Differential effects of NaCl concentration on the constitutive activity of the thyrotropin and the luteinizing hormone/chorionic gonadotropin receptors

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Abstract The TSH receptor (TSHR) and the LH/CG receptor (LHR) are members of the family of G protein-coupled receptors. Recently, point mutations conferring constitutive activity to the TSHR and LHR have been observed as a cause of toxic adenoma and familial/sporadic male pseudo-precocious puberty, respectively. When evaluated by transfection in COS-7 cells the wild-type (wt) TSHR displays definite constitutive activity towards Gs-dependent adenylylcyclase stimulation, while available evidence shows that the LHR does not. In order to compare the constitutive activity of both receptors, we performed functional studies in COS-7 cells using different assay conditions. Human TSHR and LHR cDNAs subcloned in the expression vector pSVL were transiently expressed in COS-7 cells and cAMP production was determined following incubation in a medium containing physiological concentration of NaCl [isotonic (NaCl)] or in the same medium without NaCl [hypotonic ($NaCl^-$)] or where NaCl was replaced by an isoosmolar concentration of sucrose [isotonic (sucrose)]. Cells transfected with the TSHR showed higher basal cAMP levels over cells transfected with pSVL in all conditions tested. The effect was stronger when cells were incubated in isotonic (sucrose) buffer. Cells expressing LHR exhibited a minimal increase of cAMP levels over cells transfected with pSVL in isotonic (NaCl) buffer; however, a marked increase in basal cAMP levels was observed when cells were assayed in hypotonic ($NaCl^-$) or isotonic (sucrose) buffers. Varying the pH or incubation temperature was without effect on the results obtained with both receptors. Our data show that despite extensive sequence similarity, the LH and TSH receptors differ markedly in their basal activity. The differential sensitivity of both receptors to low NaCl concentrations, suggests that the unliganded TSH receptor is less constrained than its LH homolog and may be more susceptible to activation by a wide spectrum of mutations.

Key words: Thyrotropin receptor; Luteinizing/Chorionic gonadotropin receptor; G protein-coupled receptor; Constitutive activity; cAMP; NaCl-free medium

1. Introduction

The TSH receptor (TSHR) and LH/CG receptor (LHR) together with the FSH receptor are members of a subfamily of G protein-coupled receptors (GPCR) [1–3]. These receptors share a similar structural pattern with seven transmembrane segments connected by three extra and intracellular loops, a short cytoplasmic tail and a long extracellular domains that has been shown to encode the specificity for hormone recognition and binding [1,2,4]. Both the TSH and LH receptors have the potential to activate the cAMP as well as inositolphoshate-Ca^{2+} cascades through activation of two independent G proteins, Gs-α and Gq-α, respectively [4–7]. Recently, somatic and germline mutations of the TSHR gene causing toxic adenoma or toxic thyroid hyperplasia, respectively, have been identified [8–12]. These mutations cause increased agonist-independent production of cAMP (all cases) [8–12] and inositolphosphates (some somatic mutations) [12] when evaluated by transient expression in COS-7 cells. Similar activating mutations affecting LHR have been described as a cause of familial and sporadic male pseudoprecocious puberty [13,14]. Interestingly, when tested in COS-7 cells, the wt TSHR has been shown to activate cAMP production in the absence of agonist i.e. it displays also some constitutive activity [8–11,15,16]. On the contrary, available evidence suggests that when transiently expressed in COS-7 cells the LHR, unlike TSHR, does not show any constitutive activity [13].

The present study was conducted to explore in greater detail the different behaviour of the two receptors. Our results confirm that the unliganded TSHR exhibits definite constitutive activity under conditions where the LHR is virtually silent. They demonstrate that removal of NaCl from the medium bathing COS-7 cells transfected with the cDNA constructs, unmasks strong constitutive activity of the LHR, while still increasing the basal activity of the TSHR.

2. Materials and methods

2.1. Constructs

The plasmid pSVL containing the human TSHR and mouse melanocortin receptor (MC5R) cDNAs have been previously described [17,18]. The cDNA of the human LHR in the pUC vector was a kind gift from Professor T. Minegishi (Gunma University,Japan) The cDNA was subcloned in pBluescript SK(+). A *Xho*I restriction site followed by a consensus sequence (GGAAAA) was introduced just before the first ATG by PCR with the following primers: forward primer: 5′-GCCTCGAGGGAAAAATGAAGCAGCGGTCTCGGCGG-3′ reverse primer: 5′-CCATCTCAAGCTTTCAGAGG-3′

After removing most of the 3′ non-translated region, subcloning in the pSVL expression vector was performed. The sequence was confirmed by double-strand sequencing with Sequenase Kit (version 2; USB, Cleveland, OH) according to the manufacturer's instructions.

2.2. Transient expression of TSH and LH/CG receptors cDNAs

COS-7 cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. The cells were plated at a density of 150,000 cells/dish (3 cm diameter). 24 h later, cells were transfected with the diethylaminoethyl-dextran method followed by a 2-min 10% dimethylsulfoxide shock after 3 h. Differences in the efficiency of transfection and cell survival, were responsible for the different absolute cAMP levels observed in different experiments.

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2.3. Functional assay

Cells were used 48 h after transfection for cAMP determination. Triplicate dishes were used for each condition and each experiment was repeated at least 3×; all assays always included control cells transfected with pSVL vector alone.

Results are expressed as mean±S.E. When not shown, S.E. values are so small that they fall within the symbols.

2.4. Measurement of cAMP accumulation

Experiments were performed in Krebs-Ringer-HEPES buffer (KRH) [isotonic (NaCl)], KRH where the NaCl was replaced with 280 mM sucrose [isotonic (sucrose)] and KRH without NaCl [hypotonic ($NaCl^-$)]. The culture medium was removed, cells were rinsed once with 1 ml of the appropriate buffer and preincubated for 30 min in 1 ml of the same buffer. Thereafter, cells were incubated for 60 min in fresh buffer supplemented with 25 μM of the phosphodiesterase inhibitor Rolipram (kindly provided by laboratories Jacques Logeais, Troppes, France) and the agonists under study. At the end of incubation, the medium was collected, 1 ml HCl 0.1 M was added and the samples were dried in a vacuum concentrator (Savant). Extracellular (using the hypotonic ($NaCl^-$) buffer) and intracellular (using both isotonic buffers) cAMP were measured in the medium or in cell extract, respectively, with a commercial radioimmunoassay Kit (Amersham, code 432, TRK) and expressed as pmol/dish. We choose to measure extracellular cAMP under hypotonic conditions, because >90% of the total cAMP was released in the medium in this condition. In some experiments using the isotonic (NaCl) buffer, the effect on cAMP accumulation of varying the pH of the buffer (6.5–8), or the temperature of incubation (28–42°C) was assessed.

3. Results

3.1. Constitutive activity.

3.1.1. Basal cAMP measurement Basal cAMP levels were measured in extracts from COS-7 cells transfected with increasing amounts of TSHR or LHR DNA constructs. Whether they were measured after incubation in isotonic (NaCl) or isotonic (sucrose) buffers, cAMP levels reached a plateau at 100 ng/dish of DNA transfected for both receptors (Fig. 1A,B). The absence of a consensus regarding the specific bioactivity of pure bovine TSH (reported values vary by a factor of 5–10-fold [19]) makes it impossible to compare rigorously the level of expression achieved for the two receptors in molar terms. Nevertheless, binding experiments with ^{125}I-labeled TSH and hCG were performed with COS-7 cells transfected with saturating amounts of the two constructs: similar level of expression were obtained (250,000–450,000 receptors/cell) when using the highest (more likely) value for TSH bioactivity [19] (not shown). In cells transfected with saturating amounts of the TSHR cDNA construct and incubated in isotonic (NaCl) (Fig. 1A), higher basal cAMP levels were observed with respect to cells transfected with the vector alone, confirming the constitutive activity of the wt receptor described previously [8–12,15] (Figs. 1A, 2). Under the same conditions, cells transfected with the LHR construct displayed only minimal increase of cAMP levels (Figs. 1A, 2). The TSHR caused at least a 5.5-fold cAMP increase (range 5.5–20-fold in five experiments), whereas with the LHR the effect was always smaller than 1.4-fold (range 1.1–1.4-fold) (Figs. 1A, 2). When cells were incubated in hypotonic ($NaCl^-$) buffer, basal cAMP production was higher in cells transfected with TSHR and, surprisingly, also in cells transfected with LHR (Fig. 2A). When incubated in isotonic (sucrose) buffer, cells transfected with TSHR displayed also higher levels of basal cAMP as compared with the same cells incubated in isotonic (NaCl) buffer. Similarly, cells expressing the LHR exibited a marked increase in basal cAMP production as compared with cells transfected with LHR and incubated in isotonic (NaCl) buffer (Figs. 1B, 2B). Together, this suggests that removal of NaCl from the incubation medium increases the constitutive activity of the wt TSH receptor, while simultaneously unmasking significant constitutive activity of the LH receptor. COS-7 cells transfected with the empty vector or with the DNA of the mouse melanocortin receptor (MC5R) used as

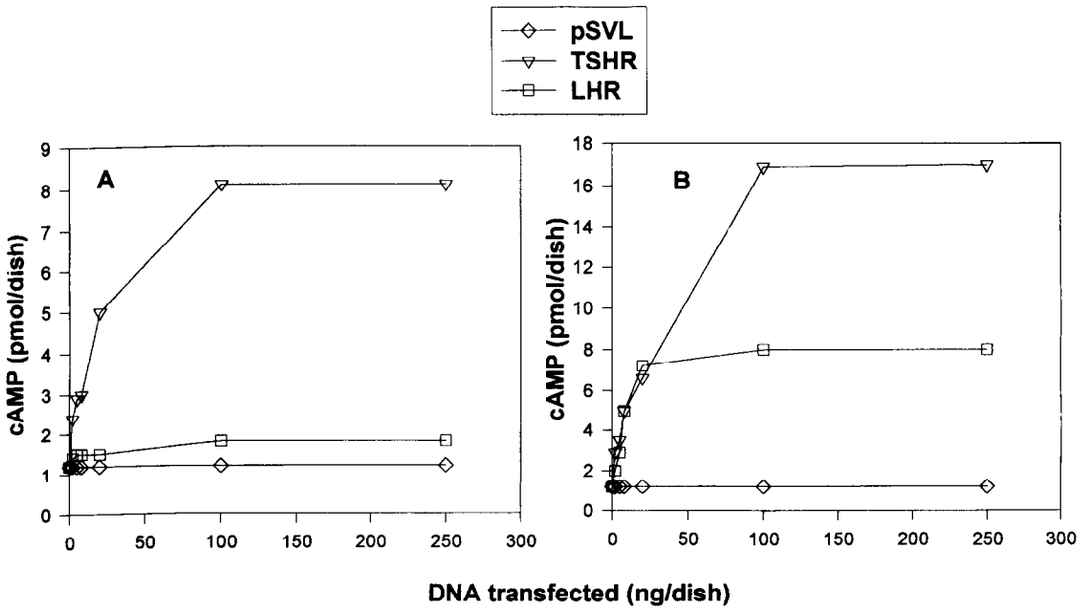

Fig. 1. Basal cAMP accumulation in COS-7 cells transfected with increasing amounts of the TSH or LH receptor DNA constructs. After incubation in isotonic (NaCl) buffer (panel A), or isotonic (sucrose) buffer (panel B) cAMP was measured as decribed in the method section. Note the different scales between the two panels.

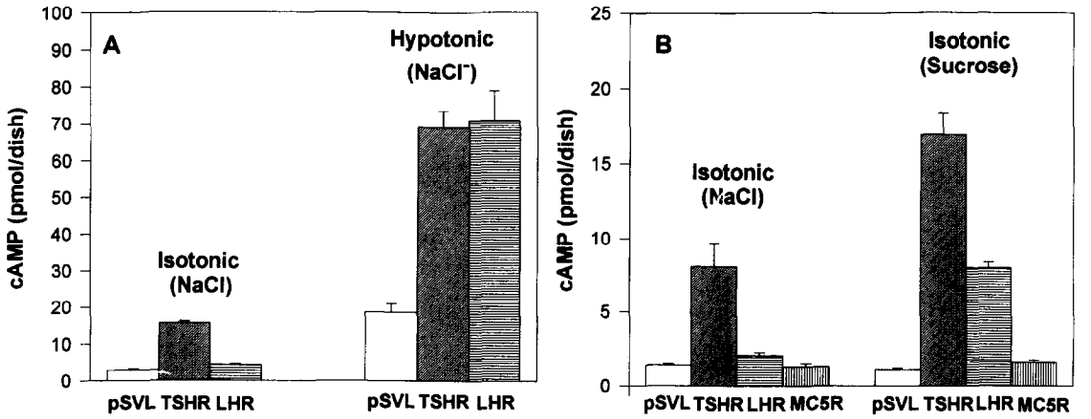

Fig. 2. Basal cAMP accumulation in COS-7 cells transfected with 100 ng/dish of the LH, TSH or MC5 receptor constructs; the empty pSVL vector served as control. cAMP was measured 48 h after transfection following incubation in isotonic (NaCl), or hypotonic ($NaCl^-$) buffers (panel A), and isotonic (NaCl) or isotonic (sucrose) buffers (panel B). Note the different scales between the two panels. Error bars are S.E.M. values of triplicates.

controls showed the same basal cAMP accumulation in both isotonic buffers (Fig. 2B).

The effect of varying the pH of the buffer (6.5–8) or the incubation temperature (28–42°C) on the cAMP production was further investigated in cells expressing TSHR, LHR or MC5R, in isotonic (NaCl) buffer. These different conditions did not affect the constitutive activity of the TSHR and did not unmask significant constitutive basal activity of the LHR or MC5R (Fig. 3).

3.2. *Biological response to bTSH, hCG and α-MSH*

cAMP accumulation in response to stimulation by bTSH, hCG and α-MSH was explored in cells transfected with TSHR, LHR and MC5R, respectively. Using isotonic (NaCl) buffer, bTSH (10 mU/ml) and hCG (1 mU/ml) increased cAMP over basal values by a factor of 12.3 (range 10–25) and 22.5 (range 18–40) for TSHR and LHR, respectively (Fig. 4). When cells were assayed in isotonic (sucrose) buffer, in the same experiment, using the same concentration of agonists as above, the TSHR and LHR were both stimulated by a factor of 1.9 only (Fig. 4). MC5R retained high sensitivity to α-MSH (40 nM) in both conditions (Fig. 4). COS-7 cells transfected with the empty pSVL vector were not stimulated by any of the three agonists (Fig. 4).

Agonist stimulated cAMP production was not affected when cells transfected with the three receptors were tested in isotonic (NaCl) buffer at different pH or temperatures of incubation (data not shown).

4. Discussion

Spontaneous mutations leading to the constitutive activation of G protein-coupled receptors have recently been implicated in a series of hereditary and acquired diseases including toxic thyroid hyperplasia [9,11,20] and hyperfunctioning thyroid adenomas (TSH receptor) [8,10,12,21], retinitis pigmentosa [22] and night blindness (rhodopsin) [23], Jansen-Type metaphysial dysplasia (PTH-PTHrP receptor) [24], autosomal dominant hypocalcaemia (calcium sensor receptor) [25] and pseudo-precocious puberty of the male (LH/CG receptor) [13,14,26,27]. Al-

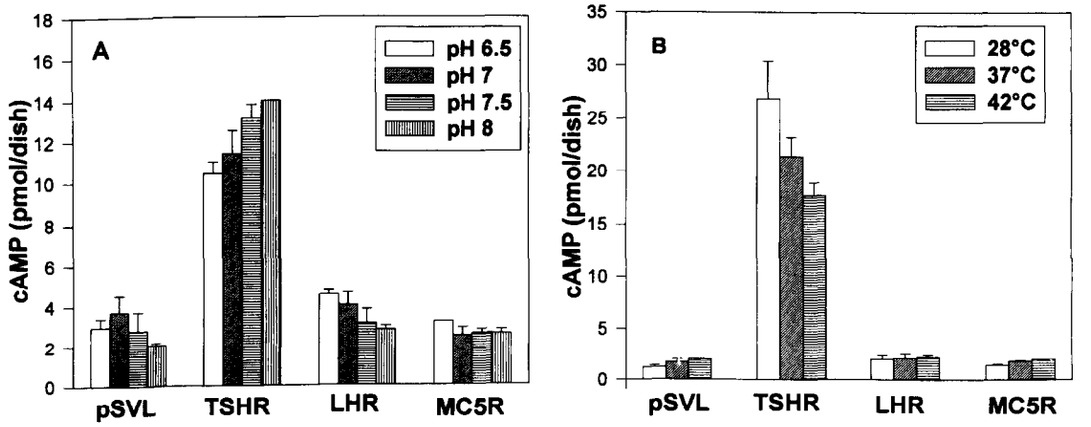

Fig. 3. Effect of pH and temperature on basal cAMP accumulated in COS-7 cells expressing TSHR, LHR or MC5R; the empty pSVL vector served as control. Cells were incubated in isotonic (NaCl) buffer with differing pH (6.5–8) (panel A) or at different temperatures (28–42°C) (panel B). Basal cAMP levels were measured as described in section 2. Note the different scales between the two panels. Error bars are S.E.M. values of triplicates.

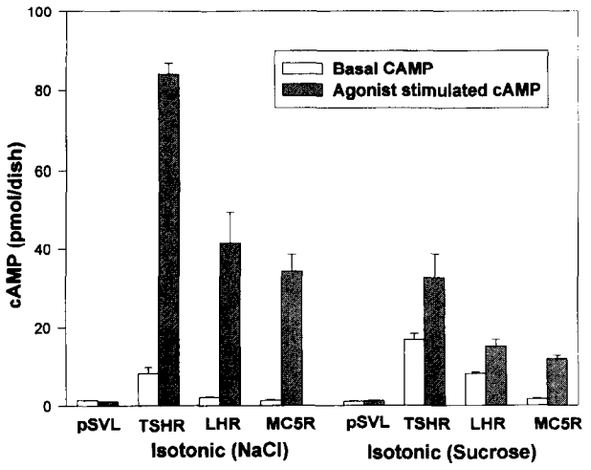

Fig. 4. Effect of NaCl on the response of COS-7 cells expressing the TSH, LH or MC5 receptors. COS-7 cells were transfected with saturating concentrations of DNA (100 ng/dish) and intracellular cAMP levels were determined under basal or agonist-stimulated conditions (bTSH 10 mU/ml, hCG 1 mU/ml, α-MSH 40 nM) following incubation in two different media as indicated: isotonic (NaCl), (left); isotonic (sucrose) (right). Error bars are S.E.M. values of triplicates.

though a series of different mutations have been demonstrated in each situation, the diversity of the gain-of-function mutations affecting the TSH receptor seems particularly high: mutations of thirteen different residues lead to receptor activation [8–12,20,28,29]. In agreement with a current model for G protein-coupled receptor activation [30], this observation led us to propose that the unliganded TSH receptor would be less constrained than others when in the inactive state [28 and MT submitted]. Giving support to this hypothesis, we and others had shown that the wt TSHR displayed significant constitutive activity, when transfected in COS-7 cells [8–12,15,16,20]. As the level of receptors achieved in COS-7 cells is several orders of magnitude higher than in thyrocytes, it remains to be demonstrated that constitutive activity of the wt TSH receptor has physiological relevance in vivo. Our present results demonstrate that, under identical experimental conditions, in a buffer containing physiological NaCl concentrations, neither the LHR nor the MC5R show significant constitutive activity towards adenylylcyclase. This confirms previous data obtained with the LHR [14,15,27] and suggests that LH and MC5 receptors are indeed subjected to a stronger silencing constraint that the TSHR. With the aim to explore whether the structure of TSHR would be more labile and, hence, more prone to autoactivation we subjected cells expressing the three receptors to incubation conditions which could destabilize the receptor structures. Whereas varying the pH or temperature was without effects, removal of NaCl from the medium (under hypotonic) (Fig. 2A) or isotonic conditions (Figs. 1B, 2B) clearly enhanced the constitutive activity of the TSH receptor while simultaneously unmasking strong constitutive activity of the LHR. As the unliganded MC5 receptor remained silent under all conditions, this allows classification of the three receptors in three categories with increasing lability: MC5R, silent; LHR, silent under normal [NaCl], active in the absence of NaCl; TSHR, already constitutively active in normal [NaCl], further activated by removal of NaCl. These observations suggest that ionic interactions play a key role in the normal silencing of the unliganded LH receptor and in keeping at a relatively low level the constitutive activity of the TSH receptor. It is likely that the highly conserved aspartate residue present in the second transmembrane segment of the vast majority of GPCRs (positions 383 and 460 in the LH and TSH receptors, respectively) is implicated in this ionic interaction [31]. Substitution of this residue in LHR [31] as well as the corresponding residue in other members of GPCR [32–34], results in a decrease in affinity for agonists and abolishes the effect of $[Na^+]$ on affinity for agonists.

Hidaka et al. [35,36], using COS-7 cells expressing TSHR or LHR failed to detect constitutive activity of the LHR in NaCl-free buffer containing sucrose. This study did not explore the behaviour of transfected cells incubated in a buffer containing physiological concentration of NaCl. The reason for the discrepancy with our results is unclear; a possible explanation being related to a lower efficiency of transfection in Hidaka et al. [35,36]. In our hands, the efficiency of transfection of COS-7 cells ranges between 40 and 50% as measured by flow cytofluorimetry using a monoclonal antibody recognizing the extracellular domain of the TSH receptor (S. Costagliola et al., to be published). In comparison with our observations, the constitutive activity observed for the TSHR by Hidaka et al. is much lower (2-fold increase of basal cAMP vs. 5–20-fold in our hands).

A blunted cAMP response was observed after stimulation of the TSHR and LHR transfected cells with bTSH and hCG, respectively, when using isotonic (sucrose) buffer. This observation suggests that the high basal activity displayed by both receptors under this condition, causes close to their full activation. This characteristic has been previously observed for some of the activating mutations of the TSHR tested in normal [NaCl] [12].

It has been recommended to incubate thyrocytes, FRTL5 cells or transfected cell lines expressing the TSH receptor in low [NaCl] in order to increase the sensitivity of assays for thyroid stimulating auto-antibodies (TSAb) [37–39]. In agreement with this practice, the affinity of the TSH receptor for binding of bovine TSH is higher in low [NaCl] [40]. One observation has been that by chosing low [NaCl] media for auto-antibody assays, one improves detection of weak TSAbs, while decreasing the range of stimulation [37,38]. This is fully compatible with the present data.

The number of receptors described, which are endowed with constitutive activity is increasing rapidly [41–47]. The structural basis responsible for this phenomenon remains unknown. The observations in the present study open the way to the identification of the residues involved in the differential behaviour of the unliganded TSH and LH receptors.

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