MOLECULAR PHARMACOLOGY, 7, 455–464

Valinomycin as an Antilipolytic Agent

Effects on Cyclic 3′,5′-Adenylic Acid, Adenyl Cyclase, Respiration, and Glucose Metabolism in Brown and White Fat Cells

JOHN N. FAIN AND SUSAN C. LOKEN[1]

Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912

(Received March 6, 1971)

SUMMARY

Valinomycin blocked the stimulation by catecholamines of lipolysis and the accumulation of both total and labeled cyclic AMP in brown and white fat cells. Under conditions when valinomycin blocked lipolysis produced by epinephrine, the ATP content of brown fat cells was not reduced. The lipolytic action of dibutyryl cyclic AMP was also blocked by valinomycin. The degradation of tritiated adenine to labeled water was stimulated by valinomycin. In white fat cell ghosts, catecholamine-stimulated adenyl cyclase activity was reduced 20% by valinomycin, and basal activity was enhanced. In white fat valinomycin inhibited both basal and insulin-stimulated conversion of glucose to carbon dioxide, lactate, and total lipids. In brown fat, however, basal glucose conversion to carbon dioxide was stimulated by valinomycin. Basal respiration in brown fat cells was doubled by valinomycin in both the presence and absence of either pyruvate or oxalacetate. The stimulation of respiration by octanoate was reduced 50% by valinomycin. These observations suggest that the antilipolytic action of valinomycin is the result of other factors in addition to the inhibition of adenyl cyclase.

INTRODUCTION

Valinomycin is a macrocyclic dodecadepsipeptide antibiotic that increases the permeability of natural (1, 2) and artificial (3) membranes to potassium but not to sodium ions. The ion-transporting antibiotic also increases active potassium uptake by rat liver mitochondria (4). Fain (5) reported that valinomycin inhibited catecholamine-stimulated lipolysis in white fat cells if potassium was present in the medium. Valinomycin stimulated the respiration of brown fat cells in the presence but not in the absence of potassium (6, 7).

Kuo and Dill (8) found that valinomycin inhibited the lipolytic action in white fat cells of norepinephrine, corticotropin, caffeine, and theophylline. They found that although 1 μM valinomycin did not alter the conversion of labeled adenine-8-^{14}C to cyclic AMP under basal conditions, it reduced by half the entry of label into cyclic AMP due to norepinephrine (0.1 μg/ml). Kuo and Dill suggested that the antilipolytic action of valinomycin resulted at least in part from inhibition of adenyl cyclase (8) These authors did not rule out other possible effects of valinomycin; however, no effect

This investigation was supported in part by United States Public Health Service Grant AM-10149 from the National Institute of Arthritis and Metabolic Diseases.

[1] Predoctoral Fellow of the National Institutes of Health (GM-36085).

on phosphodiesterase activity of white fat homogenates was observed with valinomycin.

In the present studies the effects of valinomycin on lipolysis, respiration, glucose metabolism, and conversion of adenine-8-^{3}H to cyclic AMP and to other products were investigated in isolated brown and white fat cells. White fat cell "ghosts" were used to assay adenyl cyclase activity. This report presents further evidence that valinomycin has several actions on isolated fat cells, and suggests that its antilipolytic action cannot be explained solely by direct inhibition of the adenyl cyclase system.

METHODS

Free brown and white fat cells were obtained from 130–160-g female Sprague-Dawley rats (Charles River CD strain) fed laboratory chow ad libitum. Brown fat cells were isolated by digestion of minced dorsal interscapular adipose tissue for 1 hr with 1 mg/ml of crude bacterial collagenase (*Clostridium histolyticum*, Worthington Biochemical Corporation) in a phosphate buffer medium containing 4% albumin (Armour, lot 33,407, or Pentex, lot 81) (9). Trypsin (twice crystallized, Worthington) was present at a concentration of 1 mg/ml during the isolation procedure in some of the experiments. White fat cells were isolated by the procedure of Rodbell (10) after digestion of parametrial fat pads with 0.5 mg/ml of collagenase for 40 min. The phosphate buffer contained NaCl, 128 mM; $CaCl_2$, 1.4 mM; $MgSO_4$, 1.4 mM; KCl, 5.2 mM; and Na_2HPO_4, 10 mM. It was made up daily and adjusted to pH 7.4 with NaOH after addition of regular or defatted bovine fraction V albumin powder. Defatted albumin was prepared by the procedure of Guillory and Racker (11).

For studies involving labeled adenine, the free cells were washed once and then incubated for 15 min with a 4% albumin buffer containing adenine-8-^{3}H (3.1 μCi/ml, 22.6 Ci/mmole). The labeled cells were washed once with a 1.5% albumin buffer and added to the incubation tubes containing 1.5 ml of 1.5% albumin buffer. Valinomycin was added in 10 μl of a 95% ethanol solution, and the same amount of alcohol was added to the controls.

At the end of the incubation 1 ml of medium was rapidly removed, and 8% (v/v) perchloric acid in 40% (v/v) ethanol was added to the 1 ml of medium or to the remaining cells and medium for the studies

TABLE 1

Effect of valinomycin on lipolytic action of epinephrine and on ATP and cyclic AMP in brown fat cells

Brown fat cells were isolated by digestion of brown adipose tissue for 45 min with collagenase and trypsin, 0.5 mg/ml each. After one wash the cells were labeled by incubation for 15 min in 2 ml of buffer containing adenine-8-^{3}H. Brown fat cells (10 μmoles of triglyceride per tube) were incubated for 5 or 20 min in 1.5 ml of buffer containing 4% albumin, 0.8 mM theophylline, and 2.8 mM glucose. The values are the means of five paired experiments, and the changes due to valinomycin (1 μg/ml) in the presence of epinephrine are the means ± standard errors of the paired differences. The valinomycin was added in 5 μl of ethanol and an equal volume of ethanol was added to control tubes.

Measurement	Time	Glycerol release	ATP	Labeled cyclic AMP	
				In cells	In medium
	min	*μmoles/mmole triglyceride*	*nmoles/0.1 mmole triglyceride*	*cpm/μmole triglyceride*	
Basal	5	−5.7	31	6	2
	20	−2.0	54	32	1
With epinephrine (1.3 μM)	5	5.5	45	129	20
	20	14.5	32	128	30
Change due to valinomycin (1 μg/ml) in presence of epinephrine	5	−3.2 ± 1.2	+7 ± 14	−55 ± 16	−2 ± 5
	20	−12.8 ± 3.1	+9 ± 12	−64 ± 17	−15 ± 11

shown in Table 1. ATP was determined on aliquots of the neutralized perchloric acid filtrates by the fluorometric procedures of Williamson and Corkey (12). Labeled cyclic AMP was determined by adding 0.2 ml of 5% zinc sulfate and 0.1 ml of saturated barium hydroxide to aliquots of the neutralized perchloric acid filtrates. The pH was adjusted to between 7 and 8, the mixture was centrifuged, and the entire procedure was repeated. In the other experiments the same procedure was carried out on cells or medium which had been boiled for 1 min. In some experiments aliquots of the supernatant fluid from the barium-zinc procedure were counted as described by Kuo and Dill (8), although ordinarily this fraction was chromatographed on 4 × 70 mm columns of Dowex 50 (AG50W-X8, 100–200 mesh), the cyclic AMP fraction was collected, lyophilized, and then resuspended in 0.1 ml of 50 mM Tris-HCl, pH 7.0, and a 25-μl aliquot was counted in Bray's solution (13).

Two 25-μl aliquots of the cyclic AMP fraction were analyzed for total cyclic AMP by the procedure of Goldberg *et al.* (14), using some of the modifications introduced by Kaneko and Field (15). One aliquot was incubated with active brain phosphodiesterase, and the other with boiled brain phosphodiesterase. Both samples contained 10 mM sodium phosphate (pH 7.0) and 1 mM adenosine to inhibit phosphatase activity. After conversion of cyclic AMP to 5′-AMP by phosphodiesterase, the 5′-AMP was converted to ATP, which provided energy for the phosphorylation of glucose to glucose 6-phosphate, by the procedure of Kaneko and Field (15), using enzymes obtained from Boehringer-Mannheim. The recovery of cyclic AMP added to cells and medium was approximately 30%. The sensitivity of the assay with the procedure described permitted measurement of approximately 10–100 pmoles/sample.

Adenyl cyclase activity was determined using fat cell ghosts prepared by hypotonic lysis of white fat cells by the procedure of Birnbaumer *et al.* (16) The ghosts were used immediately after preparation and were incubated at 37° in a total volume of 100 μl containing 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 30 mM KCl, 1 mM cyclic AMP, 8 mM phosphoenolpyruvate, 10 μg of pyruvate kinase, 1 mM ATP, and 1 μCi of ^{3}H-ATP (0.94 Ci/mmole; New England Nuclear). At the end of the 20-min incubation period, tubes were boiled for 3.5 min after the addition of 50 μl of a 5 mM solution of cyclic AMP. The total volume was added to a Dowex 50 column, 3 × 65 mm (AG50W-X8, 100–200 mesh). The fraction containing cyclic AMP was collected, and 0.2 ml of $ZnSO_4$ (0.2 M) and 0.1 ml of saturated $Ba(OH)_2$ were added. The pH was adjusted to 7.0–8.0. Following centrifugation the procedure was repeated, and a 1-ml aliquot was counted in 10 ml of scintillation fluid. Protein concentrations for the adenyl cyclase assays were determined according to the method of Lowry *et al.* (17).

For studies of uniformly labeled glucose-^{14}C metabolism, the cells were incubated in 17 × 100 mm polyethylene tubes containing 1.5 ml of 4% albumin buffer. All tubes containing brown fat cells were gassed with 100% O_2 and capped. White fat cells were incubated with air as the gas phase. The values for each experiment are the averages of duplicate tubes and represent changes occurring during the incubation period relative to the initial controls, which were incubated for only 5 min. Carbon dioxide was collected on rolled filter papers suspended in plastic center wells (Kontes Glass Company) attached to rubber septum stoppers, which were used to seal the tubes. At the end of the incubation period 0.2 ml of a solubilizer solution ("NCS," Nuclear-Chicago) was added to the filter paper, and 0.25 ml of 1 N H_2SO_4 was added to the medium. The filter papers were removed after 30 min and counted in 10 ml of toluene containing 0.4% Omnifluor (New England Nuclear). Label in total lipid was determined as previously described (10).

Aliquots of the medium were removed for analysis of glycerol (18) and lactate (19) at the end of the incubation. Free fatty acids were determined for the cells and remainder of the medium by a modification (hexane substituted for heptane) of the procedure of Dole and Meinertz (20). Triglyceride content was based on total fatty acids after saponification and was a convenient index

of the amount of cells added to each tube; all parameters were expressed in terms of micromoles of triglyceride.

Oxygen consumption was measured in a Gilson respirometer at 37°. Warburg side-arm respirometer flasks were treated with silicone prior to each experiment. The respirometer flasks, containing 3 ml of medium, were gassed with 100% oxygen for 3 min and equilibrated for 30 min prior to the start of the period over which respiration was measured. Carbon dioxide was absorbed by 0.2 ml of 10% KOH on rolled strips of filter paper (5 × 80 mm) in the center wells. The basal rate of oxygen consumption was linear over the entire period of the respirometer studies.

Sources of chemicals were: valinomycin (lot 860031) and *l*-epinephrine bitartrate, Calbiochem; theophylline, Mallinckrodt; glucagon-free porcine insulin, Eli Lilly and Company; oxalacetic acid, pyruvic acid, phosphoenolpyruvate, and sodium octanoate, Sigma Chemical Company; glycerokinase, pyruvate kinase, α-glycerol phosphate, and muscle lactate dehydrogenase, Boehringer-Mannheim; dibutyryl cyclic AMP, adenine-8-^{3}H, and adenine-8-^{14}C, Schwarz BioResearch; and uniformly labeled glucose-^{14}C, New England Nuclear Corporation. Valinomycin was dissolved in ethanol and diluted to 5% ethanol with water. Stock solutions were kept frozen and prepared frequently because of the instability of certain valinomycin preparations.

TABLE 2

Valinomycin-induced stimulation of label accumulation in barium-zinc filtrate of medium

Brown fat cells were isolated by incubation for 1 hr in 4 ml of albumin buffer containing 4 mg of collagenase and adenine-8-^{3}H. After one wash the cells were further labeled with tritiated adenine by incubation for 15 min in 2 ml of buffer containing adenine-8-^{3}H. The cells (average, 2.8 μmoles of triglyceride per tube, 5.2 mM K^+; 4.2 μmoles of triglyceride per tube, 0 mM K^+) were washed once and then incubated for 60 min in 1.5 ml of buffer containing 4% albumin, 0.4 mM theophylline, and 1.3 μM epinephrine. Valinomycin (1.6 μg/ml) was added in 10 μl of 5% ethanol, and the same amount of alcohol was added to the control tubes. At the end of the incubation the cells were separated from the medium, and each was treated with $Ba(OH)_2$ and $ZnSO_4$. The values are the averages of two experiments, each done in duplicate.

	Control	+ Valinomycin	Change due to valinomycin
	cpm in barium-zinc filtrates		
Cells			
5.2 mM K^+	450	470	+20
0 mM K^+	260	350	+90
Medium			
5.2 mM K^+	440	1020	+580
0 mM K^+	430	540	+110

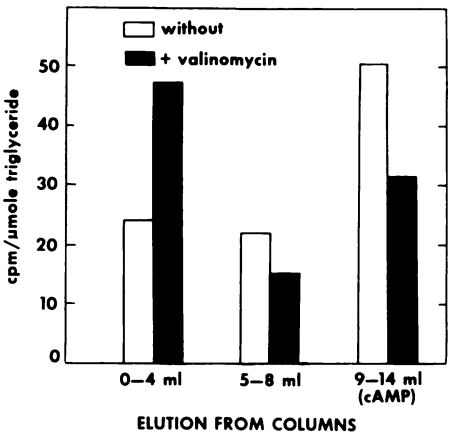

FIG. 1. *Effect of valinomycin on accumulation of tritium in barium-zinc filtrates of fat cells and medium after incubation with adenine-8-^{3}H*

Brown fat cells were isolated by incubation of tissue for 1 hr in 4 ml of 4% albumin buffer containing 4 mg of collagenase. The cells were washed, resuspended in 4 ml of 4% albumin buffer containing adenine-8-^{3}H, and incubated for 15 min. The cells were washed and incubated in 1.5 ml of 1.5% albumin buffer plus epinephrine (2.0 μM) and theophylline (0.4 mM) either with or without valinomycin (1.76 μg/ml) for 4 min. The cells and medium were boiled, and the filtrates obtained after precipitation with barium hydroxide and zinc sulfate were passed through Dowex 50-X8 columns. Each value is the mean of three paired replications. cAMP, cyclic AMP.

RESULTS

Fain (5) reported that valinomycin inhibited the lipolysis induced by epinephrine in white fat cells. The data in Table 1 indicate that valinomycin also inhibited the lipolytic action of epinephrine in brown fat

cells. The antilipolytic action of valinomycin was not associated with any detectable drop in ATP content (Table 1).

Epinephrine markedly elevated the accumulation of labeled cyclic AMP in brown cat cells after 5 min of incubation in the presence of theophylline (Table 1). The increase was sustained for 20 min. There was some leakage of cyclic AMP into the medium. Valinomycin depressed the stimulation of cyclic AMP accumulation by epinephrine (Table 1).

Kuo and Dill (8) reported that in cells previously labeled with adenine-8-^{14}C, valinomycin blocked the stimulation by catecholamines of labeled cyclic AMP accumulation in barium-zinc supernatant fractions of fat cell extracts. However, in brown fat cells labeled with tritiated adenine (Table 2), valinomycin stimulated the accumulation of label as measured by the assay of Kuo and Dill (8). Most of the increase in radioactivity due to valinomycin occurred in the medium (Table 2). In another series of experiments we chromatographed on Dowex 50 the supernatant fractions obtained from the barium-zinc procedure for purification of cyclic AMP. Valinomycin decreased the appearance of label in the cyclic AMP fraction and increased that in the first fraction eluted from the columns (Fig. 1).

In both white and brown fat cells the increased accumulation of label in barium-zinc supernatant fractions produced by valinomycin can be attributed to tritiated water. The data in Table 3 show that the entire increase in radioactivity due to valinomycin was lost if the first fraction eluted from the columns was lyophilized. The increase in appearance of tritium from position 8 of adenine was probably a result of activation of xanthine oxidase by valinomycin. The difference between our results and those of Kuo and Dill (8) can be attributed to the fact that they used ^{14}C-labeled adenine.

Since valinomycin facilitates the exchange of potassium across cell membranes, the effect of K^+ on the valinomycin-induced stimulation of the appearance of label in the barium-zinc supernatant fractions was investigated. The data in Table 2 indicate that the increased conversion of labeled adenine to water due to valinomycin was markedly reduced if potassium was omitted from the buffer used for incubation of the cells.

TABLE 3

Stimulation by valinomycin of conversion of tritium at position 8 of adenine to tritiated water

Brown or white adipose tissue was digested for 45 min with 1 mg/ml of collagenase. The cells were separated and resuspended in 1 ml of 4% albumin buffer containing tritiated adenine. After 15 min of incubation the cells were washed and then incubated in 2% albumin buffer either without or with valinomycin (1.7 μg/ml). After incubation for the specified periods, the cells and medium were boiled, treated with barium and zinc, and passed through Dowex 50-X8 columns; the first 4 ml were collected.

Experimental conditions	Radioactivity in 0–4-ml fraction	
	Before lyophilization	After lyophilization
	cpm	*cpm*
White fat cells, 60 min	3,380	200
White fat cells, 60 min, + valinomycin	11,000	<10
Brown fat cells, 5 min	240	<10
Brown fat cells, 30 min	800	100
Brown fat cells, 5 min, + valinomycin	360	<10
Brown fat cells, 30 min, + valinomycin	1,680	<10

The results in Table 4 show that valinomycin blocked the stimulation by norepinephrine and theophylline of lipolysis and the accumulation of both total and labeled cyclic AMP in white fat cells. There was a small stimulatory effect of valinomycin alone on the accumulation of both total and labeled cyclic AMP.

Kuo and Dill (8) suggested that valinomycin reduced the accumulation of cyclic AMP by inhibiting adenyl cyclase. We were able to obtain (Table 5) a significant degree of inhibition by valinomycin of the catecholamine-activated adenyl cyclase activity of ghosts prepared from white fat cells by the procedure of Birnbaumer *et al.* (16). Valinomycin alone increased basal adenyl cyclase activity (Table 5). These results are almost identical with the effects of valinomycin on cyclic AMP accumulation in intact white fat

TABLE 4

Inhibition by valinomycin of cyclic AMP accumulation and lipolysis in white fat cells

White fat cells were isolated by digestion of parametrial adipose tissue for 45 min with 1 mg/ml of collagenase. The cells were washed, incubated with adenine-8-^{3}H for 15 min, and then washed again to remove unincorporated adenine. White fat cells (50 μmoles of triglyceride per tube) were incubated for 5 or 30 min in 1.5 ml of buffer containing 4% albumin. Valinomycin to give a final concentration of 1.7 μg/ml was added in 10 μl of ethanol, and an equal volume of ethanol was added to control tubes. The concentration of theophylline was 1 mM, and that of norepinephrine was 1.3 μM.

Additions	Time	Cyclic AMP accumulation		Glycerol release
		Total	Labeled	
	min	*nmoles/mmole triglyceride*	*cpm/μmole triglyceride*	*μmoles/mmole triglyceride*
None	5	0.1	0.1	0
	30	0.1	0.6	0
Norepinephrine + theophylline	5	33.4	9.5	2.3
	30	12.5	4.5	21.4
Valinomycin	5	2.8	1.0	0
	30	1.9	1.0	0.6
Valinomycin + norepinephrine + theophylline	5	3.8	1.3	0
	30	1.6	0.5	1.0

TABLE 5

Effect of valinomycin on adenyl cyclase activity of white fat cell ghosts

Adenyl cyclase activity was determined using fat cell ghosts. The values for basal activity are the means of six experiments, while the changes due to valinomycin are the means ± standard errors of the paired differences. The concentration of valinomycin was 1.7 μg/ml, and that of norepinephrine was 0.2 mM. Approximately 90 μg of protein were present per assay tube.

Addition	Adenyl cyclase activity	
	Basal	Change due to valinomycin
	nmoles cyclic AMP formed/20 min/mg protein	
None	1.03	+0.65 ± 0.22
Norepinephrine	10.90	−2.0 ± 0.70

cells (Table 4). Ghosts are considerably less sensitive than intact fat cells to hormones (16); it is possible that they are also less sensitive to valinomycin, and that higher concentrations might have had a greater inhibitory effect.

There appear to be other effects of valinomycin besides inhibition of adenyl cyclase, since 0.8 μg/ml of valinomycin blocked the lipolytic action of dibutyryl cyclic AMP in both brown and white fat cells (Table 6). Increasing the concentration of valinomycin to 4 μg/ml (data not shown) did not result in any further decrease in lipolysis, which was almost maximally inhibited by 0.8 μg/ml (Table 6). The effects of valinomycin on glucose metabolism for the same experiments are shown in Table 7.

Lactate and total lipid production from labeled glucose by both brown and white fat cells is reduced by valinomycin. However, valinomycin had different effects in brown and white fat on the conversion of glucose to carbon dioxide (Table 7). Glucose conversion to carbon dioxide is stimulated in brown fat but inhibited in white fat by valinomycin. In brown fat the effect of valinomycin (0.8 μg/ml) is seen in both the presence and absence of dibutyryl cyclic AMP. In the presence of insulin, when glucose metabolism by brown fat cells is already nearly maximally stimulated, higher concentrations of valinomycin no longer enhance carbon dioxide production from glucose. Valinomycin at 4.0 μg/ml actually inhibited glucose oxidation to carbon dioxide by brown fat cells in the presence of insulin (Table 7).

TABLE 6

Effect of valinomycin on lipolysis in white and brown fat cells

White fat cells (average, 20.8 μmoles of triglyceride per tube) or brown fat cells (average, 7.8 μmoles of triglyceride per tube) were isolated by digestion of fat tissue with collagenase. The washed, free cells were incubated for 4 hr in 1.5 ml of buffer containing 4% albumin and 2.8 mM glucose. The final concentration of insulin was 0.17 milliunit/ml, and that of dibutyryl cyclic AMP was 1.0 mM. Valinomycin was added in 25 μl of 5% ethanol, and the same amount of 5% ethanol was added to all tubes not containing valinomycin. The basal values are means ± standard errors of three replications. Changes due to valinomycin are expressed as means ± standard errors of the paired differences.

Additions	Measurement	Free fatty acid release		Glycerol release	
		White fat	Brown fat	White fat	Brown fat
		μmoles/mmole triglyceride		*μmoles/mmole triglyceride*	
None	Basal	0 ± 0	0.5 ± 0.5	6.3 ± 3.4	4.7 ± 4.7
	Change due to valinomycin (0.8 μg/ml)	+0.4 ± 0.4	+0.5 ± 0.5	−1.2 ± 1.9	+1.4 ± 1.4
Insulin	Basal	0 ± 0	0 ± 0	9.4 ± 2.7	4.7 ± 4.7
	Change due to valinomycin (0.8 μg/ml)	+3.8 ± 3.8	0 ± 0	−4.7 ± 1.3	+0.7 ± 0.7
Dibutyryl cyclic AMP	Basal	202 ± 22	422 ± 28	96 ± 37	134 ± 23
	Change due to valinomycin (0.8 μg/ml)	−186 ± 23	−297 ± 101	−76 ± 47	−95 ± 42
Insulin + dibutyryl cyclic AMP	Basal	182 ± 21	355 ± 70	78 ± 13	144 ± 13
	Change due to valinomycin (0.8 μg/ml)	−92 ± 40	−222 ± 64	−40 ± 19	−98 ± 36

Reed and Fain (6) suggested that the increase in respiration accompanying lipolysis is due to a potassium-dependent uncoupling action by the free fatty acids released through lipolysis. They also reported a stimulation of respiration by valinomycin in the presence of potassium (6). In Table 8 some further observations on the effects of valinomycin on respiration are summarized. A fatty acid (0.9 mM sodium octanoate) stimulated the respiration of brown fat cells without added substrate, and in the presence of 10 mM oxalacetate or 10 mM pyruvate. Valinomycin doubled respiration either in the absence of added substrate or in the presence of pyruvic acid or oxalacetic acid. When valinomycin and fatty acid were present simultaneously, less oxygen was consumed than when a fatty acid alone was added to the cells. Valinomycin (2.0 μg/ml) reduced by half the respiration of brown fat cells in the presence of octanoate, using oxalacetic acid or pyruvic acid as substrate.

DISCUSSION

Kuo and Dill (8) attributed the antilipolytic effect of valinomycin in part to interference with the ability of the membrane-bound adenyl cyclase system to convert ATP to cyclic AMP. The present report shows that valinomycin has a complex mode of action that involves more than a direct effect on the adenyl cyclase system, since it also inhibited lipolysis due to dibutyryl

TABLE 7

Effect of valinomycin on glucose metabolism in white and brown fat cells

White fat cells (average, 20.8 μmoles of triglyceride per tube) or brown fat cells (average, 7.8 μmoles of triglyceride per tube) were isolated by digestion of tissue with collagenase. The washed, free cells were incubated for 4 hr in 1.5 ml of buffer containing 4% albumin and 2.8 mM glucose-^{14}C. The final concentration of insulin was 0.17 milliunit/ml, and that of dibutyryl cyclic AMP was 1.0 mM. Valinomycin was added in 25 μl of 5% ethanol, and the same amount of 5% ethanol was added to all tubes not containing valinomycin. The basal values are means ± standard errors of three paired replications. Increments due to valinomycin are expressed as means ± standard errors of the paired differences.

Additions	Measurement	Glucose conversion to CO_2		Glucose conversion to lactate		Glucose conversion to total lipid	
		White fat	Brown fat	White fat	Brown fat	White fat	Brown fat
		μmoles/mmole triglyceride		*μmoles/mmole triglyceride*		*μmoles/mmole triglyceride*	
None	Basal	5.5 ± 1.1	4.6 ± 0.1	6.8 ± 3.2	5.9 ± 0.2	12.3 ± 1.9	3.1 ± 0.3
	Change due to valinomycin						
	0.8 μg/ml	−3.1 ± 1.3	+11.4 ± 0.2	−3.7 ± 0.2	−5.9 ± 0.2	−7.9 ± 1.6	−2.4 ± 0.2
	4.0 μg/ml	−5.3 ± 1.1	+9.5 ± 0.4	−5.3 ± 2.5	−5.7 ± 0.1	−12.1 ± 2.9	−2.7 ± 0.2
Insulin	Basal	7.2 ± 1.2	48.9 ± 6.0	5.6 ± 2.2	19.4 ± 2.4	15.4 ± 3.5	40.3 ± 0.7
	Change due to valinomycin						
	0.8 μg/ml	−3.2 ± 2.2	+9.1 ± 9.8	−3.6 ± 2.0	−14.3 ± 1.2	−8.2 ± 3.1	−32.5 ± 13.2
	4.0 μg/ml	−6.5 ± 1.6	−17.5 ± 0.9	−5.7 ± 2.4	−7.7 ± 4.2	−14.7 ± 3.2	−37.7 ± 8.5
Dibutyryl cyclic AMP	Basal	2.3 ± 1.7	6.3 ± 0.3	0.2 ± 0.1	7.8 ± 2.1	3.5 ± 2.2	1.5 ± 0.1
	Change due to valinomycin						
	0.8 μg/ml	−1.8 ± 1.1	+8.9 ± 4.7	−0.6 ± 0.3	−6.5 ± 2.5	−2.5 ± 2.4	+0.7 ± 0.2
	4.0 μg/ml	−2.0 ± 0.9	+6.9 ± 0.5	−0.2 ± 0.4	−7.0 ± 2.5	−3.4 ± 1.2	−0.6 ± 0.1
Insulin + dibutyryl cyclic AMP	Basal	10.8 ± 2.2	31.0 ± 6.4	5.5 ± 1.2	22.9 ± 3.7	21.1 ± 3.1	14.1 ± 5.7
	Change due to valinomycin						
	0.8 μg/ml	−7.1 ± 2.7	+0.8 ± 4.9	−3.6 ± 1.4	−16.4 ± 8.0	−15.9 ± 4.2	−9.5 ± 7.8
	4.0 μg/ml	−10.4 ± 1.9	−14.3 ± 6.3	−5.4 ± 1.6	−20.6 ± 3.7	−20.9 ± 2.6	−12.7 ± 5.7

TABLE 8

Effect of valinomycin on respiration in the presence of oxalacetate and pyruvate

Brown fat cells were isolated by incubation of minced tissue in 5 ml of buffer containing 4% defatted albumin and 1 mg/ml of collagenase. The cells (average, 10.5 μmoles of triglyceride per flask) were added to respirometer flasks containing 3 ml of albumin buffer plus the agents and substrates indicated. The flasks were gassed with 100% oxygen for 3 min and equilibrated for 30 min. Respiration was measured for the next hour. The basal values are the means of three experiments, and the effects due to added agents are shown as the means ± standard errors of the paired differences between basal values and those obtained in the presence of added agents.

Substrate	Basal	Increment due to		
		Octanoate (0.9 mM)	Valinomycin (2 μg/ml)	Octanoate + valinomycin
	μl O_2 consumed/μmole triglyceride/hr			
None	2.3 ± 0.2	+16.4 ± 1.4	+2.6 ± 0.4	+13.7 ± 0.6
Oxalacetate (10 mM)	7.0 ± 0.3	+24.2 ± 4.1	+9.8 ± 0.7	+12.5 ± 1.2
Pyruvate (10 mM)	7.3 ± 0.9	+16.5 ± 2.0	+11.3 ± 0.4	+8.0 ± 2.2

cyclic AMP. Furthermore, valinomycin alone stimulated adenyl cyclase and cyclic AMP accumulation in white fat cells without increasing lipolysis. We have found that epinephrine alone can activate lipolysis to the same extent in the presence of theophylline, as shown in Table 4, but produces a smaller increase in cyclic AMP than was observed with valinomycin.[2]

Fain (5) suggested that the inhibition of catecholamine-stimulated lipolysis by valinomycin was due to a reduction in the total energy available for the activation of lipolysis, secondary to energy expenditure for cyclic flux of potassium. It has been shown that valinomycin rapidly depleted ATP stores in Ehrlich ascites tumor cells (21). In our studies, however, valinomycin had no significant effect on the ATP content of brown fat cells after 5 or 20 min of incubation (Table 1). This suggests that the effects of valinomycin are unrelated to total ATP content, but does not eliminate the possibility that valinomycin may divert energy which is ordinarily used for other metabolic processes into potassium flux. Since we measured only the total ATP content of fat cells, we must consider the possibility that valinomycin depletes the ATP content of a pool which accounts for only a small fraction of the total ATP. We have found a markedly antilipolytic action of carbonyl cyanide *m*-chlorophenylhydrazone, a classical uncoupler of mitochondrial oxidative phosphorylation, on brown fat cells under conditions in which the total ATP content is unaffected.[3] Thus, in intact cells, uncouplers of oxidative phosphorylation can affect energy-linked processes under circumstances in which negligible changes in total ATP content are observed.

The effects of valinomycin on lactate accumulation and glucose conversion to total lipid were similar in brown and white fat cells; in brown fat cells, however, valinomycin stimulated glucose oxidation while in white fat cells it caused inhibition. These differences in the responses of brown and white fat cells with respect to glucose oxidation are similar to those observed with oligomycin (22). It is not clear why valinomycin and oligomycin have opposing effects on glucose oxidation in brown and white fat cells, but this may be attributable to the larger number of mitochondria in brown fat cells (9). The rate of oxygen consumption of brown fat cells is far greater than that of white fat cells, and results from the larger number of mitochondria in brown fat cells.

The antilipolytic action of valinomycin is quite distinct from that of insulin. The results in Table 6 indicate that in neither brown nor white fat cells did insulin inhibit

[2] Unpublished experiments.

[3] J. N. Fain and J. W. Rosenthal, unpublished observations.

the lipolytic action of dibutyryl cyclic AMP, in agreement with the results of others for white fat cells (23, 24). Insulin is able to inhibit the lipolytic action of low concentrations of catecholamines or methylxanthines, but this effect is abolished by increasing the concentration of the lipolytic agent (25). The ability of valinomycin to inhibit the lipolytic action of dibutyryl cyclic AMP suggests that valinomycin interferes with the energy-dependent steps in the activation of triglyceride lipase by cyclic AMP, as previously suggested (5). However, we are not sure whether the mechanisms by which external addition of fairly high concentrations of dibutyryl cyclic AMP result in activation of lipolysis are identical with those by which endogenous cyclic AMP activates the triglyceride lipase.

The present results indicate that valinomycin has many effects on isolated fat cells, since glucose metabolism, respiration, adenine catabolism, and lipolysis are altered. Exactly how these effects of valinomycin are related to its ability to increase passive flux of potassium through natural and artificial membranes and accelerate the net uptake of potassium by isolated mitochondria remains to be established.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Judith W. Rosenthal for the ATP determinations.

REFERENCES

1. D. C. Tosteson, P. Cook, T. Andreoli and M. Tieffenberg, *J. Gen. Physiol.* **50**, 2513 (1967).
2. E. J. Harris, G. Catlin and B. C. Pressman, *Biochemistry* **6**, 1360 (1967).
3. P. Mueller and D. O. Rudin, *Biochem. Biophys. Res. Commun.* **26**, 398 (1967).
4. H. A. Lardy, S. N. Graven and S. Estrada-O., *Fed. Proc.* **26**, 1355 (1967).
5. J. N. Fain, *Mol. Pharmacol.* **4**, 349 (1968).
6. N. Reed and J. N. Fain, *J. Biol. Chem.* **243**, 6077 (1968).
7. J. N. Fain and N. Reed, *Lipids* **5**, 210 (1970).
8. J. F. Kuo and I. K. Dill, *Biochem. Biophys. Res. Commun.* **32**, 333 (1968).
9. J. N. Fain, N. Reed and R. Saperstein, *J. Biol. Chem.* **242**, 1887 (1967).
10. M. Rodbell, *J. Biol. Chem.* **239**, 375 (1964).
11. R. J. Guillory and E. Racker, *Biochim. Biophys. Acta* **153**, 490 (1968).
12. J. R. Williamson and B. R. Corkey, *Methods Enzymol.* **13**, 434 (1969).
13. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
14. N. D. Goldberg, J. Larner, H. Sasko and A. G. O'Toole, *Anal. Biochem.* **28**, 523 (1969).
15. T. Kaneko and J. B. Field, *J. Lab. Clin. Med.* **74**, 682 (1969).
16. L. Birnbaumer, S. L. Pohl and M. Rodbell, *J. Biol. Chem.* **244**, 3468 (1969).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
18. M. Vaughan, *J. Biol. Chem.* **237**, 3354 (1962).
19. H. Hohorst, in "Methods of Enzymatic Analysis" (H. U. Bergmeyer, ed.), p. 266. Academic Press, New York, 1965.
20. V. P. Dole and H. Meinertz, *J. Biol. Chem.* **235**, 2595 (1960).
21. C. Levinson, *Nature* **216**, 74 (1967).
22. J. N. Fain, S. C. Loken and M. P. Czech, *Biochim. Biophys. Acta* **197**, 40 (1970).
23. C. L. Hall and E. G. Ball, *Biochim. Biophys. Acta* **210**, 209 (1970).
24. M. Blecher, N. S. Merlino and J. T. Ro'Ane, *J. Biol. Chem.* **243**, 3973 (1968).
25. J. N. Fain, V. P. Kovacev and R. O. Scow, *Endocrinology* **78**, 773 (1966).