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FURTHER CHARACTERIZATION OF BOVINE THYROID PROSTAGLANDIN SYNTHASE

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Summary

1. Prostaglandin synthase activity (EC 1.14.99.1) was demonstrated in bovine thyroid homogenates.

2. The synthase was characterized and shares many characteristics of the well-studied seminal vesicle enzyme and can be inhibited by indomethacin and eicosa-5,8,11,14-tetraynoic acid.

3. The enzyme is localized in the microsomal fraction and is probably associated with the plasma membranes.

4. Thyrotropin, but no other hormone tested, increased the activity of the enzyme when added to a microsomal fraction obtained from bovine thyroid. This effect is tissue-specific since thyrotropin has no effect on bovine seminal vesicle or lung prostaglandin synthase.

5. Thyrotropin, cyclic AMP and the phosphodiesterase inhibitors, theophylline and quazodine increase enzyme activity when preincubated with bovine thyroid slices.

6. EDTA, when included in the pre-incubation mixture, enhances the thyrotropin effect on the enzyme but not the cyclic AMP, theophylline, or quazodine augmentation of enzyme activity in bovine thyroid slices. This suggests that phospholipase A is involved in the thyrotropin stimulation of prostaglandin formation.

Introduction

We have shown that thyrotropin, cyclic AMP, dibutyryl cyclic AMP and the phosphodiesterase inhibitors, theophylline and quazodine increase pros-

taglandin levels in isolated bovine thyroid cells [1,2]. Haye, Champion and Jacquemin subsequently reported that thyrotropin increases the biosynthesis of prostaglandins in pig thyroid slices [3]. In preliminary studies [4], we observed that thyrotropin stimulates prostaglandin synthase (EC 1.14.99.1) activity in bovine thyroid homogenates. By the use of a more sensitive radioactive assay we wished to confirm and extend our previous observations on the bovine thyroid enzyme.

Materials

[5,6,8,9,11,12,14,15-^{3}H] Arachidonic acid (72 Ci/mmol) and [5,6,8,11, 12,14,15-^{3}H] prostaglandin E_2 (210 Ci/mmol) were purchased from New England Nuclear. 8,11,14-eicosatrienoic acid (99%) and 5,8,11,14,17-eicosapentaenoic acid (99%) were purchased from Applied Science Laboratores. Arachidonic acid (99%), oleic acid (99%), linoleic acid (99%), linolenic acid (99%), NADPH (Type I), cytochrome *c* (Type III) from horse heart, adenosine-5'-monophosphoric acid (Type III), glutathione, reduced form, hydroquinone, cyclic AMP and dibutyryl cyclic AMP were purchased from Sigma Chemical Co. *p*-Nitrophenyl phosphate (Grade A) was purchased from Calbiochem. Theophylline was purchased from MCB Co. and quazodine was supplied by Mead Johnson and Co. Basal medium (Eagle) with Earle's Salts was purchased from GIBCO. Thyrotropin in the form of Thytropar(®) was purchased from Armour Pharmaceutical. Indomethacin was suppled by Merck and Co. and eicosa-5,8,11,14-tetraynoic acid was supplied by Dr W.E. Scott (Hoffman-LaRoche).

Methods

Tissue preparation

Bovine thyroid glands were obtained fresh from the slaughterhouse and kept on ice from the time of removal up to the time of tissue preparation.

The thyroids were trimmed of excess fat and fascia and were finely minced. For whole homogenate studies, the finely minced thyroids were homogenized in 2 volumes of cold 0.01 M Tris · HCl pH 7.3 in the "polytron" homogenizer (Brinkman) and then centrifuged at 600 × *g* for 10 min. The resultant supernatant fraction was then filtered through a fine screen mesh and used directly for the assay.

When differential centrifugation was performed, the finely minced tissue was homogenized in 0.25 M Sucrose + 0.001 M EDTA pH 7.4 in the "polytron" at low speed setting. The different fractions were obtained by the method of de Duve et al. [5] with minor modifications. The N, or nuclear fraction was sedimented by a centrifugation of 600 × *g* for 10 min. The M, or mitochondrial fraction was sedimented by 3000 × *g* for 10 minutes. The L, or lysosomal fraction was sedimented by 20 000 × *g* for 10 min and the P, or polysomal (microsomal) fraction by 100 000 × *g* for 60 min. That which did not sediment was called the S, or supernatant fraction. All the particulate fractions were brought up to volume in buffer and were frozen and thawed 3 times in a dry ice-acetone mixture to release the latent enzyme activities.

Bovine thyroid slices (0.5 mm) were prepared from a single gland with the Stadie-Riggs microtome (Thomas).

Prostaglandin synthase assay

In the prostaglandin synthase assay employed in this study, conversion of [^{3}H] arachidonic acid to [^{3}H] prostaglandin E_2 serves as the index of enzyme activity. Formation of prostaglandin E_2 was assayed by a thin layer chromatographic method [6]. Plastic coated silica gel G plates (Brinkman, 5 cm × 20 cm) were used with the solvent system chloroform/methanol/acetic acid/water (90 : 9 : 1 : 0.65). This solvent system can effectively separate the various prostaglandins from arachidonic acid [7].

A typical assay mixture contained 0.5 μCi of [^{3}H] arachidonic acid (New England Nuclear, 72 Ci/mmol) and 50 μg of arachidonic acid (as the sodium salt) with Tris · HCl buffer pH 7.3 (20 mM) in a final reaction volume of 1 ml. Enzyme protein was approximately 20 mg for studies on whole homogenate.

The reaction time varied from 3 to 6 min at 37° C at which time 4 ml of absolute ethyl ether was added and the mixture was vigorously vortexed. Then, 0.5 ml of 0.03 M HCl was added and the samples were again vortexed. Finally, 0.5 ml of 1 M HCl was added to each tube, the samples vortexed, and centrifuged in a table-top clinical centrifuge for 5 min to facilitate the separation of the two phases. The top ether layer was removed from each sample and evaporated in a 37° C water bath. The original samples were then extracted two more times with ether, and the respective ether fractions were combined.

After the ether had been evaporated, 50 μl of chloroform/methanol (1 : 1) was added to each tube. After vortexing, 30 μl was spotted on the silica plates. [^{3}H] Prostaglandin E_2 was used as a "standard", and the solvent front was allowed to migrate 14 cm. After drying, the "standard" prostaglandin E_2 plate was cut into 1 cm strips and counted directly in 10 ml of Econofluor (New England Nuclear) in a Nuclear Chicago Mark II liquid scintillation counter to locate the prostaglandin E_2 peak. All the other strips were cut in a similar manner and the results expressed as the percentage of total counts applied to the chromatogram in the prostaglandin E_2 portion.

Prostaglandin synthase activity in bovine thyroid slices

Approximately 200 mg of tissue was pre-incubated for 10 min at 37° C in 5 ml of Minimal Essential Media with Earle's Salts (GIBCO), supplemented with 1% bovine serum albumin. After this pre-incubation, thyrotropin or other compounds tested were added and the incubation time varied from 6 to 18 min. The slices were then removed, blotted and immediately homogenized in 1.2 ml of cold 0.01 M Tris · HCl pH 7.3. This homogenate was then centrifuged at 9000 × *g* for 10 min, and the supernatant used for the assay. Approximately 8 mg of enzyme protein was used per assay tube.

Indomethacin was always freshly prepared just prior to use by weighing the proper amount and adding it to saline. The exact equivalents of NaOH were then added to solubilize the indomethacin. Tris · HCl buffer was then added immediately to give a final pH of 8.0. Eicosa-5,8,11,14-tetraynoic acid was prepared by adding the exact equivalents of NaOH and subsequent heating in a 37° C water bath.

Enzyme assays a. "Marker" enzymes: cytochrome oxidase was used as a marker for the mitochondrial fraction, and was assayed by the spectrophotometric method of Cooperstein and Lazarow [8] to determine the rate of oxidation of reduced cytochrome *c*. Acid phosphatase was used as a marker for the lysosomal fraction. *p*-Nitrophenyl phosphate was used as substrate and activity measured by the release of *p*-nitrophenolate [9]. Catalase was used as a marker for peroxisomes and was assayed by the procedure of Martin and Ames [10]. Alkaline phosphatase and NADPH-cytochrome *c* reductase were used as microsomal markers. Alkaline phosphatase was assayed by the method of Brockman [11] using *p*-nitrophenyl phosphate as substrate. NADPH-cytochrome *c* reductase was assayed by following the reduction of cytochrome *c* by NADPH [12]. 5'-Nucleotidase was used as a marker for plasma membranes and was assayed by the method of Emmelot using 5'-AMP as substrate [13].

Protein was measured by the method of Lowry et al. [14].

Prostaglandin E_2 was determined by radioimmunoassay with an antibody specific for prostaglandins E developed in our laboratory [1,15]. Cross reactivities were 1.5% for prostaglandin $F_{2\alpha}$, 1.9% for prostaglandin A and 0.4% for prostaglandin B.

Results

1. The solvent system used in these studies can effectively separate prostaglandin E_2 (R_F = 0.43) from arachidonic acid (R_F = 0.86). In addition, we were able to confirm by radioimmunoassay that, in fact, authentic prostaglandin E_2 had been formed in that part of the chromatogram used for prostaglandin E_2 determination.

2. Kinetic properties of the enzyme

The bovine thyroid prostaglandin synthase was characterized; it was found that the activity increased with increased enzyme concentration in a linear fashion. Normal Michaelis-Menton kinetics were observed with a K_m of $1.6 \cdot 10^{-4}$ M and a V of 11 ng prostaglandin E_2 formed/min/mg protein. The optimal incubation temperature was 37°C, and the optimal time of incubation was 3 min. The pH optimum was between pH 7.0 and pH 7.3.

Reduced glutathione had no significant effect on bovine thyroid prostaglandin synthase activity, as opposed to its stimulatory effect in seminal vesicles [16]. Hydroquinone (0.1—0.5 mM) increased the activity by ≈25%.

3. Intracellular localization of prostaglandin synthase

Fig. 1 shows the distribution pattern for various enzymes in subcellular fractions obtained by differential centrifugation of bovine thyroid homogenate. It can be seen that the various "markers" give distinct distribution patterns. Note that prostaglandin synthase has its peak in the P fraction with an enrichment of 3 over the whole homogenate. There is also twice as much "synthase" activity in the N fraction as in the S fraction, thereby resembling the plasma membrane "marker" pattern as opposed to the microsomal "marker" pattern. The data indicate that the "synthase" is found in the polysomal (microsomal) fraction (100 000 × *g* fraction) and most probably in the plasma membrane.

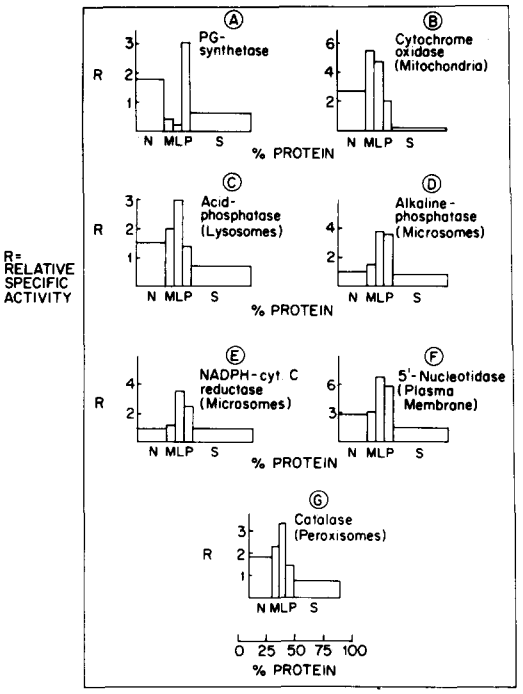

Fig. 1. Distribution patterns for various enzymes in subcellular fractions determined by differential centrifugation of bovine thyroid. Abbreviations are as follows: N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, polysomal (microsomal) fraction; S, highspeed supernatant fraction. Enzymes tested are labelled as follows: A, prostaglandin synthase; B, cytochrome oxidase; C, acid phosphatase; D, alkaline phosphatase; E, NADPH-cytochrome *c* reductase; F, 5′ nucleotidase and G, catalase. *R* on relative specific activity is plotted against % protein, where % protein is the percentage of protein in a fraction compared with the sum of the protein in all fractions.

The apparent inhibition in the M and L fractions as compared to other polysomal "marker" enzymes will be discussed later (vide infra).

4. Effect of indomethacin on enzyme activity

Fig. 2 shows a plot of thyroid "synthase" activity versus indomethacin concentration. It can be seen that indomethacin can effectively inhibit the enzyme.

Fig. 3 details an experiment where the arachidonic acid concentration was varied and the indomethacin concentration kept constant at 10 μg/ml. Activity increased as the substrate concentration was increased from 10 to 50 μg; however, no further increase was noted at 150 μg arachidonic acid, in contrast to the non-indomethacin treated control. This indicates that the inhibition by indomethacin is of the non-competitive variety.

5. Effect of eicosa-5,8,11,14-tetraynoic acid and other related fatty acids on enzyme activity

Eicosa-5,8,11,14-tetraynoic acid at a concentration of 20 μg/ml inhibited the "synthase" activity by 85% when the arachidonic acid concentration was

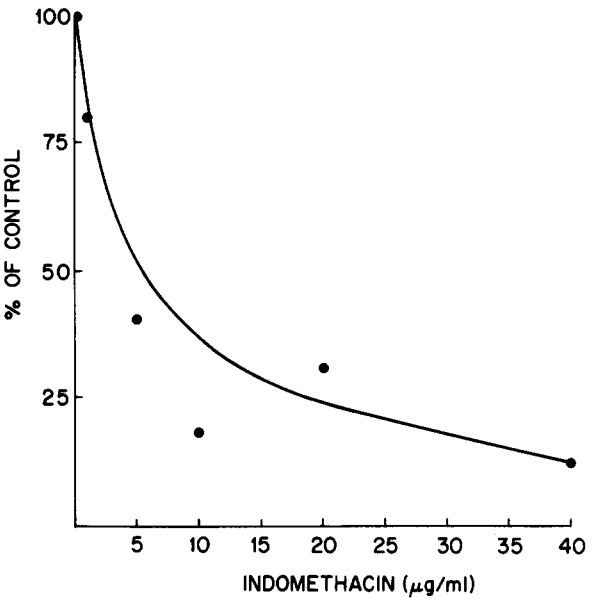

Fig. 2. Inhibition of prostaglandin synthase by varying doses of indomethacin: arachidonic acid concentration of 20 μg/ml and a polysomal enzyme fraction was used (10 mg). % of "O" indomethacin control is plotted against indomethacin concentration. The enzyme was preincubated with indomethacin for 3 min, the reaction initiated with substrate and allowed to proceed for 6 min.

2.5 μg/ml (Fig. 4). Subsequent experiments indicate that this inhibition is of the competitive reversible type.

Other fatty acids were also tested as potential inhibitors of the enzyme. The results (Fig. 4) show that 8,11,14-eicosatrienoic acid and 5,8,11,14,17-eicosapentaenoic acid can effectively inhibit the enzyme even at low concentrations. The inhibitory effects of the other fatty acids were directly related to their structural similarity to arachidonic acid (linolenic > linoleic > oleic).

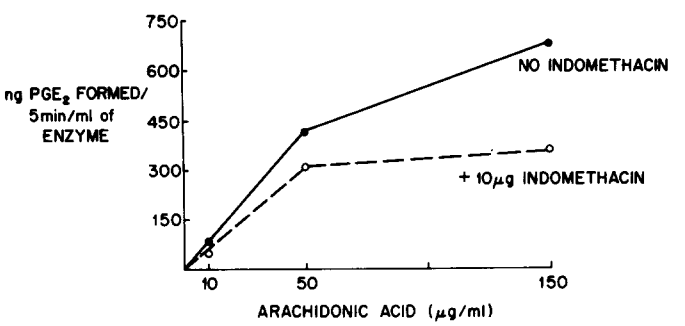

Fig. 3. Effect of varying arachidonic acid concentration on the indomethacin inhibition of prostaglandin synthase: a polysomal enzyme fraction (10 mg) was used and the arachidonic acid concentration varied from 10 to 150 μg/ml. ng prostaglandin E_2 formed/5 min/ml of enzyme was compared for the non-indomethacin control and the indomethacin (10 μg/ml) reaction. The enzyme was pre-incubated with indomethacin for 1 min at 37°C and the reaction initiated with substrate.

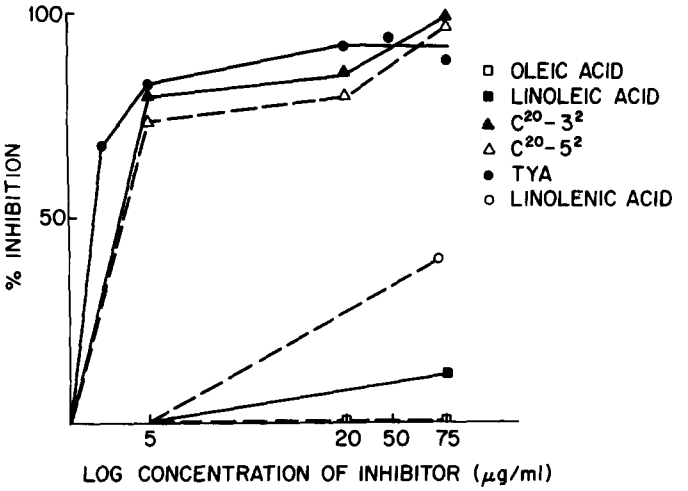

Fig. 4. Inhibition of prostaglandin synthase by fatty acids and eicosa-5,8,11,14-tetraynoic acid. A polysomal fraction (10 mg) was used for these studies. The arachidonic acid concentration was 2.5 μg/ml. The enzyme was preincubated for 1 min at 37°C with the various fatty acids, and the reaction was then initiated with arachidonic acid. C^{20}-3^2 = 8,11,14-eicosatrienoic acid; C^{20}-5^2 = 5,8,11,14,17-eicosapentaenoic acid. "Control" in 3 experiments, 0.4—0.9 ng prostaglandin E_2/min/mg protein; average value, 0.7 ± 0.3 ng prostaglandin E_2/min/mg protein.

6. Effect of thyrotropin, cyclic AMP and phosphodiesterase inhibitors on enzyme activity

a. When thyrotropin was added directly to the incubation mixture, an increase in "synthase" activity occurred (Table I). This increase was tissue- and hormone-specific.

b. Thyrotropin, dibutyryl cyclic AMP, theophylline and quazodine increased "synthase" activity when pre-incubated with thyroid slices (Tables II and III). These increases were statistically significant as determined by the student t test, with p values of $p < 0.025$ for thyrotropin, $p < 0.01$ for the cyclic nucleotide, and $p < 0.05$ for the phosphodiesterase inhibitors. The in-

TABLE I

EFFECT OF ADDING THYROTROPIN, CYCLIC AMP, OR DIBUTYRYL CYCLIC AMP DIRECTLY TO THE INCUBATION MIXTURE ON PROSTAGLANDIN SYNTHASE ACTIVITY

Incubation conditions were as outlined in Materials and Methods. A polysomal fraction was used (25 mg/ml) and the arachidonic acid concentration was 50 μg/ml.

Compound	Number of experiments	Average % increase over control*
Thyrotropin (20 munits/ml)	3	+25%
Thyrotropin (50 munits/ml)	7	+30%
Thyrotropin (100 munits/ml)	3	+19%
Dibutyryl cyclic AMP (3 mM)	2	0
Cyclic AMP (3 mM)	3	0

* "Control" in 8 experiments, 1.3—5.7 ng prostaglandin E_2/min/mg protein; average value, 3.6 ± 1.3 ng prostaglandin E_2/min/mg protein.

TABLE II

EFFECT OF THYROTROPIN ON PROSTAGLANDIN SYNTHASE ACTIVITY IN BOVINE THYROID SLICES

Incubation conditions were as outlined in Materials and Methods. Thyroid slices were pre-incubated for the designated times with 100 munits/ml thyrotropin, homogenized and then assayed for prostaglandin synthase. Arachidonic acid concentration was 30 μg/ml.

Time of pre-incubation (min)	Number of experiments	Average % increase over control*	
6	5	(4/ 5)	+35%
		(1/ 5)	−12%
12	5	(3/ 5)	+18%
		(2/ 5)	−13%
15	13	(8/13)	+30%
		(2/13)	0
		(3/13)	−26%
18	10	(7/10)	+35%
		(3/10)	0

* "Control" in 30 experiments, 1.0—7.0 ng prostaglandin E_2/min/mg protein; average value, 3.8 ± 2.0 ng prostaglandin E_2/min/mg protein.

crease for thyrotropin is hormone-specific since boiled thyrotropin failed to show this increase; neither did other hormones tested.

7. Effect of EDTA in the pre-incubation mixture on enzyme activity in thyroid slices

EDTA, when included in the pre-incubation mixture with thyroid slices, enhanced the response of the enzyme to thyrotropin (Table IV). However,

TABLE III

EFFECT OF DIBUTYRYL CYCLIC AMP, THEOPHYLLINE AND QUAZODINE ON PROSTAGLANDIN SYNTHASE ACTIVITY IN BOVINE THYROID SLICES

Compound	Time of pre-incubation (min)	Number of experiments	Average % increase over control*	
Dibutyryl cyclic AMP (3 mM)	6	7	(3/ 7)	+38%
			(1/ 7)	0
			(3/ 7)	−21%
Dibutyryl cyclic AMP (3 mM)	12	2	(2/ 2)	+22%
Dibutyryl cyclic AMP (3 mM)	18	10	(8/10)	+65%
			(2/10)	0
Theophylline (2 mM)	6	6	(5/ 6)	+66%
			(1/ 6)	−23%
Theophylline (2 mM)	12	4	(3/ 4)	+40%
			(1/ 4)	−28%
Theophylline (2 mM)	18	8	(6/ 8)	+70%
			(2/ 8)	0
Quazodine (1 mM)	6	6	(5/ 6)	+37%
			(1/ 6)	0
Quazodine (1 mM)	18	7	(5/ 7)	+36%
			(2/ 7)	0

* "Control" in 21 experiments, 1.3—6.9 ng prostaglandin E_2 min/mg/protein; average value 3.2 ± 1.2 ng prostaglandin E_2 min/mg/protein.

TABLE IV

EFFECT OF EDTA IN COMBINATION WITH THYROTROPIN, DIBUTYRYL CYCLIC AMP, THEOPHYLLINE, OR QUAZODINE IN PRE-INCUBATION MIXTURE ON PROSTAGLANDIN SYNTHASE ACTIVITY

Compound	Number of experiments	Average % increase over "control"*
Thyrotropin (100 munits/ml)	8	+28%
Thyrotropin (100 munits/ml) + 5 mM EDTA	5	+75%
Thyrotropin (100 munits/ml) + 20 mM EDTA	8	+45%
Dibutyryl cyclic AMP (3 mM)	5	+28%
Dibutyryl cyclic AMP (3 mM) + 5 mM EDTA	2	0
Dibutyryl cyclic AMP (3 mM) + 20 mM EDTA	5	0
Theophylline (2 mM)	2	+22%
Theophylline (2 mM) + 5 mM EDTA	2	+18%
Theophylline (2 mM) + 20 mM EDTA	2	0
Quazodine (1 mM)	3	+42%
Quazodine (1 mM) + 5 mM EDTA	2	+45%
Quazodine (1 mM) + 20 mM EDTA	3	0

* "Control" in 13 experiments, 2.0—6.9 ng prostaglandin E_2/min/mg protein; average value, 3.8 ± 1.4 ng prostaglandin E_2/min/mg protein.

EDTA had no stimulatory effect on the dibutyryl cyclic AMP, theophylline, or quazodine responses.

Discussion

We have previously reported that thyrotropin, dibutyryl cyclic AMP and phosphodiesterase inhibitors increase prostaglandin levels in isolated bovine thyroid cells [1,2]. This increase can be accounted for in various ways: (1) there is an activation of the prostaglandin synthase enzyme system; (2) there is an inhibition of the prostaglandin degradative system; (3) there is an increase in substrate availability; (4) there is a shift of other prostaglandins to the E form; (5) a combination of one or more of the above. This study shows that the above effectors can activate the "synthase" system, thereby causing an increase in prostaglandin levels.

The bovine thyroid enzyme was characterized in the usual kinetic fashion, and by the use of differential centrifugation and "marker" enzymes we were able to show that the "synthase" activity is associated with the polysomal (microsomes and plasma membrane) fraction. What is puzzling is the definite inhibition in the M and L fractions as compared to other known polysomal enzymes. Two attractive hypotheses to account for this phenomenon involve the enzymes catalase and phospholipase A. Catalase, which is predominantly found in the M and L fractions (Fig. 1), can effectively inhibit "synthase" activity [17]. This would then account for the observed inhibition in these fractions. Phospholipase A can effectively hydrolyze arachidonic acid from endogenous phospholipids [3]. This lysosomal enzyme could then be diluting [^{3}H] arachidonic acid with "cold" arachidonic acid, thereby spuriously decreasing conversion of [^{3}H] arachidonic acid to [^{3}H] prostaglandin E_2.

Indomethacin and eicosa-5,8,11,14-tetraynoic acid, known "synthase"

inhibitors [6,18], inhibited the thyroidal enzyme although higher concentrations of these inhibitors were necessary than for the seminal vesicle enzyme [6]. Our findings also indicate that the inhibition by eicosa-5,8,11,14-tetraynoic acid is of the reversible, competitive type. In contrast, the inhibition by indomethacin cannot be entirely overcome with increased arachidonic acid, and appears to be of the non-competitive type. Other polyunsaturated fatty acids, closely related to arachidonic acid, also inhibited the "synthase". Inhibition increased as the fatty acid more closely resembled arachidonic acid.

The present studies confirm our earlier demonstration [4] that thyrotropin, when added directly to the incubation mixture, increases prostaglandin synthase activity. This effect was hormone- and tissue-specific. This direct activation of the enzyme could then explain the rise in prostaglandin levels in a homogeneous thyroid cell preparation effected by thyrotropin [1]. Furthermore, our findings that thyrotropin, dibutyryl cyclic AMP, as well as the phosphodiesterase inhibitors enhanced "synthase" activity when pre-incubated with thyroid slices, suggest that the increase in intracellular prostaglandin levels effected by these compounds is consequent to activation of the synthase system.

If thyrotropin acts "in-vivo" by releasing endogenous arachidonic acid from phospholipids, as suggested by Haye and Champion [3] in addition to its activation of the synthase enzyme, then addition of EDTA, a known phospholipase *A* inhibitor should result in an increase of synthase activity as measured by conversion of [^{3}H] arachidonic acid to [^{3}H] prostaglandin E_2, since no "cold" arachidonic acid is released to dilute the radioactive counts. Dibutyryl cyclic AMP and theophylline do not act via the phospholipase A, thus the addition of EDTA should not enhance the conversion.

Our results confirm this hypothesis and show that EDTA enhanced the thyrotropin response but not the dibutyryl cyclic AMP, theophylline, or quazodine responses. (The lower values seen with EDTA could, in this instance, reflect tissue damage.)

These findings suggest that thyrotropin increases prostaglandin levels in slices by: (1) increasing "synthase" activity and (2) making more substrate available via activated phospholipase A.

Although the precise role of prostaglandin synthase in regulating thyroid function remains to be defined, the results reported herein reinforce our working thesis that intracellular prostaglandins may modulate thyroidal response to thyrotropin.

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