Identification of somatostatin receptors controlling growth hormone and thyrotropin secretion in the chicken using receptor subtype-specific agonists

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Abstract

Somatostatin (SRIH) functions as an endocrine mediator in processes such as growth, immune resistance and reproduction. Five SRIH receptors (sstr1–5) have been identified in mammals, where they are expressed in both the brain and peripheral tissues. To study the specific function of each receptor subtype, specific agonists (ag1–5) have been synthesized. The high degree of homology between mammalian and avian SRIH receptors suggests that these agonists might also be used in chickens. In this paper we describe two *in vitro* protocols (static incubation and perfusion system) to identify the SRIH receptors controlling the secretion of GH and TSH from the chicken pituitary. We found that basal GH or TSH secretion were never affected when SRIH or an agonist (1 μ M) were added. SRIH diminished the GH as well as the TSH response to TSH-releasing hormone (TRH; 100 nM) in

both systems. Our results have indicated that the SRIH actions at the level of the pituitary are regulated through specific receptor subtypes. In both the static and flow incubations, ag2 lowered the GH response to TRH, whereas stimulated TSH release was diminished by both ag2 and ag5. Ag3 and ag4 tended to increase rather than decrease the responsiveness of both pituitary cell types to TRH in perfusion studies. Our data have indicated that SRIH inhibits chicken pituitary function through sstr2 and sstr5. Only sstr2 seems to be involved in the control of chicken GH release, whereas both sstr2 and sstr5 inhibit induced GH secretion in mammals. The possible stimulatory action of ag3 and ag4 may point towards a species-specific function of sstr3 and sstr4.

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Introduction

The interaction of hypothalamic hormones with their respective receptor on the membrane of hypophyseal cells has been studied extensively. The initial concept of one hormone binding to a single population of receptors does not correspond to the actual situation. The presence of a diverse group of receptors enables a hormone to control different hypophyseal functions independently. Somatostatin (SRIH), a tetradecapeptide produced in the periventricular nucleus, was first isolated from sheep hypothalami (Brazeau *et al.* 1973). This peptide controls the secretion of two hypophyseal hormones: growth hormone (GH) and thyrotropin (TSH) (Brazeau *et al.* 1973, Vale *et al.* 1974, Drouin *et al.* 1976). Radioreceptor binding studies have suggested the presence of only one class of hypophyseal SRIH-binding sites (Katakami *et al.* 1985). However, molecular research indicated that SRIH acts through at least five receptor subtypes (sstr1–5). All mammalian subtypes have been cloned recently (Yamada

et al. 1992, Yasuda *et al.* 1992, Raynor *et al.* 1993a,b) and all of them are expressed in the mammalian pituitary (Bruno *et al.* 1993, O'Carroll *et al.* 1993, Hoyer *et al.* 1994, Raulf *et al.* 1994, O'Carroll & Krempels 1995).

Since SRIH and related analogues are largely non-selective in clinical receptor studies, researchers first developed semi-selective peptidyl (Patel & Srikant 1994) and later on selective non-peptidyl (Rohrer *et al.* 1998) and peptidyl agonists (Tulipano *et al.* 2001) to study the specific function of each receptor subtype. *In vitro* studies using these sstr agonists suggest that, at the level of the pituitary, SRIH controls GH and TSH secretion by binding to both sstr2 and -5 receptors (Shimon *et al.* 1997a,b, Rohrer *et al.* 1998, Tulipano *et al.* 2001). None of the studies performed demonstrated an effect of sstr1, sstr3 or sstr4 agonists on the endocrine functions of the mammalian pituitary.

The chicken has been used extensively as a model to study the control of GH secretion (hypothalamic control studies: Harvey 1993; testing of clinical GH secretagogues:

Geris *et al.* 1998, 2001). Radioreceptor assay studies indicate that pituitary SRIH receptors are comparable in birds and mammals (Harvey *et al.* 1990, Geris *et al.* 2000), and partial cDNAs for chicken SRIH receptors show a high degree of homology with their mammalian counterparts (Bossis & Porter 2001, Boardman *et al.* 2002). Therefore the synthetic non-peptidyl sstr agonists (Rohrer *et al.* 1998) can be useful to identify the receptor pathway through which SRIH controls pituitary functions in this species, as has been shown recently (Bossis & Porter 2001). This may not only lead to a better understanding of the SRIH mode of action in avian species, but it may also indicate some important evolutionary differences, since so far most studies have been performed on mammals.

In vitro static incubation and perfusion experiments were conducted to study the effect of the different agonists on basal and stimulated GH and TSH secretion. An SRIH treatment was added as a reference. In order to induce a clear response, we used TSH-releasing hormone (TRH), a well-documented stimulator of GH and TSH release in the chicken (Harvey *et al.* 1978, 1991, Geris *et al.* 2001).

Materials and Methods

Animals

All studies were performed on pituitaries from chickens of a layer strain (Hisex White), purchased as fertilized eggs from a local commercial hatchery (Euribrid, Aarschot, Belgium). Eggs were incubated in a forced-draft incubator at a temperature of 37.8 °C with increasing humidity and ventilation from day 14 onwards, with continuous lighting and a 45 ° rotation every hour. The start of incubation was called day 1. Posthatch chickens were kept in an acclimatized room with a 14 h light:10 h darkness photoperiod. Water and feed were available *ad libitum*.

Receptor-selective SRIH agonists

Non-peptidyl agonists of each of the five SRIH receptors were recently identified in combinatorial libraries constructed on the basis of molecular modelling of known peptide agonists (Rohrer *et al.* 1998). This resulted in the development of five sstr ligands which show a binding selectivity for one receptor subtype: L-797,591 or ag1 (selective for sstr1); L-779,976 or ag2 (selective for sstr2); L-796,778 or ag3 (selective for sstr3); L-803,087 or ag4 (selective for sstr4); L-817,818 or ag5 (selective for sstr5). The latter agonist is only semi-selective but sstr5-mediated effects can be demonstrated by comparison with results obtained with ag1.

Static incubations

Pituitaries ($n=64$) were collected from 1-day-old chicks and immediately transferred individually to multiwell

dishes with ice-cold M199 (Gibco, Ghent, Belgium). After collection of all pituitaries, medium was replaced and pituitaries were incubated in a CO₂ incubator at 37 °C. Medium was replaced again after 30, 90 and 150 min. Samples were collected 15 min later to analyze whether basal GH and TSH secretion were stabilized and comparable in all experimental groups ($n=8$ per group). In the next period of 15 min, SRIH (Sigma-Aldrich, St Louis, MO, USA) or a synthetic agonist (1 µM; Merck Laboratories, Rahway, NJ, USA; Rohrer *et al.* 1998) was added to the medium (group 3: SRIH; group 4: ag1; group 5: ag2; group 6: ag3; group 7: ag4; group 8: ag5). Pituitaries from groups 1 and 2 were incubated in M199 alone. After collection of all samples, TRH (100 nM; UCB, Brussels, Belgium) was added to the M199 medium with (groups 3 to 8) or without (group 2) SRIH or ag. Group 1 was used as the basal secretion control group whereas group 2 was the stimulated secretion control. Medium samples were collected 15 min later. The study was repeated 2 weeks later using animals from a different batch. All samples were stored at -20 °C until analysis of hormones levels by radioimmunoassay (RIA).

Perfusion experiments

Pituitaries from 1-day-old chicks were dissected and placed individually in perfusion chambers ($n=12$ per perfusion) at 37 °C. Each experiment consisted of five consecutive periods: (1) an equilibration period (1.5 h); (2) a basal period (0.5 h); (3) a prestimulation period (1 h); (4) a stimulation period (0.5 h); (5) a poststimulation period (2 h).

M199 was used as perfusion medium and all reagents were prepared in the same medium. Flow rate was 12 ml/h. Three independent perfusion studies were conducted and in each perfusion three conditions were tested. The first group always served as a positive control group (addition of TRH) whereas, in the other two groups, the effect of SRIH or ag1–5 on the basal or TRH-induced GH or TSH release was tested. In the first perfusion, group 1 continued with M199 in the prestimulation period, whereas groups 2 and 3 received respectively 1 µM SRIH and 1 µM ag1 ($n=4$ per group). During the stimulation period, all pituitaries were treated with 100 nM TRH, with (groups 2 and 3) or without (group 1) SRIH or ag1. Afterwards, the perfusion continued for another 2 h using medium with (groups 2 and 3) or without (group 1) SRIH or ag1 supplement. The same protocol was used in the other two perfusion experiments in which we analyzed the actions of ag2 and ag3 (perfusion 2) or ag4 and ag5 (perfusion 3). The rationale for the different perfusion periods and the duration of each period were based on previous studies investigating the hypothalamic control of GH (Darras *et al.* 1994) and TSH release (Geris *et al.* 1995, 1996) or the inhibitory effect of

thyroid hormones on the corticotropin-releasing hormone-induced TSH release (Geris *et al.* 1999).

All samples were stored at -20°C until analysis of hormone levels by RIA. The stimulation factor (SF) and the net increase after stimulation (NI) were calculated for each individual chamber (Geris *et al.* 1995). SF was calculated as the peak value (PV; i.e. the highest measured hormone concentration in response to the secretagogue) divided by the mean basal secretion value (BS) as measured in the 0.5 h basal period: $\text{SF} = \text{PV}/\text{BS}$. During the stimulation period, the increase of hormone concentration above BS was calculated for each individual time-point. The sum of all these individual net increases is called NI.

Radioimmunoassays

GH levels were measured by RIA (Darras *et al.* 1992). The sensitivity of the assay was 2 ng/ml. The intra- and interassay coefficients of variation were 4.0% and 15.5% respectively. Quantification of α -subunit concentration, the common glycoprotein subunit of TSH, was carried out according to Berghman *et al.* (1993). It has been established that the α -subunit response to TRH challenge reflects a TSH response (Berghman *et al.* 1993, Geris *et al.* 1999). The sensitivity of this assay was 0.5 ng/ml and the intra- and interassay coefficients of variation were 2.4% and 1.9% respectively. For all RIAs, chicken plasma dilution and loading tests showed good parallelism with the standard curves.

Statistics

Values represent the means $\pm$ S.E.M. Data from the static incubation experiment were analyzed by an unpaired Student's *t*-test. Due to the limited number of samples per group, the data from the perfusion experiments were analysed by the non-parametric Kruskal–Wallis test.

Results

Static incubations

After the preincubation period, mean GH and α -subunit levels were comparable in all experimental groups, indicating that a stable basal GH and TSH secretion was established. As expected, TRH induced a high GH and TSH response (Fig. 1). This increase was clearly diminished when the pituitaries were pretreated with SRIH. Only ag2 could lower the GH response to TRH, whereas both ag2 ($P=0.099$) and ag5 ($P<0.05$) affected the TSH increase (Fig. 1). Similar results were obtained when this experiment was repeated. However, this time the drop in the TSH response to TRH was significant in both the ag2 and ag5 groups ($P<0.05$).

Figure 1 The effect of SRIH (1 μM) and synthetic SRIH agonists (ag1–5; 1 μM) on the *in vitro* TRH (100 nM)-induced GH (top) or α -subunit (bottom) secretion in 1-day-old chicks. The data shown are the means $\pm$ S.E.M. ($n=3$ –4 per condition). The experiment was performed in a static incubation. * $P<0.05$, significant difference between an experimental group and the TRH control group (Student's *t*-test).

Perfusion experiments

SRIH and the different synthetic agonists did not alter basal GH secretion (illustrated for ag2 and ag3 in Fig. 2). However, the response of somatotrophs to a TRH challenge was affected depending on the reagent administered in combination with the TRH stimulus. A significantly lower SF and NI could be observed in the SRIH/TRH group compared with the TRH control condition (Table 1). This specific effect of SRIH on the somatotrophs could only be mimicked when ag2 was used as an SRIH agonist (Table 1 and Fig. 2). Although the change in SF was not statistically significant, the magnitude of this inhibition (a reduction of 28%) was comparable with that induced by an

Figure 2 The effect of ag2 and ag3 (1 μ M) on the *in vitro* TRH (100 nM)-induced GH (top) or α -subunit (bottom) secretion in 1-day-old chicks. The data shown are the means $\pm$ S.E.M. ($n=3-4$ per condition). The experiment was performed in a perfusion system. The arrows represent the start and the end of the TRH stimulation period taking into account the time needed for the medium to reach the fraction collector. The horizontal lines show the period of ag2 or ag3 administration.

Table 1 The effect of SRIH (1 μ M) and synthetic SRIH agonists (ag1–5: 1 μ M) on the *in vitro* TRH (100 nM)-induced GH secretion in 1-day-old chicks. Three separate experiments were conducted. The values shown are the means $\pm$ S.E.M. ($n=3-4$ per condition) and represent the calculated stimulation factor (SF) and the calculated net increase in GH release after the TRH stimulus (NI). A typical perfusion graph is shown in Fig. 2

	GH	
	SF	NI (ng)
TRH (control)	11.95 $\pm$ 1.47	839 $\pm$ 185
SRIH/TRH	8.16 $\pm$ 0.95*	231 $\pm$ 129*
Ag1/TRH	10.95 $\pm$ 2.82	757 $\pm$ 138 [†]
TRH (control)	11.02 $\pm$ 3.30	523 $\pm$ 38
Ag2/TRH	7.98 $\pm$ 4.30	210 $\pm$ 111*
Ag3/TRH	14.82 $\pm$ 1.74	617 $\pm$ 43 [†]
TRH (control)	16.76 $\pm$ 2.00	813 $\pm$ 106
Ag4/TRH	17.24 $\pm$ 2.88	1351 $\pm$ 272
Ag5/TRH	15.46 $\pm$ 2.67	648 $\pm$ 115 [†]

* $P<0.05$, significant difference between an experimental group and its corresponding TRH control group (Kruskal–Wallis test). [†] $P<0.05$, significant difference between both experimental groups within one perfusion experiment (Kruskal–Wallis test).

equimolar concentration of SRIH (32% reduction). Both ag3 and ag4, though not significantly, tended to increase the amount of GH secreted after the TRH challenge (NI in Table 1).

As expected, we measured higher levels of α -subunit in the perfusion samples when TRH was added to the medium (Fig. 2), indicative of the TSH-releasing activity of TRH in the chicken (Berghman *et al.* 1993). Treatment of the pituitaries with SRIH or its agonists did not alter basal TSH secretion (illustrated for ag2 and ag3 in Fig. 2). The TSH response to the TRH challenge depended on the pretreatment administered. The amount of TSH secreted after the TRH challenge was significantly lower in the SRIH/TRH group (SF and NI in Table 2). The reduction in SF was 41%. This inhibition could not be induced when SRIH was replaced by ag1, ag3 or ag4 (Table 2). Pretreatment with ag2 on the other hand resulted in similar results as in the SRIH/TRH group: a significantly lower NI and a (non-significant) 40% reduction of SF (Table 2 and Fig. 2). Also the SF in the ag5/TRH group was (non-significantly) reduced by 40% compared with the TRH control group and a non-significant drop was observed in the amount of TSH secreted after TRH challenge (NI in Table 2). In the ag3/TRH and the ag4/TRH group the response to TRH challenge was increased rather than decreased (Table 2 and Fig. 2), but only in the case of ag3 was this increase statistically significant.

Discussion

Although only one class of binding sites could be identified with a radioreceptor assay (Enjalbert *et al.* 1983, Katakami

Table 2 The effect of SRIH (1 μ M) and synthetic SRIH agonists (ag1–5: 1 μ M) on the *in vitro* TRH (100 nM)-induced α -subunit secretion in 1-day-old chicks. Three separate experiments were conducted. The data shown are the means $\pm$ S.E.M. ($n=3-4$ per condition) and represent the calculated stimulation factor (SF) and the calculated net increase in α -subunit release after the TRH stimulus (NI). A typical perfusion graph is shown in Fig. 2

	α -subunit	
	SF	NI (ng)
TRH (control)	7.25 $\pm$ 0.72	50.9 $\pm$ 1.0
SRIH/TRH	4.30 $\pm$ 0.99*	17.2 $\pm$ 7.3*
Ag1/TRH	9.30 $\pm$ 1.86 [†]	66.2 $\pm$ 19.2 [†]
TRH (control)	7.46 $\pm$ 0.46	52.1 $\pm$ 1.5
Ag2/TRH	4.46 $\pm$ 1.32	16.7 $\pm$ 8.1*
Ag3/TRH	9.38 $\pm$ 1.23	73.5 $\pm$ 6.8 [†]
TRH (control)	6.33 $\pm$ 1.93	33.0 $\pm$ 10.5
Ag4/TRH	14.07 $\pm$ 2.32	69.2 $\pm$ 10.2
Ag5/TRH	3.77 $\pm$ 0.61 [†]	22.1 $\pm$ 4.2 [†]

* $P<0.05$, significant difference between an experimental group and its corresponding TRH control group (Kruskal–Wallis test). [†] $P<0.05$, significant difference between both experimental groups within one perfusion experiment (Kruskal–Wallis test).

et al. 1985), molecular studies revealed the existence of at least five SRIH receptor subtypes (Yamada *et al.* 1992, Yasuda *et al.* 1992, Raynor *et al.* 1993a,b, O'Carroll & Krempels 1995). This makes the SRIH pathway an interesting model to study how a hormone co-ordinates its wide range of functions by binding to specific receptors. This research may not only reveal interesting insights into the endocrine relations between hormone and receptor, but may also function as an interesting tool to look at how hormones develop or specify well-defined functions through evolution.

Since mammalian pituitary cells express all five known SRIH receptor subtypes (Bruno *et al.* 1993, O'Carroll *et al.* 1993, Hoyer *et al.* 1994, Raulf *et al.* 1994, Thoss *et al.* 1995), the availability of receptor-specific agonists is indispensable to elucidate how SRIH controls GH and TSH secretion. In the last few years, both synthetic non-peptidyl (Rohrer *et al.* 1998) and peptidyl receptor-specific agonists (Patel & Srikant 1994, Patel *et al.* 1995, Tulipano *et al.* 2001) have been constructed. In this study, the chicken was used to investigate the actions of SRIH at the level of the pituitary. This choice was based on the clearly established role of SRIH in the GH and TSH secretion in this species (for reviews see Harvey 1993, Geris *et al.* 2001). Since we used non-peptidyl SRIH agonists that were constructed based on the mammalian SRIH receptor subtypes, high affinity and/or potency are not fully guaranteed for the chicken SRIH receptors. Nevertheless, earlier radioreceptor assay studies showed that the SRIH pituitary receptor family in the chicken is comparable with that in mammals (Enjalbert *et al.* 1982, Harvey *et al.* 1991, Geris *et al.* 2000) and partial cDNAs

for chicken SRIH receptors show a high degree of homology with their mammalian counterparts (Bossis & Porter 2001, Boardman *et al.* 2002). These findings suggest that the non-peptidyl SRIH agonists can be used to study the SRIH mode of action in chickens as well.

Our present data show that only *sstr2* is involved in the SRIH inhibition of TRH-stimulated GH release in birds. This corresponds with its presumed involvement in the inhibition of GH-releasing hormone (GHRH)-induced GH secretion in the chicken (Bossis & Porter 2001). Contrary to these authors, we did not observe any effect on basal GH secretion. None of the other tested agents affected basal or TRH-enhanced GH release, indicating a very specialized action of SRIH in the chicken. In rats, not only *sstr2* but also *sstr5* lowers the sensitivity of somatotrophs to an external stimulus (Rohrer *et al.* 1998, Parmar *et al.* 1999). This observation is in agreement with the presence of *sstr2* and *sstr5* mRNA in rat somatotrophs (Mezey *et al.* 1998). In the human pituitary, *sstr2* and *sstr5* act equipotently (Shimon *et al.* 1997a,b, Danila *et al.* 2001). The use of synthetic peptidyl SRIH agonists also indicates that *sstr2* and *sstr5* play a major role in the GH-suppressing activity of SRIH in mammals. Octreotide, for example, an octapeptide analogue of SRIH, is a very potent inhibitor of *in vitro* GH secretion. Since this peptide does not bind to *sstr3* and *sstr4*, these receptor subtypes can be excluded from the SRIH–GH pathway (Patel *et al.* 1995). On the other hand, injections of BIM 23014, a peptidyl agonist selective for *sstr2* and *sstr5*, result in a drop in plasma GH levels in rams (Magnan *et al.* 1992). Although the efficacy of the non-peptidyl agonists remains to be further clarified in the chicken, our results suggest that the GH-controlling role of *sstr5* may only have developed during the evolution to mammals.

Next to GH secretion, SRIH is also involved in the control of TSH release in mammals and birds (Vale *et al.* 1974, Geris *et al.* 2001, present study). In humans, SRIH controls the secretory activity of thyrotrophs through *sstr2* and *sstr5* (Shimon *et al.* 1997a,b) and *ag2* and *ag5* were found to affect the *in vitro* responsiveness of thyrotrophs to TRH equipotently (Shimon *et al.* 1997a). We showed that this controlling mechanism is also present in birds, although the *ag5*-induced drop in the TSH response to the TRH challenge was statistically not significant in the perfusion system. However, the induced effect of *ag5* on the SF was of the same magnitude as observed in the SRIH/TRH group. Moreover, two independent static incubation studies did demonstrate a significant effect of *ag5* on the TRH-induced TSH release. Recently, a partial sequence of the chicken *sstr2* (Bossis & Porter 2001) and of the presumed chicken *sstr5* were cloned (Boardman *et al.* 2002), but their cellular localization in the pituitary remains to be clarified. This kind of study could provide us with further information concerning the involvement of these receptor subtypes in the control of pituitary function in the chicken.

None of the agonists tested affected the basal secretion of GH or TSH. Also in mammals, researchers have never observed an effect at this level. Only when GH (e.g. GHRH) or TSH stimulators (e.g. TRH) were administered, did SRIH or a specific agonist alter the secretory activity of GH and/or TSH cells (Shimon *et al.* 1997a,b, Rohrer *et al.* 1998, Parmar *et al.* 1999). Bossis & Porter (2001) are the only researchers so far who have reported an inhibiting function of *sstr* agonists on basal GH secretion.

Ag1, *ag3* and *ag4* did not imitate the two functions of SRIH tested. Also, in mammals, these receptor subtypes do not seem to be involved in the GH and TSH inhibitory activity of SRIH (Patel *et al.* 1995, Rohrer *et al.* 1998). However, since *sstr1*, *sstr3* and *sstr4* mRNA is expressed in somatotrophs and thyrotrophs (Bruno *et al.* 1993, O'Carroll & Krempels 1995, Mezey *et al.* 1998), future research should look into the exact function of these receptor subtypes. Our results in the perfusion experiments did show some stimulatory effects of some of these agents on the TRH-induced GH and TSH secretion, although this observation could not be confirmed in the static incubation studies. Both *ag3* and *ag4* tended to increase the GH and TSH response to TRH without affecting basal GH or TSH release. A modest stimulatory effect of *ag4* on GH release in chicken has also been described by Bossis & Porter (2001). However, in their system the effect was observed only on basal GH release and not on GHRH-stimulated GH secretion. Certainly these stimulatory pathways are worth further exploration.

In conclusion, we present a study in which SRIH receptor selective agonists have been tested with regard to their effects on TRH-stimulated GH and TSH release in the chicken. It is the first time these agonists have been tested in relation to the regulation of TSH secretion in a non-mammalian species. As in mammals, both *sstr2* and *sstr5* are involved in the regulation of the TSH response to a TRH stimulus. Contrary to mammals, however, SRIH inhibition of the stimulated GH release was only mediated by *sstr2*, indicating some minor evolutionary changes. The possible atypical SRIH action via *sstr3* and *sstr4*, namely stimulation of the sensitivity of avian somatotrophs and thyrotrophs to TRH, deserves further investigation.

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