Intracellular cross-talk between thyrotropin receptor and A₁ adenosine receptor in regulation of phospholipase C and adenylate cyclase in COS-7 cells transfected with their receptor genes

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COS-7 cells were transiently transfected with human thyrotropin receptor (TSHR) and dog A₁ adenosine receptor (A₁R) cDNA. TSH stimulated both inositol phosphate production and cyclic AMP (cAMP) accumulation in the cells. An A₁ agonist, N⁶-(L-2-phenylisopropyl)adenosine (PIA), which is ineffective alone, significantly enhanced TSH-induced inositol phosphate production, but insignificantly inhibited TSH-induced cAMP accumulation. The PIA inhibition of cAMP accumulation was revealed by short-term treatment with the protein kinase C inhibitors, staurosporine and K252a, or long-term treatment with 12-myristate 13-acetate, suggesting that endogenous protein kinase C inhibits the A₁R-mediated inhibition of the TSHR–

adenylate cyclase system. In staurosporine-treated cells, the stimulatory and inhibitory permissive actions of PIA on TSH-induced phospholipase C and adenylate cyclase activation respectively were completely reversed by pretreatment with pertussis toxin whereas intrinsic TSH-induced effects were hardly affected by the toxin. The cross-talk between the signalling pathway for TSHR and that for A₁R was not detected in a mixture of cells expressing either TSHR or A₁R. We conclude that a single species of A₁R, via pertussis-toxin-sensitive GTP-binding proteins, not only inhibits adenylate cyclase but also stimulates phospholipase C in collaboration with an activated TSHR within a single cell expressing both types of receptor.

INTRODUCTION

In thyroid cells, thyrotropin (TSH) activates phospholipase C as well as adenylate cyclase leading to the production of intracellular second messengers, i.e. inositol 1,4,5-trisphosphate, diacylglycerol and cyclic AMP (cAMP). These three second messengers stimulate an intracellular Ca²⁺-mobilizing system, protein kinase C and protein kinase A respectively [1–3], suggesting important roles for these signalling pathways in the regulation of thyroid function [1–3]. However, compared with adenylate cyclase activity, phospholipase C activity is approximately three orders and one order of magnitude less sensitive to TSH in rat [4] and human [5] thyroid cells respectively. This reveals a major criticism of the theory of the physiological role of the phospholipase C–Ca²⁺ system in the action of TSH [3].

We have recently shown that, in rat FRTL-5 thyroid cells, adenosine and its derivatives inhibited TSH-induced adenylate cyclase activation and, in contrast, stimulated hormone-induced phospholipase C activation and subsequent Ca²⁺ mobilization [4]. As a result, in the presence of adenosine or its derivatives, TSH stimulation of both the phospholipase C–Ca²⁺ and the adenylate cyclase–cAMP systems can be detected within the physiological concentration range of TSH [4]. As thyroid gland is well innervated [1–3], adenosine may be produced by the ectonucleotidase digestion of ATP which is secreted as a co-transmitter. Indeed, exposure of FRTL-5 cells to ATP causes adenosine production in the incubation medium, suggesting the existence of ectonucleotidases in thyroid cell membranes [6]. Adenosine is also released from thyroid cells as a paracrine or autocrine factor [4]. Furthermore, goitres were formed in transgenic mice that overexpressed A₂ adenosine receptors in the thyroid, and it was demonstrated that cellular growth in the thyroid gland was stimulated *in vivo* by adenosine-induced cAMP

overproduction via the A₂ receptor leading to goitre formation [7]. These results support the idea that TSH signal transduction is modulated by adenosine *in vivo*.

However, there are three basic problems to be solved. First, which adenosine receptor subtype is responsible for the permissive action of adenosine and its derivatives? Although pharmacological specificity of adenosine receptor agonists and antagonists has suggested the involvement of A₁ adenosine receptor (A₁R) [8], it is possible that a different receptor subtype with similar ligand specificity to, but different effector specificity from, A₁R causes permissive stimulation of phospholipase C [9]. Secondly, does the co-operation between the adenosine receptor and the TSH receptor (TSHR) system occur in a single cell that expresses both systems or between cells, one expressing the adenosine receptor and the other TSHR, in a cell population that is heterogeneous with respect to receptor expression? Thirdly, does any thyroid-specific factor participate in the permissive stimulation of TSH action by adenosine. To clarify these points, in the present study COS-7 cells were transfected with TSHR cDNA derived from human thyroid and/or A₁R cDNA from dog thyroid. The results show that a single species of A₁R can couple to the adenylate cyclase and phospholipase C systems in conjunction with an activated TSHR, but without any other thyroid-specific factors, in cells that express both TSHR and A₁R.

EXPERIMENTAL

Materials

TSH, N⁶-(L-2-phenylisopropyl)adenosine (PIA) and forskolin were purchased from Sigma; staurosporine and K252a were from Kyowa Medex Co. (Tokyo, Japan); 8-[³H]cyclopentyl-1,3-

Abbreviations used: PIA, N⁶-(L-2-phenylisopropyl)adenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; TSH, thyroid-stimulating hormone (thyrotropin); TSHR, thyrotropin receptor; A₁R, A₁ adenosine receptor; PTX, pertussis toxin; Ro20-1724, 4-(3-butoxy-4-methoxybenzoyl)-2-imidazolidione; cAMP, cyclic AMP; PMA, phorbol 12-myristate 13-acetate.

Figure 1 Effects of TSH and forskolin on inositol phosphate production and cAMP accumulation

COS-7 cells transfected with TSHR cDNA (a and c) or co-transfected with TSHR and A₁R cDNAs (b and d) were incubated alone (control) or with TSH (100 nM) or forskolin (25 µM) in the presence (■) or absence (□) of 100 nM PIA in HEPES-buffered medium containing 10 mM LiCl, 0.1 mM Ro20-1724 and 0.5 unit/ml adenosine deaminase. Results are means ± S.E.M. of three values of a representative experiment. Data are expressed as percentages of basal values in the absence of TSH and PIA taken as 100% for inositol phosphate production (a and b) and as cell content (nmol/mg of protein) for cAMP accumulation (c and d). Two separate experiments gave similar results. *Significant effect of PIA ($P < 0.01$).

dipropylxanthine (CPX) (109.2 Ci/ml) came from Du Pont–New England Nuclear. Human TSHR cDNA [10] and dog A₁R cDNA [11] were generously provided by Dr. G. Vassart (Université Libre de Bruxelles), pCDL-SRα 296 vector (SRα) [12] by Dr. Y. Takebe (National Institute of Health, Tokyo, Japan) and pertussis toxin (PTX) by Dr. M. Ui (Tokyo Uni-

versity). Radioimmunoassay of cAMP used a Yamasa cAMP assay kit which was a gift from Yamasa Shoyu Co. (Choshi, Chiba, Japan). The sources of all other reagents were as described previously [4,6,8].

Cell culture and cDNA transfection

COS-7 cells were cultured on 10 cm dishes in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum in a 5% CO₂ atmosphere at 37 °C. The cells were harvested for transfection experiments by using 0.05% trypsin in 0.53 mM EDTA (Gibco). A₁R cDNA inserted in an SRα vector [13] or TSHR cDNA inserted in a pSVL vector [10] or a combination of the two was transfected into COS-7 cells suspended in Mg²⁺- and Ca²⁺-free PBS (20 µg of DNA in 0.8 ml) by electroporation (0.3 kV, 500 µF). The cells (about 2 × 10⁶) were cultured for 2 days on 24-well plates in inositol-free Dulbecco's modified Eagle's medium supplemented with [³H]inositol (2 µCi/ml) and 10% fetal calf serum. In Figure 2, where time-course experiments were performed, 6 µCi/ml [³H]inositol was used. For [³H]CPX-binding experiments, [³H]inositol was omitted from the medium.

Assay of inositol phosphate and cAMP

The cells were washed twice with Hepes-buffered medium [10 mM Hepes (pH 7.5), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 2.5 mM NaHCO₃, 5 mM glucose and 0.1% (w/v) BSA (fraction V)] and then incubated for 30 min unless otherwise stated at 37 °C, with the same medium containing 10 mM LiCl, 0.1 mM Ro20-1724, 0.5 unit/ml adenosine deaminase and the agents to be tested in a final volume of 0.5 ml. The reaction was terminated by the addition of 0.1 ml of 1 M HCl. The contents of cAMP and inositol phosphate were measured in the same HCl extract. cAMP was measured by radioimmunoassay. [³H]Inositol mono-, bis- and tris-phosphates were separated from inositol and glycerophosphoinositol on Dowex 1 (X8; formate form) columns as described previously [4]. Except for the experiment in Figure 2, total inositol phosphates were measured as an index of phospholipase C activity.

Table 1 Effect of protein kinase C inhibitors and long-term phorbol 12-myristate 13-acetate (PMA) treatment on the action of PIA

COS-7 cells were co-transfected with both TSHR and A₁R cDNA. In Experiment 1, the cells were incubated with TSH (100 nM), forskolin (25 µM) or vehicle in the presence or absence of PIA (100 nM). The incubation medium was further supplemented with nothing (control), staurosporine or K252a. In Experiment 2, the cells were treated with PMA or its vehicle (control) for 24 h, then incubated with or without TSH (100 nM) in the presence or absence of PIA. Other experimental conditions were as for Figure 1. Changes in inositol phosphate production are presented as percentage above the basal value each obtained without TSH under the respective conditions, and changes in cAMP accumulation as nmol/mg of protein. Values in parentheses indicate effects of PIA expressed as percentage of the values obtained in the absence of PIA. Results shown are means ± S.E.M. of quadruplicate samples from a representative experiment. Two separate experiments gave similar results. *Effect of PIA is significant ($P < 0.01$). ND, not determined.

	TSH-induced inositol phosphate production (% above basal value)		TSH-induced cAMP accumulation (nmol/mg)		Forskolin-induced cAMP accumulation (nmol/mg)	
	– PIA	+ PIA	– PIA	+ PIA	– PIA	+ PIA
Experiment 1						
Control	143 ± 4	261 ± 10* (183)	14.4 ± 0.6	14.7 ± 0.5 (102)	17.3 ± 0.4	13.2 ± 0.3* (77)
Staurosporine (1 µg/ml)	151 ± 15	283 ± 8* (187)	19.9 ± 0.6	14.9 ± 0.2* (75)	20.5 ± 1.2	15.3 ± 0.2* (74)
K252a (1 µM)	207 ± 10	333 ± 15* (161)	16.5 ± 0.6	13.0 ± 0.2* (81)	ND	ND
Experiment 2						
Control	50 ± 9	110 ± 3* (220)	11.1 ± 0.3	10.7 ± 0.3 (96)	ND	ND
Long-term PMA (1 µg/ml)	42 ± 7	87 ± 7* (207)	7.5 ± 0.3	5.7 ± 0.1* (76)	ND	ND

Figure 2 Time-course effect of TSH and PIA on inositol phosphate production and cAMP accumulation

COS-7 cells co-transfected with TSHR and A_1R cDNA were incubated in the presence of staurosporine ($1 \mu\text{g/ml}$) alone ($\circ$), or with 100 nM PIA ($\bullet$), 100 nM TSH ($\triangle$) or a combination ($\blacktriangle$) of PIA (100 nM) and TSH (100 nM) for the indicated times. Other experimental conditions were as for Figure 1. Results are means $\pm$ S.E.M. of three values of a representative experiment. Data are expressed as percentages of the initial values for inositol

$[^3\text{H}]\text{CPX}$ binding

The cells were washed twice with Hepes-buffered medium, then incubated in the same medium with 1 nM $[^3\text{H}]\text{CPX}$ (about 10000 c.p.m.) in the presence or absence of $1 \mu\text{M}$ CPX for the indicated times at 37°C . The cells were again washed twice with ice-cold Hepes-buffered medium, then membrane-bound $[^3\text{H}]\text{CPX}$ was extracted with 5% trichloroacetic acid (0.5 ml). Radioactivity in the extract was measured by a liquid-scintillation counter. Specific binding was defined as the radioactivity displaced by the addition of $1 \mu\text{M}$ unlabelled CPX.

Data presentation

All experiments were performed in triplicate or quadruplicate with more than three different batches of cells. The results are presented as means $\pm$ S.E.M. of three or four values in a representative experiment unless otherwise stated.

RESULTS

A single species of A_1R can mediate not only inhibition of adenylate cyclase but also stimulation of phospholipase C when both enzymes are activated by TSH

Native and pSVL vector- or $\text{SR}\alpha$ vector-transfected COS-7 cells showed no response to TSH (results not shown). However, in cells transfected with TSHR cDNA, TSH clearly stimulated both inositol phosphate production (Figure 1a) and cAMP accumulation (Figure 1c) via phospholipase C and adenylate cyclase respectively. Forskolin also induced cAMP accumulation to a similar extent to that induced by TSH (Figure 1c). Addition of PIA, an A_1 agonist, hardly affected these enzyme activities under basal and TSH- or forskolin-stimulated conditions (Figures 1a and 1c), indicating almost no activity of functional A_1R in native COS-7 cells.

When the cells were co-transfected with both TSHR and A_1R cDNA, PIA, which was ineffective alone, enhanced TSH-induced phospholipase C activation (Figure 1b) in a similar way to that in FRTL-5 cells [4]. Unexpectedly, the A_1 agonist did not inhibit TSH-induced cAMP accumulation, whereas the action of forskolin was clearly inhibited by the A_1 agonist (Figure 1d). In relation to this, it should be noted that protein kinase C has been reported to phosphorylate several components of the adenylate cyclase system, e.g. the α -subunit of G_i -protein [14] and adenylate cyclase catalytic subunit [15], which in turn may inhibit the action of PIA on TSH-induced activation of adenylate cyclase. To examine this possibility, we first used the protein kinase C inhibitors, staurosporine and K252a. As shown in Table 1 (Experiment 1), in the presence of these inhibitors, PIA significantly inhibited cAMP accumulation induced by TSH, as in the case of forskolin. The role of protein kinase C was further investigated by using cells pretreated for 24 h with a high dose of PMA ($1 \mu\text{g/ml}$) to down-regulate the enzyme [16]. Under such conditions also, PIA significantly inhibited TSH-induced cAMP accumulation (Table 1, Experiment 2). These results suggest that the activation of protein kinase C caused by TSH plays a suppressive role in A_1R -mediated inhibition of the TSHR-adenylate cyclase system. As shown in Table 1, the short-term treatment of the cells by protein kinase C inhibitors slightly increased the basal values (in the absence of PIA) but the long-term treatment by PMA slightly decreased them. As the changes

phosphate production (a to c); the initial values (c.p.m.) for inositol mono- (a), bis- (b) and tris- (c) phosphate were 989 ± 35 , 432 ± 55 and 1165 ± 7 respectively. For cAMP response (d), the data are expressed as cell content (nmol/mg of protein).

Figure 3 Effect of PTX on the actions of TSH and PIA

COS-7 cells were co-transfected with TSHR and A₂R cDNA, pretreated with (b and d) or without (a and c) PTX (100 ng/ml) for 18 h, and then incubated with staurosporine (1 μ g/ml) and the indicated doses of TSH in the presence (●) or absence (○) of PIA (100 nM). Inositol phosphate production (a and b) and cAMP accumulation (c and d) were measured. Other experimental conditions were as for Figure 1. Results are expressed as percentages of the basal values obtained in the absence of TSH and PIA taken as 100% in (a) and (b), and as cell content (nmol/mg of protein) in (c) and (d). The PTX treatment did not significantly change the basal value of either inositol phosphate or cAMP. Insets in (a) and (c) show dose-dependent effects of PIA on TSH-induced inositol phosphate production and cAMP accumulation respectively where the cells were incubated with the indicated dose of PIA in the presence or absence of 100 nM TSH. Changes in activity induced by TSH are presented as percentages of those in the absence of PIA.

are small and not entirely consistent with the predicted changes in protein kinase C activity, they may reflect non-specific actions of these agents [17] rather than protein kinase C inhibition or depletion.

On the basis of the above results, the following experiments (Figure 2–4) were performed in the presence of staurosporine. Figure 2 shows time-dependent effects of TSH and PIA on the production of three species of inositol phosphate (Figures 2a–2c) and cAMP accumulation (Figure 2d). TSH increased levels of all the three species of inositol phosphate, but their time courses were somewhat different from those generally found for Ca²⁺-mobilizing hormones, which for inositol bis- and tris-phosphates, especially trisphosphate, show rather sharp peaks, with a transient increase in their levels [18]. In contrast, in the present system, TSH induced a slow increase with no saturation within 30 min for all three labelled inositol phosphates (Figures 2a–2c). This may reflect no or a very low level of desensitization caused by TSH in its receptor-system coupling to phospholipase C.

Consistent with this, in FRTL-5 thyroid cells also, TSH has been shown to induce a slow increase in the levels of inositol bis- and tris-phosphates for at least 15 min of incubation [19]. As shown in Figures 2(a)–2(c), PIA enhanced TSH-induced production of the three inositol phosphates, although alone it hardly affected their production during the whole period of the experiment. The enhancement of TSH-induced inositol phosphate production by PIA was accompanied by inhibition of TSH-induced cAMP accumulation (Figure 2d).

PTX-sensitive G-protein is involved in A₂R-mediated action but not in TSHR-mediated action

Figure 3 shows the effect of PIA on the dose–response curves of TSH-induced inositol phosphate production (Figure 3a) and cAMP accumulation (Figure 3c). Consistent with the findings in thyroid cells [4,5], a half-maximal effective dose of TSH is about two orders of magnitude higher for phospholipase C activation

Figure 4 Absence of reciprocal modulation by PIA of TSH-induced effects in the mixture of cells expressing either TSHR or A₁R

Cells were transfected with SRα vector (SRα cells), TSHR cDNA (TSHR cells), A₁R cDNA (A₁R cells) or with TSHR and A₁R cDNA (TSHR + A₁R cells). Mixtures of TSHR cells and A₁R cells in equal proportions (a, c and e) and TSHR + A₁R cells and SRα cells in equal proportions (b, d and f) were used. The cell mixtures were incubated in the presence of staurosporine (1 μg/ml), without (control) or with the indicated doses of TSH or 25 μM forskolin, and inositol phosphate production and cAMP accumulation were measured. Other experimental conditions and data presentation are as for Figure 1. In (e) and (f), the time course of [3H]CPX binding is shown. The cells were incubated with 1 nM [3H]CPX for the indicated times in the presence (○) or absence (●) of 1 μM CPX. At 20 min, when binding was saturated, 1 μM CPX was added to the incubation medium in some experiments (▲); [3H]CPX binding was decreased to the level of non-specific binding, indicating that the binding is reversible. *Significant effect of PIA ($P < 0.01$).

than for adenylate cyclase activation. PIA alone had no effect on the basal activity, but significantly enhanced TSH-induced inositol phosphate production and inhibited hormone-induced cAMP accumulation. The dose-dependent modulation by PIA of the actions of TSH (insets in Figures 3a and 3c) shows that the A₁ agonist as slightly more effective in adenylate cyclase inhibition than phospholipase C enhancement. As shown in Figures 3(b) and 3(d), PTX treatment completely inhibited the PIA-induced effects, whereas the toxin hardly influenced those induced by TSH, suggesting that the expressed A₁R couples to a PTX-sensitive G-protein(s) to exert both phospholipase C stimulation and adenylate cyclase inhibition.

Cross-talk between TSHR and A₁R occurs when both receptors are expressed in the same cell

As we cannot expect 100 % transfection efficiency of cDNA into the cells, the co-transfected cells with TSHR and A₁R cDNA may be a mixture of at least four groups of cells, i.e. cells expressing TSHR but not A₁R, A₁R but not TSHR, both TSHR and A₁R, and neither TSHR nor A₁R. We therefore cannot rule out the possibility that the cross-talk between the TSHR and A₁R systems may occur as a result of paracrine interactions between the cells, one expressing TSHR and the other expressing A₁R, rather than as a result of intracellular cross-talk between the TSHR and A₁R systems expressed in a single cell. To clarify this point, we prepared cells transfected with either TSHR cDNA (TSHR cells) or A₁R cDNA (A₁R cells) and then mixed the two groups of cells in equal proportions. As shown in Figures 4(a) and 4(c), the cell mixture did not show any significant effect of PIA on either TSH-induced inositol phosphate production or cAMP accumulation. Forskolin-induced cAMP accumulation without TSHR activation was significantly inhibited by the A₁ agonist probably via A₁R. In contrast results consistent with those in Figures 2 and 3 were obtained even when the cells co-transfected with both TSHR and A₁R cDNA (TSHR + A₁R cells) were mixed with cells that were transfected with only SRα vector (SRα cells) to equalize the number of TSHR and A₁R expressed per unit cell population in a culture well with that in the mixture of TSHR and A₁R cells. There were no significant differences in binding of A₁ antagonist [3H]CPX (Figures 4e and 4f) and the rate of PIA inhibition of forskolin-induced cAMP accumulation (Figures 4c and 4d) between the two cell mixtures, showing that the extent of A₁R expression in the mixture of TSHR cells and A₁R cells was the same as that in the mixture of TSHR-A₁R cells and SRα cells. On the other hand, TSH-induced inositol phosphate production and cAMP accumulation were higher in the former mixture (Figures 4a and 4c) than in the latter (Figures 4b and 4d). Taken together, these results suggest only a negligible contribution, if any, of cell-cell interaction in the modulation of actions of TSH by PIA.

DISCUSSION

In this paper, we report the following new observations. The modulation of TSH action by an A₁ agonist, PIA, can occur in cells other than thyroid cells if the appropriate receptors are expressed. Thus PIA, which is ineffective alone, inhibits TSH stimulation of adenylate cyclase and enhances TSH-induced phospholipase C in COS-7 cells co-transfected with TSHR and A₁R. In this event, a single molecular species of A₁R can couple to the two effector systems in collaboration with an activated TSHR. Inhibition of TSH-induced adenylate cyclase activation by PIA was, however, observed only when endogenous protein kinase C was inhibited or depleted, suggesting that protein kinase C attenuates the A₁R-mediated inhibition of adenylate cyclase. Finally, the cross-talk occurs predominantly between activated TSHR and A₁R systems in single cells transfected with both TSHR and A₁R but not between cells expressing either TSHR or A₁R.

In FRTL-5 thyroid cells, PIA also enhances α₁-adrenergic receptor- [20] and P₂-purinergic receptor- [8,21] as well as TSHR-mediated phospholipase C activation. Similarly, adenosine and its derivatives potentiates phospholipase C activation induced by IgE in RBL2H3 cells [22] and ATP and bradykinin in the smooth muscle cell line [23,24]. In NG108-15 cells, enkephalin, somatostatin, α₂-agonist and carbachol have also been shown to enhance P₂-receptor agonist or bradykinin-induced phospholipase C activation and Ca²⁺ mobilization in a PTX-sensitive manner

[25–27]. These results further support the idea that the present findings of the cross-talk between the TSHR and A_1R systems may represent a universal mechanism mediated by a PTX-sensitive G_i/G_o -protein, via which the Ca^{2+} -mobilizing receptor agonist-dependent phospholipase C is synergistically or permissively stimulated by the adenylate cyclase-inhibiting receptor agonists.

As shown in Figure 1, forskolin-induced but not TSH-induced cAMP accumulation was inhibited by PIA, suggesting that the failure of the inhibitory action of PIA in the latter case was caused by the action of TSH itself. In fact, when protein kinase C, which is located downstream of the TSH-induced phospholipase C cascade, was inactivated or desensitized, PIA inhibition of TSH-induced cAMP accumulation was restored. Previous reports have already suggested that the α -subunit of G_i -protein [14] and the catalytic subunit of adenylate cyclase [15] are phosphorylated by protein kinase C and thereby inactivated. On the other hand, in these cases all the G_i -protein-mediated inhibitory mechanisms, including the inhibition of the forskolin-induced action, were attenuated by protein kinase C. In contrast, our result (Table 1) show that the action of PIA on forskolin-induced cAMP accumulation was hardly affected by staurosporine, a protein kinase C inhibitor. This suggests the involvement of a different mechanism from that suggested in previous reports [14,15] for the protein kinase C attenuation of A_1R -mediated inhibition of the TSHR–adenylate cyclase system in transfected COS-7 cells. In relation to this, the presence of an unusual subtype of adenylate cyclase in COS-7 cells has been reported, which is activated by $\beta\gamma$ -subunits of G_i/G_o -protein in the presence of $G_{s\alpha}$ under specific conditions [28], although it has not been investigated whether this $\beta\gamma$ -subunit-dependent stimulation is influenced by protein kinase C.

The results in the present study, under conditions in which protein kinase C is inactivated, are consistent with those of pharmacological studies of FRTL-5 cells [4,8] and clearly indicate that a single species of A_1R can mediate not only potentiation of TSH-induced phospholipase C activation but also inhibition of hormone-induced adenylate cyclase activation. In similar studies, we have recently found that A_1R expressed in Chinese hamster ovary cells transfected with its cDNA couples to three signal-transducing systems, i.e. adenylate cyclase-inhibiting and phospholipase C and A_2 -stimulating systems [13].

The main objective of the present study was to clarify whether the modulation of TSH-induced responses by A_1 agonists occurs through intracellular cross-talk between the signalling pathway for TSHR and that for A_1R within a single cell or through intercellular communication in heterogeneous cell populations. As mentioned above, similar cross-talk between Ca^{2+} -mobilizing hormone receptor and G_i/G_o -protein coupled receptor systems in the regulation of phospholipase C activity has been reported in various cell types [4,20–27] which were cloned and hence assumed to be homogeneous. As far as regulatory mechanisms of cell function are concerned, however, they may be variable under the influence of intracellular or extracellular stimuli even in the cloned cell lines. A good example is the cell-cycle-dependent change in receptor functions [29]. We cannot therefore rule out the possibility that the observed phenomena occurred as the result of interactions among functionally heterogeneous cells. However, the lack of PIA effect on the action of TSH in the mixture of TSHR-transfected and A_1R -transfected cells suggests that the cell–cell interaction is not a major cause, if at all, of the modulation of the TSH-induced responses by a signal produced via A_1R . Hence we conclude that the cross-talk between the TSHR and A_1R systems occurs predominantly through interactions between the signalling pathways within a single cell.

The molecular mechanism of PIA modulation of TSH-induced responses, especially phospholipase C activation, remains unclear. The effects of PIA were completely reversed by PTX, suggesting the involvement of PTX-sensitive G-proteins, G_i/G_o . On the other hand, intrinsic responses induced by TSH are PTX-insensitive. In analogy with other transmembrane types of receptor, TSHR may couple to G_s - and G_q -proteins to activate adenylate cyclase and phospholipase C respectively. The mechanism of inhibition of G_s -protein-induced adenylate cyclase activation by G_i/G_o -protein has been explained as the α -subunit liberated from the activated G_s -protein being inhibited by interacting with $\beta\gamma$ -subunits derived from the receptor-stimulated G_i/G_o -protein [30]. If phospholipase C activity is attenuated by cAMP-dependent phosphorylation [31], the PIA potentiation of TSH-induced phospholipase C can be simply explained by the recovery of attenuated phospholipase C activity through the decrease in cAMP level mediated by PIA-induced G_i/G_o -protein activation. However, the results obtained in our studies suggest that this mechanism is unlikely. First, PIA enhanced TSH-induced phospholipase C activation under conditions in which PIA did not change the cAMP concentration (Figure 1). Secondly, in FRTL-5 cells, similar PIA enhancement of α_1 -adrenergic as well as P_2 -purinergic receptor-mediated phospholipase C activation was independent of cellular cAMP level [20,21]. On the other hand, $\beta\gamma$ -subunits of G_i/G_o -protein have been recently shown to stimulate directly β_2 -type phospholipase C [32,33]. This mechanism, however, cannot explain the permissive nature of PIA-dependent stimulation of phospholipase C observed in the present study in which the A_1 agonist alone, which activates G_i/G_o -protein to release $\beta\gamma$ -subunits, did not induce enzyme activation. Interaction between a G-protein(s), which couples to phospholipase C in a stimulatory fashion, and G_i/G_o -protein, which couples to adenylate cyclase in an inhibitory fashion, may play a role in the modulation of phospholipase C activity as reported in a turkey erythrocyte system [34] and discussed previously [4,35].

The inhibitory effect of PIA on TSH-induced cAMP accumulation was much weaker in COS-7 cells transfected with receptors than in FRTL-5 thyroid cells. PIA inhibited TSH-induced cAMP accumulation by only 30% at most even in the presence of protein kinase C inhibitors. This may be partly explained by the low population of cells successfully co-transfected with both TSH and adenosine receptors. Another possible cause is, as discussed above, the possible presence in COS-7 cells of the unusual subtype of adenylate cyclase that is activated by $\beta\gamma$ -subunits with the aid of $G_{s\alpha}$ [28]. The rate of PIA inhibition of cAMP accumulation induced by the ordinary type of adenylate cyclase might be reduced by this $\beta\gamma$ -dependent stimulation of the unusual adenylate cyclase subtype. Thus the reconstructed system is not ideal for further investigation of quantitative aspects of cross-talk between the TSHR and A_1R signalling pathways. Nevertheless, with the aid of protein kinase C inhibitors, the transfected COS-7 cells successfully reproduced basic features of the modulation of TSH action by adenosine receptor agonists. In addition, in some preliminary experiments, overexpression of β - and γ -subunits of G_i/G_o -protein in COS-7 cells clearly enhanced TSH-induced activation of phospholipase C. Thus the transfected COS-7 cell system is still useful at least for further characterization of the mechanism by which stimulation of the A_1R – G_i/G_o system enhances TSH-induced phospholipase C activation.

In conclusion, in COS-7 cells co-transfected with TSHR and A_1R , a single species of A_1R , through a PTX-sensitive G-protein(s), couples to phospholipase C in a stimulatory fashion and to adenylate cyclase in an inhibitory fashion in collaboration

with an activated TSHR. This cross-talk probably occurs between TSHR and A₁R systems in a single cell expressing both types of receptor.

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