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EFFECTS OF DEOXYFRENOLICIN ON ISOLATED ADIPOSE CELLS—II

LIPOLYSIS, ADENOSINE 3′,5′-MONOPHOSPHATE LEVELS, AND COMPARISON WITH THE EFFECTS OF VITAMIN K_5

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Abstract—Deoxyfrenolicin, an antifungal naphthoquinone, inhibited lipolysis induced by norepinephrine, corticotropin, caffeine or theophylline in isolated adipose cells. It was unexpectedly found that deoxyfrenolicin augmented still higher the intracellular adenosine 3′,5′-monophosphate (cyclic AMP) levels that were maximally elevated by theophylline plus norepinephrine or corticotropin. Deoxyfrenolicin was without effect on the basal levels of cyclic AMP. Vitamin K_5, not vitamin K_1, was found to mimic not only the effects of deoxyfrenolicin on inhibiting lipolysis and augmenting cyclic AMP levels elevated by hormones but also its effects on stimulating glucose oxidation by adipose cells. Vitamin K_1 could not counteract the effects of deoxyfrenolicin or vitamin K_5. No addition was observed for the effects of deoxyfrenolicin and vitamin K_5.

It seemed that the mode of action of deoxyfrenolicin on adipose cells is similar to that of vitamin K_5, of which inhibition of electron transport or oxidative phosphorylation may be one of the sites of action.

IN A PRECEDING report,[1] the stimulatory effect of deoxyfrenolicin on the utilization of glucose and fructose by isolated adipose cells were studied and compared with those of insulin and some proteases mimicking insulin effects *in vitro*.[2–4] Since insulin[5] and those proteases[6] were reported to block the lipolysis mediated by lipolytic hormones or by phosphodiesterase inhibitors, it was the object of the present study to investigate the antilipolytic action of deoxyfrenolicin. In order to elucidate the possible mode of action of deoxyfrenolicin, its effects on adenosine 3′,5′-monophosphate (cyclic AMP) levels in adipose cells were examined and compared with that of insulin and vitamin K_5. The possible influence of deoxyfrenolicin on the activities of adenyl cyclase, phosphodiesterase and lipase was also studied in the homogenates of adipose tissue or its isolated cells.

MATERIALS AND METHODS

Adenine-8-^{14}C (52 mc/m-mole), cyclic AMP, adenine, adenosine, AMP, ADP and ATP were purchased from Schwarz; ATP-U-^{14}C (0·05 mc/0·0605 mg) and cyclic AMP-U-^{14}C (5 μc/0·0322 mg) from New England Nuclear; adenine-2,8-^{3}H (5·2 c/m-mole) from International Chemical and Nuclear; vitamins K_1 and K_5 from

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Calbiochem; filipin from Upjohn. Other materials employed in the present study were the same as in the previous report.[1]

Preparation of isolated adipose cells, determination of free fatty acid release and conversion of radioactive glucose to CO_2 by the cells were essentially the same as reported by Rodbell.[7]

Adenyl cyclase was assayed by measuring the formation of either cyclic AMP-2,8-^{3}H or cyclic AMP-8-^{14}C by free adipocytes that were preincubated with glucose in the presence of either adenine-2,8-^{3}H or adenine-8-^{14}C.[8] Briefly, fat pads pooled from 10 male Sprague–Dawley rats, weighing 110–130 g, were incubated for 1 hr at 37°, with shaking, in 4 ml of albumin–bicarbonate medium[1] containing 40 μmoles glucose and either 40 μc adenine-2,8-^{3}H or 10 μc adenine-8-^{14}C. Another 40 μmoles glucose and either 20 μc adenine-2,8-^{3}H or 5 μc adenine-8-^{14}C were then added to the incubation mixture together with 7 mg collagenase. The digestion was completed in 40 min. The dispersed adipocytes were filtered through a nylon cloth and were washed free of excess radioactive adenine with 30 ml of medium five times and finally suspended in an appropriate volume of albumin–bicarbonate medium containing 3 mM theophylline. One-ml aliquots of the adipocyte suspension were incubated for varying times at 37°, with shaking, in transparent plastic tubes (12 × 150 mm, Falcon). At the end of incubation, the medium was quickly separated with a syringe from the adipose cells floating to the surface. One ml of fresh incubation medium was then added back to the cells. Lipid extraction[7] was performed on both fractions (cells and medium) and the organic layer was removed with a syringe attached to an aspirator. The tubes were placed in a vacuum oven at room temperature for 20 min to remove residual organic solvent. The precipitates (mostly albumin) were removed by centrifugation and cyclic AMP-2,8-^{3}H or cyclic AMP-8-^{14}C in the clear aqueous supernatants was purified, without passage of Dowex resins, by the $BaSO_4$ method of Rodbell[9] and Krishna *et al.*[10] Cyclic AMP represented exclusively the radioactivity present in the $BaSO_4$-supernatant, as verified by paper chromatography[11] using the following solvent systems: isopropanol–concentrated NH_4OH–water (7:1:2, v/v); isopropanol–concentrated NH_4OH–0·1 M H_3BO_3 (6:1:3, v/v); 95% ethanol–1 M ammonium acetate, pH 5·0 (7:3, v/v). About 0·2 μmole each of unlabeled adenine, adenosine, cyclic AMP, AMP, ADP and ATP were spotted along with the $BaSO_4$-supernatants from each experiment as carriers and markers. In typical experiments in which adipose cells were incubated with norepinephrine for 20 min, about 10 per cent of the starting radioactivity was found in the clear aqueous layer after lipid extraction, of which about 8 per cent was recovered in the cyclic AMP fraction.

Adenyl cyclase[11] in the tissue or cell homogenates was assayed by the conversion of ATP-U-^{14}C to cyclic AMP-U-^{14}C and phosphodiesterase[11] by the disappearance of cyclic AMP-U-^{14}C. Lipase[12] was assayed by the hydrolysis of Ediol.

RESULTS

The dose–response curves of the norepinephrine-induced lipolysis by isolated adipose cells, incubated in the presence of varying concentrations of insulin and deoxyfrenolicin, are illustrated in Fig. 1. At a low concentration of norepinephrine (0·05 μg/ml), the induced lipolysis was almost completely depressed by insulin at a concentration as low as 50 microunits/ml or by deoxyfrenolicin as low as 3 μg/ml.

The inhibition of lipolysis by insulin or deoxyfrenolicin was progressively reversed by increasing concentrations of norepinephrine; more than 90 per cent of the lipolysis inhibited by insulin (up to 2000 microunits/ml) or by deoxyfrenolicin (3 μg/ml), was recovered in the presence of norepinephrine at 0·5 μg/ml. Only 74 per cent and 40 per cent were recovered with deoxyfrenolicin at concentrations of 9 and 30 μg/ml in the presence of norepinephrine at 0·5 μg/ml.

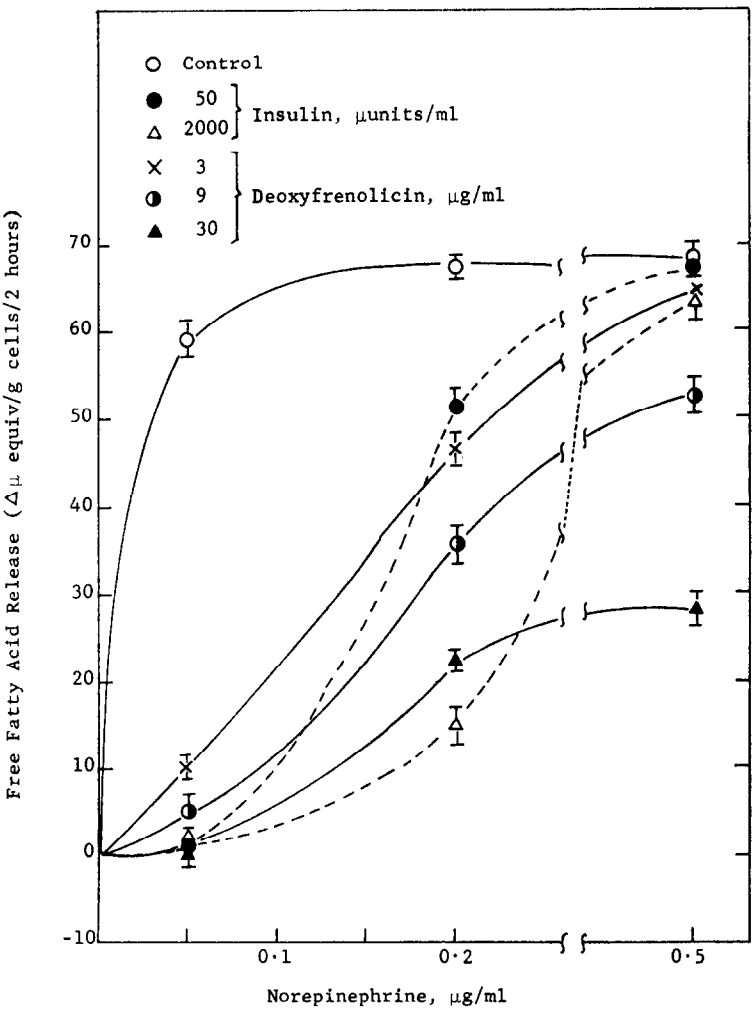


FIG. 1. The concentration-dependent inhibition by deoxyfrenolicin on norepinephrine-induced lipolysis in isolated adipose cells. Free adipocytes were incubated for 2 hr in 1 ml of albumin–bicarbonate medium in the presence of varying concentration of norepinephrine, deoxyfrenolicin and insulin, as indicated. Glucose was omitted from the incubation medium used for lipolytic studies. Each treatment was performed in triplicate and the means (± S.E., expressed as vertical bars) are presented. The free fatty acid released was taken as the difference between the treated and the control (untreated) cells. Little or no free fatty acid was released by the incubated adipose cells in the absence of lipolytic agents.

Data pertaining to the inhibitory effects of deoxyfrenolicin on lipolysis induced by other lipolytic agents are presented in Table 1. The lipolysis mediated by either lipolytic hormones (norepinephrine and corticotropin) or by phosphodiesterase inhibitors (caffeine and theophylline) was inhibited by deoxyfrenolicin. A higher concentration of deoxyfrenolicin was required to inhibit lipolysis in the co-presence of hormone and phosphodiesterase inhibitors.

TABLE 1. INHIBITORY EFFECTS OF DEOXYFRENOLICIN ON LIPOLYSIS*

Lipolytic agents	Free fatty acid released ($\Delta\mu$equiv./g cells/2 hr)	
	Deoxyfrenolicin (15 μg/ml)	
	−	+
None	0·0 ± 0·5	0·3 ± 0·7
Norepinephrine (0·05 μg/ml)	56·0 ± 0·7	1·5 ± 0·6
Corticotropin (0·05 μg/ml)	68·0 ± 0·2	5·0 ± 2·0
Caffeine (1 mM)	57·3 ± 2·5	6·2 ± 0·9
Theophylline (1 mM)	70·6 ± 2·4	8·5 ± 0·5
Norepinephrine + caffeine	78·5 ± 1·4	13·5 ± 1·2
Norepinephrine + theophylline	82·0 ± 0·1	15·0 ± 1·8
Corticotropin + caffeine	84·5 ± 1·9	18·2 ± 3·4
Corticotropin + theophylline	86·5 ± 0·6	18·9 ± 1·7

* Free adipocytes were incubated in 1 ml of glucose-free medium for 2 hr under the conditions indicated. Each treatment was performed in triplicate and the means (± S. E.) are presented.

Insulin[13, 14] was recently reported to lower intracellular cyclic AMP levels in adipose cells, at least in part through inhibition of adenyl cyclase. The opposite to insulin effects of lipolytic hormones on the cyclic AMP levels and adenyl cyclase activity were also reported.[14–16] The results obtained by use of the new method for assaying adenyl cyclase directly in the intact adipose cells in the present study, as shown in Fig. 2, confirmed these phenomena. It was unexpected, however, to find that deoxyfrenolicin, in spite of its marked antilipolytic action, augmented still more the intracellular cyclic AMP levels as elevated by norephinephrine. Deoxyfrenolicin also augmented still higher the intracellular cyclic nucleotide levels as elevated by corticotropin (Table 2). Furthermore, in the presence of norepinephrine or corticotropin, deoxyfrenolicin at 50 μg/ml augmented it to an even higher value than deoxyfrenolicin at 20 μg/ml. The cyclic AMP levels in the incubation medium (extracellular) were only slightly affected by deoxyfrenolicin.

Filipin[17] was previously shown to stimulate sugar utilization and inhibit lipolysis in isolated adipose cells. It was also found that filipin facilitated the leakage of cyclic AMP from cells to incubation medium.* The data shown in Table 3 clearly indicate that filipin at 0·07 mM (a concentration optimal for sugar utilization and inhibiting lipolysis) counteracted the effects of deoxyfrenolicin on elevating intracellular cyclic AMP levels; i.e. filipin markedly lowered the cyclic nucleotide levels in the cells incubated with norepinephrine or corticotropin and thus concomitantly elevated the nucleotide levels in the medium.

* J. F. KUO, unpublished observation.

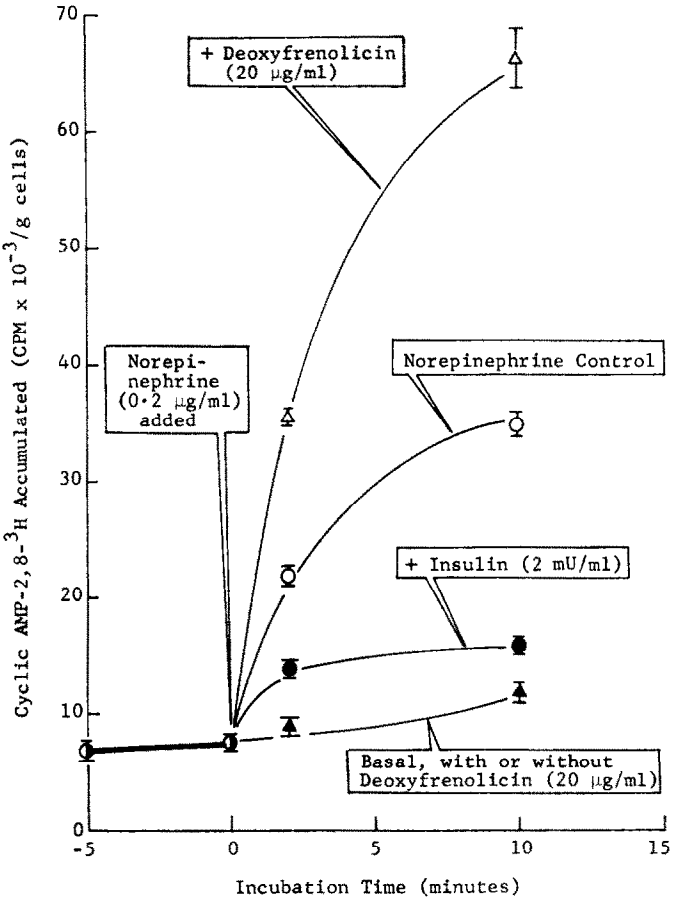


FIG. 2. Comparison of the effects of deoxyfrenolicin and insulin on the intracellular cyclic AMP levels of isolated adipose cells incubated with 3 mM theophylline in the absence (basal) and presence of norepinephrine. The free adipocytes prelabeled with adenine-2,8-^{3}H were incubated for 5 min with or without insulin and deoxyfrenolicin prior to addition of norepinephrine. Each treatment was performed in triplicate and the means (± S.E., expressed as vertical bars) are presented.

TABLE 2. EFFECTS OF DEOXYFRENOLICIN ON THE CYCLIC AMP LEVELS IN THE MEDIUM AND ISOLATED ADIPOSE CELLS INCUBATED WITH NOREPINEPHRINE AND CORTICOTROPIN*

Hormones	Cyclic AMP-8-^{14}C accumulated (cpm × 10^{-3}/g cells/20 min)					
	Deoxyfrenolicin (μg/ml)					
	0		20		50	
	Cells	Medium	Cells	Medium	Cells	Medium
None	39·6 ± 2·8	22·5 ± 0·7	34·9 ± 5·4	25·5 ± 4·3	39·0 ± 5·7	35·5 ± 0·8
Norepinephrine (0·5 μg/ml)	118·1 ± 9·7	60·5 ± 5·5	340·4 ± 33·9	66·7 ± 1·7	448·8 ± 1·2	80·4 ± 7·1
Corticotropin (0·5 μg/ml)	120·5 ± 7·7	61·2 ± 4·7	347·7 ± 11·8	73·1 ± 7·2	489·9 ± 18·5	74·0 ± 2·2

* Free adipocytes prelabeled with adenine-8-^{14}C were incubated with 1 mM theophylline in the presence and absence of additives, as indicated. The incubation time was 20 min. Each treatment was performed in triplicate and the means (± S. E.) are presented.

The influences of deoxyfrenolicin on adenyl cyclase, phosphodiesterase and lipase were studied in a series of experiments in which deoxyfrenolicin (up to 50 μg/ml) was added directly to the assay mixture containing homogenates of fat pads or isolated adipose cells. In parallel experiments, fat pads were incubated for 10 min with deoxyfrenolicin (up to 50 μg/ml), and the activities of the above-mentioned enzymes

TABLE 3. EFFECTS OF DEOXYFRENOLICIN ON THE CYCLIC AMP LEVELS IN THE MEDIUM AND ISOLATED ADIPOSE CELLS INCUBATED WITH OR WITHOUT NOREPINEPHRINE AND FILIPIN*

Additives	Accumulation of cyclic AMP-8-^{14}C (cpm $\times$ 10^{-3}/g cells/10 min)			
	Control		Filipin (0·07 mM)	
	Cells	Medium	Cells	Medium
None	9·1 ± 0·8	15·7 ± 0·7	26·8 ± 0·9	72·2 ± 4·7
Deoxyfrenolicin (20 μg/ml)	11·0 ± 0·9	18·4 ± 0·1	28·3 ± 3·4	40·4 ± 3·1
Norepinephrine (1 μg/ml)	61·2 ± 2·5	30·5 ± 1·9	38·4 ± 2·7	81·6 ± 7·1
Norepinephrine + deoxyfrenolicin	199·3 ± 3·7	48·5 ± 0·3	67·3 ± 9·5	130·0 ± 4·2

* Experimental conditions were the same as for Table 2. Each treatment was performed in triplicate and the means (± S. E.) are presented.

in the homogenates were assayed and compared with that in the homogenates prepared from the control pads (not incubated with deoxyfrenolicin). In any case, deoxyfrenolicin was found to be without detectable effects on the activities of these enzymes.

Since deoxyfrenolicin is a napthoquinone derivative,[18] it is interesting to examine the possible effects of vitamin K_1 (phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone) and its analog, vitamin K_5 (4-amino-2-methyl-1-naphthol hydrochloride), on isolated adipose cells. The results shown in Fig. 3 indicate that vitamin K_5, like deoxyfrenolicin, augmented still higher the intracellular cyclic AMP levels elevated by norepinephrine. Vitamin K_1 was without effect. In the absence of hormone, deoxyfrenolicin, vitamins K_1 and K_5 were all without effect, however. Furthermore, as shown in Table 4, the synergistic effects of deoxyfrenolicin and vitamin K_5 on norepinephrine action in augmenting cyclic AMP levels were not counteracted by vitamin K_1. No addition in the stimulatory effects of deoxyfrenolicin and vitamin K_5 was observed when they were present together.

As already shown, vitamin K_5 mimicked deoxyfrenolicin in its effects on cyclic AMP levels; it seemed that it also mimics deoxyfrenolicin in stimulating glucose oxidation and inhibiting lipolysis.[1] The results presented in Fig. 4 indicate that this is the case, Vitamin K_1 was without effect.

DISCUSSION

In a preceding report,[1] deoxyfrenolicin has been shown to stimulate glucose oxidation by isolated adipose cells to a greater extent than insulin. However, the synthetic process (e.g. lipogenesis) was more effectively stimulated by insulin. This difference in pattern of sugar utilization suggested that there exists a basically different

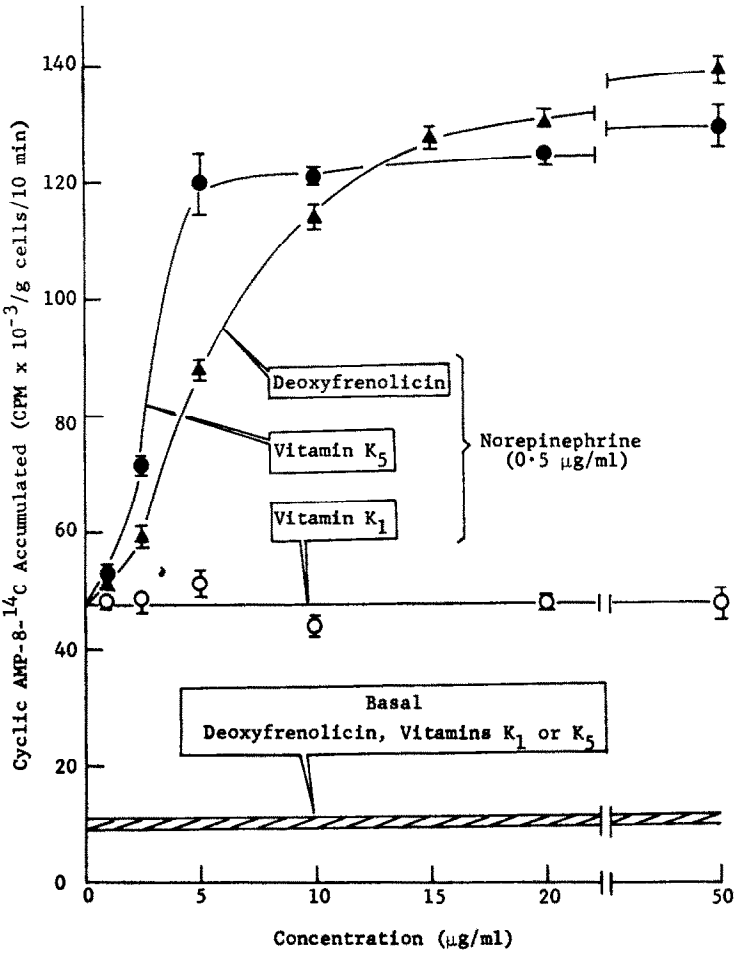


FIG. 3. Comparison of the effects of deoxyfrenolicin, vitamins K_1 and K_5 on intracellular cyclic AMP levels of isolated adipose cells incubated for 10 min with 1 mM theophylline in the presence and absence of norepinephrine. Free adipocytes were prelabeled with adenine-8-^{14}C. Each treatment was performed in triplicate and the means (± S. E.) are presented.

TABLE 4. EFFECTS OF DEOXYFRENOLICIN, VITAMINS K_1 AND K_5 ON INTRACELLULAR CYCLIC AMP LEVELS WHEN PRESENT SINGLY OR IN COMBINATION*

Additives	Accumulation of cyclic AMP-8-^{14}C (cpm × 10^{-3}/g cells/10 min)
None	13·8 ± 0·6
Norepinephrine (0·5 μg/ml)	52·1 ± 4·4
Norepinephrine + deoxyfrenolicin (20 μg/ml)	95·3 ± 0·5
Norepinephrine + vitamin K_1 (100 μg/ml)	49·4 ± 0·1
Norepinephrine + vitamin K_5 (10 μg/ml)	89·2 ± 3·5
Norepinephrine + deoxyfrenolicin + vitamin K_1	100·5 ± 0·4
Norepinephrine + deoxyfrenolicin + vitamin K_5	79·5 ± 3·5
Norepinephrine + vitamin K_1 + vitamin K_5	89·1 ± 3·5
Norepinephrine + deoxyfrenolicin + vitamin K_1 + vitamin K_5	90·3 ± 2·7

* Experimental conditions were same as for Table 2. Each treatment was performed in quadruplicate and the means (± S. E.) are presented.

mode of action for these agents. In support of this contention, it has been shown that deoxyfrenolicin synergistically augments the norepinephrine-elevated cyclic AMP levels while insulin acts to depress them. It is well recognized that cyclic AMP is the common cellular mediator of hormone actions.[19] In the case of lipolysis in adipose cells,[12, 19–22] lipolytic agents augment the cellular levels of cyclic AMP,[14–26] whereas the effects of deoxyfrenolicin on norepinephrine-elevated cyclic AMP levels were quite unexpected, since deoxyfrenolicin, like insulin, is a potent antilipolytic agent.

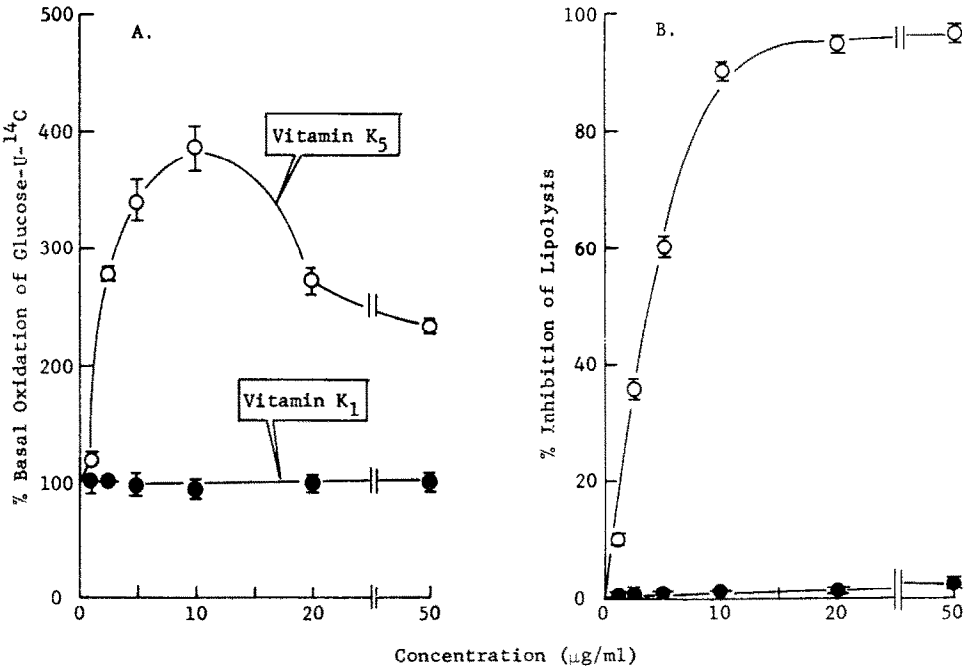


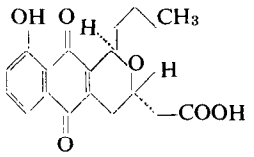
FIG. 4. Comparison of the effects of vitamins K_1 and K_5 on the oxidation of glucose-U-^{14}C and inhibition of norepinephrine-induced lipolysis. Free adipocytes were incubated for 2 hr in 1 ml of medium with either 0·1 μc (1 μmole) glucose-U-^{14}C (A), or norepinephrine, 0·2 μg/ml (B). The basal rate of glucose-U-^{14}C oxidation was 0·35 μmole/g cells per 2 hr. Each treatment was performed in triplicate and the means (± S. E.) are presented.

As this study shows, the antilipolytic action of deoxyfrenolicin and vitamin K_5 is due neither to the direct inhibition of adenyl cyclase and lipase nor to the direct activation of phosphodiesterase. Therefore, there must be a point (or points), other than the above three enzymes, at which deoxyfrenolicin and vitamin K_5 exerted their antilipolytic action directly or indirectly.

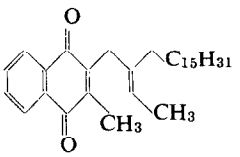
At least two possibilities could account for this. First, the concentration of cyclic AMP required for the activation of lipase is very narrow and critical and excess concentration of cyclic AMP will cause a reduction in lipase activity.[12] Second, lipase may be inhibited in a feedback fashion by excess free fatty acid accumulated within the cells as a result of "overactivation" of lipase by the sharp rise in cyclic AMP elicited by deoxyfrenolicin or vitamin K_5. Butcher *et al.*[14] and we* have found that a considerable time lag exists between the elevation of cyclic AMP and the

* J. F. KUO and I. K. DILL, unpublished observation.

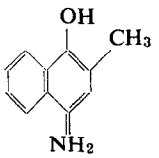
release of free fatty acid from adipose cells to the incubation medium after the cells were exposed to the lipolytic agents.



Deoxyfrenolicin



Vitamin K_1



Vitamin K_5

The role of vitamin K_1 in mitochondrial oxidative phosphorylation has appeared elsewhere.[23–25] In contrast to vitamin K_1, vitamin K_5 was found to possess antimicrobial activities.[26, 27] Gershbein[28] reported a differential effect of vitamins K_1 and K_5 on carbohydrate metabolism of rat diaphragm. These observations, coupled with the results from the present study with adipose cells, make it appear that both vitamin K_5 and deoxyfrenolicin act differently than vitamin K_1. Since respiration-linked energy stores are required for the activation of lipase,[29, 30] the antilipolytic action of deoxyfrenolicin and vitamin K_5 can be explained alternatively by their interfering with oxidative phosphorylation. From this reasoning, one may also explain the extraordinarily high rate of sugar oxidation elicited by deoxyfrenolicin and vitamin K_5. Due to their possible interference with oxidative phosphorylation and the concomitant high rate of conversion of ATP to cyclic AMP, the only way to relieve this drain may be through substrate level phosphorylation.

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