHONDRIA IN BUFFER 1 ON THE ENERGY-DEPENDENT PYRIDINE NUCLEOTIDE TRANSHYDROGENASE REACTION AND THE ATP-DRIVEN REVERSE ELECTRON FLOW REACTION IN SUBSEQUENTLY PREPARED SUBMITOCHONDRIAL PARTICLES

Submitochondrial particles were prepared from control mitochondria, mitochondria incubated in buffer 1, and mitochondria isolated from glucagon-treated rats. The transhydrogenase reaction was begun by the addition of 2 mM ATP. Results are corrected for the nonenergy-linked transhydrogenase reaction. The reverse electron flow reaction was initiated by addition of 0.5 mM NAD^+. Values are the mean ± S.E. Significance was determined according to Dunnett's test of least significant difference.

	Mitochondria			
	Control ($n = 10$)	Control + buffer 1 ($n = 10$)	Glucagon ($n = 5$)	Glucagon + buffer 1 ($n = 5$)
Energy-dependent transhydrogenase (nmol/min per mg)	28.0 ± 4.0	39.0 ± 4.0 [a]	36.0 ± 3.0 [b]	39.0 ± 5.0 [a]
Reverse electron flow (nmol/min per mg)	6.1 ± 0.9	9.5 ± 1.2 [a]	7.6 ± 1.1 [b]	10.6 ± 1.6 [a]

[a] $P < 0.01$.
[b] $P < 0.05$.

the respiratory inhibitor, rotenone. It is evident that increasing amounts of rotenone abolished the stimulation of pyruvate carboxylation (CO_2 fixation). Supplementation of the incubation with succinate circumvented the inhibition of rotenone.

TABLE II

ENERGY DEPENDENCE OF THE BUFFER 1 EFFECT

Control mitochondria received no treatment or were incubated in buffer 1, as described in Materials and Methods except the incubation medium also included rotenone in the concentrations indicated. The pyruvate carboxylation (CO_2 fixation) assay medium was as described in Materials and Methods, except 1 mM succinate was included. Where indicated, 1 mM succinate was added to buffer 1 in addition to rotenone. Values are the mean ± S.E. of four experiments. Significance was determined using Dunnett's test of least significant differences ([a] $P < 0.05$, [b] $P < 0.01$). All groups incubated with succinate in buffer 1 were significantly stimulated ($P < 0.01$).

Rotenone (μM) additions to buffer 1	CO_2 fixation (nmol/min per mg) (buffer 1)	Buffer 1 + 1 mM succinate
0	35 ± 6 [b]	41 ± 4
33	30 ± 6 [a]	36 ± 5
66	22 ± 6	42 ± 2
137	17 ± 4	36 ± 6
167	15 ± 2	34 ± 6
Control mitochondria	18 ± 5	

It has been proposed [30] that the glucagon effect on mitochondrial energization is the result of membrane stabilization rather than a direct stimulation. To test whether the buffer 1 treatment was a true stimulation, freshly isolated control mitochondria were assayed for pyruvate carboxylation before and after a separate aliquot was treated with buffer 1. Control mitochondria did not significantly age during the time course of these experiments (before 15 ± 1; after, 14 ± 2 nmol CO_2/min per mg); also buffer 1-treated mitochondria showed a significant stimulation of function above control values (22 ± 2 nmol (CO_2/min per mg; $P < 0.001$; $n = 5$)

Dependence on Mg^{2+}, P_i and K^+

To determine the ionic composition of the incubation medium necessary for activation of mitochondria, mitochondria were incubated in buffer 1 in which the concentration of either Mg^{2+}, P_i or K^+ was varied. It can be seen from Table III that Mg^{2+} (0–5 mM) was not necessary in the medium in order to stimulate CO_2 fixation. In contrast, the uncoupler-dependent ATPase was insignificantly elevated when Mg^{2+} was absent but became significantly greater with increasing Mg^{2+} concentrations, to 5 mM. Similarly, increasing the

TABLE III

EFFECT OF ALTERING ION CONCENTRATIONS IN INCUBATIONS

Control mitochondria received either no treatment or incubation in buffer 1 as described in Materials and Methods, except the Mg^{2+}, P_i or K^+ composition of the medium was altered as indicated. In the K^+-free medium P_i was neutralized with NaOH. Assays were as described in Materials and Methods. Values for the uncoupler-dependent ATPase are presented for assays with (+) and without (−) the uncoupler dinitrophenol. Values are the mean ± S.E. of five experiments. Significance was determined using Dunnett's test of least significant differences ([a] $P < 0.01$; [b] $P < 0.05$).

Buffer 1 ionic content (mM)		ATPase (nmol/min per mg)		CO_2 fixation (nmol/min per mg)	ATP/ADP
		+	−		
Mg^{2+}	Control	267 ± 29	23 ± 2	18 ± 3	1.27 ± 0.21
	0	316 ± 31	19 ± 1	30 ± 4 [b]	2.12 ± 0.04
	2.5	362 ± 19 [a]	19 ± 1	31 ± 4 [b]	2.92 ± 0.35 [a]
	5.0	399 ± 7 [a]	16 ± 1	34 ± 4 [b]	3.06 ± 0.33 [a]
P_i	Control	229 ± 48	26 ± 2	15 ± 2	1.03 ± 0.20
	0	321 ± 56	26 ± 3	15 ± 1	0.67 ± 0.20
	2.5	362 ± 43 [a]	27 ± 1	21 ± 2	1.15 ± 0.08
	5.0	420 ± 36 [a]	30 ± 1	26 ± 2 [a]	2.27 ± 0.09 [a]
	10.0	437 ± 36 [a]	30 ± 2	31 ± 2 [a]	2.67 ± 0.27 [a]
K^+	Control	254 ± 55	23 ± 2	16 ± 1	1.03 ± 0.2
	0	262 ± 35	32 ± 7	13 ± 1	1.63 ± 0.11
	5.0	394 ± 65	23 ± 9	17 ± 2	1.78 ± 0.25 [a]
	10.0	445 ± 55 [b]	32 ± 8	24 ± 3 [a]	2.86 ± 0.47 [a]
	20.0	463 ± 49 [b]	13 ± 4	25 ± 2 [a]	2.77 ± 0.51 [a]
	30.0	474 ± 65 [b]	24 ± 5	27 ± 2 [a]	2.67 ± 0.27 [a]

concentration of Mg^{2+} from 0 to 5 mM resulted in a higher ATP/ADP ratio. On the other hand, P_i had to be included in the incubation buffer in order to elicit a significant stimulation of either CO_2 fixation or the uncoupler-dependent ATPase. This effect was enhanced as P_i was increased to 10 mM. Like P_i, K^+ was necessary in the incubation medium to elicit a stimulation of either CO_2 fixation or uncoupler-dependent ATPase. Furthermore, a minimum concentration of 10 mM was required to elicit a significant effect on CO_2 fixation and the uncoupler-dependent ATPase.

It has been reported that hepatic mitochondria isolated from glucagon-treated rats have an increased content of Mg^{2+} and K^+ [14,15]. Although it has been reported that P_i is also elevated [31], this has not been confirmed [32] (Hamman, H.C. and Haynes, R.C., Jr., unpublished data). The data in Table IV show that mitochondria had elevated levels of Mg^{2+}, K^+ and P_i after incubation in buffer. Both phosphate and magnesium content remained above control levels after four washes in 0.3 M sucrose, but potassium content fell within the control range after one wash. There was no effect of either buffer 1 incubation or washing on citrulline formation. There was poor correlation between the observed increase in intramitochondrial contents of Mg^{2+}, K^+ or P_i and a stimulation of either the uncoupler-dependent ATPase or CO_2 fixation. For instance, the uncoupler-dependent ATPase remained significantly elevated after one wash in 0.3 M sucrose, but K^+ content was within the control range. Furthermore, P_i and Mg^{2+} content remained above the control even when the functions studied returned to control levels. On the other hand, an elevated ATP/ADP ratio corresponded well with the stimulation of both CO_2 fixation and uncoupler-dependent ATPase. These data represent a loss of stimulation due to washing rather than aging, since in freshly diluted (5 mg/ml) control mitochondria assayed concurrently with the washed, buffer 1-treated mitochondria, these values remained constant throughout the time course of the experiment.

TABLE IV

EFFECT OF SUCCESSIVE WASHES ON IONIC CONTENT, UNCOUPLER-DEPENDENT ATPase, PYRUVATE CARBOXYLATION (CO_2 FIXATION) AND ATP/ADP RATIO OF BUFFER 1-TREATED MITOCHONDRIA

Control mitochondria received no treatment (control) or incubation in buffer 1, as described in Materials and Methods. Treated mitochondria were then washed repetitively in 0.3 M sucrose. After each wash, aliquots were assayed for Mg^{2+}, K^+, P_i, adenine nucleotides, citrulline formation, and CO_2 fixation and uncoupler-dependent ATPase. The uncoupler-dependent ATPase was assayed in the presence (+) and absence (−) of the uncoupler, dinitrophenol. Values are the mean ± S.E. of five experiments. Significance was determined using Dunnett's test of least significant difference ([a] $P < 0.01$; [b] $P < 0.05$).

	Control	Number of washes following buffer 1 treatment				
		0	1	2	3	4
K^+ (nmol/mg)	170 ± 6	250 ± 11 [a]	150 ± 8	150 ± 3	140 ± 4	130 ± 3 [a]
P_i (nmol/mg)	14 ± 1	33 ± 4 [a]	32 ± 3 [a]	27 ± 1 [a]	25 ± 1	27 ± 4 [a]
Mg^{2+} (nmol/mg)	33 ± 1.0	61 ± 1.5 [a]	44 ± 0.6 [a]	39 ± 0.9 [b]	38 ± 0.6 [b]	36 ± 0.4 [b]
ATP/ADP	2.0 ± 0.3	5.1 ± 1.8 [a]	3.2 ± 0.2	2.2 ± 0.3	1.7 ± 0.1	1.2 ± 0.04
Citrulline formation (nmol/min per mg)	16 ± 2	17 ± 1.9	19 ± 2	16 ± 2	15 ± 2	13 ± 2
CO_2 fixation (nmol/min per mg)	23 ± 2	29 ± 3 [b]	28 ± 2	25 ± 3	22 ± 2	18 ± 2
Uncoupler-dependent ATPase (+)	211 ± 56	422 ± 63 [b]	378 ± 49 [b]	317 ± 47	282 ± 41	227 ± 31
(−)	20 ± 4	27 ± 4	41 ± 7 [b]	28 ± 3	24 ± 5	31 ± 5

Although multiple washings resulted in a return to the original control values for both uncoupler-dependent ATPase and CO_2 fixation, this probably did not indicate a selective loss of function as is illustrated in Fig. 2. It is evident that although the rate of CO_2 fixation fell for treated mitochondria with each successive wash, the stimulation relative to control levels was retained if control mitochondria were similarly washed. A similar decline in activity of glucagon-treated mitochondria was observed after multiple washings (data now shown).

Effect of altering ATP/ADP ratios on stimulation by buffer 1

The data presented in Table IV suggest that an increase in ATP/ADP ratios may correlate with the activity of the uncoupler-dependent ATPase and CO_2 fixation. To delineate more clearly the correlation between the stimulation and ATP/ADP ratios, mitochondria were incubated in buffer 1 plus increasing concentrations of rotenone. As may be seen from Fig. 3, there was no correlation ($r^2 = 0.007$) between ATP/ADP ratios and

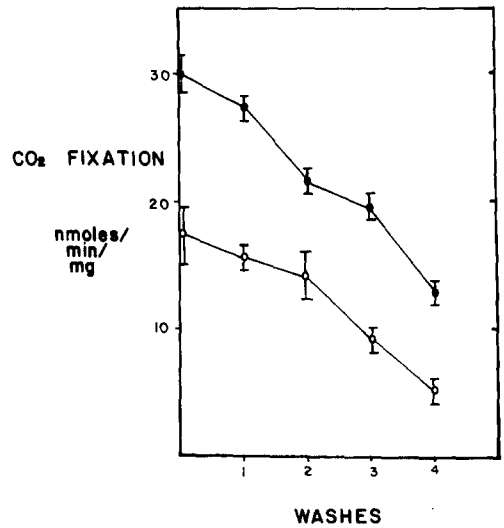

Fig. 2. Effect of successive washes on control mitochondria and buffer 1-treated mitochondria. Control mitochondria (open circles) or buffer 1-treated (closed circles) were repetitively washed in 0.3 M sucrose. Mitochondrial samples were assayed immediately after each wash. Values are the mean ± S.E. of five experiments. All comparisons between treated and control mitochondria were significant ($P < 0.001$). Analysis was by Student's paired, two-tailed t-test.

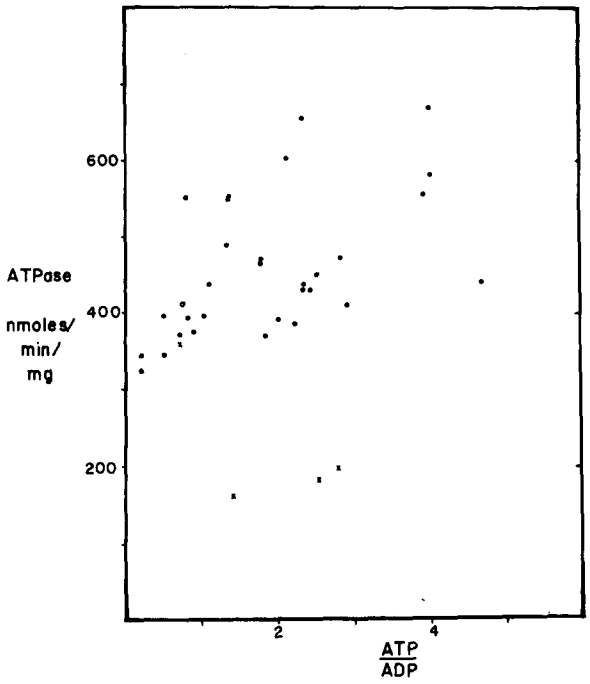

Fig. 3. Influence of ATP/ADP ratios on buffer 1 stimulation of the uncoupler-dependent ATPase. Control mitochondria received no treatment (×) or incubation in buffer 1 (○) as described in Materials and Methods except rotenone was included in the incubation mixture at 0, 33, 66, 137 or 167 nM. Mitochondria were then assayed for uncoupler-dependent ATPase activity and adenine nucleotide content. Data are from four separate experiments.

stimulation of the uncoupler-dependent ATPase when the ATP/ADP ratio was altered by this technique.

Another approach to the question of the importance of the ATP/ADP ratio was to perform the incubation in the presence of increasing concentrations of oligomycin to inhibit ATP generation and the development of high ATP/ADP ratios. Intramitochondrial adenine nucleotides were assayed after this incubation. CO_2 fixation and citrulline formation were then assayed in the presence of oligomycin, FCCP and rotenone and excess ATP (1 mM). Under these conditions, ATP formation will not be rate controlling for the reactions. ATP will be used, and ADP will be formed, but ATP content will remain high and relatively uninfluenced by the intrinsic ATP/ADP ratio. As can be seen from Table V, increasing amounts of

TABLE V

THE EFFECT OF OLIGOMYCIN ON BUFFER 1 TREATMENT

Mitochondria were incubated at 0°C in buffer 1 supplemented with the indicated quantity of oligomycin. Assay conditions were as described in Materials and Methods except the assay medium was supplemented with 0.4 μM FCCP, 1.0 mM ATP, 0.5 μg/ml rotenone and 0.5 μg/ml oligomycin. Values are the mean ± S.E.; significance was determined by Dunnett's test of least significant difference, $n = 6$.

	CO_2 fixation (nmol/min per mg)	Citrulline formation (nmol/min per mg)	ATP/ADP
Control	18 ± 1	33 ± 5	1.67 ± 0.36
Glucagon	49 ± 4 [a]	52 ± 6 [a]	–
Buffer 1 + oligomycin (μg/ml)			
0	34 ± 3 [b]	21 ± 4 [a]	13.77 ± 3.0 [a]
0.1	35 ± 4 [c]	21 ± 5 [a]	7.0 ± 1.55 [a]
0.5	34 ± 4 [c]	22 ± 4 [a]	5.22 ± 1.54 [a]
1.0	36 ± 4 [c]	24 ± 5 [a]	5.22 ± 1.55 [a]
5.0	42 ± 5 [b]	28 ± 7	0.25 ± 0.05 [a]
10.0	40 ± 6 [b]	29 ± 7	0.21 ± 0.02 [a]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.02$.

oligomycin reduced the ATP/ADP ratio to values below that found in the control mitochondria prior to assay. Net nucleotide content was unchanged (data not shown). It can also be seen from Table V that, under these conditions of assay, mitochondria isolated from glucagon-treated rats showed a stimulation of both pyruvate carboxylation and citrulline formation. Incubation in buffer 1 also stimulated CO_2 fixation as assayed under these conditions, but did not stimulate citrulline formation nor was citrulline formation enhanced by the increased ATP/ADP ratio, as has been proposed [33]. Indeed, the converse appeared to be true for incubated mitochondria, as there was a depression of the rate of citrulline synthesis in the presence of an elevated ATP/ADP ratio following this treatment. Surprisingly, increasing concentrations of oligomycin tended to nullify this effect.

There is a large amount of literature indicating that citrulline formation is regulated by the intramitochondrial *N*-acetylglutamate content

TABLE VI

THE STIMULATION OF CITRULLINE FORMATION BY INCUBATION OF MITOCHONDRIA IN BUFFER 1 SUPPLEMENTED WITH GLUTAMATE

Control mitochondria or mitochondria from glucagon-treated rats received no incubation or an incubation in buffer 1 supplemented with 40 mM glutamate. Values are the means ± S.E. of five experiments. Significance was determined according to Dunnett's test of least significant difference.

Treatment	Citrulline synthesis (nmol/min per mg)	*N*-Acetylglutamate (nmol/mg)
Control	13 ± 4	0.9 ± 0.05
Control + buffer 1 + glutamate	24 ± 3 [b]	2.5 ± 0.4 [b]
Glucagon	27 ± 5 [c]	1.1 ± 0.1
Glucagon + buffer 1 + glutamate	42 ± 3 [a]	2.9 ± 0.4 [a]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.

[33–37]. In agreement with such a possibility, Table VI shows that, when *N*-acetylglutamate levels were elevated following an incubation supplemented with glutamate, the rate of citrulline formation was also elevated and not significantly different from that seen in mitochondria isolated from glucagon-treated rats. However, if elevation of the intramitochondrial *N*-acetylglutamate content is the mechanism by which glucagon stimulates citrulline formation, there should be close correspondence between rates of citrulline formation and intramitochondrial *N*-acetylglutamate content. In confirmation of an earlier report from this laboratory [11], we did not find this to be the case. For instance, the *N*-acetylglutamate levels in buffer 1-treated control and glucagon-treated mitochondria were not significantly different, whereas the rate of citrulline formation by the glucagon-treated mitochondria was approximately twice that found in the control group.

Discussion

The purpose of this report is to describe the stimulation of certain mitochondrial functions by exposure to a buffered medium containing K^+, P_i and Mg^{2+} and to compare the stimulation with that effected by glucagon administration to intact animals or hepatocytes. Glutaminase activity was not assayed due to the phosphate dependence of the enzyme [12]. Similarly, P_i-ATP exchange and swelling in the presence of K^+, ATP and valinomycin were not assayed, since these activities are presumably a reflection of the activity of the mitochondrial ATPase. The mitochondrial proton gradient and membrane potential are presumably increased, since mitochondrial respiration was stimulated following buffer 1 treatment [21]. In all functions studied, other than the ureogenic function of citrulline formation, the mitochondria, treated in vitro, responded with rates similar to those assayed in mitochondria isolated from glucagon-treated rats. Although both phosphate and potassium were necessary to elicit these stimulations, there was a poor correlation between the maintenance of elevated mitochondrial ion levels and enhanced activities. The stimulation of mitochondrial functions induced by this treatment is a stable alteration insofar that it is present following repeated washes with 0.3 M sucrose and is retained in submitochondrial particles prepared from such mitochondria. The fact that the stimulation may be induced in isolated mitochondria suggests that the effect is a dynamic one on mitochondrial energization rather than a passive protection against mitochondrial aging, as has been recently proposed to explain stimulations effected by glucagon [30]. Mitochondrial respiration is required for the development of the activated state as the stimulation is blocked by increasing concentrations of rotenone in the incubation. On the other hand, although inclusion of oligomycin in the incubation inhibited ATP synthesis, as evidenced by the altered ATP/ADP ratio, the stimulation of pyruvate carboxylation was unaffected if ATP was provided in the assay. This is clear evidence that a change in the intrinsic ATP/ADP ratio of the mitochondria is not the driving force, nor is it even necessary, for either the stimulation of pyruvate carboxylation or the uncoupler-dependent ATPase. The ratios measured were assayed in mitochondria following the incubation and prior to assay for mitochondrial functions, in which ATP (and the uncoupler FCCP) was present in

excess. Although the ATP/ADP ratio during the assay is not known, it is reasonable to assume that the generation of the observed stimulation of function occurred during the incubation. The rise in the ATP/ADP ratio seen after an incubation in buffer 1 without oligomycin is probably the result of an increased mitochondrial 'energization'.

The mechanism by which either glucagon or the incubation in buffer 1 activates a variety of functions is not known. Armston et al. [38] have reported that assay in hypotonic medium mimics many of the glucagon effects on mitochondria. We have previously reported this same phenomenon [1,21] except we do not find citrulline formation to be stimulated by hypotonicity. The effect of hypotonicity is not similar to the stimulation of functions found after incubation in buffer 1 [21] in that it does not persist when mitochondria are returned to isosmotic medium.

We have shown [21] that matrix water content does not change after the incubation in buffer 1, nor does light scattering measured at 520 nm. Similarly, we have been unable to measure a change in matrix volume or light scattering in mitochondria isolated from glucagon-treated rats (Hamman and Haynes, unpublished data). This is in contrast to a report by Halestrap et al. [39] of an increase in mitochondrial volume following glucagon treatment. Hatefi et al. [40] recently presented evidence suggesting that membrane energization leads to a change in enzyme conformation, resulting in an increased enzyme-substrate affinity, as reflected by changes in V_{max}/K_m. The reactions studied were (i) nucleoside triphosphate-$^{33}P_i$ exchange, (ii) ATP synthesis, (iii) nicotinamide nucleotide transhydrogenation and (iv) ATP-driven electron transfer from succinate to NAD. The latter three have been shown to be stimulated by both glucagon [13,17] and incubation in buffer 1 (this paper). The possibility exists that the stimulations that we describe are effected similarly. Perhaps an enzyme conformational change is further modified by a high potassium phosphate environment, enhancing enzyme-substrate affinity and thereby stimulating the reaction rate. In support of this, Lacey et al. [12] have shown that glucagon treatment of rats results in an increased V_{max}/K_m for the phosphate dependence of glutaminase. Such a possibility is currently under investigation.

Mitochondrial citrulline synthesis is unaffected by incubation in buffer 1. If, however, glutamate is included in the incubation medium, stimulation of citrulline synthesis occurs at rates comparable to those seen after glucagon treatment of the intact animal. Similarly, Meijer and Van Woerkom [34] have reported that the rate of citrulline production in isolated mitochondria is significantly elevated in the presence of glutamate. Their buffer system was also supplemented with potassium and phosphate. Preliminary data from this laboratory (data not shown) indicate that an incubation in 0.3 M mannitol plus 60 mM glutamate will result in an increased intramitochondrial *N*-acetylglutamate content and also a stimulation of the rate of citrulline formation, indicating that extramitochondrial potassium and phosphate play no role in this stimulation. Because citrulline formation appears to be unaffected by a incubation in buffer 1, we propose that the hormonal control mechanism for this function is different from that of the other functions that are stimulated by an incubation in buffer 1. A similar conclusion, based on differential effects of glucagon, has been forwarded by Verhoeven et al. [41]. In agreement with Verhoeven et al., our present data indicate that after glucagon treatment of intact rats, in contrast to isolated hepatocytes [11], a small increase in intramitochondrial *N*-acetylglutamate accompanies an increased rate of citrulline synthesis. However, since there is a poor correlation between mitochondrial content of *N*-acetylglutamate and rates of citrulline formation, these data do not support the widely held hypothesis that glucagon stimulates citrulline formation via an increase in intramitochondrial *N*-acetylglutamate levels [36,37,41]. These data suggest that glucagon is not acting directly on the mitochondria to stimulate citrulline synthesis, but rather is altering the concentration of extramitochondrial substrates or cofactors, such as glutamate and/or ornithine. The possibility that ornithine content or transport may be rate controlling is especially interesting in light of recent data of Cohen et al. [42] which suggest that ornithine transcarbamylase can be rate limiting for citrulline synthesis.

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