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ACETYLENIC INHIBITORS OF SHEEP VESICULAR GLAND OXYGENASE

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SUMMARY

Biosynthesis of prostaglandins could be stopped by several acetylenic acids. They interfered with the oxygenase activity of the sheep vesicular gland enzymic system in two distinct patterns: an instantaneous, concentration-dependent effect and a time-dependent, destructive effect. Constants for both effects are reported. No measurable oxygen consumption was observed with the acetylenic analogs. Nevertheless, both oxygen and hydroperoxide were required for the inhibition to occur, whereas the inhibitory action could be blocked by the presence of diethyldithiocarbamic acid. The results support the concept of a highly reactive intermediate in the mechanism of oxygenase action.

INTRODUCTION

Various groups have reported that the biosynthesis of prostaglandin from arachidonic acid by sheep vesicular gland enzyme could be inhibited by a number of fatty acid analogs[1–3]. In addition, inhibition was observed with the acetylenic analog of arachidonic acid, eicosa-5,8,11,14-tetraynoic acid[4]. In continuing our study of the oxygenase activity[5] of the sheep vesicular gland, we directed our attention to the inhibitory characteristics of various acetylenic acids to elaborate on the mechanism of interaction of the hydrocarbon chain with the active site of the enzyme.

MATERIALS AND METHODS

Materials

Sheep vesicular gland acetone powder was generously donated by the Upjohn Company, eicosa-5,8,11,14-tetraynoic acid was a gift from Hoffmann-La Roche whereas the other acetylenic acids used were kindly provided by Dr F. D. Gunstone. Glutathione peroxidase was isolated and purified as previously described[6]. Arachidonic acid was purchased from the Hormel Institute. All other chemicals, reagent grade, were obtained from common commercial sources. Deionized distilled water was used for the preparation of all solutions.

The enzyme used in these studies was prepared by suspending the acetone powder of sheep vesicular gland to a concentration of 50 mg/ml in 0.1 M Tris–HCl (pH 8.5)

using a small Dounce ball-type homogenizer. Activation was accomplished by adding 0.1 M phenol to a final concentration of 0.67 mM and maintaining the mixture at room temperature for 30 min.

Oxygen absorption measurement

All oxygen absorption measurements were done on a Yellow Springs Instrument Company Model 53 Oxygen Monitor equipped with a Model 5301 Bath Assembly. The monitor was attached to a Beckman Model 100502 Linear-Log Ten-Inch Potentiometric Recorder and all readings were made using the % transmittance setting of the recorder. Oxygen absorption measurements were made using the "O_2" position of the monitor and a 10-mV recorder setting in addition to a voltage offset attached to the recorder. A constant temperature of 30 ± 0.5 °C was maintained with a Haake Type F Thermoregulated incubator bath. In most experiments, the total final volume of the reaction mixture in the sample chamber was 3.0 ml. Additions were made through the side of the electrode holder.

Determination of GSH peroxidase activity

A procedure for the colorimetric determination of GSH peroxidase activity, designed to assay very small quantities of enzyme, was modified from the approach reported by Smith and Lands[6]. An incubation sample was prepared by mixing 0.40 ml 0.1 M Tris–HCl buffer (pH 7.4), 50–100 nmoles 15-hydroperoxy-5,8,11,14-eicosatetraenoic acid, 300 nmoles GSH and a 1- to 3-μl aliquot of GSH peroxidase preparation. The mixture was incubated at room temperature and 100-μl aliquots were removed with 30-s intervals and added to 1.7 ml of 5,5′-dithio-bis-(2-nitrobenzoic acid) (1.7 μmoles) with efficient mixing. The zero time point was determined by removing a 100-μl aliquot from the incubation mixture prior to the addition of enzyme and adding it to 5,5′-dithio-bis-(2-nitrobenzoic acid). Controls containing no enzyme were needed because of slow loss of mercaptan in the system. The absorbance at 413 nm of the assay solution above that for a reference solution containing 1.7 ml 5,5′-dithio-bis-(2-nitrobenzoic acid) and 25 nmoles peroxide was determined using either a Beckman Model B or DB-G spectrophotometer. Since a rate of color change due to thionitrobenzoate production (ΔA 413 nm/min) of approx. 0.010 can be measured accurately, amounts as low as 35 munits of peroxidase can thus be assayed. One unit is a μmole/ml per min.

Establishment of the triple bond location

The ozonolysis procedure was a modification of that described by Gensler and Schlein[7]. A mixture of measured amounts of oleic, *trans*-vaccenic, Δ^{10}-yne-18:1, Δ^{13}-yne-18:1 and either stearic or palmitic acid (about 0.2 μmole each) in glacial acetic acid (0.5 ml) was oxidized with a Supelco Micro-Ozonizer for 9 min. After evaporation of approx. 80% of the solvent in a current of nitrogen, 6 drops of 30% H_2O_2 and 4 drops of water were added and the mixture allowed to stand overnight. After the addition of saturated salt solution (0.3 ml), the mixture was extracted twice with light petroleum (1.5 ml). The combined light petroleum extract was washed with saturated salt solution. Pentadecanoic acid (0.10 μmole) was added, followed by evaporation of solvent. The residue was dissolved in methanol (0.5 ml), to which BF_3 in methanol (0.75 ml, 14% BF_3) was added. The mixture was heated on a steam bath for 5 min,

and after cooling, the esters were extracted by adding 1–2 ml of water and shaking with 3–4 ml light petroleum. The organic layer was washed with saturated salt solution, dried over sodium sulfate–sodium bicarbonate (2:1, w/w), decanted and concentrated to dryness under nitrogen. A few drops of CS_2 were added and aliquots were analyzed by gas–liquid chromatography using a 9-ft glass column (4 mm internal diameter) packed with 10% EGS on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa). A column temperature of 193 °C and nitrogen carrier flow rate of 60 ml/min were used. Recovery of the reference saturated acid (16:0 or 18:0) was greater than 90% for the overall treatment.

RESULTS AND DISCUSSION

The oxygenation of arachidonic acid by sheep vesicular gland fatty acid oxygenase was found to be inhibited by various acetylenic acids. Among these inhibitors, eicosa-5,8,11,14-tetraynoic acid, the acetylenic analog of arachidonic acid, exhibited two distinct types of interference: an instantaneous, concentration-dependent effect and a time-dependent destructive effect. Incubating the enzyme with the tetraynoic acid for different times prior to the addition of substrate resulted in a progressive loss of enzyme activity (Figs 1A and 1B). The values on the ordinate in Fig. 1B show that, at a constant substrate level, increasing the concentration of the tetraynoic acid immediately decreased the rate of oxygen uptake. The time-dependent effects

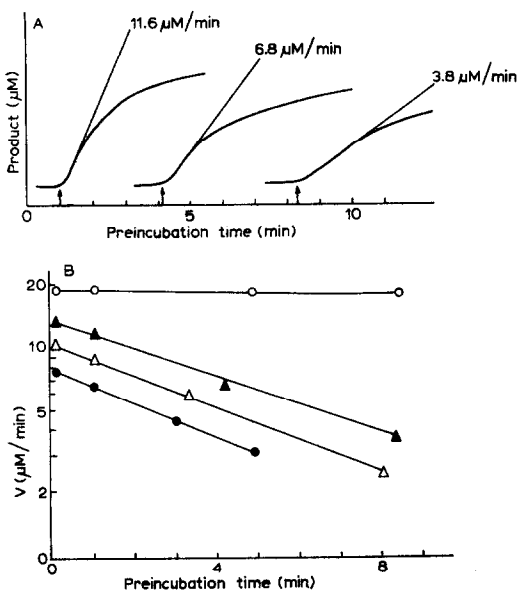

Fig. 1. Oxygenation of arachidonic acid by sheep vesicular gland oxygenase after preincubation of the enzyme with eicosa-5,8,11,14-tetraynoic acid. A. Phenol-activated acetone powder preparation of sheep vesicular gland (2 mg) was added to an assay mixture containing 0.66 mM phenol and eicosatetraynoic acid (6 nmoles). After different times of preincubation, arachidonic acid (45 nmoles) was added as indicated and the rate of oxygen consumption measured. B. Log initial velocity (v) was plotted *versus* incubation time of the oxygenase with eicosatetraynoic acid. ○–○, o μM tetraynoic acid; ▲–▲, 2 μM; △–△, 5 μM; ●–●, 7.5 μM.

TABLE I

EFFECTS OF EICOSATETRAYNOIC ACID AND ARACHIDONIC ACID ON SHEEP VESICULAR GLAND OXYGENASE

Phenol-activated oxygenase (2 mg) was added to an assay mixture containing 0.66 mM phenol and the indicated concentration of eicosa-5,8,11,14-tetraynoic acid at 30 °C. After the indicated times of incubation with the tetraynoic acid, arachidonic acid (35 μM final concentration) was added and the reaction rate (μM/min = nmoles/ml per min) determined from continuous measurements of oxygen uptake with an oxygen electrode.

Eicosatetraynoic acid		*Rate (μM/min) after 20:4 addition*	
Concn (μM)	*Rate (μM/min)*	*Incubation time:*	
		<3 min	*>8 min*
0	0.0	38.0 (1.0′)	39.5 (8.5′)
10	0.0	11.0 (2.1′)	3.0 (9.6′)
20	0.0	8.2 (1.5′)	1.6 (10.0′)

appeared to be irreversible since the lost enzymic activity could not be recovered upon diluting the inhibitor to a concentration of 5 μM, which initially has less inhibitory effect *per se*. The results for this substrate analog appeared similar to the phenomenon of self-catalyzed destruction noted for fatty acids that are substrates[6,8]. On the other hand, the analog is not an effective substrate since no oxygen consumption was observed with eicosatetraynoic acid alone with the oxygenase (Table I).

In order to gain a greater understanding of the mechanism of tetrayne inhibition, other factors known to influence the destruction of oxygenase activity by reactive acids were studied. For example, when the enzyme system was kept under anaerobic conditions with the tetrayne, the time-dependent loss of enzyme activity was eliminated (Table II). This result suggested that oxygen was essential for the time-dependent loss of the vesicular gland oxygenase activity that was caused by the tetrayne.

TABLE II

OXYGENASE ACTIVITY AFTER AEROBIC AND ANAEROBIC INCUBATION WITH EICOSA-5,8,11,14-TETRAYNOIC ACID

A solution (550 μl) of the tetrayne (192 nmoles) in 0.1 *M* Tris buffer (pH 8.5) containing 0.55 mM phenol was placed in the top of a Thunberg tube while the bottom of the tube contained 10 mg of phenol-activated oxygenase preparation of sheep vesicular gland in 0.275 ml of 0.1 M Tris buffer. The tube was evacuated for 3 min with a vacuum pump. The contents of the tube were then mixed and incubated for various times. After incubation, the tube was opened and allowed to air equilibrate for 30 s by vigorous shaking. Next, a 200-μl aliquot was assayed for residual enzyme activity by adding the aliquot to a solution of arachidonic acid (80 μM) in 0.1 *M* Tris buffer containing 0.66 mM phenol and determining the reaction rate as before with a final tetrayne concentration of 21 μM. An analogous aerobic experiment was run where the contents of the Thunberg tube were equilibrated with air prior to incubation.

Experimental conditions	*Eicosatetraynoic acid concn (μM)*	*Activity (μM/min)*		
		Incubation time:		
		1.2 min	*11.7 min*	*29.2 min*
Aerobic	0	35.0		32.6
	21	12.1	6.8	1.6
Anaerobic	21	15.8	15.5	15.3

TABLE III

EFFECT OF GSH PEROXIDASE AND EICOSA-5,8,11,14-TETRAYNOIC ACID ON THE OXYGENATION OF ARACHIDONIC ACID BY VESICULAR GLAND OXYGENASE

Phenol-activated oxygenase (2 mg) was added to an assay mixture containing 0.66 mM phenol, 0.66 mM GSH, GSH peroxidase (15.7 units) and/or tetrayne (10 μM). After incubating for t min, the GSH peroxidase activity was reversed by the addition of *N*-ethylmaleimide (5 mM final concentration). After an additional minute, arachidonic acid (50 μM final concentration) was added and oxygen uptake measurements were carried out as before.

GSH peroxidase (units)	*N-Ethyl-maleimide (mM)*	*Eicosa-5,8,11, 14-tetraynoic acid concn (μM)*	*Rate (μM/min)*		
			Incubation time:		
			1 min	*5 min*	*10 min*
—	—	—	36.7	36.8	36.5
15.7	—	—	<1.0		0.0
15.7	5	—	36.0		35.2
—	—	10	10.1	4.0	1.8
15.7	—	10	<1.0		<1.0
15.7	5	10	17.6	18.8	18.8

Glutathione peroxidase in the presence of glutathione (GSH) was reported earlier to inhibit the oxygenation of arachidonic acid by the sheep vesicular gland enzyme system[9]. This inhibition could be reversed by removal of the GSH by adding *N*-ethylmaleimide. Consequently, GSH peroxidase was used to determine whether it could protect the vesicular gland oxygenase from the destructive effect of the tetrayne. Table III shows the effects of added GSH peroxidase and/or tetrayne on the vesicular gland enzyme system. Incubating both agents together with vesicular gland oxygenase for various times, followed by inactivation of the GSH peroxidase with *N*-ethylmaleimide, resulted in a full recovery of the expected oxygenase activity without any time-dependent destruction by tetrayne. These results suggest that the presence of a lipid peroxide as well as oxygen was necessary for the tetrayne to destroy the vesicular gland oxygenase. Thus the time-dependent loss of enzyme seems analogous to the self-catalyzed destruction caused by substrates[8] even though no measurable oxygen consumption was observed. The similarity of the time-dependent destructive actions of

TABLE IV

EFFECT OF DIETHYLDITHIOCARBAMIC ACID ON THE INHIBITION BY EICOSA-5,8,11,14-TETRAYNOIC ACID

Phenol-activated oxygenase (13 mg) was treated with diethyldithiocarbamic acid (9.1 mM final concentration) and/or tetrayne (76 μM final concentration). After the indicated incubation time, an aliquot containing 3 mg of enzyme was added to an assay mixture containing 0.66 mM phenol and 80 μM arachidonic acid and oxygen uptake measurements were carried out as before.

Run	*Diethyldithio-carbamic acid concn (mM)*	*Eicosa-5,8,11, 14-tetraynoic acid concn (μM)*	*Rate (μM/min)*			
			Incubation time:			
			1 min	*4 min*	*8 min*	*14 min*
1	—	—	10.9	11.1	11.7	11.4
2	9.1	—	8.5	9.2	9.7	11.4
3	—	76	8.0	7.3	6.0	4.0
4	9.1	68		7.0	8.5	9.4

TABLE V

SUMMARY OF INHIBITORY CHARACTERISTICS OF VARIOUS ACETYLENIC ACIDS ON SHEEP VESICULAR GLAND OXYGENASE

The K_{I_i} and k_2' were obtained as described in the text. The k_2' values are mean ± S.E. and the figures in parentheses indicate the number of experiments included.

Acid	K_{I_i} *(μM)*	k_2' *(min^{-1})*
Δ10a-18:1	10 (20)	0.082 ± 0.008 (10)
Δ13a-18:1	25 (6)	0.151 ± 0.010 (9)
Δ9a, 12a-18:2	0.6 (8)	0.266 ± 0.023 (9)
Δ5a,8a,11a,14a-20:4	2.5 (25)	0.172 ± 0.014 (18)

tetraynoic acid and arachidonic acid (substrate) was further confirmed by experiments with diethyldithiocarbamic acid. Diethyldithiocarbamic acid has been shown to inhibit the oxygenase reaction (and its concommitant destruction) of sheep vesicular glands[10]. As with arachidonic acid, the progressive loss of oxygenase activity due to incubation with tetrayne could also be prevented by added diethyldithiocarbamic acid. Run 2 in Table IV shows that pretreating the vesicular gland enzyme preparation with an inhibitory level (9.1 mM) of diethyldithiocarbamic acid and subsequent dilution to 0.18 mM allowed recovery of nearly all of the original activity. Run 3 shows the time dependent loss of enzyme activity due to incubation with tetraynoic acid. However, when the enzyme was treated with tetrayne in the presence of diethyldithiocarbamic acid (Run 4), the time-dependent inhibition by the ynoic acid was prevented. Since eicosa-5,8,11,14-tetraynoic acid exhibits both an instantaneous, concentration-dependent inhibition as well as a time-dependent destruction of the vesicular gland oxygenase, the term K_{I_i} will be used to describe the inhibition constant for the instantaneous, reversible effect and k_2' will be used for the first order rate constant for oxygenase destruction caused by the inhibitor. When the oxygenase activity was plotted *versus* incubation time of the oxygenase with tetrayne, a rate of loss of enzyme activity was obtained (Fig. 1B). This time-dependent inactivation of oxygenase, characterized by an apparent first order rate constant (k_2'), was evaluated by averaging the slopes for loss of enzyme activity shown in Fig. 1B and is given in Table V. The k_2' value appeared to be independent of both substrate and inhibitor concentrations at the levels tested. When the enzymic activities such as in Fig. 1B were measured at or

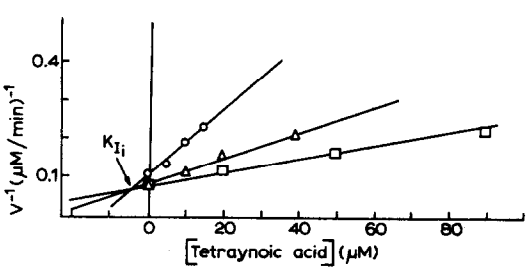

Fig. 2. Reversible inhibition of vesicular gland oxygenase by eicosatetraynoic acid. The reaction velocities corresponding to zero incubation time with the inhibitor and enzyme are expressed as product formation (μM/min) and the substrate concentration as μM. ○–○, 10 μM arachidonic acid; △–△, 30 μM; □–□, 50 μM.

extrapolated to zero incubation time, they provide the initial velocity values for estimating the instantaneous effect on enzymic activity. The reciprocal of these initial reaction velocities plotted *versus* inhibitor concentration[11] provided a K_{I_i} value for the tetraynoic acid from the intersection point of the various lines (Fig. 2). The position of the intersection point indicates that the instantaneous inhibition was competitively reversed with higher substrate concentrations.

In order to determine whether other ynoic acids exhibit the same reversible concentration-dependent and irreversible time-dependent destructive properties with oxygenase as eicosa-5,8,11,14-tetranoic acid does, the inhibitory characteristics of other acids were investigated. Among fourteen isomeric C_{18}-acetylenic acids tested, only three showed the time-dependent effects. These were Δ10a-18:1, Δ13a-18:1 and Δ9a,12a-18:2. Their respective K_{I_i} and k_2' values are listed in Table V. Since the possibility remained that an impurity in the octadecynoic acids might be responsible for the observed inhibition, the acids were purified by preparative thin-layer chromatography on silica gel H in heptane–isopropyl ether–acetic acid (60:40:3, by vol.). Identical results were again obtained with these purified acids. Furthermore, to unambiguously locate the acetylenic bond position and affirm the isomeric homogeneity of the octadecynoic acids, oxidative ozonolysis[7] was followed by esterification of the resulting dicarboxylic acids. Comparing the gas chromatographic behavior of these diesters with those obtained from the ozonolysis and methanolysis of oleic and *trans*-vaccenic acids (Table VI) provided no evidence for impurities and conclusively confirmed the original assignment of the location of the triple bond.

The observed time-dependent loss of oxygenase activity suggests that even though these acetylenic acids do not lead to any measurable oxygen consumption, they may still be serving in a manner analogous to substrate in facilitating a self-catalyzed destruction of oxygenase[6]. In the case of the acetylenic acids, however, the observed appreciable k_2' value has a negligible k_3 counterpart. This phenomenon could be interpreted in terms of the mechanism proposed earlier[6] if low levels of the hydroperoxide were bound to the product site and certain acetylenic acids were configurationally appropriate for inducing formation of the proposed planar tetroxide. In such a situation, the substrate analogs might form metastable intermediates that lead to destructive side reactions at the enzymic site without measurable amounts of oxygen being consumed. The concept that the 10 and 13 isomers may be configurationally suitable for intermediate formation whereas others, especially the 9 and 12 are not, was unexpected. Interestingly another shift of selectivity by one carbon atom was

TABLE VI

RETENTION TIMES AND EQUIVALENT CHAIN LENGTHS OF THE DIESTERS RESULTING FROM THE OZONOLYSIS OF UNSATURATED ACIDS

For experimental conditions, see text.

Unsaturated acid	*Ozonolysis product* $MeO_2C(CH_2)_nCO_2Me$ *(n)*	*Retention time (min)*	*Equivalent chain length*
Δ9c-18:1	7	7.80	17.77
Δ10a-18:1	8	9.88	18.70
Δ11t-18:1	9	12.60	19.63
Δ13a-18:1	11	20.64	21.50

recently observed in comparing *cis*-ethylenic and acetylenic thiol esters in acyltransferase reactions[12]. In that case, an examination of molecular models showed that the 10-acetylenic isomer may be configurationally equivalent to the *cis*-9-ethylenic acid. The relatively low K_{I_t} and relatively high k_2' values for the acetylenic acids suggest that they might very efficiently serve as site-destructive agents on the oxygenases that lead to prostaglandin formation *in vivo*. Since the acetylenic acids were not appreciably oxidized, they may act even more effectively than the naturally-occurring polyunsaturated acids which also cause the loss of enzyme[5]. Such an action might be considered as an ultramicrosurgical removal of an enzyme reaffirming an earlier suggestion[9] that synthesis of new protein could be a significant feature in regulating the continued production of prostaglandins in tissues.

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