Postępy Nauk Medycznych, t. XXIV, nr 11, 2011

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TSH signal transduction in thyroid cells of Nthy-ori 3-1 line**

Przekazywanie sygnału TSH w komórkach tarczycy linii Nthy-ori 3-1

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S u m m a r y

The paper characterizes proteins and intracellular signaling activating under TSH stimulation in thyroid cells of Nthy-ori 3-1 line. Solubilized cell membrane proteins from Nthy-ori 3-1 cells were characterized by immunoblotting with antibodies against TSH receptor and G-protein α and β subunits. Intracellular G-protein-dependent signal transmission was investigated by assaying adenylate cyclase activity and intracellular Ca^{2+} concentration under TSH stimulation. TSH receptor and all four classes of G protein: G_s, G_q, G_i, and $G_{12/13}$ and the G protein subunits β were present in membrane fraction of Nthy-ori 3-1 cells. In accordance with the presence of these proteins known to participate in signal transduction in thyroid cells, TSH treatment led to adenylate cyclase stimulation and intracellular Ca^{2+} rise. The results show, however, that the response of Nthy-ori 3-1 thyroid cells to TSH stimulation as adenylate cyclase activation and intracellular Ca^{2+} mobilization is weaker than that of thyroid cells in primary culture or transfected CHO cells expressing TSH receptor.

Key words: TSH, adenylate cyclase, calcium, protein G, thyroid

S t r e s z c z e n i e

W poniższej pracy badano białka błonowe oraz wewnątrzkomórkowe szlaki sygnalizacyjne uruchamiane pod wpływem TSH w komórkach tarczycy linii Nthy-ori 3-1. Białka w solubilizowanych błonach komórkowych charakteryzowano metodą immunoblotingu z użyciem przeciwciał przeciwko receptorowi TSH i podjednostkom α i β regulatorowego białka G. Sygnalizację wewnątrzkomórkową uruchamianą przez TSH badano przez pomiar aktywności cyklazy adenylanowej i pomiar stężenia wewnątrzkomórkowego Ca^{2+}. Wykazano występowanie w błonach komórek linii Nthy-ori 3-1 receptora TSH oraz wszystkich czterech podstawowych klas białka G: G_s, G_q, G_i i $G_{12/13}$ jak również występowanie podjednostek Gβ. Stwierdzono, że egzogenny TSH aktywuje procesy zachodzące z udziałem wykazanych białek, a więc stymuluje aktywność cyklazy adenylanowej i wzrost wewnątrzkomórkowego stężenia jonów Ca^{2+}. Poziom tej stymulacji w komórkach Nthy-ori 3-1 jest jednak niższy niż w komórkach tarczycy z pierwotnej hodowli oraz transferowanych komórkach CHO prezentujących receptor TSH.

Słowa kluczowe: TSH, cyklaza adenylanowa, wapń, białko G, tarczyca

INTRODUCTION

In vitro models that preserve a functional characteristics of the normal thyroid gland have been a challenging objective of recent experimental thyroidology. The earliest experimental thyroid models were based on organ culture or tissue slice preparations or, alternatively, short-term cell suspensions (1).The first permanently growing normal cell line named FRTL originated from rat thyroid (2). Many authors have studied human thyroid cell growth and expression of differentiated functions in primary cultures and thyroid cancer cell lines, but for a long time, normal human cell lines were unavailable. The cell line named Nthy-ori 3-1 was derived from normal thyroid follicular epithelium of an human adult (3). The cells were transfected with a plasmid encoding the SV40 large T gene. The resultant immortalized cell line has retained an epithelial morphology. It was active in the iodide trapping assay, but this activity was much lower compared with that of human thyroid follicular cells after extended passage. Initial re-

**This study was supported by Grant 521-1-25-01-11 from the Medical Centre of Postgraduate Education.

search on newly established thyroid cell lines focuses on thyroglobulin detection, quantification, and hormonal regulation of its production, because this protein is the marker of thyroid tissue unequivocally defining the thyroid character of the cells. Assay of thyroglobulin production by Nthy-ori 3-1 cell line has confirmed the specific function of this line. Its thyroglobulin production, however, was about 10-fold lower than that of primary normal thyroid cell culture (3). The transfected human thyroid follicular cell line has been an attractive model of tumorigenesis (4, 5). In other studies this cell line has been used as a control. For example, RNA from Nthy-ori 3-1 cells stably transfected with a plasmid carrying the Pax8/PPARG fusion gene was used as a positive control in a clinical assay for the detection of such rearrangements in patients (6).

MATERIAL AND METHODS

Cell culture

Nthy-ori 3-1, normal human differentiated thyroid cells (European Collection of Human Cell Cultures), were grown in 5% CO_2 at 37°C in RPMI 1640 AQmedia with 10% FBS and 1% penicillin – streptomycin solution (all medium components from Sigma-Aldrich). The medium was changed every 3 days.

TSH receptor and G protein estimation

The cells were detached with trypsin/EDTA (GIBCO BRL), or with non-enzymatic Cell Dissociation Solution (Sigma), washed and frozen. The frozen sample of $1.2x10^8$ cells were suspended in 3 ml of homogenization buffer, 20 mM Tris pH 7.4, 50 mM NaCl, 10 mM EDTA, 10 mM EGTA and protease inhibitor cocktail (Roche) and homogenized in glass/teflon homogenizer. The homogenates were centrifuged for 30 min at 800 x g. The supernatants were further centrifuged for 30 min at 25000 x g. After washing, the membrane pellets were resuspended in 90 μl of the above buffer with 1.5% Triton X 100, stirred for 3 h on ice, centrifuged for 1 h at 60 000 x g and supernatant adjusted to 100 μl. Proteins (20 μg corresponding to $1x10^6$ cells per well) were separated in nonreducing or reducing conditions by electrophoresis on 10% polyacrylamide gel 0.1% SDS in a Mini Protean apparatus (Bio Rad, Richmond CA). Dual color protein standards (Bio-Rad) were included in each gel. After electrophoresis proteins were blotted onto nitrocellulose membranes (Whatman, Dassel, Germany), blots were rocked for 1 h with 5% defatted milk in PBS and incubated overnight in the cold room with a antibody suitably diluted in PBS, 0.2% BSA, 0.1% Tween20. The following antibodies were used for specific protein recognition: anti-TSH receptor subunit A (A9, Advanced Targeting System, dilution 1:1500), TSH receptor subunit B (peptide 398-415, dilution 1:2000), anti-$G\alpha_s$ (Abcam, dilution 1:2000), anti-$G\alpha_{q/11}$ (Santa Cruz, dilution 1:100), anti-$G\alpha_i$ (Santa Cruz, dilution 1:100), anti-$G\alpha_{13}$ (Santa Cruz, dilution 1:100) and anti-$G\beta$ (Santa Cruz, dilution 1:500). The blots were then rinsed and incubated for 2 h at room temperature with the appropriate horsereadish peroxidase-conjugated second antibodies diluted 1:4000 (Dako). The signal was developed with Super Signal West-Pico chemiluminescent substrate (Pierce) and exposed against Kodak X-ray film.

Adenylate cyclase assay

For cAMP measurements 1 x 10^5 cells per well were seeded into 12-well plate and cultured overnight. Before the assay the medium was aspirated and 0.5 ml of fresh medium without atibiotic or FBS, containing 0.5 mM 3-isobutyl-1-methyl-xantine (IBMX) (Sigma-Aldrich) and the indicated in figure 2 concentration of bovine TSH (Sigma-Aldrich) was added. Control samples were without TSH. Samples were incubated for 0, 30 or 120 minutes at room temperature, then the medium was aspirated and the reaction was stopped with 250 μl 0.1M HCl. Concentration of cellular cAMP in non-stimulated and TSH stimulated samples was estimated with a cAMP enzyme immunoassay kit (Sigma Aldrich) according to the supplier's instruction.

Measurements of intracellular Ca^{2+} concentration in single cells

The cells grown on the glass coverslips in flexi Perm (Greiner Bio-One GmbH) were rapidly washed with Tyrode solution containing (in mM): 144 NaCl, 5 KCl, 1.5 $CaCl_2$ 1 $MgCl_2$, 0.43 NaH_2PO_4, 10 Hepes, 11 glucose, pH 7.4. For estimation of changes of intracellular Ca^{2+} concentration cells were incubated with 2.5 μM Indo-1 acetoxymethyl ester for 15 min at 37°C and after incubation rinsed in Tyrode solution. Then cells on the coverlips were placed on superfusion chamber mounted on the stage of inverted microscope (Nicon) equipped for epifluorescence and perfused with Tyrode solution at 37°C or 24°C. Changes in intracellular Ca^{2+} concentration were traced by monitoring the ratio of 405 nm to 495 nm Indo-1 fluorescence obtained from the output of Dual Channel Ratio Fluorometer (Biomedical Instrumentation Group, University of Pennsylvania). Once a stable baseline of fluorescence was obtained, the stimulator, TSH (Sigma-Aldrich) or ATP (Boehringer) were added to 10 mU/ml and 100 μM, respectively, from 100-fold concentrated stock solution. In some experiments two minutes before TSH application the purinergic receptor agonist N-(L-2-phenylisopropyl)-adenosine (PIA) (Sigma-Aldrich) was added.

RESULTS

Membrane protein characterization

Staining of electrophoreticaly separated Nthy-ori 3-1 cell membrane proteins with antibodies against TSH receptor revealed the presence of intact receptor as well as both A (MW about 50 kDa) and B (MW about 40 kDa) receptor subunits in membrane preparation (fig. 1A). Moreover, several α subunits of heterotrimeric G proteins were found: two isoforms of $G\alpha_s$

of 45 and 42 kDa, four isoforms of $G\alpha_q$, with that of 42 kDa predominant, $G\alpha_i$ of 41 kDa, and $G\alpha_{13}$ of 45 kDa, which indicated the presence of four principal classes of G protein in Nthy-ori 3-1 cells. Additionally the presence of protein G subunit β was observed (fig. 1B).

Adenylate cyclase assay

In Nthy-ori 3-1 cells 100 mU/ml TSH induced rapid accumulation of cAMP within 30 minutes and then progressive decrease of its concentration. A smaller dose of TSH, 10 mU/ml, gave lower but sustained accumulation of cAMP, whereas 1 mU/ml TSH did not increase cAMP concentration above the basal level (fig. 2).

Measurements of intracellular Ca^{2+} concentration

10 mU/ml TSH induced a transient increase of intracellular Ca^{2+} concentration in cells pretreated with PIA (fig. 3A). However, such effect was seen only in a few percent of investigated cells. The response was observed exclusively at 37°C. In contrast, ATP stimulation resulted in pronounced increase of intracellular Ca^{2+} concentration in all cells both at room temperature and at 37°C (fig. 3B).

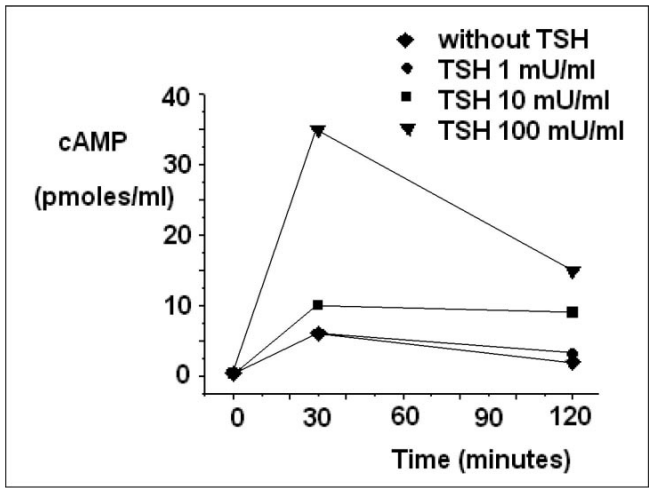

Fig. 2. Time course of cAMP accumulation in untreated Nthy-ori 3-1 cells and cells treated with 1 mU/ml, 10 mU/ml and 100 mU/ml of TSH, respectively.

DISCUSSION

TSH regulates thyroid function through a G protein-coupled TSH receptor present on thyrocytes membranes. Global gene expression profiling of the response of the rat thyroid cell line FRTL5 to TSH stimulation has identified a total of 123 TSH-regulated genes (7). In the plasma membrane TSH receptor undergoes posttranslational modification: it is proteolycally cleaved from a single-polypeptide form to dimeric form in which the two subunits A and B are held together by disulphide bonds (8). We found here that, similarly to the data for thyroid tissue, the membrane fraction of Nthy-ori 3-1 cells contains both A and B subunit of TSH receptor and all four principal G protein types, active in normal thyroids (8): G_s, G_q, G_i and $G_{12/13}$, as well as the G protein β subunits.

TSH receptor-dependent activation of the G_s/adenylate cyclase pathway mediates increased endocytosis of thyroglobulin from colloid, hormone secretion, growth and differentiation of thyroid cells, iodide uptake and organification via transcription of the peroxidase and sodium iodide transporter genes. Measurement of the amount of intracellular cAMP in the presence of a phosphodiesterase inhibitor, such as IBMX, directly reflects its accumulation. The most popular system for

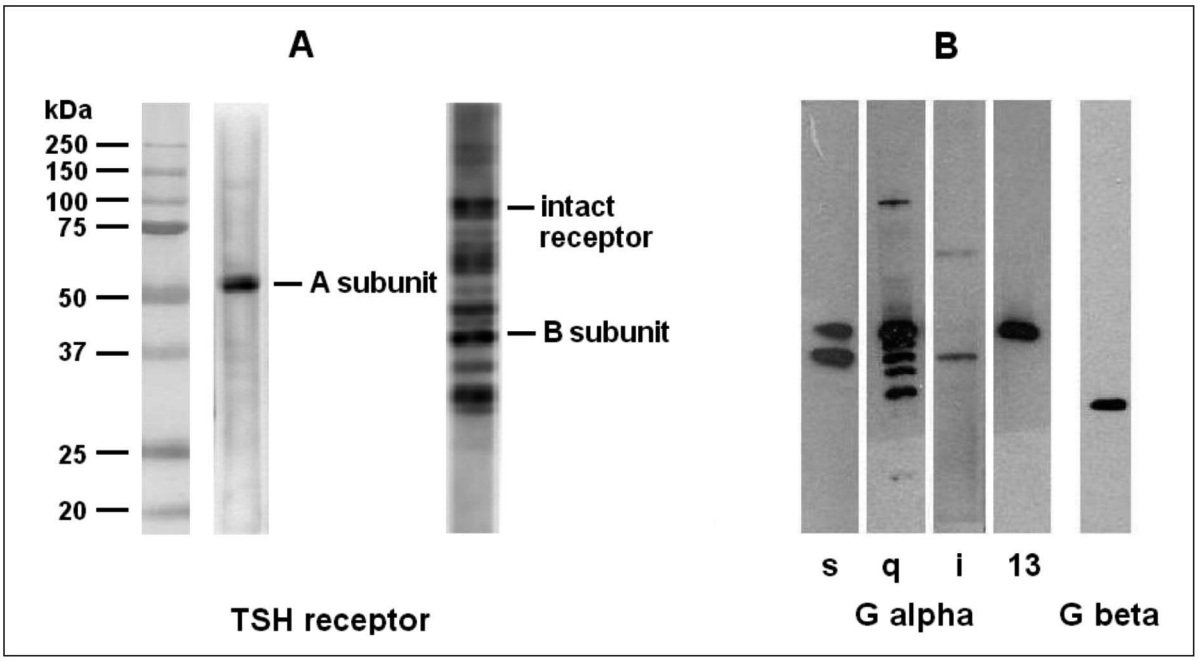

Fig. 1. Expression of TSH receptor and G proteins in plasma membrane of Nthy-ori 3-1 cells. Proteins are visualized on electroblots with antibodies directed against subunit A and B of the TSH receptor and G protein subunits: $G\alpha_s$, $G\alpha_q$, $G\alpha_i$, $G\alpha_{13}$ and Gβ.

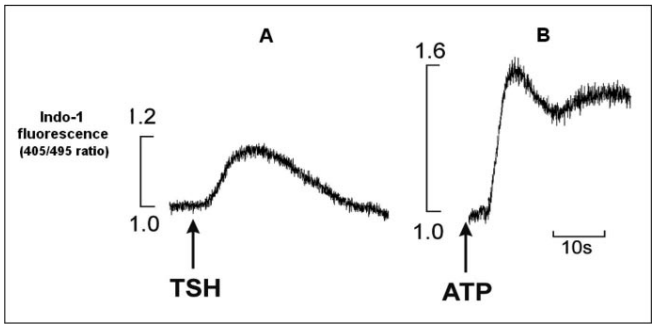

Fig. 3. Intracellular Ca^{2+} concentration in Nthy-ori 3-1 cells treated with 10 mU/ml TSH (A) or 100 μM ATP (B).

adenylate cyclase study is enzyme linked immunosorbent assay, where the amount of basal cAMP level is subtracted from the amount cAMP after TSH stimulation, and this method was used here. We found that TSH at concentration of 10 mU/ml or higher stimulated adenylate cyclase in Nthy-ori 3-1 cells. However, this effect is lower than one reported for other systems, e.g., in primary culture of thyroid cells (10), FRTL5 cells (11) and CHO cells expressing TSH receptor (12), where a stimulatory effect was already seen at the 1 mU/ml of TSH and the level of cAMP remain elevated for at least 2 h.

TSH can also induce the $G\alpha_q$ and $G\beta\gamma$-mediated stimulation of phospholipase C, leading to mobilization of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate. This second pathway stimulates the generation of H_2O_2 by directly regulating dual oxidases, enzymes crucial for all thyroid peroxidase-catalyzed reactions. A report about a familiar TSH receptor L653V mutation has provided *in vivo* evidence that the inositol phosphates/Ca^{2+} cascade mediates TSH-regulated iodination (13). Calcium signaling of thyrocytes is additionally modulated by TSH through calcium binding protein expression (14). In the present study we focused on the effect of TSH on intracellular Ca^{2+} and we found that TSH induced transient increase of intracellular Ca^{2+}concentration only in a few percent of cells. Moreover, this effect was noticed only at 37°C in cells pretreated with PIA, a purinergic receptor agonist. A non-uniform increase of Ca^{2+} concentration in individual cells was previously reported for primary culture of human thyroid cells (15, 16) and CHO cells expressing human TSH receptor (17). Hai *et al.* have postulated that the efficiency of the TSH stimulated pathways leading to intracellular Ca^{2+} increase depends on the extent of cell-cell contacts in cell culture. *In vivo*, where the thyrocytes are confluent this pathway is fully active (18). They link this dependency with TSH receptor maturation; cell-cell contacts control the TSH receptor cleavage by matrix protease and the TSH receptor cleavage is required for full activation of the phospholipase C pathway by TSH. In the present experiments cells were grown to about 80% confluency, but in spite of that the percentage of TSH-stimulated cells was much lower than previously reported indicating that stimulation of the Ca^{2+} concentration increase by TSH in Nthy-ori 3-1 cells is reduced. We studied also the effect of ATP, another regulator of thyroid function. *In vivo* ATP can be released from the autonomic innervation of the gland, from capillary endothelial cells or possibly from thyrocytes themselves (19). It stimulates increase of Ca^{2+} concentration via G protein-coupled P2Y purinergic receptors and such a stimulation we also found in Nthy-ori 3-1 cells.

Thyroid follicular cells are polar. The basolateral part of the cell, in thyroid tissue connected with the basement membrane, contains Na/K ATPase, Na^+/I^- symporter, TSH, P2Y and EGF receptors and is responsible for cell – environment interaction. When cells are cultured in plastic vessels, access to this region and its contact with cell milieu must be more difficult than it is in the tissue.

The apical part of the cell is involved in secretion of thyroglobulin into the follicular lumen, its iodination by peroxidase and in first stages of secretion of thyroid hormones: endocytosis of thyroglobulin from colloid followed by its hydrolysis. In the cytosol iodide and thyroglobulin are transported from basolateral to the apical domain and transport of thyroid hormones proceeds in the opposite direction.

In the follicle the cells are fixed by tight junctions. The polarity of cells cultured in a monolayer is disturbed, even if they are derived from a proper tissue. Trypsin or the non-enzymatic dissociation reagents used for release of the cells from their substratum completely destroy the polarity which also affects the transport pathways. After attachment to a plastic surface the cell polarity must be reconstructed. These disturbances seem not to be so drastic in non polar cells, such as CHO cells over-expressing TSH receptor. Thyroid cells from primary cultures are probably less disturbed than those from established culture and the polarity disturbances may be responsible for decreased stimulation by TSH.

In conclusion, the thyroid cells from Nthy-ori 3-1 line reveled lower sensitivity to TSH stimulation manifested by weaker adenylate cyclase activation and sporadic increase of intracellular Ca^{2+} concentration. These results should be taken into consideration when Nthy-ori 3-1 cells are going to be use as normal thyroid cells.

BIBLIOGRAPHY

1. Pulvertaft RJV, Davies JR, Weiss L, Wilkinson JH: Studies on tissue cultures of human pathological thyroids. J Pathol Bacteriol 1959; 77: 19-32.

2. Ambesi-Impiombato FS, Parks LAM, Coon HG: Culture of hormone dependent functional epithelial cells from rat thyroids. Proc Natl Acad Sci USA 1980; 77: 3455-3459.

3. Lemoine NR, Mayall ES, Jones T et al.: Characterization of human thyroid epithelial cells immortalized *in vitro* by simian virus 40 DNA transfection. Br J Cancer 1989; 60: 897-913.
4. Powell JG, Wang X, Allard BL et al: The PAX8/PPARγ fusion oncoprotein transforms immortalized human thyrocytes through a mechanism probably involving wild-type PPARγ inhibition. Oncogene 2004; 23: 3634-3641.
5. Lee JJ, Au AYM, Foukakis T et al.: Array-CGH identifies cyclin D1 and UBCH10 amplicons in anaplastic thyroid carcinoma. Endocrine-Related Cancer 2008; 15: 801-815.
6. Algeciras-Schimnich A, Milosevic D, McIver B et al.: Evaluation of the Pax8/PPARG translocation in follicular thyroid cancer with a 4-color reverse transcription PCR assay and automated high – resolution fragment analysis. Clin Chem 2010; 56: 391-98.
7. Calebiro D, Filippis T, Lucchi S et al.: Selective modulation of protein kinase A I and II reveals distinct roles in thyroid cell gene expression and growth. Mol Endocrinol 2006; 20: 3196.
8. Misrahi M, Milgrom E: Cleavage and shedding of the TSH receptor. Eur J Endocrinol 1997; 137: 599-602.
9. Laugwitz KL, Allgeier A, Offermans S et al.: The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. Proc Natl Acad Sci USA 1996; 93: 116-120.
10. Bidey SP, Marshall NJ, Ekins RP: Characterisation of the cyclic AMP response to thyrotropin in monolayer cultures of normal human thyroid cells. Acta Endocrinol (Copenh) 1981; 98: 370-376.
11. Bidey SP, Chiovato L, Day A et al.: Evaluation of the rat thyroid cell strain FRTL-5 as an *in vitro* bioassay system for thyrotropin. J Endocrinol 1984; 101: 269-276.
12. Perret J, Ludgate M, Libert F et al.: Stable expression of the human TSH receptor in CHO cells and characterization of differentially expressing clones. Biochim Biophys Res Commun 1990; 171: 1044-1050.
13. Grasberger H, VanSande J, Mahameed AHD et al.: A familial thyrotropin (TSH) receptor mutation provides in vivo evidence that the inositol phosphates/Ca cascade mediates TSH action on thyroid hormone synthesis. J Clin End Metab 2007; 92: 2816-2820.
14. Lorenz S, Eszlinger M, Paschke R et al.: Calcium signaling of thyrocytes is modulated by TSH through calcium binding protein expression. Biochim Biophys Acta 2010; 1803: 352-360.
15. Yanagita Y, Okajima F, Sho K et al.: An adenosine derivative cooperates with TSH and Graves' IgG to induce Ca mobilization in single human thyroid cells. Mol Cellular Endocrinol 1996; 118: 47-56.
16. D'Arcangelo D, Silletta MG, Francesco AL et al.: Physiological concentrations of thyrotropin increase cytosolic calcium levels in primary cultures of human thyroid cells. J Clin End Metab 1995; 80: 1136-1143.
17. Metcalfe RA, Findlay C, Robertson WR et al.: Differential effect of thyroid-stimulating hormone (TSH) on intracellular free calcium and cAMP in cells transfected with the human TSH receptor. J Endocrinol 1998; 157: 415-424.
18. Vu MT, Radu A, Ghinea N: The cleavage of thyroid-stimulating hormone receptor is dependent on cell-cell contacts and regulates the hormonal stimulation of phospholipase C. J Cell Mol Med 2009; 13: 2253-2260.
19. Kochukov MY, Rithie AK: A $P2X_7$ receptor stimulates plasma membrane trafficking in the FRTL rat thyrocyte cell line. Am J Physiol Cell Physiol 2004; 287: C992-C1002.

otrzymano/received: 12.09.2011
zaakceptowano/accepted: 17.10.2011

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