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Modification of TSH-stimulated adenylate cyclase activity of bovine thyroid by manipulation of membrane phospholipid composition with a nonspecific lipid transfer protein

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The lipid composition of bovine thyroid plasma membranes was modified using the nonspecific lipid transfer protein from bovine liver. Incubation of plasma membranes with transfer protein and phosphatidylinositol-containing liposomes caused a strong, concentration dependent, inhibition of TSH-stimulated adenylate cyclase activity. Other phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidic acid were two to four times less effective as inhibitors of TSH-stimulation. The phosphatidylinositol-induced inhibition was not reversed when more than 80% of phosphatidylinositol incorporated was removed using phosphatidylinositol-specific phospholipase C. Incorporation of phosphatidylinositol in plasma membranes provoked no significant change in the fluorescence anisotropies of the fluorophores 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(14-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), indicating that the inhibition was not due to changes in membrane fluidity. At phosphatidylinositol concentrations causing a 66% reduction in TSH-stimulated adenylate cyclase activity cholera toxin- and forskolin-stimulated activity as well as basal activity were decreased by maximally 10%. Since TSH binding to bovine thyroid plasma membranes was not affected it is suggested that phosphatidylinositol can act as a negative modulator of the TSH activation of adenylate cyclase and this probably by interfering with the coupling between the occupied TSH receptor and the stimulatory GTP-binding regulatory protein of the adenylate cyclase complex.

Introduction

The membrane-bound enzyme adenylate cyclase forms a transmembrane complex composed of at least three distinct classes of protein components: hormone receptors, the guanine-nucleotide-binding regulatory protein(s) (G-proteins) and the catalyst [1,2] and is regulated by interaction of these components in a relatively unperturbed membrane (for reviews, see Refs. 1–4). Optimal hormonal control appears to be influenced by the

Abbreviations: $G_{s\alpha}$, α-subunit of stimulatory GTP binding regulatory protein; TSH, thyroid-stimulating hormone or thyrotropin; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; SUV, sonicated small unilamellar lipid vesicles; buffer A, 0.25 M sucrose in 20 mM Tris-HCl buffer (pH 7.4); DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

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physicochemical state and composition of the lipid environment of the components of the adenylate cyclase system [5–8].

More specifically, a requirement for specific membrane lipids has been suggested in TSH-responsive adenylate cyclase system [9–12]. As part of a systematic study of lipid–protein interactions involved in TSH-action we previously studied the relationship between bovine thyroid plasma membrane composition and its 'structural' and 'fluidity' characteristics [13]. In the present study, we describe the effect of incorporation of various phospholipids in thyroid plasma membranes on TSH-stimulated adenylate cyclase activity. Phospholipids were incorporated using bovine liver nonspecific transfer protein (ns-TP) and small unilamellar vesicles (SUV). Transfer protein has been shown to promote the net transfer of lipid molecules between lipid vesicles and biological membranes [8,14,15] under relatively nonperturbing conditions, thus reducing the ambuigity in the interpretation of the data.

Materials and Methods

Materials. L-3-Phosphatidyl[2-^{3}H]inositol (15.6 Ci/mmol); L-1,2-di[1-^{14}C]palmitoyl-3-phosphatidylcholine (117 mCi/mmol); L-1,2-di[1-^{14}C]palmitoyl-3-phosphatidic acid (117 mCi/mmol); L-1,2-dioleoyl-3-phosphatidyl-L-[3-^{14}C]serine (31 mCi/mmol); L-1,2-dioleoyl-3-phosphatidyl[2-^{14}C]ethanolamine (50 mCi/mmol); glycerol [9,10-^{3}H]trioleate (397 mCi/mmol) and glycerol [1-^{14}C]trioleate (60 mCi/mmol) were obtained from Amersham. Phospholipids were purchased from Sigma. Thyrotropin came from Armour Laboratories. Phosphatidylinositol-specific phospholipase C was purified from *Staphylococcus aureus* as described by Low and Finean [16] and was a generous gift of Dr. Martin Low (Oklahoma Medical Research Foundation). Aldrich was the source of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was obtained from Molecular Probes Inc. [α-^{32}P]ATP was provided by NEN Research Products. Highly purified TSH (15–20 IU/mg) came from UCB, Bioproducts and was radioiodinated by means of the iodinated beads system from NEN, which uses the lactoperoxidase method. Following iodination, ^{125}I-TSH was separated from unreacted iodide by chromatography on Sephadex G-100.

Preparation of plasma membranes. Plasma membranes with a protein to phospholipid ratio (w/w) of 3.91 ± 0.15 were prepared from fresh bovine thyroid glands as described previously [17]. They were stored in liquid N_2 at $-180\,^{\circ}C$ immediately after preparation.

Chemical analysis. Protein concentration was estimated by the procedure of Lowry et al. [18] using bovine serum albumin as the standard. Phospholipid phosphorus was assayed according to Rouser et al. [19]. Cholesterol was determined by the method of Hanel and Dam [20]. The phospholipid composition of plasma membranes was assayed by performing two dimensional thin-layer chromatography on precoated silicagel 60 plates (Merck, 10×10 cm) on a Bligh and Dyer [21] lipid extract of plasma membranes using the following solvent systems: (a) chloroform/methanol/methylamine 40% (13:6:1.5, v/v) and (B) chloroform/acetone/methanol/acetic acid/water (100:40:20:30:10, v/v) [22].

Lipid vesicles. Small unilamellar vesicles (SUV) were prepared according to the method of Johnson and Zilversmit [23] except that the lipid suspension was sonicated with a Braun-Sonic 300 homogenisator (Quigley-Rochester, Inc.) using a 4.0 mm diameter titanium probe. SUV were prepared in buffer A (0.25 M sucrose in 20 mM Tris-HCl buffer (pH 7.4)). Before incubation with bovine thyroid plasma membranes and nonspecific transfer protein, SUV were centrifuged at $10\,000 \times g$ for 30 min to remove multilamellar liposomes.

Lipid transfer assay. The nonspecific lipid transfer protein from bovine liver was prepared by the method of Crain and Zilversmit [24]. Lipid transfer activity was determined by measuring the transfer of labelled lipid from SUV to bovine thyroid plasma membranes in buffer A. Exchange activity was expressed in units representing the transfer of 1% phosphatidylcholine of total SUV PC from [^{14}C]PC/cholesterol SUV (0.15 μmol phospholipid, cholesterol to phospholipid molar ratio of 0.4) to bovine thyroid plasma membranes (0.6 μmol phospholipid) in a total volume of 0.5 ml in 1 h at 37 °C. In a typical assay bovine

thyroid plasma membranes (0.6 μmol phospholipid) were incubated with labelled phospholipid-containing SUV (0.15 μmol phospholipid, cholesterol to phospholipid molar ratio of 0.4) and 13–14 units of transfer protein for 1 h at 37 °C in a total volume of 0.5 ml. SUV contained also [^{14}C]triolein or [^{3}H]triolein as a nonexchangeable marker. Incubations were always performed in buffer A. Exchange was terminated by centrifugation at $10000 \times g$ for 30 min. Bovine thyroid plasma membranes were washed three times with buffer A. The percentage lipid transfer was calculated from the radioactivity counted in the pellet (bovine thyroid plasma membranes) as well as in the supernatant. A correction for cosedimentation of vesicles was done by quantitation of the nonexchangeable marker [^{14}C]triolein or [^{3}H]triolein. It was assumed that there was no return of label from the membranes to SUV.

Modification of bovine thyroid plasma membranes by phospholipid incorporation. Phospholipids were incorporated in bovine thyroid plasma membranes (0.6 μmol phospholipid) using the same conditions as described for the lipid transfer assay. Control incubations contained bovine thyroid plasma membranes with or without transfer protein only. After incubation the mixture was centrifuged at $10000 \times g$ for 30 min. Bovine thyroid plasma membranes were washed three times with the appropriate buffer. The washed plasma membranes were then suspended in an appropriate volume of buffer and aliquots were taken for adenylate cyclase assay, binding assay, anisotropy measurements and Bligh and Dyer lipid extraction.

Adenylate cyclase assay. Adenylate cyclase activity was measured by adapting the methodology of Salomon [25]. Incubations were performed for 30 min at 30 °C in a final volume of 60 μl containing: approx. 100 μg protein; approx. $3 \cdot 10^6$ cpm of [α-^{32}P]ATP (30–35 Ci/mmol); 5 mM creatine phosphate; 50 IU/ml creatine phosphokinase; 25 mM Tris-acetate buffer (pH 7.4); 5 mM magnesium acetate; 0.5 mM ATP; 0.05 mM cAMP; 1 mM dithiothreitol; 0.1 mg/ml bovine serum albumin; 0.01 mM GTP; 10 mM theophyline. The reaction is terminated by the addition of 110 μl stopping solution (45 mM ATP and 1.3 mM cAMP in 2% (w/v) sodium laurylsulfate (pH 7.5)), 50 μl [8-^{3}H] 3′,5′-cAMP ($2 \cdot 10^5$ cpm/ml) and heating for 3 min in a boiling water bath.

TSH binding assay. TSH binding was assayed in 40 mM Tris-acetate buffer (pH 6.0); 0.25% bovine serum albumin; approx. 100000 cpm ^{125}I-TSH (about 1 ng of protein) and 20 μg of membrane protein in a final volume of 100 μl. Specific binding of 20 μg of membranes gave a value that is on the linear part of the binding curve when cpm bound is plotted against amount of membrane protein. Incubations were performed for 1 h at 4 °C. The incubation was terminated by addition of 1 ml 40 mM Tris-acetate (pH 6.0) containing 2.5% bovine serum albumin. Bound radioligand was separated from free ^{125}I-TSH by filtration through 0.45 μm cellulose acetate filters placed in a 12-place vacuum filtration manifold VFM3 (Amicon). The filters were presoaked in 2.5% bovine serum albumin and the wash buffer was 40 mM Tris-acetate (pH 6.0) containing 2.5% bovine serum albumin. Nonspecific binding as measured by the addition of a 10000-fold excess of unlabelled TSH ($4 \cdot 10^{-6}$ M) amounted to 7% of the specific binding.

Fluorescence measurements. Diphenylhexatriene was made up in tetrahydrofuran at a concentration of $4 \cdot 10^{-4}$ M. For labelling, this solution was diluted 1000-fold by adding to vigourously stirred buffer A. A stock solution of $4 \cdot 10^{-7}$ M TMA-DPH was prepared by diluting 1000-fold a $4 \cdot 10^{-4}$ M TMA-DPH solution in in chloroform with buffer A. DPH and TMA-DPH were added to plasma membranes at a ratio of one molecule fluorophore for every 500 phospholipid molecules. The final lipid phosphorus concentration was always adjusted to 50 μM. This was important in securing constant 'dilute' anisotropy values. Anisotropy measurements were performed at 37 °C on a SLM 4800 spectrofluorometer. Temperature was controlled by Lauda thermostated water-bath and measured inside the cuvette with an AD590 probe (Analog Devices). For both fluorophores the excitation wavelength was 360 nm and Schott KV399 filters were used in both emission beams.

Results

Phospholipid incorporation in thyroid plasma membranes

As shown in Tables I and II, the nonspecific

TABLE I

LIPID TRANSFER FROM SUV TO BOVINE THYROID PLASMA MEMBRANES CATALYZED BY TRANSFER PROTEIN

For incubation conditions see Materials and Methods. Lipid transfer is expressed as percent of the indicated radioactive labelled lipid. SUV compositions are expressed as molar ratios. Cholesterol/phospholipid molar ratios were determined on a Bligh and Dyer lipid extract of bovine thyroid plasma membranes. Results are averages ± S.E. of duplicate determinations of three separate experiments. CH, cholesterol; PL, phospholipid; ns-TP, nonspecific lipid transfer protein.

SUV	ns-TP	% lipid transfer	CH/PL
[^{14}C]PC/CH (1:0.4)	−	1.1 ± 0.2	0.40 ± 0.04
	+	13.5 ± 1.2	0.38 ± 0.03
[^{14}C]PC/PI/CH (1:1:0.8)	−	3.4 ± 0.3	0.39 ± 0.05
	+	27.0 ± 1.9	0.37 ± 0.03
PC/[^{3}H]PI/CH (1:1:0.8)	−	11.1 ± 1.0	0.38 ± 0.04
	+	42.4 ± 2.8	0.37 ± 0.03

lipid transfer protein of bovine liver was able to promote the exchange of anionic (PS,PI,PA) as well as zwitterionic (PC,PE) phospholipids. Anionic phospholipids exhibited nearly the same transfer activity which was 2–3-times higher than that observed with zwitterionic phospholipids (Table III). The transfer of zwitterionic phospholipids (PC), however, was significantly (2-fold) enhanced when acidic phospholipids (PI) were present in the donor vesicles (Table I). The spontaneous, non-protein-mediated transfer of acidic phospholipids was, respectively, 2- and 10-times higher than that of PE and PC (Table III). Transfer of a particular phospholipid occurring in the absence of transfer protein was always less than 25% of the total transfer observed in the presence of transfer protein. The donor vesicles in all experiments had a cholesterol to phospholipid molar ratio of 0.4, a ratio identical to that of bovine plasma membranes [13]. Incubation of bovine thyroid plasma membranes with SUV of this composition in the presence of transfer protein did not affect the molar ratio of phospholipids to cholesterol in the plasma membranes (Table I). The data in Table II demonstrate that a net change in phospholipid composition of bovine thyroid plasma membranes occurred after incubation with SUV and transfer protein. In all experiments the plasma membrane phospholipid content did not differ significantly between control incubations and those containing SUV and transfer protein indicating that no net transfer of total phospholipid mass occurred. The phospholipid composition of plasma membranes was also not affected after incubation with transfer protein in the absence of exogenous phospholipids. Incubation with SUV and transfer protein resulted in a decrease in the percentage of sphingomyelin and an increase in the percentage of phosphatidylcholine (Table II; $P < 0.05$, Student's t-test). The percentage phosphatidylethanolamine only increased after incubation with phosphatidylethanolamine-containing SUV and transfer protein. Incubation of bovine thyroid

TABLE II

PHOSPHOLIPID COMPOSITION OF BOVINE THYROID PLASMA MEMBRANES AFTER INCUBATION WITH SUV AND TRANSFER PROTEIN

For incubation conditions see Materials and Methods. The phospholipid composition of plasma membranes was assayed on a Bligh and Dyer lipid extract of plasma membranes as described in Materials and Methods. Control incubations contained bovine thyroid plasma membranes with either no additions or with transfer protein alone. SUV compositions are expressed as molar ratios. All values are expressed as percent of total phospholipid phosphorus. All data are average values ± S.D. for three separate experiments. CH, cholesterol; SPH, sphingomyelin; PA, phosphatidic acid.

SUV	PI + PS	SPH	PC + lysoPC	PE + lysoPE	PA
None	13.0 ± 0.9	23.0 ± 2.2	39.6 ± 3.1	24.4 ± 1.5	–
PC/CH (1:0.4)	11.9 ± 0.8	21.8 ± 1.8	43.2 ± 3.2	23.1 ± 1.3	–
PC/PE/CH (1:1:0.8)	10.6 ± 0.9	19.4 ± 2.1	43.1 ± 2.4	26.9 ± 1.1	–
PC/PI/CH (1:1:0.8)	18.3 ± 1.8	18.0 ± 2.3	42.7 ± 3.2	21.0 ± 1.8	–
PC/PS/CH (1:1:0.8)	18.2 ± 1.6	17.9 ± 1.6	43.0 ± 2.3	20.9 ± 1.1	–
PC/PA/CH (1:1:0.8)	10.9 ± 1.2	18.7 ± 1.5	42.9 ± 2.5	22.7 ± 1.7	4.8 ± 0.4

TABLE III

INHIBITION OF TSH STIMULATED ADENYLATE CYCLASE ACTIVITY BY VARIOUS PHOSPHOLIPIDS

For incubation conditions see Materials and Methods. Control incubations contained bovine thyroid plasma membranes with either no additions or with transfer protein alone. The final concentration of TSH was 50 mU/ml. This TSH concentration is situated at the beginning of the plateau of the dose-response curve. Percent inhibition was determined relative to control incubations. SUV compositions are expressed as molar ratios. Results are means ± S.E. of duplicate determinations of four different experiments. CH, cholesterol; PA, phosphatidic acid; ns-TP, nonspecific lipid transfer protein.

SUV	ns-TP	% lipid transfer	cAMP (pmol/mg protein per 30 min)	Inhibition (%)
–	–	–	0.60 ± 0.04	–
–	+	–	0.60 ± 0.04	< 1
PC/CH (1:0.4)	–	1.1 ± 0.2	0.59 ± 0.03	2
	+	13.5 ± 1.2	0.51 ± 0.04	14
PC/PI/CH (1:1:0.8)	–	11.0 ± 1.0	0.50 ± 0.06	17
	+	42.4 ± 2.8	0.20 ± 0.03	66
PC/PE/CH (1:1:0.8)	–	5.1 ± 0.4	0.58 ± 0.03	3
	+	19.3 ± 1.6	0.54 ± 0.04	10
PC/PS/Ch (1:1:0.8)	–	10.7 ± 0.9	0.56 ± 0.03	7
	+	41.6 ± 2.6	0.44 ± 0.05	26
PC/PA/CH (1:1:0.8)	–	10.0 ± 0.8	0.56 ± 0.03	7
	+	41.0 ± 2.5	0.43 ± 0.05	28

plasma membranes with phosphatidylcholine/cholesterol SUV containing another phospholipid resulted in plasma membranes enriched in that particular phospholipid (Table II). Furthermore, the percentage increase of a certain phospholipid coincided always very well with that calculated from the amount which was incorporated of that particular phospholipid. For different plasma membrane preparations the amount of a particular phospholipid transferred from bovine thyroid plasma membranes to SUV by transfer protein was variable. These differences might be due to variations in the ratio of right-side out to inside-out vesicles in different plasma membrane preparations, because phospholipids on the inside surface of the membrane vesicles appear to be less exchangeable than the outside phospholipids [26]. Electron micrographs of thyroid plasma membrane preparations revealed that the plasma membranes were almost exclusively present under the form of closed vesicular structures. The percentage right-side out vesicles in bovine thyroid plasma membranes preparations was estimated to be approximately 80% as determined by inhibition of the ecto-enzyme AMPase after treatment with the diazonium salt of sulfanilic acid (data not shown).

Effect of modification of membrane phospholipid composition on adenylate cyclase activity

Incubation of bovine thyroid plasma membranes with lipid-exchange proteins only did not affect TSH stimulation. On the other hand incubation of plasma membranes with lipid-exchange proteins and phosphatidylinositol-containing liposomes led to a signifiant decrease in TSH-stimulated adenylate cyclase activity ($P < 0.001$) (Table III). Incubation of plasma membranes with 13–14 units of transfer protein and phosphatidylserine- or phosphatidic acid-containing SUV resulted in approximately the same percentage lipid transfer as with PI-containing SUV (approx. 41%), but provoked a much smaller decrease in TSH-stimulated adenylate cyclase activity (approx. 27% vs. 66%) (Table III). The percentage inhibition of TSH-stimulated adenylate cyclase activity varied linearly with the amount of each phospholipid incorporated (Fig. 1). The data were fit to straight lines by the method of linear regression. The correlation coefficient was always around 0.8 and the correlation was significant at a $P <$ 0.005 level. Calculation of the slopes of the regression lines revealed that phosphatidylinositol was 2.5-times more effective as inhibitor of TSH

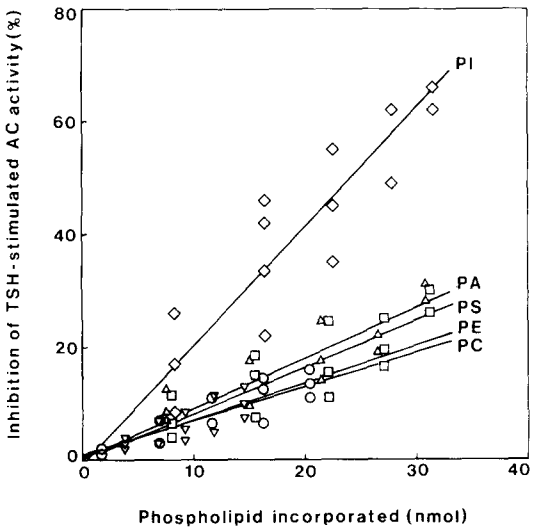

Fig. 1. Specificity of phospholipid inhibition of TSH-stimulated adenylate cyclase activity. Bovine thyroid plasma membranes (0.6 μmol phospholipid) were incubated with SUV containing the indicated phospholipid PX (0.15 μmol phospholipid, PC/PX/CH, 1:1:0.8 mol/mol) and 0; 4.5; 7.5; 11 and 13.5 units of transfer protein. Controls contained bovine thyroid plasma membranes with the respective amount of units of transfer protein. The phospholipids tested for their inhibitory effect were: phosphatidylinositol (◇), phosphatidic acid (△), phosphatidylserine (□), phosphatidylethanolamine (▽) and phosphatidylcholine (○).

stimulation than phosphatidic acid or phosphatidylserine and 3.5-times more effective than phosphatidylcholine or phosphatidylethanolamine.

Reversibility of PI effect

In order to investigate the reversibility of the inhibitory effect of PI an attempt was made to restore the stimulated adenylate cyclase activity by removal of PI. Specific removal of PI from PI modified plasma membranes can be achieved by treatment with either PI specific phospholipase C from *Staphylococcus aureus* [16] or by incubation with transfer protein and phosphatidylcholine SUV [8]. Incubation of PI-modified plasma membranes with transfer protein and phosphatidylcholine SUV removed 60% of the incorporated PI (measured by removal of [^{3}H]phosphatidylinositol) but did not affect the inhibition of the TSH stimulation (Table IV). Similarly, treatment with PI-specific phospholipase C resulted in hydrolysis of about 86% of the incorporated PI but

TABLE IV

REMOVAL OF PHOSPHATIDYLINOSITOL FROM PI-MODIFIED PLASMA MEMBRANES

PI was incorporated in bovine thyroid plasma membranes as described in Materials and Methods. The PI-modified plasma membranes (0.12 μmol phospholipid) were then incubated either with PI-specific phospholipase C (3 μg protein/ml) or with 15 units transfer protein and PC/CH-SUV (0.6 μmol phospholipid; 1:0.4; mol/mol) for 1 h at 37°C in a total volume of 0.5 ml. After this incubation the procedure as described in Materials and Methods was followed. Results are means ± S.E. of duplicate determinations of three separate experiments. CH, cholesterol; ns-TP, nonspecific lipid transfer protein.

Treatment	% [^{3}H]PI removed	cAMP (pmol/mg protein per 30 min)
PI-specific phospholipase C		
–	1	0.13 ± 0.01
+	86	0.12 ± 0.01
ns-TP + PC/CH-SUV		
Control	1	0.16 ± 0.02
–	5	0.16 ± 0.02
+	60	0.15 ± 0.01

did not relieve the inhibition of TSH stimulation (Table IV). Thus, the inhibitory effect seemed not to be reversed by removal of the incorporated PI. Incubation of plasma membranes with transfer protein and phosphatidylcholine SUV after treatment with PI-specific phospholipase C did not result in any additional removal of the incorporated PI (results not shown). These data therefore suggest that the inhibition is due to a nonremovable pool of phosphatidylinositol. A possibility to be considered is that the lack of reversibility is due to removal of a critical endogenous PI pool during enzyme treatment. Incubation of unmodified plasma membranes with PI-specific phospholipase C, however, did not affect TSH-stimulated adenylate cyclase activity (data not shown).

Site of action of incorporated PI

Incorporated PI might affect one of the sequential events in the activation process of adenylate cyclase by TSH. In the first place inhibition of TSH stimulation could be due to PI affecting TSH receptor binding. Therefore various phospholipids were incorporated and tested for their ability to affect the binding of ^{125}I-TSH to bovine thyroid

TABLE V

INFLUENCE OF SOME EFFECTORS ON ADENYLATE CYCLASE ACTIVITY IN UNMODIFIED AND PI-MODIFIED BOVINE THYROID PLASMA MEMBRANES

For incubation conditions see Materials and Methods. Percent inhibition was determined relative to control incubations which contained bovine thyroid plasma membranes with transfer protein alone. Agonists were added to the incubation mixture to give the indicated final concentrations. Results are averages ± S.E. of duplicate determinations of three different experiments. CH, cholesterol.

Agonist	cAMP (pmol/mg protein per 30 min)			% inhibition	
	Control	PC/CH-SUV	PC/PI/CH-SUV	PC/CH-SUV	PC/PI/CH-SUV
–	0.29 ± 0.03	0.28 ± 0.03	0.27 ± 0.02	3	7
TSH (50 mU/ml)	0.59 ± 0.04	0.51 ± 0.04	0.20 ± 0.03	14	66
Cholera toxin (5 μM)	1.80 ± 0.16	1.57 ± 0.14	1.58 ± 0.15	13	12
Forskolin (10 μM)	1.30 ± 0.14	1.14 ± 0.11	1.16 ± 0.12	12	11

plasma membranes. From these experiments it appeared that none of the phospholipids incorporated were able to significantly affect the TSH-receptor binding (data not shown). Cholera toxin stimulation was inhibited only 12% in PI-modified plasma membranes and this under conditions in which TSH stimulation was inhibited by 66% (Table V; $P < 0.05$). This indicates that the PI inhibition is not situated at the level of the interaction between $G_{s\alpha}$ and the catalyst of adenylate cyclase. Furthermore, under the same conditions basal adenylate cyclase as well as activation of adenylate cyclase by the diterpene forskolin were inhibited to a similar extent (approx. 10%) (Table V), indicating that the PI-inhibition does not act at the level of the catalyst.

Previous studies have shown that hormonal stimulation of adenylate cyclase might be affected by changes in membrane fluidity [27–30]. In order to study the influence of phospholipid incorporation on the fluidity of bovine thyroid plasma membranes we measured the steady-state fluorescence anisotropy (r_s) of DPH, known to probe the hydrophobic interior of the membrane [31] and TMA-DPH, located at the hydrophobic/hydrophylic interface of the membrane [32]. Incorporation of various phospholipids in bovine thyroid plasma membranes by transfer protein did not significantly ($P > 0.05$) affect the fluorescence anisotropy of TMA-DPH (Table VI). PI-modification of plasma membranes resulted in a 3% change in DPH anisotropy whereas incorporation of phosphatidylserine and phosphatidic acid induced changes of approximately 5% in DPH anisotropy (Table VI).

TABLE VI

EFFECT OF PHOSPHOLIPID-INCORPORATION IN PLASMA MEMBRANES BY TRANSFER PROTEIN ON MEMBRANE FLUIDITY

For incubation conditions see Materials and Methods. Control incubations contained either bovine thyroid plasma membranes alone or bovine thyroid plasma membranes and transfer protein. SUV compositions are expressed as molar ratios. Results are means ± S.E. of duplicate determinations of three separate experiments. All measurements were performed at 37 °C. CH, cholesterol; PA, phosphatidic acid.

SUV	r_s (DPH)	r_s (TMA-DPH)
None	0.173 ± 0.005	0.224 ± 0.004
PC/CH (1:0.4)	0.168 ± 0.005	0.224 ± 0.004
PC/PI/CH (1:1:0.8)	0.178 ± 0.006	0.229 ± 0.005
PC/PE/CH (1:1:0.8)	0.177 ± 0.005	0.230 ± 0.003
PC/PS/CH (1:1:0.8)	0.181 ± 0.006	0.228 ± 0.005
PC/PA/CH (1:1:0.8)	0.183 ± 0.006	0.229 ± 0.003

Discussion

The ability of a nonspecific lipid transfer protein, purified from bovine liver [24], to introduce phospholipids into membranes, was used to modify the phospholipid composition of bovine thyroid plasma membranes. In order to gain further information concerning the role of the lipid environment on TSH receptor expression, the correlation between this modification and TSH receptor binding and stimulation of adenylate cyclase activity was evaluated.

Phospholipid incorporation in thyroid plasma membranes by transfer protein hardly affected its phospholipid content indicating that the observed changes in phospholipid composition must be ascribed to phospholipid exchange rather than to adsorption of liposomes to the plasma membrane vesicles. Moreover, we always corrected the modified phospholipid composition for cosedimentation of SUV with bovine thyroid plasma membranes by quantitation of the non-exchangeable marker triolein. The transfer of acidic phospholipids (PI,PA,PS) in the presence of 13–14 units of transfer protein was approximately 2-times higher than that of PE. The addition of an equimolar amount of PI to PC vesicles provoked a 2-fold increase in PC transfer activity. A similar enhancement of phospholipid exchange by transfer protein when acidic phospholipids are present in donor or acceptor vesicles has been reported previously [24,33]. It has been argued that addition of acidic phospholipids may perturb the PC bilayer structure due to electrostatic repulsion, thus causing a greater spacing between phospholipid headgroups and as a consequence an enhanced lipid transfer activity [33]. The spontaneous non-protein-mediated transfer of the acidic phospholipids was nearly ten times higher than that of PC. This observation might also be explained by a less condensed or tightened membrane structure in the presence of acidic phospholipids.

Incubation of bovine thyroid plasma membranes with lipid-exchange proteins and PI-containing liposomes resulted in a marked decrease in TSH-stimulated adenylate cyclase activity. Since during such an incubation a considerable part of the plasma membrane lipids was substituted by PI the decrease in TSH stimulation is most likely due to the incorporation of PI into bovine thyroid plasma membranes. The latter incubation also resulted in both an increase in the amount of PC and a decrease in the amounts of PE and sphingomyelin. This might suggest that PI-inhibition could be the result of PE- or sphingomyelin-depletion or PC-enrichment. However, incubation of bovine thyroid plasma membranes with transfer protein and phosphatidylcholine/cholesterol SUV or SUV containing phosphatidylserine or phosphatidic acid always resulted in both sphingomyelin- and PE-depletion as well as PC-enrichment in the membrane, but only the PI-containing SUV caused such a large decrease in TSH stimulation. A similar specific for PI has been reported previously for the isoproterenol-stimulated adenylate cyclase of turkey erythrocytes [8].

In an attempt to restore the enzyme activity, the PI-modified plasma membranes were treated with PI-specific phospholipase C or were incubated with transfer protein and PC/CH-SUV. Incubation with PC/CH-SUV resulted in a removal of 60% of the incorporated PI, while treatment of PI-modified plasma membranes with PI-specific phospholipase C resulted in hydrolysis of 86% of the incorporated PI. However, neither method used for the removal of incorporated PI reversed the inhibition of TSH-stimulated adenylate cyclase activity. Also a combination of both methods or addition of higher amounts of phospholipase C did not result in an additional removal of incorporated PI and a subsequent enhancement of TSH-stimulated adenylate cyclase activity. Assuming that phospholipid 'flip-flop' in bovine thyroid plasma membranes is similar to that reported for rat erythrocytes [26], it is reasonable to suggest that the PI introduced into plasma membranes by protein-mediated lipid transfer is located almost entirely on the outer halve of the phospholipid bilayer of the plasma membrane vesicles.

These data support the view that inhibition of TSH stimulation is due to a nonremovable pool of PI located at the outer leaflet of the plasma membrane phospholipid bilayer. In this respect it is interesting to mention the difference in susceptibility to release by a PI-specific phospholipase C of certain PI-bound plasma membrane enzymes [34–36]. The differential release of these enzymes has been ascribed to their association with different populations of PI molecules, one being inaccessible to PI-specific phospholipase C. In a similar way, the nonremovable pool of PI in our experiments might also be shielded from phospholipase C attack due to an intimate association with some plasma membrane proteins such as for instance a component of the adenylate cyclase system. Treatment with PI-specific phospholipase C might also result in removal of endogenous PI which might lead to an irreversible inhibition of TSH stimulation. This is however unlikely since

treatment of unmodified plasma membranes with PI-specific phospholipase C did not affect TSH stimulation. PI might interfere with one or more of the sequential events between initial TSH binding and ultimate activation of the catalyst of adenylate cyclase. Binding experiments demonstrated that TSH binding to bovine thyroid plasma membranes was not affected by PI-incorporation. Thus, PI appears to act as an inhibitor of TSH-stimulated cyclase at a step beyond TSH binding. Basal as well as cholera toxin- and forskolin-stimulated adenylate cyclase activity were inhibited by approximately 10% in PI-modified plasma membranes, under conditions in which TSH stimulation was decreased by 66%. These results indicate that the PI effect does not interfere with the activating coupling between the α-subunit of the stimulatory GTP binding regulatory protein ($G_{s\alpha}$) and the catalyst nor does it act at the level of the catalyst itself. Thus, by elimination, it appears that PI diminishes the TSH-stimulated adenylate cyclase activity by interfering with the efficiency of the activating coupling between the occupied TSH receptor and the stimulatory GTP binding regulatory protein. Earlier studies revealed that the receptor-adenylate cyclase interaction might be significantly altered by manipulations which change the membrane fluidity [28,37]. However, in our system incorporation of PI in bovine thyroid plasma membranes showed no significant change in both DPH- and TMA-DPH anisotropies, indicating that the PI-effect is due to a direct interaction of this phospholipid with a component of the adenylate cyclase system rather than a general sensitivity to changes in bulk membrane fluidity.

Several lines of evidence support the concept that adenylate cyclase is influenced by the composition of its membrane environment [8,38,40]. More specifically, the involvement of membrane phospholipids has been suggested in TSH receptor binding [11,12,41,42,45,46] and TSH-stimulated adenylate cyclase activity [9,11,43,44]. With regard to receptor binding it has been shown that treatment of thyroid membranes or tissue with phospholipase A_2 [11,12,45] or phospholipase C [12,46] enhanced TSH receptor binding. Addition of acid phospholipids to thyroid plasma membranes (PI $\approx$ PA > PS), on the other hand, diminished TSH receptor binding [41,42]. According to Aloj et al. [42] this PI-induced inhibition of TSH binding is the result of a direct interaction with the hormone whereas Omodeo-Sale et al. [41] believe that this inhibition is due to an interaction of acidic phospholipid with plasma membranes. This latter conclusion was based on the observation that inhibition took place when membranes were preincubated with phospholipids and subsequently separated by centrifugation prior to TSH binding. Similar experiments, reported by Aloj et al. [42], did not support this conclusion. In these experiments preincubation of membranes with acidic phospholipids were, however, performed at 0°C whereas Omodeo-Sale et al. [41] conducted their experiments at 24°C. Our results indicate that at these elevated temperatures, even in the absence of transfer protein, phospholipids are incorporated into thyroid plasma membranes. Using binding conditions identical to those reported by Omodeo-Sale our results, however, show that incorporation of PI, in the absence or presence of transfer protein, has no effect on TSH binding. An explanation to be considered is that incomplete removal of PI liposomes from plasma membranes after the preincubation period partially blocks TSH binding due to direct interaction with the hormone. In the turkey erythrocyte system PI incorporation has also no effect on β-adrenergic receptor binding [8].

Several studies have shown that treatment of thyroid membranes with phospholipases also caused a decrease in TSH-stimulated adenylate cyclase activity [9,11,43] and subsequent addition of phosphatidylcholine and to a lesser extent phosphatidylserine partially restored hormone response [9]. Also treatment of membranes with Lubrol PX caused a loss of their TSH response, addition of phosphatidylcholine again partially restored activity [9]. This phospholipid requirement for hormone responsiveness appears, however, to be tissue-specific since it has been previously reported by Levey [47] that phosphatidylinositol specifically restores norepinephrine responsiveness of Lubrol PX solubilized cat myocardial adenylate cyclase. From these studies it would appear that at least in thyroid plasma membranes, phosphatidylcholine is a critical phospholipid for the hormone responsiveness of adenylate cyclase.

More recent reconstitution experiments [48] have shown that phosphatidylcholine is indeed important for the productive regulatory interaction of the catalytic protein of rabbit hepatic adenylate cyclase with the stimulatory GTP binding regulatory protein. These observations, taken together with our results, therefore suggest that phospholipids may play a dual role in the regulation of adenylate cyclase activity. Phosphatidylcholine being probably an essential component for TSH stimulation and phosphatidylinositol, if present in the outer half of the phospholipid bilayer, acting as a negative modulator of the coupling between the occupied receptor and the stimulatory GTP binding regulatory protein. A similar modulatory action of PI has been suggested previously [8] for the β-adrenergic receptor mediated regulation of adenylate cyclase in turkey erythrocytes.

References

1 Rodbell, M. (1980) Nature 284, 17–22.
2 Ross, E.M. and Gilman, A.G. (1980) Annu. Rev. Biochem. 49, 533–564.
3 Gilman, A.G. (1984) Cell 36, 577–579.
4 Northup, J.K. (1985) in Molecular Mechanisms of Transmembrane Signalling (Cohen, P. and Houslay, M.D., eds.), Vol. 4, pp. 91–116, Elsevier, Amsterdam.
5 Rimon, G., Hanski, E. and Levitski, A. (1980) Biochemistry 19, 4451–4460.
6 Klein, I., Moore, L. and Pastan, I. (1978) Biochim. Biophys. Acta 506, 42–53.
7 Bakardjieva, A., Galla, H.J. and Helmreich, E.J.M. (1979) Biochemistry 18, 3016–3023.
8 McOsker, C.C., Weiland, G.A. and Zilversmit, D.B. (1983) J. Biol. Chem. 258, 13017–13026.
9 Yamashita, K. and Field, J.B. (1973) Biochim. Biophys. Acta 304, 686–692.
10 Dacremont, G., De Baets, M., Kaufman, J.M., Elewaut, A. and Vermeulen, A. (1984) Biochim. Biophys. Acta 770, 142–147.
11 Moore, W.V. and Wolff, J. (1974) J. Biol. Chem. 249, 6255–6263.
12 Amir, S.M., Goldfine, I. and Ingbar, S.H. (1976) J. Biol. Chem. 251, 4693–4699.
13 Depauw, H., De Wolf, M., Van Dessel, G., Hilderson, H.J., Lagrou, A. and Dierick, W. (1985) Biochim. Biophys. Acta 814, 57–67.
14 Barsukov, L.I., Kulikov, V.I., Simakova, I.M., Tikhonova, G.V., Ostrovskii, D.N. and Bergelson, L.D. (1978) Eur. J. Biochem. 90, 331–336.
15 Dyatlovitskaya, E.V., Lemenovskaya, A.F. and Bergelson, L.D. (1979) Eur. J. Biochem. 99, 605–612.
16 Low, M.G. and Finean, J.B. (1977) Biochem. J. 162, 235–240.
17 Dierick, W. and Hilderson, H.J. (1976) Arch. Int. Physiol. Biochim. 75, 1–11.
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
19 Rouser, G., Siakotos, A.N. and Fleischer, S. (1966) Lipids 1, 85–86.
20 Hanel, H.K. and Dam, H. (1955) Acta Chim. Scand. 9, 677–682.
21 Bligh, E.G. and Dyer, W.I. (1959) Can. J. Biochem. Physiol. 37, 911–919.
22 Yavin, E. and Zutra, A. (1977) Anal. Biochem. 80, 430–437.
23 Johnson, L.W. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 165–175.
24 Crain, R.C. and Zilversmit, D.B. (1980) Biochemistry 19, 1433–1439.
25 Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
26 Bloj, B. and Zilversmit, D.B. (1981) Mol. Cell. Biochem. 40, 163–172.
27 Whetton, A.D., Gordon, L.M. and Houslay, M.D. (1983) Biochem. J. 212, 331–338.
28 Houslay, M.D., Dipple, I. and Gordon, L.M. (1981) Biochem. J. 197, 675–681.
29 Hanski, E., Rimon, G. and Levitzki, A. (1979) Biochemistry 18, 846–853.
30 Dipple, I. and Houslay, M.D. (1978) Biochem. J. 174, 179–190.
31 Lentz, R.B., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521–4528.
32 Prendergast, F.G., Haugland, R.P. and Callahan, P.J. (1981) Biochemistry 20, 7333–7338.
33 Dicorleto, P.E. and Zilversmit, D.B. (1977) Biochemistry 16, 2145–2150.
34 Shukla, S.D., Coleman, R., Finean, J.B. and Michell, R.H. (1980) Biochem. J. 187, 277–280.
35 Low, M.G. and Zilversmit, D.B. (1980) Biochemistry 19, 3913–3918.
36 Low, M.G. and Finean, J.B. (1978) Biochim. Biophys. Acta 508, 565–570.
37 Whetton, A.D., Gordon, L.M. and Houslay, M.D. (1983) Biochem. J. 210, 437–449.
38 Engelhard, V.H., Glaser, M. and Storm, D.R. (1978) Biochemistry 17, 3191–3200.
39 Lad, P.M., Preston, M.S., Welton, A.F., Nielsen, T.B. and Rodbell, M. (1979) Biochim. Biophys. Acta 551, 368–381.
40 Hebdon, G.M., Levine, H., Sahyoun, N.E., Schmitges, C.J. and Cuatrecasas, P. (1981) Proc. Natl. Acad. Sci. USA 78, 120–123.
41 Omodeo-Sale, F., Brady, R.O. and Fishman, P.H. (1978) Proc. Natl. Acad. Sci. USA 75, 5301–5305.
42 Aloj, S.M., Lee, G., Grollman, E.F., Beguinot, F., Consiglio, E. and Kohn, L.D. (1979) J. Biol. Chem. 254, 9040–9049.
43 Yamashita, K., Bloom, G., Rainard, B., Zor, U. and Field, J.B. (1970) Metabolism 19, 1109–1118.
44 Macchia, V., Tamburrini, O. and Pastan, I. (1970) Endocrinology 86, 787–792.
45 Haye, B. and Jacquemin, C. (1970) FEBS Letters 18, 47–52.
46 Macchia, V. and Wolff, J. (1970) FEBS Lett. 10, 219–221.
47 Levey, G.S. (1971) J. Biol. Chem. 246, 7405–7410.
48 Ross, E.M. (1982) J. Biol. Chem. 257, 10751–10758.