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Comparison of Effects of Glucagon and Valinomycin on Rat Liver Mitochondria and Cells

ROBERT C. HAYNES, JR., JAMES C. GARRISON, AND RUSSELL K. YAMAZAKI

Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22903

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SUMMARY

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It has been shown previously that treatment of rats with glucagon, epinephrine, or cortisol alters the mitochondria of the liver so that the isolated organelles utilize pyruvate at an accelerated rate as measured by disappearance, decarboxylation, or carboxylation of pyruvate. It is reported here that valinomycin added at approximately 10 nM to isolated mitochondria partially mimicked these hormone treatments by stimulating carboxylation and decarboxylation of pyruvate. Valinomycin added in concentrations several times greater than those required for stimulation inhibited carboxylation, apparently by depleting mitochondrial ATP, but these same high levels continued to stimulate decarboxylation. The stimulations by this antibiotic were not duplicated by dinitrophenol, suggesting that they depend on the ability of valinomycin to act as an ionophore rather than as an uncoupler. Although similar, the mitochondrial stimulations resulting from treatment with glucagon and addition of valinomycin were additive, suggesting nonidentity of their mechanisms. In another action that resembled that of glucagon, valinomycin (10 nM) stimulated glucose synthesis from lactate in isolated liver cells. This effect of valinomycin was shown not to be mediated through the agency of adenosine cyclic 3′,5′-monophosphate.

INTRODUCTION

In the course of studies on the control of gluconeogenesis, we found that the addition of valinomycin in appropriate concentrations to isolated rat liver mitochondria accelerated the decarboxylation of pyruvate and the fixation of $^{14}CO_2$ by the mitochondria. The similarity of these effects to those seen in mitochondria prepared from rats treated with glucagon, epinephrine, or cortisol (1) prompted us to examine some of the characteristics of these actions of valinomycin and contrast them with those produced by glucagon. These experiments form the basis of this report.

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MATERIALS AND METHODS

Male rats of the Wistar strain, weighing 100–300 g, were used. Liver cells were isolated by the technique of Berry and Friend, modified as previously described (2). Rats treated with glucagon were anesthetized with pentobarbital and given 20 μg of glucagon

via the tail vein in 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.4, 6 min before livers were removed and chilled in 10% sucrose; control rats received buffer only. Mitochondria were prepared by homogenizing livers (10%, w/v) in a buffered sucrose solution of the following composition: 0.3 M sucrose, 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 650 × *g* for 10 min, and the precipitated material was discarded. The supernatant fluid was then centrifuged at 9000 × *g* for 10 min, and the mitochondrial pellet was resuspended in the buffered sucrose solution. The mitochondria were used without further purification, as their ability to fix CO_2 declines with washing. All operations were performed at 4°. When the effects of hormone treatment were studied, mitochondria were pooled from two rats serving as controls and compared with a similar preparation from two rats treated with glucagon.

Studies of mitochondrial swelling were carried out in two ways. Aliquots were removed at intervals from the mitochondrial incubation mixture, and the absorbance at 520 nm was determined immediately, using a Gilford 300-N spectrophotometer, or mitochondrial incubations were made in cuvettes maintained at 37° in a Gilford 2400 spectrophotometer, and readings of absorbance (520 nm) were made at intervals.

Stock solutions of antibiotics, 1 mg/ml in 100% ethanol, were diluted each day in 1% (v/v) ethanol (isolated cells) or 50% (v/v) ethanol (mitochondrial incubations) before addition to the incubation media. Incubation flasks without valinomycin received appropriate volumes of dilute ethanol.

The carboxylation of pyruvate in mitochondria was determined by measuring the rate at which $^{14}CO_2$ was fixed into acid-stable compounds in the presence of pyruvate. Previously we found that the radioactive products formed in similar incubations were malate, fumarate, and citrate (3). The rate of the pyruvate dehydrogenase reaction in mitochondria was estimated by following the decarboxylation of [1-^{14}C]pyruvate. In earlier experiments the quantitative relationships between utilization of pyruvate by the mitochondria, fixation of CO_2, and decarboxylation of pyruvate were described (1).

All mitochondrial incubations were made at 37° in a medium containing 203 mM sucrose, 5 mM sodium pyruvate, 12 mM $KHCO_3$, 12.5 mM $MgCl_2$, 2.5 mM K_2SO_4, 8 mM potassium phosphate, 3.4 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 0.7 mM EDTA at pH 7.4; the osmolality was 310 mOsM, and the concentration of K^+ was 33 mM. Mitochondrial protein ranged from 0.6 to 0.9 mg/ml.

In assays of CO_2 fixation, the reaction was terminated by the addition of trichloroacetic acid, the precipitated protein was removed by centrifugation, and the acid-stable ^{14}C was determined in an aliquot of the supernatant fluid by scintillation counting after residual $^{14}CO_2$ had been removed by bubbling with CO_2 for 2 min.

Decarboxylation was measured by trapping $^{14}CO_2$ in phenethylamine after the incubation reaction had been stopped by the addition of 1 M H_2SO_4 containing 4 mM 2,4-dinitrophenylhydrazine. Unless otherwise noted, pyruvate carboxylation assays were run for 5 min and pyruvate decarboxylation assays were run for 10 min. Oxygen utilization was measured with a Clark electrode (Yellow Springs No. 5331), and K^+ disappearance, with Beckman K^+-specific electrode 39047.

Protein was determined by the method of Lowry *et al.* (4). ATP, malate, and citrate were assayed fluorometrically (5–7). Glucose was determined by the alkaline ferricyanide method on a Technicon AutoAnalyzer. This technique was validated by occasional checks using a glucose oxidase method (Glucostat, Worthington), and values obtained with the two methods agreed to within 5%.

Cyclic adenylate (adenosine cyclic 3′,5′-monophosphate) was assayed by the protein binding method of Gilman (8).

Materials. Valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone, and DL-palmitoylcarnitine were purchased from Calbiochem; gramicidin D, gramicidin J (S), and glucagon, from Sigma; collagenase, from Worthington; lactate, from Schwarz/Mann; and 2,4-dinitrophenylhydrazine, from Eastman. Sodium [^{14}C]bicarbonate was purchased

from Amersham/Searle, and [1-^{14}C]pyruvic acid, from New England Nuclear. Nigericin was generously donated by Dr. Henry Lardy, and A-204 and monazomycin, by Dr. David Wong.

RESULTS

Effect of valinomycin on carboxylation and decarboxylation of pyruvate. The upper portion of Fig. 1 shows that the addition of 2–10 nM valinomycin stimulated fixation of CO_2 both in mitochondria from rats serving as controls and in mitochondria from glucagon-treated animals. The same preparations of mitochondria were used to follow the activity of the pyruvate dehydrogenase complex, and Fig. 1 (bottom) shows that the addition of valinomycin in increasing concentrations stimulated the decarboxylation of pyruvate, again both in control mitochondria and in mitochondria from glucagon-treated animals. Although carboxylation of pyruvate was inhibited by high levels of valinomycin, decarboxylation of pyruvate was accelerated as the concentration of valinomycin was increased. In other experiments this stimulation was observed to persist at the highest level of valinomycin tested, 72 nM.

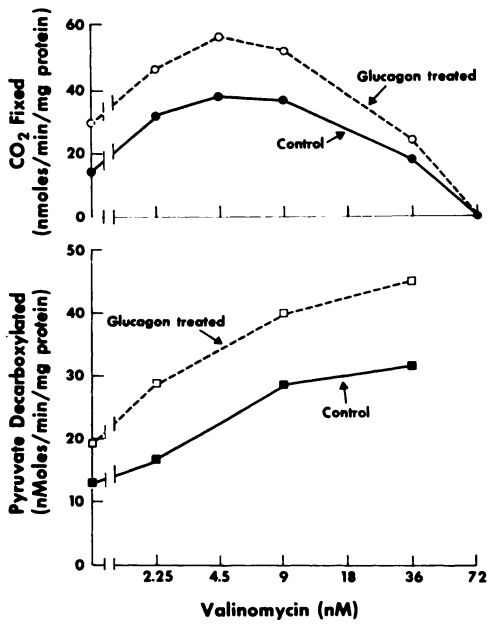

FIG. 1. *Effect of valinomycin on carboxylation and decarboxylation of pyruvate*

Each point represents the mean of duplicate incubations.

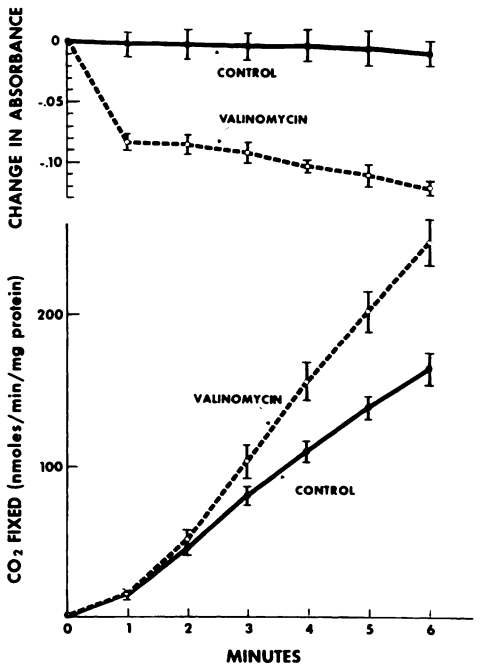

FIG. 2. *Time course of valinomycin effect on fixation of CO_2 and mitochondrial swelling*

Mitochondria were incubated with valinomycin (4 nM) or an ethanol control solution added immediately after the initial reading. Aliquots were removed at 1-min intervals and absorbance at 520 nm was measured. The mean initial absorbance was 2.243. Values are the means and standard errors of four replications.

The effects of valinomycin on the time course of CO_2 fixation and on the absorbance of the suspension at 520 nm (an estimate of mitochondrial volume) are shown in Fig. 2. It can be seen that swelling, as noted by the decline in absorbance, occurred promptly following the addition of valinomycin and persisted throughout the period of incubation.

Adam and Haynes (1) found that most of the additional CO_2 fixed by mitochondria isolated from hormone-treated rats was accounted for by the malate and citrate that accumulated during the incubation. Analogously, the data in Table 1 show that the increased carboxylation of pyruvate caused by the addition of valinomycin also resulted in a greater formation of malate and citrate.

TABLE 1

Effect of valinomycin on fixation of CO_2 and accumulation of malate and citrate

Incubations were conducted as described in MATERIALS AND METHODS. The reactions were terminated by addition of perchloric acid, the deproteinized extracts were neutralized with $KHCO_3$, and aliquots of the supernatant fluid were taken for fluorometric analyses. Each value is the mean of duplicate incubations.

Time	Valinomycin (4.5 nM)	Malate	Citrate	Malate + citrate	CO_2 fixed
min		*nmoles/mg protein*	*nmoles/mg protein*	*nmoles/mg protein*	*nmoles/mg protein*
5	0	66	23	89	140
5	+	167	69	236	303
10	0	128	71	199	260
10	+	243	226	469	570

As anticipated, valinomycin required the presence of potassium ion in the incubation medium for stimulation of the carboxylation of pyruvate, and increased oxygen consumption and uptake of potassium ion occurred when valinomycin acted on mitochondria incubated in the medium used in these studies (data not shown).

Other ionophores. Other ionophores were tested for their ability to stimulate fixation of CO_2 and decarboxylation of pyruvate. We found gramicidin J (S) inactive at all dose levels studied. Gramicidin D and monazomycin showed significant but small (20–25%) stimulation.

The monocarboxylic antibiotics nigericin and A-204 increase the permeability of membranes to monovalent cations and generally inhibit the effects of ionophores such as valinomycin. They can cause contraction of mitochondria and inhibit oxidation of pyruvate (9, 10). Experiments were performed to determine whether the valinomycin-induced acceleration of pyruvate metabolism would be antagonized by these agents (Fig. 3). Both compounds blocked basal rates of fixation of CO_2 and decarboxylation of pyruvate in preparations of mitochondria from glucagon-treated as well as from untreated rats. Contraction of the mitochondria, as indicated by increased absorbance at 520 nm, took place at concentrations of these

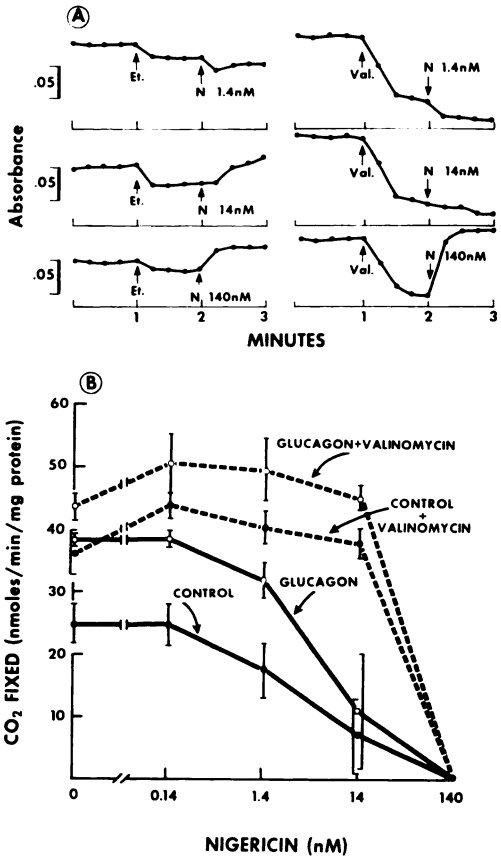

FIG. 3. *Effects of nigericin on light scattering and CO_2 fixation by mitochondria incubated with and without valinomycin*

A. The absorbance measurements (520 nm) of mitochondrial suspensions are plotted to show the effects of additions of nigericin and valinomycin. At Et. 10 μl of 50% ethanol were added; at N nigericin was added to give concentrations as noted. At Val. valinomycin was added to give a final concentration of 9 nM. Antibiotic-induced changes in absorbance with mitochondria from glucagon-treated rats were not significantly different from those of control mitochondria shown here.

B. The fixation of CO_2 by control and glucagon-stimulated mitochondria is shown as a function of the concentration of nigericin. The dashed lines indicate incubations in the presence of 9 nM valinomycin. Values are the means and standard errors of four replications.

antibiotics that inhibited basal rates of metabolism. At concentrations 10-fold greater than those inhibiting basal rates, the monocarboxylic antibiotics reversed the ac-

TABLE 2

ATP content of mitochondria related to dose of valinomycin and fixation of CO_2

Incubations were conducted for 5 min as described under MATERIALS AND METHODS for the determination of CO_2 fixation. Parallel incubations were carried out and terminated by the addition of perchloric acid, and ATP was determined in the deproteinized, neutralized extracts. ATP levels before inculations were 7 ± 1.5 nmoles/mg of protein. Values are the means and standard errors of four determinations.

Valinomycin	CO_2 fixed	ATP
nM	*nmoles/min/mg protein*	*nmoles/mg protein*
0	35 ± 6	9 ± 1
0.9	51 ± 6	10 ± 1
9	54 ± 5	9 ± 1
90	4 ± 4	2 ± 0.2
900	0.1 ± 0.02	2 ± 0.4

TABLE 3

Effect of uncouplers on fixation of CO_2 and decarboxylation of pyruvate

Incubations were conducted as described in MATERIALS AND METHODS, with additions as indicated. Values are the means of duplicate determinations.

Conditions	CO_2 fixed	Decarboxylation
	nmoles/min/mg protein	*nmoles/min/mg protein*
Control mitochondria	20.1	11.5
+100 μM DNP[a]	0.1	11.3
+1 μM Cl-CCP	0.2	9.1
+72 nM valinomycin	0.5	24.2
Mitochondria from glucagon-treated rats	36.9	19.8
+100 μM DNP	0.3	18.3
+1 μM Cl-CCP	0.3	15.1
+72 nM valinomycin	1.2	45.0

[a] DNP, 2,4-dinitrophenol; Cl-CCP, carbonyl cyanide *m*-chlorophenylhydrazone.

celerated metabolism of pyruvate and the swelling induced by valinomycin, as others have reported (10). In experiments not shown, essentially identical results were obtained using A-204.

Uncouplers as potential stimulators. Since ATP is a substrate in the pyruvate carboxylase reaction and valinomycin can uncouple oxidative phosphorylation (11, 12), the inhibition of fixation of CO_2 at high levels of valinomycin is probably due to depletion of ATP within the mitochondria. Table 2 shows values of ATP found at the end of 5-min incubations of mitochondria with various concentrations of valinomycin. It is evident that higher doses of valinomycin did produce a decline in the level of ATP.

Because ionophores lead to uncoupling of oxidative phosphorylation as they accelerate energy-requiring ion transport, we tested two classical uncouplers, 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone, to see whether they would mimic the effects of valinomycin on fixation of CO_2 and decarboxylation of pyruvate. At no concentration of these compounds was stimulation of fixation of CO_2 observed in either control or glucagon-stimulated mitochondria. Table 3 shows that uncoupling concentrations of either compound completely inhibited the fixation of CO_2, presumably because ATP was depleted in the mitochondria. However, the rate of the decarboxylation reaction was only slightly affected, in contrast to the stimulation found with uncoupling doses of valinomycin.

Stimulation of fixation of CO_2 with pyruvate dehydrogenase inhibited. In order to investigate the role of the pyruvate dehydrogenase reactions, mitochondria were incubated in the presence of sodium arsenite to block these reactions, together with DL-palmitoylcarnitine as a source of acetyl-CoA. As shown in Table 4, these additions blocked decarboxylation of pyruvate. Valinomycin effectively stimulated fixation of CO_2 in this system, demonstrating that this effect of valinomycin was not secondary to its effect on oxidation of pyruvate via the pyruvate dehydrogenase reaction.

Valinomycin as stimulator of gluconeogenesis. If the rate of pyruvate carboxylation is limiting for the entire sequence of gluconeogenesis, its stimulation should result in an acceleration of glucose synthesis. The finding that valinomycin enhanced the rate of CO_2 fixation in mitochondria suggested that it might increase the rate of glucose synthesis in a more intact system. To test this possibility, we incubated liver cells isolated from fasted rats in Krebs-Ringer-bicarbonate solution containing 20 mM L-lactate. Under these conditions the cells synthesize glucose at rates comparable to perfused rat liver and

respond to L-epinephrine, glucagon, cyclic adenylic acid, or dibutyryl cyclic adenylic acid with increased rates of glucose synthesis (2). Figure 4 presents the effects of various concentrations of valinomycin on the rate of gluconeogenesis in liver cells isolated from fasted rats. Notice that 30 nM valinomycin stimulated synthesis of glucose about 35% ($p < 0.002$) and that increasing the valinomycin concentration to 100–300 nM inhibited glucose production. Furthermore, concentrations of valinomycin that stimulated and inhibited gluconeogenesis were similar to those that stimulated and inhibited fixation of CO_2 in isolated mitochondria. In order to determine whether the inhibitory effects of high levels (100–300 nM) of valinomycin were due to the uncoupling of oxidative phosphorylation, the respiratory rates of cell suspensions were measured in the presence of various concentrations of valinomycin and 2,4-dinitrophenol. Cellular respiration was stimulated about 45% by 300 nM valinomycin and about 47% by 50 μM 2,4-dinitrophenol. In contrast, concentrations of valinomycin which stimulated production of glucose, 10–30 nM, had no measurable effect on respiration.

Since cyclic adenylate is thought to mediate some hormonal stimulation of gluconeogenesis in liver (13), the effect of valinomycin on the level of this nucleotide in liver cells was measured. Valinomycin had no detectable effect on the concentration of cyclic adenylate in the cells after 7 or 30 min of incubation, whereas glucagon increased the level of the nucleotide 15-fold (from 1.6 to 25 pmoles/mg of protein). In addition, in experiments not presented, valinomycin at 10–30 nM had no effect on glycogenolysis, a process that has been shown to be quite sensitive to intracellular levels of the cyclic nucleotide (14).

TABLE 4

Effect of valinomycin and inhibition of pyruvate dehydrogenase on fixation of CO_2

Incubations were conducted as described in MATERIALS AND METHODS. Mitochondrial protein concentration ranged from 0.68 to 0.72 mg/ml. Values are the means and standard errors of three determinations.

$NaAsO_2$ (0.2 mM) + palmitoyl-carnitine (30 μM)	Valino-mycin (0.9 nM)	CO_2 fixed	Pyruvate decarboxylated
		nmoles/min/mg protein	*nmoles/min/mg protein*
0	0	28.7±1.5	7.3 ±0.1
0	+	48.7±3.5 $p<0.025$	
+	0	30.7±3.6	0.03±0.1
+	+	50.7±1.1 $p<0.025$	0.03±0.1

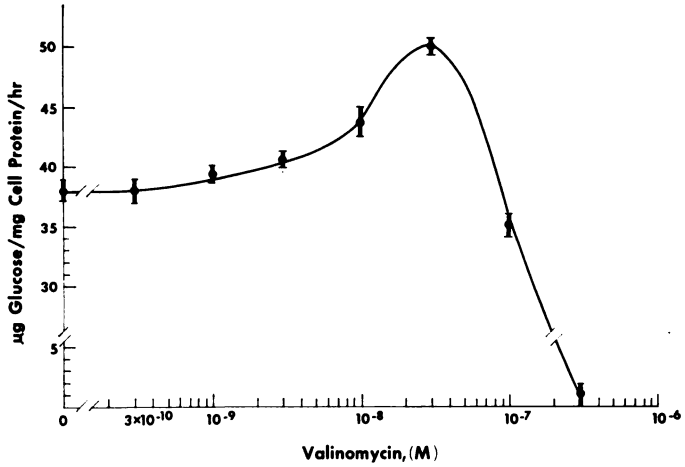

FIG. 4. *Effect of various concentrations of valinomycin on glucose synthesis from L-lactate in isolated liver cells*

Cells were isolated from rats fasted for 24 hr, and incubated with 20 mM L-lactate together with the indicated valinomycin concentrations for 1 hr. Each point is the mean of four or five determinations.

DISCUSSION

The data presented in this paper show that valinomycin (at certain dose levels) and treatment with glucagon produced similar responses in preparations of rat liver. That is, both increased the rates of carboxylation and decarboxylation of pyruvate in isolated mitochondria, and both stimulated the synthesis of glucose in isolated liver cells. The maximal effects of the hormone and ionophore on mitochondria were additive, suggesting that they do not act in an identical fashion. In stimulating glucose synthesis in isolated cells, the agents were partially additive (data not shown). Nevertheless, the similarities of these responses do suggest the importance of considering what is known of valinomycin actions in an attempt to understand the effects of the ionophore in this system and also to generate conceptual models that might help to elucidate the action of glucagon.

Valinomycin has the following known actions on mitochondria: (a) it accelerates the utilization of energy by mitochondria, as evidenced by its stimulation of hydrolysis of ATP and increased oxygen consumption; (b) it stimulates the uptake of K^+ by mitochondria and, after a steady state is reached where no net transfer of K^+ occurs, continues to produce an increased rate of flux of the ion (15); (c) it facilitates the passage of anions, including phosphate and substrates, into the mitochondria; and (d) it causes swelling of mitochondria, which has been shown to be an expansion of the matrix (16).

Which of these actions of valinomycin, if any, is responsible for the increased rates of fixation of CO_2, decarboxylation of pyruvate, and synthesis of glucose? Could glucagon conceivably act in an analogous manner? Stimulation of oxidative processes by valinomycin could, in theory, accelerate the fixation of CO_2 in two ways. Valinomycin in appropriate concentrations can enhance oxidative phosphorylation (12). This could lead to a higher level of ATP within the mitochondria, in turn stimulating the pyruvate carboxylase reaction as more substrate (ATP) became available. This mechanism can be rejected by the finding (Table 2) that valinomycin in stimulatory doses did not increase the level of ATP. A second potential mechanism is that increased oxidation of pyruvate elevates the levels of acetyl-CoA in the mitochondria, resulting in the activation of pyruvate carboxylase. This mechanism can be ruled out, since blockade of the pyruvate dehydrogenase group of reactions with arsenite, together with the addition of palmitoylcarnitine to generate acetyl-CoA, permitted valinomycin to stimulate CO_2 fixation (Table 4). The possibility remains that valinomycin stimulated oxidation of palmitoylcarnitine, producing additional acetyl-CoA, which in turn activated pyruvate carboxylase. The determination of mitochondrial levels of acetyl-CoA will be required for a definitive test of this hypothesis.

The second action of valinomycin listed, the increased transport of K^+ into the mitochondria, could lead to activation of pyruvate carboxylase, which requires monovalent cations (17). Opposing this explanation is the observation that, as valinomycin facilitates the passage of K^+ into mitochondria, there is an accompanying influx of water so that the concentration of K^+ within the mitochondria does not increase appreciably (18). In regard to the concept of control related to ion fluxes, Friedmann (19) demonstrated that cyclic adenylate produces rapid but brief fluxes of Ca^{2+}, Na^+, and K^+ in the perfused rat liver. It is not clear at present whether these ionic redistributions are related to the mitochondrial changes produced by glucagon and other hormones that stimulate synthesis of glucose.

The valinomycin-induced flux of K^+ facilitates the entry of phosphate and organic anions into the mitochondria, as demonstrated by the observation (20) that valinomycin stimulated oxygen consumption in mitochondria uncoupled by classic uncouplers; it increased state 3 respiration when substrate concentrations were 3 mM but not when they were 10 mM, and it stimulated the uptake of phosphate and oxidative phosphorylation. It is therefore possible that valinomycin enhances the rate of pyruvate entry into the mitochondria, permitting both the pyruvate carboxylase and pyruvate dehydrogenase reactions to proceed more rapidly by providing an increased availability of substrate within the organelles.

Finally, the expansion of the mitochondrial matrix that occurs as a response to valinomycin may allow pyruvate greater access to the two enzymes which metabolize it. This correlates well with the observation that substrate oxidations (except those of succinate and β-hydroxybutyrate) and carboxylation and decarboxylation of pyruvate are accelerated in hypotonic media, which, like valinomycin, produce an expansion of the mitochondrial matrix (1, 21, 22). The reverse situation—i.e., inhibition of metabolism—has been shown to accompany mitochondrial contraction induced by nigericin (11).

In summary: the stimulation of oxidation and increased concentrations of K^+ within the mitochondria are judged to be unlikely as the mechanisms through which valinomycin stimulates fixation of CO_2. However, the evidence available does not entirely exclude these two possibilities. The two alternatives that appear more probable are the facilitation of anion entry secondary to K^+ flux and an increased access of pyruvate to its enzymes of metabolism as a result of spatial changes secondary to expansion of the matrix. These two mechanisms, in the absence of contradictory evidence, are both situations in which control is exerted by availability of substrate. They therefore resemble the hypothesis for the effect of glucagon on pyruvate metabolism (1), which proposes that control of fixation of CO_2 occurs via the rate of transfer of pyruvate into the mitochondria.

The degree of relevance of valinomycin as a model for the action of glucagon is, of course, uncertain. These experiments reported in this paper are considered by us to be heuristic in regard to the study of the hormonal control of gluconeogenesis, suggesting as they do the possibility that mitochondrial ion fluxes or configurational changes in the matrix of the mitochondria are potential mechanisms through which glucagon and other hormones stimulating gluconeogenesis might act.

ACKNOWLEDGMENT

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