Immunoglobulins from Graves' disease patients stimulate phospholipase A₂ and C systems in FRTL-5 and human thyroid cells

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We have studied the effects of immunoglobulin G from Graves' disease patients on phospholipase A₂ (PLA₂) and C (PLC) systems in FRTL-5 and human thyroid cells. Immunoglobulin G (IgG) from Graves' disease patients stimulated arachidonic acid (AA) release in a time- and dose-dependent manner. In FRTL-5 thyroid cells, removal of external calcium had no significant effect on the IgG (20 μg/ml)-induced AA release in FRTL-5 thyroid cells. U-73122 (3 μmol/l), a PLC inhibitor, and quinacrine (100 μmol/l) but not U-26384 (5 μmol/l), PLA₂ inhibitors, blocked the IgG-induced (20 μg/ml) AA release in FRTL-5 thyroid cells. Immunoglobulin G (100 μg/ml) also stimulated accumulation of inositol-1,4,5-triphosphate (IP₃) in a time- and dose-dependent (20–300 μg/ml) manner in FRTL-5 cells. Immunoglobulin G from Graves' disease patients induced a significant increase of IP₃ production (p = 0.01) compared to IgG from normal subjects. Removal of external calcium had no significant effect on the IgG-induced IP₃ production. The PLC inhibitor U-73122 completely blocked IgG-induced IP₃ production from FRTL-5 thyroid cells. Also, in human thyroid cells, IgG from Graves' disease patients induced a significant increase of AA release (p = 0.001) and IP₃ production (p = 0.004) compared to the IgG from normal subjects. These data indicate that IgG from Graves' disease patients induced PLA₂ activity that was PLC dependent, a pattern referred to as sequential activation. Our studies suggest that IgG from Graves' disease patients activates PLA₂ and PLC systems in FRTL-5 and human thyroid cells. These signal transduction pathways could be involved in the pathogenesis of Graves' disease and future studies are warranted to investigate this area.

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Graves' disease is an autoimmune disorder associated with increased production and secretion of thyroid hormones (1). The thyroid gland in these patients contains a large number of lymphocytes, is diffusely enlarged and is highly vascular. In typical cases the patients have circulating antibodies that can be shown to stimulate thyroid cells in vivo and in vitro and to compete for TSH binding to the TSH receptor (1, 2). Adams and Purves (3) discovered that the injection of serum from a patient with Graves' disease exerted stimulating activity upon the thyroid glands of guinea pigs by causing a sustained release of protein-bound ¹³¹I. This long-acting thyroid stimulator (LATS) was subsequently found to be associated with immunoglobulin G (IgG) (4). Immunoglobulin G affects thyroid function and growth by a mechanism that is still partially unclear, but it is believed to stimulate thyroid function via the TSH receptor and thought to act, like TSH, by increasing the cellular levels of cAMP, the main intracellular regulator of growth and differentiation in thyroid cells (5–9). Thyroid growth, however, can also be induced by an IgG that does not affect cAMP production, suggesting that other transduction mechanisms might account for the effects (10–12).

Thyrotropin has been reported to stimulate arachidonic acid (AA) release and inositol-1,4,5-triphosphate (IP₃) production in FRTL-5 thyroid cells (13). Di Cerbo et al. (14) have reported that IgG from Graves' disease patients caused a significant increase of AA release compared to normal subjects in FRTL-5 thyroid cells. However, it is unclear whether AA release was due to direct action of IgG on phospholipase A₂ (PLA₂) or indirectly through the phospholipase C (PLC) system in FRTL-5 thyroid cells. Furthermore, the effects of IgG

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from Graves’ disease patients on AA release and IP₃ production have not been studied in human thyroid cells, which potentially could interact differently than cultured rat thyroid cells.

Recently, 1-[6-[[175-3-methoxyestra-1,3,5(10)-tri-en-17-y]amino]hexyl]-1H-pyrrole-2,3-dione (U-73122), an anionomeric compound that inhibits PLC-mediated events in hematopoietic cells (15), has been shown to effectively inhibit PLC activities in GH₃ pituitary cells (16). N-[3-(Dimethylamino)propyl]-3-methoxy-N,n-methylestra 2,5(10)-dien-17β-amine (U-26384), an inhibitor of PLA₂ activity in myocardial cells (17, 18), was demonstrated to be a potent inhibitor of ATP-stimulated AA release in FRTL-5 thyroid cells (19). Using these two inhibitors, the present study was performed to investigate the effects of IgG from Graves’ patients on the PLA₂ and PLC systems in FRTL-5 cells and human thyroid cells, thereby giving further insight into the role of these signal transduction pathways in the pathophysiology involved in Graves’ disease.

Materials and methods

Reagents

Tris (hydroxymethyl)aminomethane, ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St Louis, MO). Coon’s modified F-12 medium was obtained from Biofluids (Rockville, MD). U-73122, U-73343 (an inactive analog of U-73122) and U-26384 (PLA₂ inhibitor) were provided by the Upjohn Company (Kalamazoo, MI). Thyrotropin (TSH) was obtained from Rhone-Poulec Rorer (Collegeville, PA). Myo-[5H]-inositol and [3H]-arachidonic acid were purchased from DuPont/NEN (Boston, MA). Thyrotropin-binding inhibiting IgGs (TBII) were measured by TSH receptor antibody kits from Kronus (San Clemente, CA). Other reagents were obtained from Gibco (Grand Island, NY).

Patients

Sera were obtained from seven patients with active Graves’ disease (three men and four women aged 18–60 years) and seven normal subjects. Each patient gave informed consent. Active Graves’ disease was defined as diffuse thyroid enlargement, high levels of free thyroid hormones and undetectable levels of TSH, with classic symptoms and signs of thyrotoxicosis. Thyrotropin-binding inhibiting IgG (TBII) were positive in all Graves’ disease patients (36 ± 9%; range 17–80%) and negative (less than 15%) in normal subjects. Normal subjects included age- and sex-matched blood donors.

Rat thyroid cell culture

Continuously proliferating, TSH-dependent. FRTL-5 cells (ATCC CRL 8305, Baltimore, MD) were cultured in 75-cm² flasks in Coon’s modified Ham’s F-12 medium containing 0.1 mmol/l non-essential amino acids, six-hormone (6H) mixture of insulin (10 µg/ml), cortisol (10⁻⁸ mol/l), transferrin (5 µg/ml), glycyrrhizin, histidyl-lysine acetate (2 ng/ml), somatostatin (10 ng/ml), bovine TSH (1 µIU/ml) and 5% calf serum in 5% CO₂ at 37°C in a humidified incubator (20). Cells were fed twice weekly and passed every 7–8 days using a trypsin–collagenase–chicken serum mixture. When cells were 80% confluent, 6H was replaced by medium deprived of TSH (5H) and cells were maintained for an additional 5–7 days before an experiment. Cells were used between their 9th and 15th passages. Fresh 5H medium was added 24 h before each measurement.

Human thyroid cell culture

Thyroid tissue was obtained from patients with multinodular goiter undergoing subtotal thyroidectomy or from normal extranodular tissue excised from patients undergoing subtotal thyroidectomy for thyroid nodules. Each patient gave informed consent. The tissue was minced finely with sterilized instruments, suspended in phosphate-buffered saline and digested with 4 mg/ml collagenase at 37°C for 45–60 min with constant stirring. The dispersed cells were separated from residual tissue by transferring the cell suspension into another sterile tube after the larger fragments had settled. Fresh collagenase solution was added to the remaining tissue for another 45–60 min at 37°C. The cell suspension was centrifuged at 600 g for 10 min and the pellet was washed three times with RPMI 1640 medium containing 10% fetal bovine serum, 2 mmol/l glutamine and 100 IU/ml penicillin–100 µg/ml streptomycin. The cells were then plated in flasks in the above medium and incubated at 37°C in a humidified atmosphere of 5% CO₂ (21). The medium was changed every 3rd day. When the cells became confluent after 10 days of culture, they were removed by trypsinization (0.05% trypsin in 0.02% EDTA). The cell suspension was washed three times with medium and transferred into the wells of six-well Costar plates.

Immunoglobulin G purification

Immunoglobulin G purification was performed by the method of Reader et al. (22). The serum was applied to protein-A-Sepharose CL4B columns (Pharmacia Biotech Inc., Piscataway, NJ) and the bound IgG was washed, eluted with 0.1 mol/l glycine–0.1 mol/l NaCl (pH 2.9), concentrated, dialyzed overnight against saline solution and measured by protein assay kit from Bio-Rad Laboratories (Hercules, CA).

Arachidonic acid release

Cells were seeded in six-well plates. When cells were
75% confluent, media were removed and replaced with 6H medium containing 10 mmol/l HEPES and fatty acid-free BSA (1 mg/ml). This medium also contained $[^3]$H]arachidonic acid (0.1 µCi/ml). After overnight incubation, cells were washed three times with HBSS(+), pH 7.4, containing 10 mmol/l HEPES and fatty acid-free BSA (1 mg/ml). After the cells were stimulated with test agents, the medium was collected, placed in scintillation fluid and counted in a liquid scintillation spectrometer.

Inositol phosphate generation

Cells were grown in six-well plates and incubated with $[^3]$H]myoinositol (2 µCi/ml) for 48 h. Cells were washed twice with HBSS, treated with LiCl (10 mmol/l) for 10 min at 37°C (to inhibit inositol phosphate hydrolysis) and rewashed. Test agents were then added using the time indicated. Production of IP$_3$ was measured using Dowex AG 1-XS resin column chromatography, as previously described (16). The amount of IP$_3$ formed was expressed as the percentage of the control.

Statistical analysis

Comparisons among groups were performed using one-factor analysis of variance with post hoc testing, using the Statview II program (Abacus Concepts, Berkeley, CA).

Results

Effects of IgG on cAMP formation from FRTL-5 thyroid cells

Cyclic AMP was measured in the extracellular fluid with a commercial RIA kit as reported previously (14). The IgG from Graves’ disease patients induced more cAMP formation (257 ± 66% of the basal 0.42 ± 0.02 pmol/well, N = 7) than did IgG from normal subjects (122 ± 5.2%, N = 7).

Effects of IgG on AA release from FRTL-5 thyroid cells

In cells prelabeled with AA, IgG induced AA release in a time- and dose-dependent manner. An early increase in release of AA induced by IgG (20 µg/ml) was reached at 5 min (216 ± 49% of the control value of 103 ± 3%, mean ± SEM, N = 7), remained stable until 10 min and then reached another peak at 30 min (310 ± 53% of the control, N = 7). The IgG from Graves’ disease patients induced more AA release (216 ± 49% of the control, N = 7) than did IgG from normal subjects (146 ± 12, N = 7). However, the differences between Graves’ disease patients and normal subjects did not achieve statistical significance (p = 0.2).

Removal of the external calcium by using Ca$^{2+}$-free buffer (HBSS(−)) and addition of 1 mM EGTA had no significant effect (186 ± 15, mean ± SEM, N = 7, p > 0.05) on the IgG-induced (20 µg/ml) AA release at 5 min. U-73122 added 2 min prior to IgG (20 µg/ml) suppressed (120 ± 8% of the control, N = 7) IgG-induced AA release. Quinacrine (100 µmol/l) significantly suppressed (p < 0.05, N = 7) Graves’ IgG-induced AA release. U-26384 did not significantly inhibit IgG-induced AA release (220 ± 54% of the control) (Fig. 1).

Fig. 1. Effects of external calcium. U-26384 and U-73122 on IgG-induced arachidonic acid (AA) release from FRTL-5 cells. Removal of external calcium was carried out by using calcium-free buffer (HBSS(−)) and the addition of 1 mM EGTA. Cells were incubated with IgG (20 µg/ml) either in the presence (+) or absence (−) of external calcium: for controls, cells were treated under the same conditions except for the lack of IgG addition. U-26384 (5 µmol/l), U-73122 (3 µmol/l) and quinacrine (100 µmol/l) were added 2 min prior to IgG (20 µg/ml) and the AA produced was measured after 5 min of incubation with IgG. In this representative experiment, the AA data points are the average of seven cases for two different experiments, with the error bars representing the standard error of the mean.

Effects of IgG on IP$_3$ production from FRTL-5 thyroid cells

In cells preincubated with $[^3]$H]myoinositol, IgG promptly increased IP$_3$ production. The IP$_3$ maximal response occurred at 60 s and returned close to the baseline at 300 s. The IP$_3$ increase was dose-dependent, with the maximal increase being 278 ± 32% of the control (102 ± 2%, mean ± SEM) at a dose of 300 µg/ml. The IgG from Graves’ disease patients (100 µg/ml) induced a significant increase (169 ± 6% of the control, mean ± SEM, N = 7, p = 0.01) of IP$_3$ production compared to the IgG from normal subjects (106 ± 5% of the control, N = 7).

Removal of the external calcium by using Ca$^{2+}$-free buffer (HBSS(−)) and addition of 1 mM EGTA, had no significant effect (159 ± 10% of the control, p > 0.05) on the IgG-induced (100 µg/ml) IP$_3$ production. U-73122 (3 µmol/l) added 2 min prior to IgG
Fig. 2. Effects of external calcium and U-73122 on IgG-induced inositol-1,4,5-triphosphate (IP₃) production from FRTL-5 cells. Removal of external calcium effect was carried out by using calcium-free buffer (HBSS(-)) and 1 mmol/l EGTA. U-73122 (3 μmol/l) was added 2 min prior to IgG (100 μg/ml) and the IP₃ produced was measured after 60 s of incubation with IgG. In this representative experiment, the IP₃ data points are the average of seven cases for two different experiments, with the error bars representing the standard error of the mean.

(100 μg/ml) completely inhibited IgG-induced IP₃ production (Fig. 2). The inactive analog, U-73343, did not inhibit the IgG-induced IP₃ production (168 ± 5% of the control).

Effect of IgG on AA release and IP₃ production from human thyroid cells

In cells prelabeled with AA, IgG (20 μg/ml) induced a significant increase in AA release (224 ± 15% of the control value of 102 ± 2%, mean ± SEM, p < 0.001) (Fig. 3). The maximal release of AA reached was observed at 5 min. At a greater concentration (100 μg/ml), IgG from normal subjects increased AA release (140 ± 6% of the control), but the response was greater in Graves' disease patients (N = 7, p < 0.001).

In cells prelabeled with [³H]myoinositol. Graves' disease IgG (100 μg/ml) significantly increased IP₃ production (168 ± 5% of the control value of 102 ± 2%, mean ± SEM, p < 0.001). The IgG from normal subjects increased IP₃ production non-significantly (128 ± 8% of the control, N = 7).

Discussion

A variety of hormones and neurotransmitters elicit their biological responses by activating PLC-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂). Activation of the PLC system leads to hydrolysis of PIP₂ into IP₃ (23–27), which then mobilizes Ca²⁺ from intracellular stores (24, 28, 29) and may also mediate external Ca²⁺ influx (24, 28, 29). Arachidonic acid can be generated via two major pathways, one of which is PLA₂ and the other involves another PIP₂ metabolite, diacylglycerol (DAG), which is catalyzed by DAG lipase to liberate AA. Diacylglycerol also activates protein kinase (PKC), which participates in PLA₂-mediated AA release (30–33).

In the presence of external Ca²⁺, IgG from Graves' disease patients induced AA release from FRTL-5 cells in a time- and dose-dependent manner. The IgG from Graves' disease patients induced more AA release than the IgG from normal subjects; although the differences between these two groups did not achieve statistical significance in FRTL-5 cells (perhaps due to the large so), they did when human thyrocytes were utilized.

Removal of external Ca²⁺ had no significant effect on the IgG-induced AA release. These results, in general, are similar to those reported by Di Cerbo et al. (14) but differ from the results of TSH in the same cells (13). Di Cerbo et al. (34) showed that Graves' disease IgG stimulates PLA₂; in fact, the Graves' IgG that could stimulate adenyl cyclases, PLA₂, and triitated thymidine incorporation tended to correlate with the highest likelihood of those patients having ophthalmopathy and having the largest goiter and the highest serum thyroid hormone levels. Kosugi et al. (35, 36) have also demonstrated that Graves' IgG stimulated inositol phosphate and cAMP production in Cos 7 cells transfected with the rat TSH receptor cDNA. The alanine at position 623 in the third cytoplasmic loop (residues 605–625) was shown to be important in mediating PIP₂, but not cAMP, signal transduction.

U-26384 inhibits PLA₂ activity in vitro (17) and phosphatidylcholine degradation and AA release in cultured rat myocytes (17, 18). Previous work in this...
laboratory described the inhibitory effects of this agent on ATP-induced AA release and ³1 efflux (19).

In the present study, U-26384 did not block IgG-induced AA release from FRTL-5 thyroid cells. However, quinacrine, an inhibitor of PLA₂ activity in porcine prathyroid cells (37) and rat normal uterine cells (38), significantly suppressed IgG-induced AA release from FRTL-5 thyroid cells, indicating that multiple PLA₂ isoforms could be involved in this process. Furthermore, down-regulation of PKC with phorbol-12-myristate-13-acetate (PMA) (200 ng/ml/24 h) significantly inhibited AA release induced by IgG from Graves’ patients (data not shown), indicating involvement of the DAG–PKC system in this process.

In the presence of external Ca⁺⁺, IgG from Graves’ disease patients stimulated accumulation of IP₃ in a time- and dose-dependent manner in FRTL-5 thyroid cells. These results are similar to those reported previously in the same cells by using TSH (13). Removal of external Ca⁺⁺ had no significant effect on the IgG-induced IP₃ production. These results are similar to those reported in the same cells by using ATP (19).

U-73122 is an aminosteroid compound that has a selectively inhibitory effect on different agonist-induced PLC-mediated biological activities, including IP₃ production (15, 16) and Ca⁺⁺ (13, 15, 16, 19, 39), in a variety of cells without affecting PKC (40) and PLA₂ (15, 19) activities. Previous work in this laboratory described the inhibitory effects of this agent on TRH-induced IP₃ production. Ca⁺⁺ mobilization and prolatin secretion in GH₃ pituitary cells (16) and on ATP and TSH effects on FRTL-5 cells (13, 19).

In our current study, U-73122 inhibition of IgG-induced IP₃ production provides evidence that PLC mediates IgG signaling in FRTL-5 thyroid cells, results that are similar to those found with TSH (13).

These data indicate that IgG-induced PLA₂ activity requires prior activation of PLC. This pattern of enzyme activation, reported in a variety of cells, is referred to as sequential activation (33, 41, 42). However, our results must be considered preliminary because inhibitors may not be as specific as originally believed, and further studies in this area are required.

Also, in the presence of external Ca⁺⁺, IgG from Graves’ disease patients significantly stimulated release of AA and accumulation of IP₃ in human thyroid cells. The IgG from Graves’ disease patients induced significantly more of an increase in AA release and IP₃ production than did the IgG from normal subjects.

An inactive analog of U-73122, U-73343 (in which succinimide replaces maleimide), showed no effect compared to U-73122 on IP₃ formation and AA release in FRTL-5 thyroid cells, indicating that the maleimide group in U-73122 may determine the inhibitory effect of this aminosteroid.

In summary, IgG from Graves’ disease patients stimulated AA release and IP₃ production in FRTL-5 and human thyroid cells. U-73122 blocked IgG-induced AA release and IP₃ production. Also, quinacrine but not U-26384 blocked IgG-induced AA release. The IgG effects were calcium independent.

Our studies suggest that IgG from Graves’ disease patients stimulates the PLA₂ and PLC systems in FRTL-5 and human thyroid cells and provides evidence for alternative pathways for thyroidal stimulation, other than cAMP production. A proposed model to help explain these results may involve Graves’ IgG binding to a TSH receptor that activates PLC via a G protein-mediated process. Further studies are warranted to investigate the potential role of these non-classical signal transduction pathways in the pathophysiology of Graves’ disease.

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