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CORRECTION

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The affiliation for Andrea G. Lania was given incorrectly.

Correct affiliations are as shown below.

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The authors apologise for this mistake.

Specific roles of G_i protein family members revealed by dissecting SST5 coupling in human pituitary cells

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Summary

Despite intensive investigation over the past 20 years, the specific role played by individual Gi protein family members in mediating complex cellular effects is still largely unclear. Therefore, we investigated the role of specific Gi proteins in mediating somatostatin (SS) effects in somatotroph cells. Because our previous data showed that SS receptor type 5 (SST5) carrying a spontaneous R240W mutation in the third intracellular loop had a similar ability to inhibit intracellular cAMP levels to the wild-type protein but failed to mediate inhibition of growth hormone (GH) release and cell proliferation, we used this model to check specific receptor–G-protein coupling by a bioluminescent resonance energy transfer analysis. In HEK293 cells, wild-type SST5 stimulated the activation of $G\alpha_{i1–3}$ and $G\alpha_{oA,\ B}$, whereas R240W SST5 maintained the ability to activate $G\alpha_{i1–3}$ and $G\alpha_{oB}$, but failed to activate the splicing variant $G\alpha_{oA}$. To investigate the role of the selective deficit in $G\alpha_{oA}$ coupling, we co-transfected human adenomatous somatotrophs with SST5 and a pertussis toxin (PTX)-resistant $G\alpha_{oA}$ ($G\alpha_{oA(PTX\text{-}r)}$) protein. In PTX-treated cells, $G\alpha_{oA(PTX\text{-}r)}$ rescued the ability of the selective SST5 analog BIM23206 to inhibit extracellular signal-related kinase 1/2 (ERK1/2) phosphorylation, GH secretion and intracellular cAMP levels. Moreover, we demonstrated that silencing of $G\alpha_{oA}$ completely abolished SST5-mediated inhibitory effects on GH secretion and ERK1/2 phosphorylation, but not on cAMP levels. In conclusion, by analysing the coupling specificity of human SST5 to individual $G\alpha_i$ and $G\alpha_o$ subunits, we identified a crucial role for $G\alpha_{oA}$ signalling in human pituitary cells.

Key words: G proteins, Somatostatin receptor 5, GH-secreting pituitary adenomas

Introduction

The heterotrimeric guanine nucleotide binding proteins, known as G proteins, composed of α, β and γ subunits, are ubiquitous and crucial signalling molecules which transduce signals from G-protein-coupled receptors (GPCRs) to downstream intracellular effectors. The distinct α subunits can be divided into four major subfamilies represented by $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$. Proteins of the G_i class, which includes several protein substrates for pertussis toxin (PTX) ADP ribosylation such as $Gi_{1\text{-}3}$ and $Go_{A,B}$, are involved in adenylyl cyclase inhibition, ion channel modulation and phosphatase activation, but the specific role played by individual G proteins in activating specific effector molecules is still largely unclear.

The inhibitory effects of somatostatin (SS) on hormone secretion and cell proliferation are mediated by a family of five different GPCRs (SST1-5) coupled to multiple PTX-sensitive G proteins (reviewed in Ben-Shlomo and Melmed, 2010). All five subtypes are coupled to adenylyl cyclase inhibition and some have also been found to reduce calcium entry by modulating L-type Ca^{2+} and K^+ channels (Yang and Chen, 2007), all these events being involved in the inhibition of hormone release. The anti-proliferative effects of SS are mainly mediated by SST2 and 5 by tyrosine phosphatase activation and inhibition of ERK1/2 phosphorylation, respectively (Lopez et al., 1997; Buscail et al., 1995; Cordelier et al., 1997; Peverelli et al., 2009). The high density of SSTs, in particular SST2 and 5, on human GH-secreting adenomas has been used clinically to successfully treat patients with acromegaly (Gueorguiev and Grossman, 2011), but the specific G proteins and the molecular mechanisms involved in intracellular signal transduction of these receptors have not been completely clarified.

We have previously investigated human SST5 receptor structural domains mediating intracellular signalling pathways in the rat pituitary cell line GH3, focusing on the BBXXB domain in the third intracellular loop and the DRY motif in the second intracellular loop (Peverelli et al., 2008; Peverelli et al., 2009). Our data indicated that residues D136 and R137 in the DRY motif are critical for SST5 signalling since their substitution abolished all intracellular responses. Conversely, mutations in the BBXXB domain, and in particular the naturally occurring mutant R240W identified in an acromegalic patient resistant to SS analogues (Ballarè et al., 2001), resulted in a receptor with retained ability to inhibit intracellular cAMP levels similarly to the wild-type but failed to mediate the inhibition of GH release

and cell proliferation. These data suggested that the R240W mutation caused the loss of coupling with specific but yet unidentified G proteins involved in the anti-proliferative and anti-secretory effects of SST5.

In order to identify the Gα protein family members involved in these effects, we employed a BRET biosensor that monitors the conformational rearrangements between probes inserted in heterotrimeric G protein complex (Galés et al., 2006; Busnelli et al., 2012; Saulière et al., 2012). In particular, the GDP/GTP exchange that occurs during receptor activation is translated into a decrease in the energy transfer between the donor (*Renilla* luciferase, Rluc, fused to a specific Gα subunit) and the acceptor (a variant of green fluorescent protein, GFP10 fused to the $G\gamma_2$ subunit). By this approach we found that R240W SST5 failed to activate $G\alpha_{oA}$ protein with respect to wild-type SST5, while the activation of $G\alpha_{i1,\ i2,\ i3}$ and G_{oB} was maintained. The role of $G\alpha_{oA}$ protein in the transduction of SST5 signalling was confirmed in PTX pretreated human somatotrophs in which a PTX-resistant $G\alpha_{oA}$ was able to rescue the SST5 mediated inhibition of ERK1/2 activation and GH secretion. Accordingly, both effects were abrogated in $G\alpha_{oA}$ silenced cells. On the contrary, although $G\alpha_{oA}$ was able to mediate a reduction of cAMP levels, other G proteins were involved in this effects, as demonstrated by persistence of cAMP inhibition in $G\alpha_{oA}$ silencing experiments.

By demonstrating the coupling specificity of human SST5 to individual $G\alpha_i$ and $G\alpha_o$ subunits, our data revealed a crucial role for $G\alpha_{oA}$ signalling in human pituitary somatotrophs.

Results

G proteins activated by wild-type SST5

The BRET biosensor is schematically represented in Fig. 1A. The bioluminescent energy transfer occurs between the energy donor (Rluc) inserted within the Gα subunit amino acid sequence, and the acceptor (GFP10) fused to the N-terminus of Gγ2 subunit (Galés et al., 2006). To investigate the specific ligand-induced G protein activation by wild-type SST5, we co-transfected HEK293 with wild-type SST5, Gα-RLuc, Gγ2-GFP10 and complementary Gβ1 subunit. In particular, we tested six different Gα-RLuc subunits ($G\alpha_{i1,\ i2,\ i3,\ q,\ oA,\ oB}$) by transfecting the corresponding plasmids (described in Saulière et al., 2012; Busnelli et al., 2012). A significant ($P<0.001$ versus PBS treated cells) BRET signal ratio decrease was obtained following wild-type SST5 activation by specific agonist BIM23206 (100 nM) with the $G\alpha_{i1,2,3}$ and $G\alpha_{oA,B}$ subunits, whereas no decrease was observed with $G\alpha_q$, as shown in Fig. 1B.

These data indicate that human SST5 activates all $G\alpha_i$ subtypes subunits including both isoforms of $G\alpha_o$, without affecting $G\alpha_q$. Although all experiments were performed using similar expression levels of donor (RLuc) and acceptor (GFP10; see Materials and Methods), the maximal amplitude of BIM23206-promoted BRET signals detected with the different $G\alpha_i$ $G\alpha_o$ donor probes do not necessarily reflect differences in maximal coupling efficiency due to intrinsic differences in intramolecular rearrangements in the single Gα-RLuc constructs (Saulière et al., 2012). Therefore, in this biomolecular assay, the maximal amplitude of BRET signal was not informative of how efficiently a receptor is coupled to a single Gα subunit.

G proteins activated by R240W SST5

In previous studies we reported that R240W mutation did not change receptor expression at the plasma membrane, nor its binding profile or ability to inhibit intracellular cAMP levels,

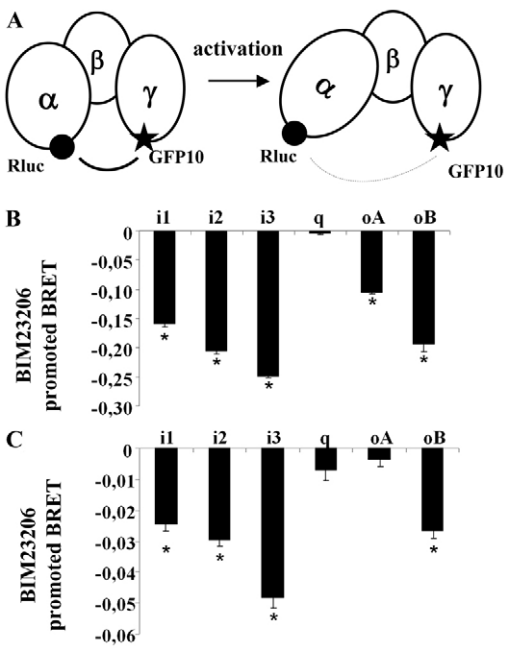

Fig. 1. BRET measurements of G protein activation following BIM23206 stimulation. (**A**) BRET was measured between Rluc (the donor, represented as a circle) and GFP10 (the acceptor, represented as a star) introduced into the α-helical domain of the indicated Gα subunits and the N-terminal domain of Gγ2, respectively. BIM23206-induced Gα activation leads to a conformational rearrangement of the heterotrimeric G-protein complex that corresponds to a decrease in BRET ratio. BRET was measured in HEK293 cells co-expressing wild-type SST5 (**B**) or R240W SST5 (**C**) together with GFP10-Gγ2, Gβ1 and Rluc-tagged Gα subunits: αi1, αi2, αi3, αq, αoA, αoB. The results shown here represent the differences in BRET signal after BIM23206 (100 nM) or PBS stimulation, and are expressed as mean values ± s.d. One-way ANOVA by Dunnett's test was used to determine the statistical differences between SST5-agonist-promoted BRET in the presence of the indicated Gα proteins and untreated controls (baseline) *$P<0.01$.

while it prevented the ability to mediate the inhibition of GH release and cell proliferation (Ballarè et al., 2001; Peverelli et al., 2008; Peverelli et al., 2009). Efficient localization at the plasma membrane was now further confirmed in HEK 293 cells (supplementary material Fig. S1). As all these data suggest that the R240W mutation causes a perturbation in the coupling with specific G proteins mediating the anti-proliferative and anti-secretory effects of SST5, we co-transfected HEK-293 cells with R240W SST5, together with Gα-RLuc ($G\alpha_{i1,\ i2,\ i3,\ q,\ oA,\ oB}$), Gγ2-GFP10 and complementary Gβ1. As observed for wild-type SST5, BIM23206 activated R240W SST5/$G\alpha_{i1,\ i2,\ i3}$ and G_{oB} complexes, as indicated by the significant decrease in BRET signal ($P<0.001$ versus PBS-treated cells; Fig. 1C). Interestingly, no significant activation of G_{oA} was detected.

It is worth noting that, compared to the wild type, the R240W mutant displayed a reduction of the BRET signal for all the Gα isoforms. This general reduction was not caused by different transfection efficiency, as revealed by the very similar and reproducible values of fluorescence for GFP10-Gγ2 (25,402±108.4) and total luminescence for Gα-Rluc constructs (mean 37,269±986.5 arbitrary units) in the different experiments, suggesting that, in addition to the inability to activate G_{oA}, the mutant receptor showed a reduced capability to reach G protein selective active conformations.

G_{oA} and G_{oB} expression in adenomatous somatotrophs

G_i proteins are expressed ubiquitously, whereas G_o proteins show a more restricted expression pattern. In particular, no data are available on the expression of the two isoforms of G_o in somatotrophs, that are well characterized targets of SST5-mediated action. Therefore, before investigating the role of G_o isoforms in the pituitary, we performed preliminary experiments by RT-PCR analysis on tissue samples obtained from human somatotroph adenomas ($n=8$). Our data showed that both $G\alpha_{oA}$ and $G\alpha_{oB}$ transcripts were expressed in all the samples analysed (supplementary material Fig. S2).

$G\alpha_{oA(PTX\text{-}r)}$ restored the ability of SST5 to inhibit ERK1/2 phosphorylation in PTX-pretreated somatotrophs

The anti-proliferative effects of SS are mediated by SST5 through inhibition of ERK1/2. To investigate the role of G_{oA} protein in mediating SST5 signalling, we transfected cultured somatotrophs from four human adenoma samples with SST5 receptor alone or together with mutated PTX-resistant G_{oA} ($G\alpha_{oA(PTX\text{-}r)}$). Transfected cells were incubated for 16 h with 100 ng/ml PTX, and finally stimulated in fresh medium with or without the SST5 selective agonist BIM23206. Western blot analysis (Fig. 2A) showed a significant reduction (56±7%, $P<0.01$ versus basal) in ERK1/2 phosphorylation by BIM23206, that was abolished by the PTX-induced blockade of all the endogenous PTX-sensitive G proteins (5±5%, $P=0.6$ versus basal). When we co-transfected $G\alpha_{oA(PTX\text{-}r)}$, the inhibitory effect (51±9%, $P<0.01$ versus basal) was maintained after PTX treatment (62±2%, $P<0.01$ versus basal and $P<0.01$ versus corresponding SST5-transfected cells), suggesting that this G protein was able to mediate the reduction in ERK1/2 phosphorylation. The rescue effect of $G\alpha_{oB(PTX\text{-}r)}$ protein was less pronounced (16±4% of ERK1/2 inhibition after PTX treatment, $P<0.05$).

$G\alpha_{oA(PTX\text{-}r)}$ restored the ability of SST5 to inhibit GH secretion in PTX pretreated somatotrophs

To further analyse the role of G_{oA} on the biological responses elicited by SST5 activation, we investigated the regulation of GH release from cultured adenomatous somatotroph cells ($n=6$). As shown in Fig. 2B, the exposure of cells transfected with SST5 to the specific agonist BIM23206 (10 nM) caused a slight but significant reduction in basal GH release (16±13% inhibition, $P<0.05$ versus basal), while in PTX pretreated cells BIM23206 was ineffective. As observed for the inhibition of ERK1/2 phosphorylation, the expression of $G\alpha_{oA(PTX\text{-}r)}$ restored the ability of SST5 to reduce basal GH secretion (13±4%, $P<0.05$ versus corresponding basal). On the contrary, $G\alpha_{oB\ (PTX\text{-}r)}$ was not able to rescue this effect.

$G\alpha_{oA}$ silencing abolishes the inhibitory effects of SST5 on ERK1/2 phosphorylation and GH secretion in somatotrophs

In order to evaluate whether $G\alpha_{oA}$ is essential for ERK1/2 phosphorylation and GH secretion inhibition in somatotrophs, we used a specific siRNA selectively targeting this isoform. RT-PCR analysis was performed to test the efficiency and the specificity of silencing. As shown in Fig. 3A, after 72 h transfection with $G\alpha_{oA}$ siRNA, $G\alpha_{oA}$ transcript was undetectable, whereas no alterations of $G\alpha_{oB}$ mRNA was observed. A negative control siRNA, i.e. a non targeting sequence without significant homology to the sequence of human transcripts, was used in all experiments. To test ERK1/2 activation, cultured adenomatous somatotrophs from three adenoma samples were transfected with negative control or $G\alpha_{oA}$ siRNA for 72 h and then stimulated with BIM23206 for 10 min (Fig. 3B). As expected, the exposure of cells transfected with negative control siRNA to BIM26203 resulted in a reduction in ERK1/2 phosphorylation (52±10% inhibition, $P<0.05$ versus basal). On the contrary, $G\alpha_{oA}$ silencing completely abolished the ability of BIM23206 to inhibit ERK1/2 phosphorylation, indicating that this isoform is required for SST5-mediated inhibition of this proliferative pathway.

We next investigated by siRNA technique the role of $G\alpha_{oA}$ in mediating GH release inhibition (Fig. 3C). As expected, stimulation of cells transfected with negative control siRNA with

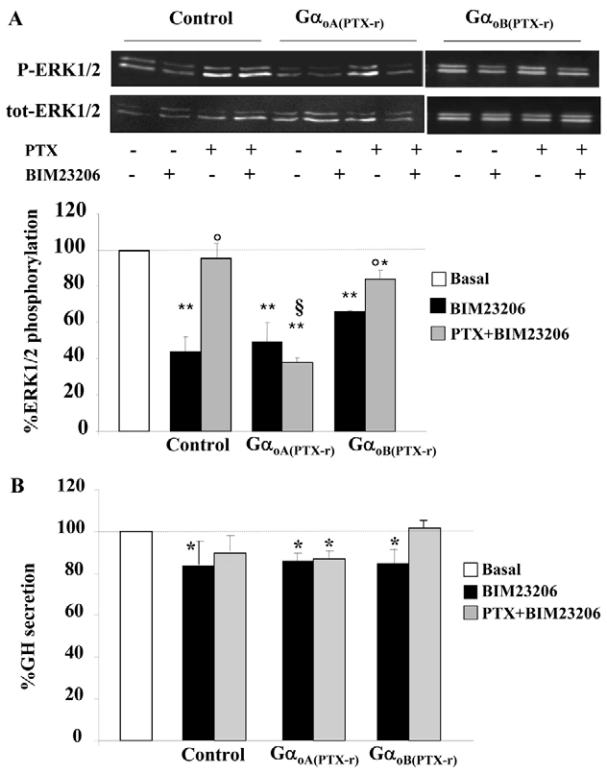

Fig. 2. $G\alpha_{oA(PTX\text{-}r)}$ restored the ability of BIM23206 to inhibit ERK1/2 phosphorylation and GH secretion in PTX-treated adenomatous somatotroph cells. (**A**) A representative immunoblot of ERK1/2 phosphorylation demonstrating that the SST5-mediated reduction of ERK1/2 by BIM23206 (10 nM for 10 min) observed in SST5-transfected cells was abolished after PTX treatment and restored in $G\alpha_{oA(PTX\text{-}r)}$-transfected cells. The rescue effect of $G\alpha_{oB(PTX\text{-}r)}$ protein was less pronounced. The graph shows the quantification of phospho-ERK1/2 normalized to total ERK1/2 (mean values ± s.d. from four independent experiments). $^*P<0.05$, $^{**}P<0.01$ versus corresponding basal, $^{\S}P<0.01$ versus corresponding SST5-transfected cells, $^{\circ}P<0.05$ versus BIM23206; *t*-test. (**B**) $G\alpha_{oA(PTX\text{-}r)}$ restored the ability of BIM23206 to inhibit GH secretion in PTX-treated adenomatous somatotroph cells. Cells were transfected for 48 h with human wild-type SST5, $G\alpha_{oA(PTX\text{-}r)}$ or $G\alpha_{oB(PTX\text{-}r)}$ and incubated for 3 h with fresh medium containing BIM23206. In SST5-transfected cells, BIM23206 caused a slight but significant reduction of basal GH release. After PTX pretreatment, no significant effect of BIM23206 on GH release was observed, whereas transfection with $G\alpha_{oA(PTX\text{-}r)}$, but not $G\alpha_{oB(PTX\text{-}r)}$, restored GH responsiveness to BIM23206. $^*P<0.05$ versus corresponding basal value; *t*-test.

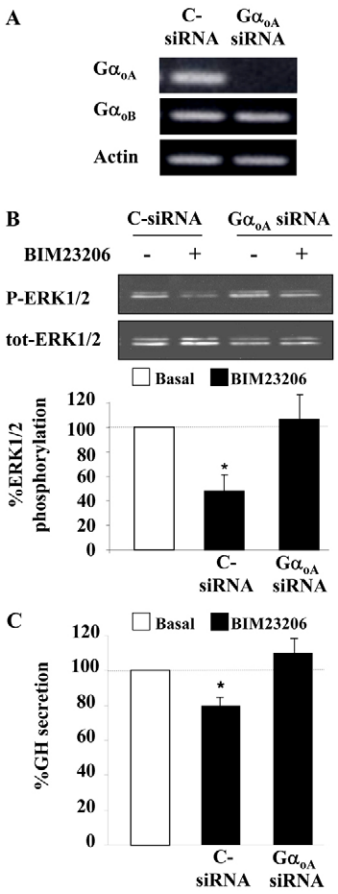

Fig. 3. GαoA is required for the inhibitory effects of SST5 on ERK1/2 phosphorylation in pituitary somatotroph cells. (**A**) RT-PCR analysis was performed after transfection with negative control siRNA or $G\alpha_{oA}$ siRNA for 72 h. $G\alpha_{oA}$ transcript was expressed in control cells and undetectable in $G\alpha_{oA}$-silenced cells. No alterations of $G\alpha_{oB}$ mRNA was observed. Negative control siRNA (C-siRNA) is a non targeting sequence without significant homology to the sequence of human transcripts. (**B**) Representative immunoblot of ERK1/2 phosphorylation demonstrating that SST5-mediated inhibition of ERK1/2 by BIM23206 (10 nM, 10 min) was present in C-siRNA and abolished in $G\alpha_{oA}$-siRNA-transfected cells. The graph shows the quantification of phospho-ERK1/2 normalized to total ERK1/2 (mean values ± s.d. from three independent experiments). $*P<0.05$ versus corresponding basal value; $**P<0.01$ versus corresponding basal value; *t*-test. (**C**) $G\alpha_{oA}$ is required for SST5-mediated inhibition of GH release. $G\alpha_{oA}$ silencing abolished the inhibitory effect of selective SST5 agonist BIM23206 on GH secretion in adenomatous somatotroph cells. Cells were incubated with negative control siRNA or $G\alpha_{oA}$ siRNA for 72 h and treated with BIM23206 for 3 h. GH was measured in the culture medium. Values are means ± s.d. from three independent experiments; each determination was done in triplicate. $*P<0.05$ versus corresponding basal value; *t*-test.

BIM23206 for 3 h reduced GH secretion in culture medium (18±9%, $P<0.05$ versus basal). This effect was completely lost in $G\alpha_{oA}$-silenced cells, demonstrating the essential role of this protein in mediating the anti-secretion effect of SST5.

Role of $G\alpha_{oA}$ in SST5-mediated inhibition of intracellular cAMP levels

Since little is known about the ability of G_o to inhibit adenylyl cyclase, we tested the ability of $G\alpha_{oA}$ and $G\alpha_{oB}$ to reduce forskolin-stimulated intracellular cAMP levels, that reflects the

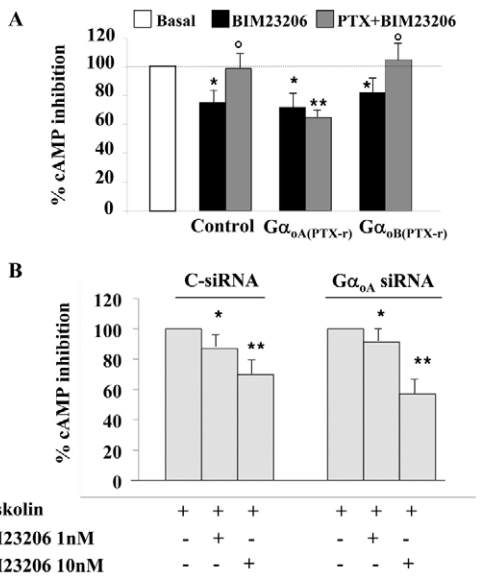

Fig. 4. Role of $G\alpha_o$ in SST5-mediated adenylyl cyclase inhibition. (**A**) 48 h after transfection, cells were pre-incubated with IBMX for 30 min, and subsequently with 1 μM forskolin with or without BIM23206 (10 nM) for 30 min. In cells transfected with SST5, the selective agonist BIM23206 (10 nM) inhibited forskolin-stimulated cAMP accumulation whereas this effect was abolished after PTX pretreatment. Co-transfection of SST5 and $G\alpha_{oA(PTX\text{-}r)}$, but not $G\alpha_{oB(PTX\text{-}r)}$, rescued the ability of SST5 to inhibit forskolin-stimulated intracellular cAMP levels. $*P<0.05$, $**P<0.01$ versus corresponding basal value; $^{\circ}P<0.05$ versus BIM23206; *t*-test. (**B**) $G\alpha_{oA}$ is not required for SST5-mediated inhibition of forskolin-stimulated cAMP accumulation. Cells were incubated with negative control siRNA or $G\alpha_{oA}$ siRNA for 72 h, pre-incubated with IBMX for 30 min and treated with forskolin with or without BIM23206 (1 nM or 10 nM) for 30 min. The inhibitory effect of BIM23206 on intracellular cAMP levels in adenomatous somatotroph cells was maintained in $G\alpha_{oA}$-silenced cells. Values are means ± s.d. from three independent experiments, each determination was performed five times. $*P<0.05$, $**P<0.01$ versus corresponding basal value; *t*-test.

reduction in adenylyl cyclase activity in the presence of phosphodiesterase inhibitors. Experiments were performed in cultured cells from three GH-secreting adenomas (Fig. 4A). As expected, BIM23206 (10 nM) inhibited forskolin-stimulated cAMP accumulation in cells transfected with SST5 (25±8% inhibition, $P<0.05$), while this effect was abolished after PTX pretreatment. Co-trasfection of SST5 and $G\alpha_{oA(PTX\text{-}r)}$, but not $G\alpha_{oB(PTX\text{-}r)}$, rescued the ability of SST5 to inhibit forskolin-stimulated intracellular cAMP levels (35±5% inhibition in cell transfected with $G\alpha_{oA(PTX\text{-}r)}$ and pretreated with PTX, $P<0.01$). To test whether other G proteins are involved in this effect, we silenced $G\alpha_{oA}$ protein in cultured cells from three GH-secreting adenomas (Fig. 4B). We observed a dose-dependent reduction in forskolin-stimulated intracellular cAMP levels both in negative control and $G\alpha_{oA}$ siRNA transfected cells (30±6% and 43±8%, at 10 nM, respectively, $P<0.05$), demonstrating that $G\alpha_{oA}$ is not essential to inhibit adenylyl cyclase activity in pituitary somatotrophs.

Discussion

This study identified the specific role played by different G_i protein family members in mediating the inhibitory signals of SST5 in human somatotroph cells. Previous studies on receptor–G-protein

complexes performed by co-immunoprecipitation found that SSTs are coupled to different G proteins ($G\alpha_{i\ 1,\ 2,\ 3}$, $G\alpha_o$), although the specific coupling between individual members of the same G-protein family and different SST subtypes expressed in the tissues analysed remained largely undefined (Law et al., 1993; Brown and Schonbrunn, 1993; Gu and Schonbrunn, 1997). In particular, no data are available on SST5 specific coupling.

First, by using a novel BRET biosensor, here we show that SST5 activates $G\alpha_{i\ 1,\ 2,\ 3}$ and $G\alpha_{oA,\ B}$ in living cells. Although the role of G_i proteins in mediating adenylyl cyclase inhibition is well established, few information are available on the role of G_o proteins in target tissues, such as somatotrophs. Our previous results (Peverelli et al., 2009) suggested that cAMP-independent mechanisms are involved in the regulation of GH secretion and cell proliferation in a pituitary cell model, since SST5 third intracellular loop mutants, such as the naturally occurring R240W mutant, lost the ability to inhibit hormone secretion and cell growth, though maintaining the ability to reduce intracellular cAMP levels. By BRET experiments we now demonstrated that this mutant displays a reduction of G protein activation that, in the case of the $G\alpha_{oA}$, results in a complete loss of activation, suggesting that this isoform might play an essential role in SST5 signal transduction.

A single gene encodes for both $G\alpha_o$ subunits, and its alternative splicing produces two mRNA products, $G\alpha_{oA}$ and $G\alpha_{oB}$, which differ in the alternative use of the two final exons 7/8 and 9/10, respectively, thus coding for proteins with different C-terminal primary sequences (Hsu et al., 1990). The extreme C terminus of the G alpha protein is one of the most important domains involved both in the selectivity of activation by GPCRs (Blahos et al., 1998; Sasamura et al., 2000), and in the downstream activation of different effectors (Masters et al., 1988). In agreement with data reported in literature, our results suggest that the C-terminal regions of the two splice variants $G\alpha_{oA}$ and $G\alpha_{oB}$ are differentially involved in SST5 binding and activation of different effectors. Furthermore, our data demonstrate that R240 in the third intracellular loop of SST5 is critical for its coupling with $G\alpha_{oA}$ but not with $G\alpha_{i(1–3)}$ and $G\alpha_{oB}$, supporting the view that different regions of GPCR are required for different G protein interaction, as previously demonstrated for other GPCRs (Wu et al., 1995; Beqollari et al., 2009).

In contrast to G_i proteins, which are expressed ubiquitously, the expression of G_o is restricted to the central and peripheral nervous system, endocrine cells and cardiomyocytes. Although our group has previously demonstrated by western blot that G_o proteins are expressed in significant amounts in human pituitary tumours (Ballaré et al., 1997), the expression of the two splicing variants $G\alpha_{oA/B}$ was not assessed. In this work, RT-PCR data on tissue samples from human somatotroph adenomas showed that both $G\alpha_{oA}$ and $G\alpha_{oB}$ transcripts are expressed in all the samples analysed.

Second, to test the hypothesis that $G\alpha_{oA}$ may play an essential role in SST5 signal transduction in somatotrophs, that represents a well characterized target of SS action, we transfected primary cell cultures from somatotroph adenomas with $G\alpha_{oA(PTX\text{-}r)}$, a $G\alpha_{oA}$ resistant to PTX, and we analysed the effects triggered by SST5 after PTX treatment. By this approach we demonstrated that the inhibition of ERK1/2 phosphorylation and GH secretion was completely rescued by $G\alpha_{oA(PTX\text{-}r)}$, suggesting its direct involvement in the generation of SST5-mediated inhibitory signals.

Third, although the employ of $G\alpha_{o(PTX\text{-}r)}$ constructs demonstrated that G_{oA} is sufficient to mediate the inhibitory effects of SST5 in the absence of other endogenous PTX-sensitive G proteins, this experimental approach did not provide any information about the possible involvement of other G proteins in the same pathways. To address this issue, we transfected somatotroph cells with a specific siRNA targeting $G\alpha_{oA}$ but not $G\alpha_{oB}$ isoform. Our results showed that in the absence of $G\alpha_{oA}$ no inhibition of ERK1/2 phosphorylation and GH secretion was induced by SST5 agonist, demonstrating that in the absence of $G\alpha_{oA}$ the other endogenous G proteins expressed in somatotrophs are not able to transduce these signals.

The last two findings support previous data obtained in different cell systems indicating that SST5 is associated with the inhibition of ERK phosphorylation (Buscail et al., 1995; Cordelier et al., 1997; Peverelli et al., 2009) and firstly demonstrate that the generation of this anti-proliferative pathway is dependent on receptor coupling with $G\alpha_{oA}$. However, the involvement of $G\alpha_{oA}$ in inhibiting ERK1/2 is most likely a cell specific event, since it has been reported that in other cell systems, such as CHO cells, $G\alpha_o$ activates ERK1/2 phosphorylation via PKC-dependent mechanism (van Biesen et al., 1996). Moreover, the first somatic mutation for GNAO1 (R243H), recently described in breast cancer (Kan et al., 2010), renders the protein constitutively active and promotes NIH3T3 cells growth by increasing Src-dependent activation of STAT3 (Garcia-Marcos et al., 2011).

Although no direct effector for $G\alpha_{oA}$ has been characterized to date, it has been demonstrated that inactive form of $G\alpha_o$ directly interacts with Rap1GAP (GTPase activating protein for the small GTPase Rap1) (Jordan et al., 1999). Activation of $G\alpha_o$ would release Rap1GAP, leading to the inhibition of Rap activity. We hypothesize that this mechanism in somatotrophs would negatively modulating signalling by Rap1, with a consequent inhibition of B-Raf/MEK/ERK1/2. Further studies are needed to investigate the molecular mechanisms and the effectors downstream of $G\alpha_{oA}$ in different cell systems.

Overall, our results suggest a crucial role for G_{oA} in mediating the anti-secretion effect of SST5. We previously demonstrated that SST5 R240W mutant was not able to induce any inhibitory effect on intracellular calcium levels and GH release, while it retained the ability to inhibit cAMP accumulation, suggesting that the absent inhibition of hormone release may be attributable to the failure of this receptor to reduce calcium influx (Peverelli et al., 2009). The present data indicate that the R240W mutant receptor activates $G\alpha_i$ but not $G\alpha_{oA}$, supporting the view that G_o proteins are involved in calcium influx reduction, resulting in decreased hormone exocytosis, consistent with previous data supporting a cAMP-independent and G_o-mediated inhibition of calcium channels by SS (Degtiar et al., 1997).

Finally, contradictory data are available in the literature about the ability of G_o to inhibit adenylyl cyclase (Kobayashi et al., 1990; Wong et al., 1992). Our results first suggest that $G\alpha_{oA}$ is able to mediate inhibition of adenylyl cyclase activity in GH-secreting adenoma cells after SST5 activation, but its role is not essential for this effects, since it was maintained after $G\alpha_{oA}$ silencing. Therefore, other members of $G\alpha_{i/o}$ family seems to be involved in adenylyl cyclase activity inhibition. These results are in agreement with our previous data demonstrating the ability of

R240W SST5 to mediate reduction of intracellular cAMP levels, in the absence of $G\alpha_{oA}$ activation (Peverelli et al., 2009).

In conclusion, a more complete understanding of the functional properties of SST5 and how it activates target G proteins is of extreme importance to future drug discovery. The present results might provide a basis for identification of SSTs functional selective ligands (Urban et al., 2007; Schonbrunn, 2008; Cescato et al., 2010), effective in specifically couple with $G\alpha_{oA}$. Additional studies are needed to investigate whether $G\alpha_{oA}$ reduced expression or genetic alterations might be associated with pituitary tumour resistance to pharmacologic treatment with SS analogues.

Materials and Methods

Constructs

Human wild-type and R240W SST5 cDNAs were amplified starting from previous constructs as template (Peverelli et al., 2008) and subcloned into multiple cloning site of pRc/CMV expression vector (Invitrogen). The sequence of all constructs was verified by dideoxynucleotide sequencing. Preliminary immunofluorescence results confirmed that wild-type and R240W SST5 are correctly expressed and targeted to the plasma membrane in HEK293 cells (data not shown), as we previously showed in other cell types (Peverelli et al., 2008). The plasmids encoding for wild-type and R240W SST5 fused to DsRed2 fluorescent protein were previously described in Peverelli et al. (Peverelli et al., 2008).

All G protein subunits coding plasmids for BRET experiments (Galphaq-97-Rluc, Galphai1-91-Rluc, Galphai2-91-Rluc, Galphai3-91-Rluc, GalphaoA-91-Rluc8 or GalphaoB-91-Rluc8) were previously described (Busnelli et al., 2012; Saulière et al., 2012). $G\alpha_{oA}$ C351I ($G\alpha_{oA(PTX\text{-}r)}$) and $G\alpha_{oB}$ C351I ($G\alpha_{oB(PTX\text{-}r)}$) plasmids were purchased from Missouri S&T cDNA Resource Center.

Fluorescence microscopy

HEK293 cells were transiently co-transfected with same amounts (7 μg) of SST5-DsRed2 and R240W SST5-DsRed2 alone or with the same constructs used for BRET experiments: Galphai1-91-Rluc (4 μg) or GalphaoA-91-Rluc8 (4 μg), GFP10-Gγ2 (5 μg) and Gβ1 (4 μg). Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and washed several times in PBS and once with H_2O, and the glass coverslips were mounted with MOWIOL. The cells were analysed using an LSM 510 META confocal laser-scanning microscope (Zeiss, Jena, Germany) and the following filter set: HeNe543, HFT 488/543 (dichroic) and emission filter LP560.

RT-PCR analysis of $G\alpha_{oA/B}$ expression

RNA was extracted using standard methods (Trizol, Invitrogen, S.R.L., Italy) from GH-secreting adenomas ($n=8$) in order to verify the expression of $G\alpha_{oA/B,}$ as well as from cultured cells in silencing experiments. To examine the expression of $G\alpha_{oA}$ and $G\alpha_{oB}$ transcripts, 3 μg of total RNA were reverse transcribed (Promega, Madison WI, USA) and 5 μl of the cDNA was subjected to PCR using a common upstream primer located in exon 3–4 (5′-AGAAAGGCTGACGCCAAGAT-3′) and two specific downstream primers located in exon 7 (5′-AGTCGAAGAGCATG-AGAGAC-3′) and 9 (5′-TGGACGTGTCTGTGAACCAT-3′), amplifying $G\alpha_{oA}$ or $G\alpha_{oB}$, respectively. The β-actin transcript was used as a control.

Cell cultures and transfection

HEK-293 cells were cultured in DMEM containing 10% fetal calf serum at 37℃ in a humidified atmosphere of 95% air, 5% CO_2. Transient transfections of G protein subunits and SST5 were performed using polyethylenimine (PEI linear, MW 25000, Polysciences Europe GmbH, Eppelheim, Germany) according to the instructions by the manufacturer.

Human pituitary cells were obtained from 12 GH secreting adenomas surgically removed by the trans-sphenoidal route. The study was previously approved by the local ethics committee. Informed consent was obtained from all subjects involved in the study.

Tissues were enzymatically dissociated in DMEM containing 2 mg/ml collagenase (Sigma-Aldrich, St Louis, MO) at 37℃ for 2 h, as previously described (Lania et al., 2004). Transient transfections of SST5, $G\alpha_{oA}$ and $G\alpha_{oB}$ were performed in cultured cells using JetPEI (Polyplus transfection, San Marcos, CA) according protocols previously optimized in our lab (Peverelli et al., 2012). Western blotting was performed in each experiment to control the expression level of SST5 in transiently transfected cells.

$G\alpha_{oA}$ silencing in pituitary adenoma cells

$G\alpha_{oA}$ gene silencing was performed in GH-secreting pituitary adenoma cells using specific custom siRNA and siPORT NeoFX transfection agent according to manufacturer's instructions (Ambion, Austin, TX, USA). In order to obtain the best efficiency of silencing, three different siRNAs purchased from Ambion were tested. Preliminary experiments to determine the optimal concentration of siRNAs and the kinetics of silencing were performed. RT-PCR was performed to verify that $G\alpha_{oA}$ silencing does not affect $G\alpha_{oB}$ transcript. A negative control siRNA, a non targeting sequence without significant homology to the sequence of human transcripts, was used in each experiment. $G\alpha_{oA}$ silencing was verified by RT-PCR analysis in each silencing experiment.

BRET assay

To detect the activation of the different Gα subunits by BRET experiments, HEK293 cells were co-transfected with either wild-type or R240W SST5 and Gαq-97-Rluc, Gαi1-91-Rluc, Gαi2-91-Rluc, Gαi3-91-Rluc, GαoA-91-Rluc8, GαoB-91-Rluc8 constructs in the presence of plasmids encoding for GFP10-Gγ2 and Gβ1. To avoid possible variations in the BRET signal resulting from fluctuation in the relative expression level of donors and acceptors, we set up transfection conditions in which comparable protein expression levels were maintained constant and similar values of total luminescence were obtained for all Gα-Rluc constructs. In particular, for transfection we used a 20 μg mix of DNA containing: 4 μg Gα-Rluc, 5 μg Gγ2-GFP10, 4 μg Gβ1 and 7 μg receptor. Forty-eight hours after transfection, cells were washed twice, detached and resuspended with PBS $MgCl_2$ 0.5 mM at room temperature. They were then distributed in a white 96-well microplate (100 μg of proteins per well; Optiplate, Perkin Elmer, Monza, Italy), and incubated in the presence or absence of BIM23026 100 nM for 2 min before substrate addition. The BRET between Rluc/Rluc8 and GFP10 was measured immediately after the addition of the Rluc substrate coelenterazine 400a (5 μM), using an Infinite F500 reader plate (Tecan, Milan, Italy) that allows the sequential integration of light signals detected with two filter settings (Rluc/Rluc8 filter 370–450 nm; GFP10 filter 510–540 nm). The data were recorded and the BRET signal was calculated as the ratio between GFP10 emission and the light emitted by Rluc/Rluc8. The changes in BRET induced by the ligand were expressed on graphs as 'BIM23206-promoted BRET' using the formula: ligand-promoted BRET = (emission GFP10 BIM23206/emission Rluc BIM23206) − (emission GFP10 PBS/emission Rluc PBS).

Immunoblotting analysis of p42/44

Pituitary cells were transfected with wild-type or mutated SST5 for 48 h, incubated with 100 ng/ml PTX (Sigma Aldrich, St Louis, MO) for 16 h in serum-starved medium and then stimulated with 10% FBS with or without 10 nM BIM23206 for 10 min. In silencing experiments, cells were silenced for 72 h with $G\alpha_{oA}$ siRNA or negative control siRNA before stimulation with 10% FBS containing medium with or without 10 nM BIM23206 for 10 min. As previously described (Peverelli et al., 2009), cells were then lysed in lysis buffer in the presence of protease inhibitors. Proteins were separated on SDS/polyacrylamide gels and transferred to a nitrocellulose filter. To detect phosphorylated p42/44 proteins, 1:2000 dilution of anti-phospho-p42/44 antibody (Cell Signaling, Danvers, MA) and an anti-rabbit HRP-linked antibody were used. The presence of total p42/44 was analysed by stripping and reprobing with anti-total p42/44 antibody (1:1000, Cell Signaling, Danvers, MA). Chemiluminescence was detected using the ChemiDoc-IT Imaging System (UVP, Upland, CA) and analysed using the image analysis program NIH ImageJ.

GH release

GH-secreting adenoma cells were transfected for 48 h with SST5, $G\alpha_{oA(PTX\text{-}r)}$ or $G\alpha_{oB(PTX\text{-}r)}$ or silenced for 72 h with $G\alpha_{oA}$ siRNA or negative control siRNA and then incubated for 3 h with fresh medium containing 10 nM BIM23206. Human GH was measured in culture medium using specific immunoassays (Perkin Elmer, Finland), according to the manufacturer's instructions.

cAMP assay

GH-secreting adenoma cells were transfected with for 48 h with SST5, $G\alpha_{oA(PTX\text{-}r)}$ or $G\alpha_{oB(PTX\text{-}r)}$ or silenced for 72 h with $G\alpha_{oA}$ siRNA or negative control siRNA. To quantify the inhibition of forskolin-induced cAMP accumulation, transfected cells were pre-incubated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min, and subsequently with 1 μM forskolin with or without BIM23206 (1 nM or 10 nM) for 30 min at 37℃. At the end of incubation, the medium was removed and intracellular cAMP was measured. Cells were lysed and assayed by enzymatic immunoassay (cAMP-Glo Assay, Promega, Madison, WI, USA) according to the instruction of the manufacturer. Experiments were repeated at least three times and each determination was performed five times.

Data analysis

The results are expressed as the means ± s.d. All data were tested *a priori* for normal distribution by using D'Agostino and Pearson omnibus normality test (GraphPad Prism 5.0). A paired two-tailed Student's *t*-test was used to detect the significance between two series of data. $P<0.05$ was accepted as statistically significant. One-way ANOVA by Dunnett's test was used to determine the statistical differences between SST5-agonist-promoted BRET in the presence of the different Gα proteins and untreated controls.

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