0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(9):4031–4041

doi: 10.1210/en.2003-0439

Endogenous Hypothalamic Somatostatins Differentially Regulate Growth Hormone Secretion from Goldfish Pituitary Somatotropes *in Vitro*

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Using Southern blot analysis of RT-PCR products, mRNA for three different somatostatin (SS) precursors (PSS-I, -II, and -III), which encode for SS_{14}, goldfish brain (gb)SS_{28}, and [Pro^2]SS_{14}, respectively, were detected in goldfish hypothalamus. PSS-I and -II mRNA, but not PSS-III mRNA, were also detected in cultured pituitary cells. We subsequently examined the effects of the mature peptides, SS_{14}, gbSS_{28}, and [Pro^2]SS_{14}, on somatotrope signaling and GH secretion. The gbSS_{28} was more potent than either SS_{14} or [Pro^2]SS_{14} in reducing basal GH release but was the least effective in reducing basal cellular cAMP. The ability of SS_{14}, [Pro^2]SS_{14}, and gbSS_{28} to attenuate GH responses to GnRH were comparable. However, gbSS_{28} was less effective than SS_{14} and [Pro^2]SS_{14} in diminishing dopamine- and pituitary adenylate cyclase-activating polypeptide-stimulated GH release, as well as GH release resulting from the activation of their underlying signaling cascades. In contrast, the actions of a different 28-amino-acid SS, mammalian SS_{28}, were more similar to those of SS_{14} and [Pro^2]SS_{14}. We conclude that, in goldfish, SSs differentially couple to the intracellular cascades regulating GH secretion from pituitary somatotropes. This raises the possibility that such differences may allow for the selective regulation of various aspects of somatotrope function by different SS peptides. (*Endocrinology* 144: 4031–4041, 2003)

THE SOMATOSTATIN (SS) neuropeptide family is comprised of multiple genes and gene products. In mammals, there are two biologically active forms of SS, SS_{14} and its N-terminal extension mammalian (m)SS_{28}, both of which are derived from the same precursor molecule, prosomatostatin (PSS)-I (reviewed in Ref. 1). The cDNAs for PSS-I have also been cloned from numerous nonmammalian species, including chicken, frog, and several teleost fish, including anglerfish, rainbow trout, sturgeon, and goldfish (Ref. 2, reviewed in Ref. 3). Similar to mPSS-I, goldfish PSS-I is capable of yielding SS_{14}. In fact, the SS_{14} derived from goldfish PSS-I is identical to mSS_{14} in amino acid sequence (4). However, unlike mammalian PSS-I, goldfish PSS-I does not contain a monobasic Arg cleavage site capable of yielding SS_{28}. Although there is a cleavage site capable of yielding a 26-amino-acid SS (4), a SS_{26} has not been isolated.

In addition to PSS-I, goldfish possess two additional PSSs, PSS-II and PSS-III. Goldfish brain (gb)SS_{28}, which is contained within PSS-II, differs from mSS_{28} in two ways. First, in addition to differing by eight amino acids in the N terminus, it contains [Glu^1, Tyr^7, Gly^{10}]SS_{14} at its C terminus (4). Second, it is a separate gene product, rather than an alternate cleavage product (4). Although goldfish PSS-II contains cleavage sites capable of generating both 14- and 28-amino-acid peptides, studies in anglerfish have shown that the 14-amino-acid SS and the 28-amino-acid SS are separate, independent products of PSS-I and PSS-II, respectively (5–8). As such, it is believed that goldfish PSS-II yields only gbSS_{28}.

The third goldfish PSS, PSS-III, contains a potential cleavage site for a 14-amino-acid peptide but not for a 28-amino-acid peptide (4). There are, however, Arg monobasic cleavage sites capable of yielding 24- and 29-amino-acid peptides. However, a [Pro^2]SS_{14} has been identified in Russian sturgeon (9), making the occurrence of [Pro^2]SS_{14} in goldfish a likely possibility. Phylogenetic analysis suggests that PSS-III is related to the [Pro^2, Met^{13}]SS_{14} precursor in frog, and cortistatin in mammals (4, 10).

In mammals, SS_{14} and SS_{28} are differentially expressed throughout the central nervous system, peripheral nervous system, and most of the major organs of the body (11–13), whereas cortistatin is expressed primarily in the cerebral cortex and hippocampus (14, 15). Similarly, in several nonmammalian vertebrates, including frog (16), coho salmon (17), rainbow trout (17, 18), sturgeon (2), and goldfish (4), differential distribution of PSS-I, PSS-II, and PSS-III have been reported.

The SSs act through a family of G protein-coupled receptors. In mammals, five SS receptor subtypes ($sst_{1–5}$) have been identified. Each subtype is capable of interacting with a distinct set of intracellular signaling systems (reviewed in Ref. 19). All five sst's bind SS_{14} and mSS_{28} with high affinity; however, sst_5 exhibits some selectivity for mSS_{28}. Although all five receptor subtypes are expressed in the pituitary, sst_2 and sst_5 are believed to be the

Abbreviations: AA, Arachidonic acid; AC, adenylate cyclase; 8Br-cAMP, 8-bromo-cAMP; c, chicken; DA, dopamine; DiC8, dioctanoyl glycerol; gb, goldfish brain; K_{ir}, inwardly rectifying K^+ channel; m, mammalian; NO, nitric oxide; PACAP, pituitary adenylate cyclase-activating polypeptide; PKA, protein kinase A; PKC, protein kinase C; PSS, prosomatostatin; s, salmon; SKF-38393, (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride; SNP, sodium nitroprusside; SS, somatostatin; sst, SS receptor; TPA, tetradecanoyl phorbol acetate.

primary regulators of somatotrope function (20). In goldfish, 8 sst's ($gfsst_{1A, 1B, 2, 3A, 3B, 5A, 5B, 5C}$) have been cloned from brain tissues (21–24). Similar to the situation in mammals, pharmacological characterization of $gfsst_{5A}$ has shown that although it binds SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ with high affinity, it displays some selectivity for 28-amino-acid SSs (22). Consistent with mammalian studies, $gfsst_2$ and $gfsst_5$ mRNA is predominantly expressed in the pituitary, compared with other brain regions (22, 24).

The differential expression of sst subtypes is clearly one of the means by which functional specificity is achieved in the SS/sst system. However, whether (and how) the different SS peptides contribute to the selective regulation of cell function in tissues where more than one isoform are present is poorly understood. To begin exploring this possibility, we searched for PSS mRNA in goldfish hypothalamus and pituitary. We subsequently examined the ability of the mature peptides, SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$, to regulate basal GH secretion and cAMP production. In addition, we examined the ability of these three SS peptides to inhibit GH release stimulated by several different goldfish neuroendocrine regulators, as well as GH secretion resulting from the pharmacological activation of their respective intracellular signaling cascades. In particular, we used GnRH, which stimulates GH release in a protein kinase C (PKC)-dependent manner, and dopamine (DA) and pituitary adenylate cyclase (AC)-activating polypeptide (PACAP), which act through AC/cAMP/protein kinase A (PKA)-sensitive mechanisms (reviewed in Ref. 25). The concept of SS isoform functional selectivity was further tested by comparing the apparent intracellular mechanisms mediating mSS_{28} and $gbSS_{28}$ inhibition of GH release.

Materials and Methods

Animals and cell preparation

All animal maintenance and experimental protocols used in this study were approved by the University of Alberta, Biological Sciences Animal Care Committee in accordance with national guidelines. Common goldfish (*Carassius auratus*, 8–13 cm in length) were purchased from Aquatic Imports (Calgary, Alberta, Canada) and maintained in flow-through aquaria (1800 liters) at 16–20 C as previously described (26). As needed, goldfish were anesthetized in 0.05% tricaine methanesulfonate and euthanized by cervical transection. Pituitaries were subsequently excised and dispersed using a previously described trypsin/DNase dispersion protocol (26).

Pituitaries from male and female goldfish at different stages of the reproductive cycle were used in this study. The magnitude of SS_{14} inhibition of basal GH release has previously been reported to vary throughout the seasonal reproductive cycle (27). However, when all available data, collected over the last 4 yr, concerning SS_{14} regulation of basal GH release were pooled and analyzed according to the time of year, these changes were not significant (Yunker, W. K., unpublished data). In the present study, SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ inhibition of basal GH release was always significant, regardless of gonadal state. Nevertheless, where possible, the effects of the different SS peptides, either alone or against a specific secretagogue, were compared simultaneously to control for possible seasonal variation.

Reagents and test substances

All media contained M-199 (Invitrogen, Burlington, ON or Sigma-Aldrich, St. Louis, MO) with 0.1 g/liter L-glutamine, 26 mM $NaHCO_3$, 25 mM HEPES, 100 mg/liter streptomycin, and 100,000 U/liter penicillin (pH adjusted to 7.2). Dispersion media contained Hanks' salts and 0.3% BSA (fraction V, Calbiochem, San Diego, CA). Plating media (for overnight incubation) contained Earle's salts and 1% horse serum (Invitrogen). Testing media was the same as dispersion media except that BSA was reduced to 0.1%. In instances in which cells were depolarized with 30 mM KCl, equimolar substitution of KCl for NaCl was employed to maintain osmolarity.

Distilled, deionized water was used to prepare stock solutions of $[Pro^2]SS_{14}$, $gbSS_{28}$ (synthesized by Dr. J. Rivier), SS_{14}, mSS_{28}, $mPACAP_{38}$, salmon (s)GnRH ([Trp^7, Leu^8]GnRH), chicken (c)GnRH-II ([His^5, Trp^7, Tyr^8]GnRH, Peninsula Laboratories, Belmont, CA), and CsCl. 8-bromo-cAMP (8Br-cAMP), forskolin, (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride (SKF-38393), tetradecanoyl phorbol acetate (TPA, Research Biochemicals Inc., Natick, MA), dioctanoyl glycerol (DiC8), and A23187 (Calbiochem) were dissolved in dimethyl sulfoxide. Ionomycin (Calbiochem) and arachidonic acid (AA, Sigma-Aldrich) were dissolved in ethanol. Sodium nitroprusside (SNP, Calbiochem) was dissolved in testing medium immediately before use. Aliquots of concentrated stock solutions were stored at either room temperature or −20 C. Final concentrations were achieved by dilution in testing medium. Final concentrations of dimethyl sulfoxide and ethanol never exceeded 0.1% and had no effect on basal GH release (28), $[Ca^{2+}]_i$ (29), or ionic currents in identified goldfish somatotropes (30).

Trizol reagent, Taq DNA polymerase, and SuperScript II RNase H^- reverse transcriptase were purchased from Invitrogen. Hybond nylon membrane, Rediprime II random prime labeling system, and [α-^{32}P]deoxy-CTP were purchased from Amersham Biosciences (Buckinghamshire, UK), and the QIAquick nucleotide removal kit was obtained from QIAGEN (Mississauga, ON).

RT-PCR and Southern blot analysis

Total RNA was extracted from freshly excised hypothalamus and pituitary, as well as dispersed pituitary cells that had been cultured overnight, using Trizol RNA isolation reagent (Invitrogen). Total RNA was reverse transcribed into cDNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen). PCR amplifications were carried out using primers specific for PSS-I, -II, and -III mRNA (4). The primer sets were:

SS1-F2 (5′-GCGTATCCAGTGCGCACTGGC-3′) and SS1-R2 (5′-GTGAAAGTTTTCCAGAAGAA-3′) for PSS-I mRNA, SS2-F1 (5′-CGAATCACAGCTACAAAGAGTC-3′) and SS2-R1 (5′-CAAGCGAGGGCCTGAGCAGG-3′) for PSS-II mRNA, SS3-F1 (5′-GGAGCTACAAGACTTCAAC-3′) and SS3-R1 (5′-CTGTGTCAGAGTAAGTCCACG-3′) for PSS-III mRNA.

PCR conditions were denaturation for 1 min at 95 C, annealing for 1 min at either 51 C for SS_{14} or 54 C for SS_{28} and $[Pro^2]SS_{14}$, and extension at 73 C for 1 min for a total of 30 cycles, with a final extension of 5 min at 73 C (4). The reactions were then electrophoresed on 1% agarose gels, transferred to Hybond nylon membranes (Amersham Biosciences) by capillary transfer and fixed by baking at 80 C for 2 h. The membranes were prehybridized at 65 C for 1 h in a hybridization solution containing 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, 1 mM EDTA (pH 8.0), and 1% BSA. The membranes were then transferred into fresh hybridization solution to which [α-^{32}P]deoxy-CTP-labeled probe was added. Probes for SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ were labeled using the Rediprime II random prime labeling system (Amersham Biosciences) and purified using the QIAquick nucleotide removal kit (QIAGEN) according to the manufacturer's instructions. Hybridization was carried out overnight at 65 C. The membranes were subsequently washed twice with wash solution containing 0.04 M $NaHPO_4$ (pH 7.2), 1% SDS, 1 mM EDTA (pH 8.0) at 65 C and exposed to a Phosphorscreen (Molecular Dynamics, Sunnyvale, CA) for 1 h. The screen was scanned using a PhosphorImager 445 SI (Molecular Dynamics) and analyzed using the IMAGEQUANT software (Molecular Dynamics). As a negative control, PCRs were performed in the absence of cDNA to examine cross-contamination of samples. As an internal control of the reverse transcription step, PCR amplification was carried out for 35 cycles of 94 C for 1 min, 50 C for 1 min, and 73 C for 1 min with primers designed on the basis of β-actin partial cDNA sequence in goldfish (31) (unpublished sequence, GenBank accession no. AF079831).

Static incubation experiments assessing GH release

Following dispersion, cells were plated at a density of 0.25×10^6/well in 24-well plates (Falcon Primaria, Becton Dickinson Labware, Franklin

Lakes, NJ) and cultured overnight at 28 C, 5% CO_2 and saturated humidity (26). The next day, following a rinse in testing media, cells were cultured in the presence of natural ligands and/or pharmacological agents for 2 h (26). Experiments were performed in either triplicate or quadruplicate on each plate, and each experiment was repeated a minimum of three times, using different cell preparations each time. The testing media was subsequently removed and stored at −26 C until GH content was measured using a previously validated RIA (32). Hormone release (nanograms per milliliter) was normalized as a percentage of the mean basal control value (average = 969 ± 65.8 ng/ml) and compared using ANOVA followed by least significant difference multiple comparisons. Differences were considered significant when $P < 0.05$. All secretagogues employed in this study caused significantly elevations in GH release relative to basal control values. Hormone release results are presented as mean ± SEM. Regression lines and IC_{50}s were calculated using SigmaPlot version 7.0 (SPSS Inc., Chicago, IL).

Static incubation experiments assessing cAMP

Freshly dispersed pituitary cells were plated and cultured overnight using the same procedure as described above. The next day, cells were washed with clear testing media (testing media without phenol red) and subsequently cultured for 2 h in clear testing media supplemented with varying concentrations of one of the three different SS isoforms. Experiments were performed in triplicate on each incubation plate, and each experiment was repeated a minimum of three times, using different cell preparations each time. Following drug treatment, 800 μl of clear testing media were collected to assess cAMP release, and cellular cAMP was extracted by lysing the cells with 1 ml distilled, deionized water and subsequent 30-sec sonication. All samples, released and cellular, were placed in a boiling water bath for 10 min to denature phosphodiesterases. Samples were then acetylated and assayed for cAMP content using a cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). To facilitate pooling of data from replicate experiments, cAMP levels (picomoles per milliliter) were normalized as a percent of the mean basal control value (average = 6.58 ± .77 pmol/ml for released and 0.15 ±.01 pmol/ml for cell content) and compared using ANOVA followed by least significant difference multiple comparisons. Differences were considered significant when $P < 0.05$. Results are presented as mean ± SEM. Regression lines were calculated using SigmaPlot version 7.0 (SPSS Inc.).

Results

Expression of PSS-I, PSS-II, and PSS-III mRNA in the hypothalamus and pituitary

To assess whether pituitary cells may be exposed to SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$, we examined the expression of their prohormone mRNA in the hypothalamus and pituitary. Because the hypothalamic neurons that directly innervate the teleost pars distalis (33) are removed by trypsin/DNase dispersion (26), the inclusion of both pituitary fragments and primary pituitary cell cultures enabled us to examine PSS mRNA expression in pituitary preparations that still contained hypothalamic nerve terminals, and preparations devoid of such terminals. The mRNA for all three PSSs was detected in the hypothalamus following Southern blot analysis of RT-PCR products (Fig. 1). Interestingly, although PSS-I and PSS-II mRNA were also present in dispersed pituitary cells, no PSS mRNA was detected in pituitary fragments (Fig. 1).

SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ on basal GH release

Previously, SS_{14} has been shown to dose-dependently reduce GH release from both goldfish pituitary fragments and dispersed pituitary cell preparations (27, 34); similarly, $[Pro^2]SS_{14}$ has been shown to reduce basal GH release from pituitary fragments (4). However, results obtained from pituitary fragment preparations may be complicated by SS acting on hypothalamic neuronal terminals present within the anterior pituitary (33). Here we demonstrate that all three SS isoforms can dose-dependently diminish basal GH release by acting at the level of the pituitary cell (Fig. 2A). SS_{14} and $[Pro^2]SS_{14}$ produced similar dose-response curves that were markedly different from that of $gbSS_{28}$. IC_{50} estimates establish that $gbSS_{28}$ is more potent than either SS_{14} or $[Pro^2]SS_{14}$ in reducing basal GH release (Table 1). Maximal inhibition, as calculated by regression analysis, was 71.90 ± 5.06% for SS_{14}, 72.73 ± 0.46% for $[Pro^2]SS_{14}$, and 53.24 ± 6.18% for $gbSS_{28}$.

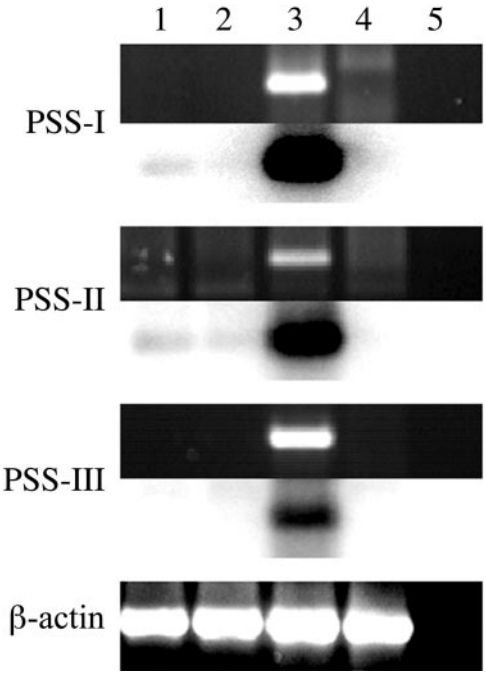

FIG. 1. Southern blot analysis (*below*) of RT-PCR (*above*) for PSS-I, -II, and -III mRNA. The different lanes represent the following: 1 and 2, dispersed pituitary cells; 3, hypothalamus; 4, pituitary fragments; 5, negative control. PCR for goldfish β-actin was used as an internal control for the reverse transcription step.

SS_{14}, $[Pro^2]SS_{14}$ and $gbSS_{28}$ actions on cAMP and cAMP/PKA-dependent GH secretion

In goldfish, as in other vertebrate systems, cAMP/PKA-dependent signaling cascades have been implicated in the regulation of basal GH secretion (35–37). Here we examined the ability of SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ to modulate both released and intracellular basal cAMP levels in naïve pituitary cells. All three isoforms significantly reduced intracellular cAMP levels. However, although SS_{14} and $[Pro^2]SS_{14}$ were equal in their ability to maximally reduce intracellular cAMP levels, $gbSS_{28}$ was significantly less effective (Fig. 2B). In contrast, none of the three SS isoforms tested significantly altered the levels of released cAMP (Fig. 2C). Significant reductions in basal GH release (Fig. 2A) did not directly correlate with significant decreases in either intra- or extracellular cAMP levels (Fig. 2, B and C).

We subsequently examined the ability of maximally effective concentrations of $[Pro^2]SS_{14}$ (100 nM; Fig. 2A) and

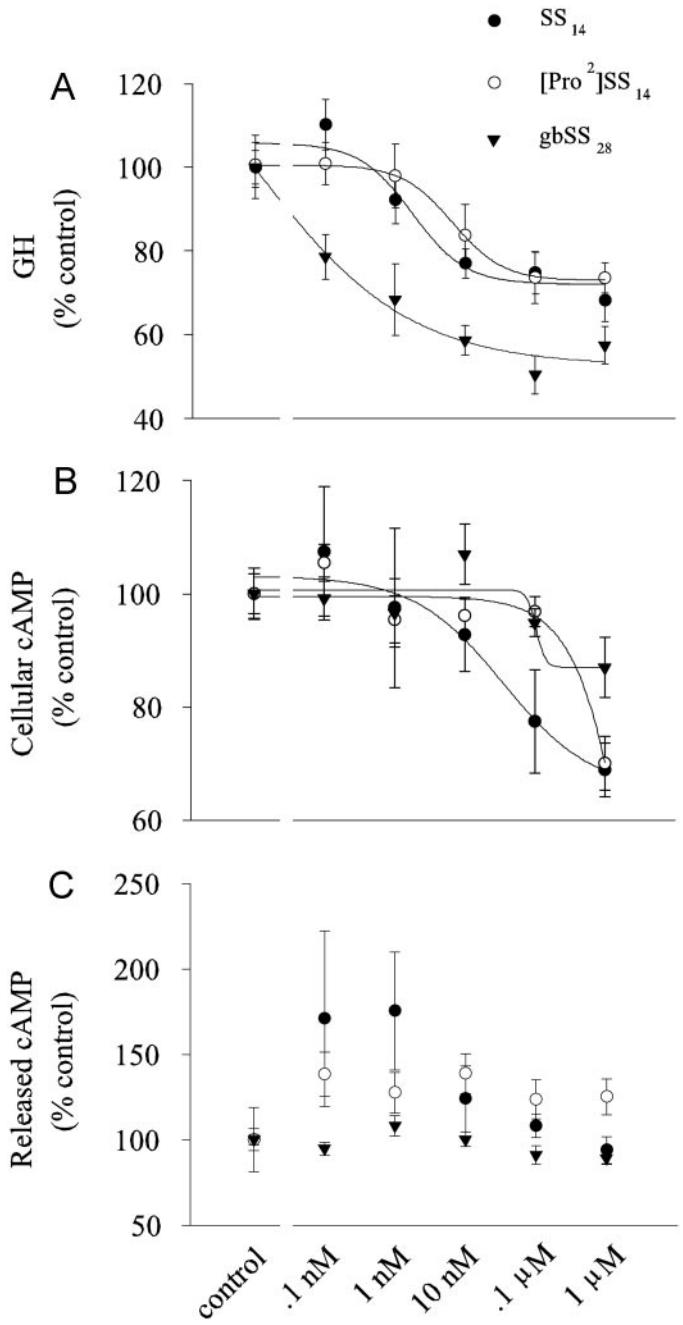

FIG. 2. Dose-dependent actions of SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ on basal GH secretion (A), intracellular cAMP (B), and released cAMP (C).

$gbSS_{28}$ (10 nM; Fig. 2A) to affect cAMP/PKA-dependent GH secretion, and compared these findings with those previously observed with a maximally effective concentration of SS_{14} (1 μM; Table 1) (27, 38). Activation of PACAP and DA-D1 receptors have been shown to stimulate GH secretion from goldfish pituitary cells through cAMP/PKA-dependent mechanisms (reviewed in Ref. 25). Accordingly, treatments with maximal stimulatory concentrations of the DA D1-agonist SKF-38393 (1 μM) (28) and PACAP (10 nM) (39) significantly elevated GH release in this study (Fig. 3A). Similarly, direct activation of the cAMP/PKA cascade with the adenylate cyclase activator forskolin (1 μM) and the cell permeant cAMP analog 8Br-cAMP (1 mM) resulted in significant increases in GH secretion (Fig. 3A). As had been demonstrated for SS_{14} (27), $[Pro^2]SS_{14}$ abolished the ability of PACAP, SKF-38393, forskolin, and 8Br-cAMP to induce GH release (*i.e.* GH responses to treatment with SS plus secretagogue were not significantly different from responses to SS alone; Fig. 3A). In contrast, $gbSS_{28}$ only partially reduced the GH responses to SKF-38393 and PACAP and did not alter GH responses to forskolin or 8Br-cAMP (Fig. 3B). These results establish that $gbSS_{28}$, unlike SS_{14} and $[Pro^2]SS_{14}$, does not act distal to cAMP formation to suppress GH responses. To further test this hypothesis, we examined the ability of all three SSs to inhibit AA-stimulated GH release. Previous work from this laboratory has shown that AA mediates DA-stimulated GH secretion subsequent to cAMP formation (40). Although both SS_{14} and $[Pro^2]SS_{14}$ inhibited GH responses to 50 μM AA (Fig. 4, A and B), $gbSS_{28}$ had no effect (Fig. 4C). These data support the hypothesis that these three goldfish SSs differ in their ability to act subsequent to cAMP formation to inhibit GH secretion.

TABLE 1. Summary of the effects of four different SS isoforms on basal and stimulated GH secretion from primary cultures of dispersed goldfish pituitary cells

Treatment	Effects on GH release in static incubation			
	SS_{14}	$[Pro^2]SS_{14}$	$gbSS_{28}$	mSS_{28}[a]
Basal GH secretion				
SS alone	↓	↓	↓	↓
IC_{50}	1.73 nM	6.69 nM	0.16 nM	
Maximally effective concentration	1 μM	100 nM	10 nM	
SS plus 5 mM CsCl	↓	↓	↔	
Stimulated GH secretion				
GnRH Cascade				
sGnRH	X[b]	X	X	X
cGnRH-II	↓[b]	X	X	X
DiC8	↓[b]	↓	↓	↓
TPA	↓[b]	↓	↔	↓
DA/PACAP cascade				
PACAP	X[c]	X	↓	X
SKF-38393	X[b]	X	↓	X
Forskolin	X[b]	X	↔	↓
8Br-cAMP	X[b]	X	↔	↓
AA	↓	↓	↔	↓
NO cascade				
SNP	X	X	↔	X
Ca^{2+} ionophores				
A23178	↓[b]	↓	↔	
Ionomycin	↓[b]	↓	↓	
Depolarization				
30 mM KCl	X	X	X	

X, Abolished; ↓, reduced; ↔, not affected.
[a] mSS_{28} contains SS_{14} within its C terminus.
[b] Taken from Ref. 27.
[c] Taken from Ref. 38.

SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ on PKC-dependent GH secretion

In goldfish, PKC-dependent transduction mechanisms act independently of the cAMP/PKA pathway to stimulate GH secretion. Two endogenous GnRHs, sGnRH and cGnRH-II, have been shown to evoke GH release through activation of PKC, and SS_{14} is known to inhibit GH responses to PKC

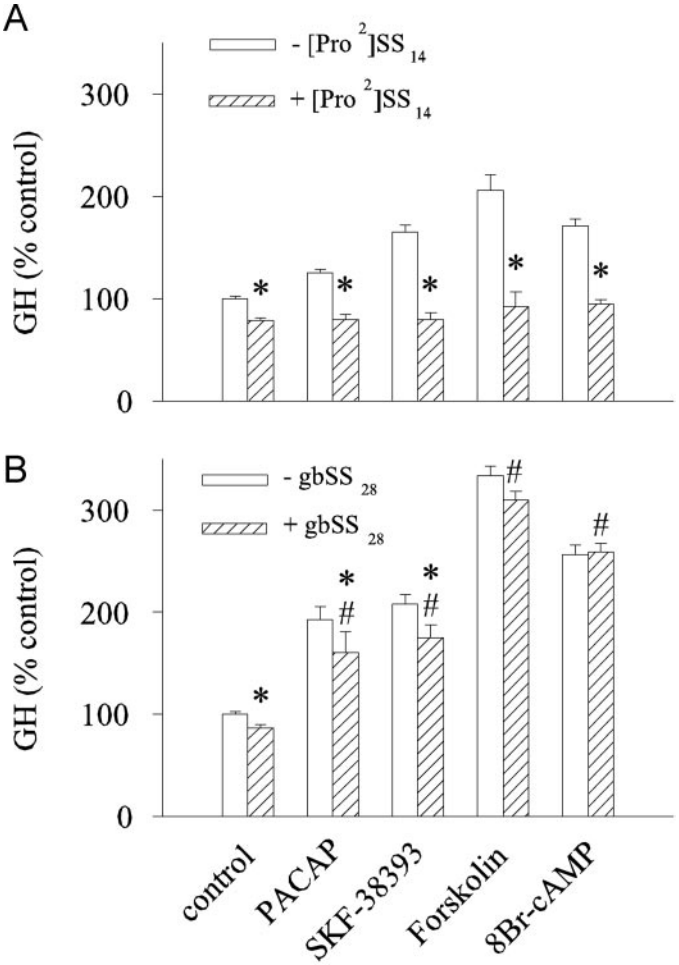

FIG. 3. Effects of $[Pro^2]SS_{14}$ (A) and $gbSS_{28}$ (B) on PACAP-, DA-D1-, and 8Br-cAMP-stimulated GH release. An *asterisk* (*) represents a significant reduction in GH release, compared with the non-SS-exposed column of the pair. A *number sign* (#) represents a significant difference, compared with the SS-treated control.

activation (reviewed in Ref. 25). To compare the ability of different SSs to affect PKC-dependent GH release, the effects of $[Pro^2]SS_{14}$ and $gbSS_{28}$ on GH responses to maximal stimulatory concentrations of sGnRH (100 mM) (26), cGnRH-II (100 mM) (26), and two PKC activators, TPA (100 nM) (41) and DiC8 (100 μM) (41) were examined. In the presence of 100 nM $[Pro^2]SS_{14}$ or 10 nM $gbSS_{28}$, the GH-releasing ability of both cGnRH-II and sGnRH were completely abolished, and the responsiveness to DiC8 was significantly reduced (Fig. 5A). These observations are similar to those previously observed with SS_{14} (27) (Table 1). GH responses to TPA were also greatly reduced by $[Pro^2]SS_{14}$ (Fig. 5A), as was reported for SS_{14} (Table 1). In contrast, $gbSS_{28}$ was completely ineffective against TPA-stimulated GH release (Fig. 5B). These observations reveal a surprising difference in the ability of these three goldfish SSs to affect TPA-sensitive but not DiC8-induced GH secretion.

SSs and their effects on nitric oxide-mediated GH release

Nitric oxide (NO) signaling has been implicated in both the GnRH and DA signaling pathways regulating GH secretion

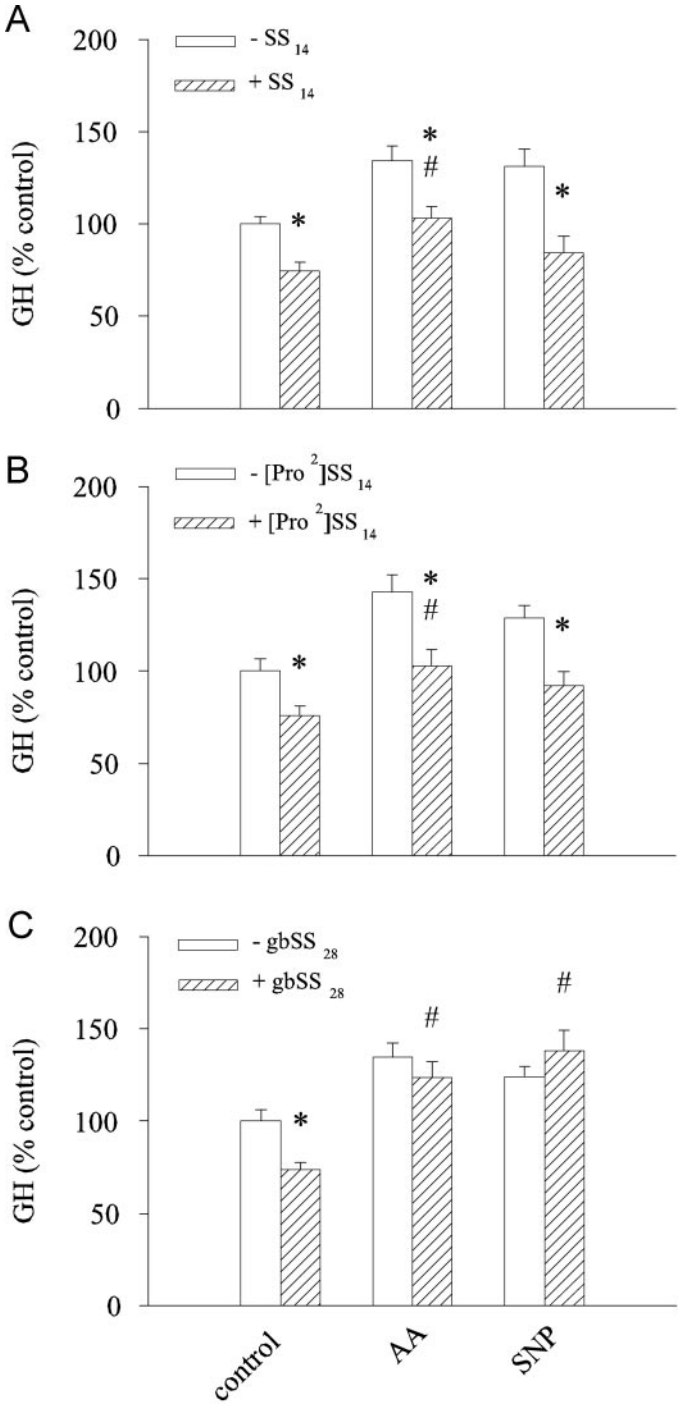

FIG. 4. Effects of SS_{14} (A), $[Pro^2]SS_{14}$ (B), and $gbSS_{28}$ (C) on AA- and SNP-stimulated GH release. An *asterisk* (*) represents a significant reduction in GH release, compared with the non-SS-exposed column of the pair. A *number sign* (#) represents a significant difference, compared with the SS-treated control.

in goldfish (42, 43). Consequently, we examined the effectiveness of the goldfish SSs against NO-stimulated GH release, using maximally effective concentrations of SNP (100 μM) (42), an NO donor. In this study, SNP significantly increased GH release, and this stimulation was abolished by both SS_{14} and $[Pro^2]SS_{14}$ (Fig. 4, A and B). However, $gbSS_{28}$ had no effect on SNP-stimulated GH secretion (Fig. 4C).

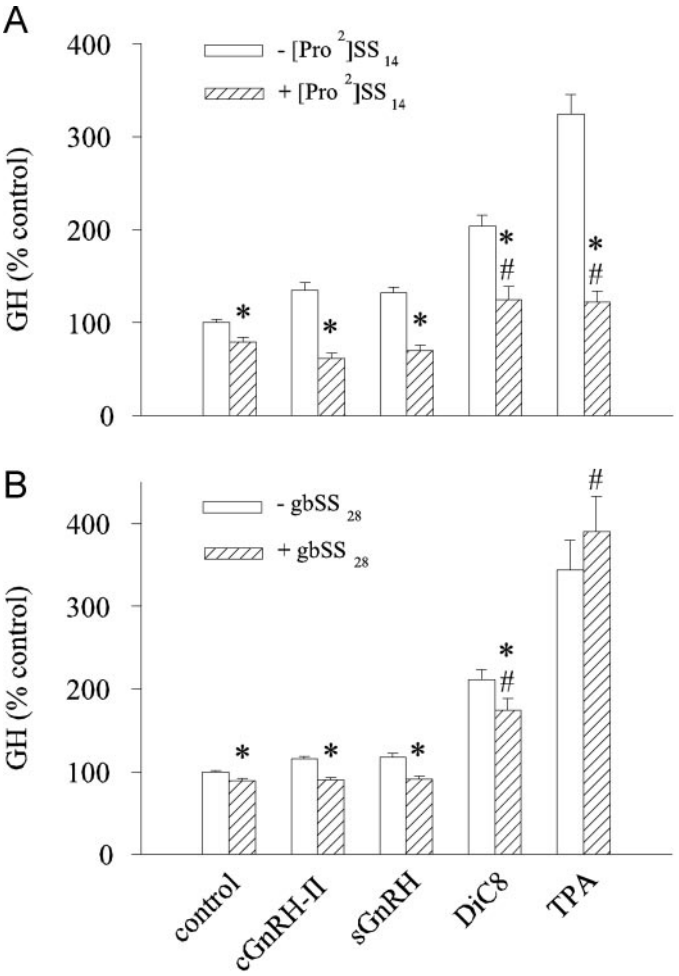

FIG. 5. Effects of $[Pro^2]SS_{14}$ (A) and $gbSS_{28}$ (B) on GnRH- and PKC-stimulated GH release. An *asterisk* (*) represents a significant reduction in GH release, compared with the non-SS-exposed column of the pair. A *number sign* (#) represents a significant difference, compared with the SS-treated control.

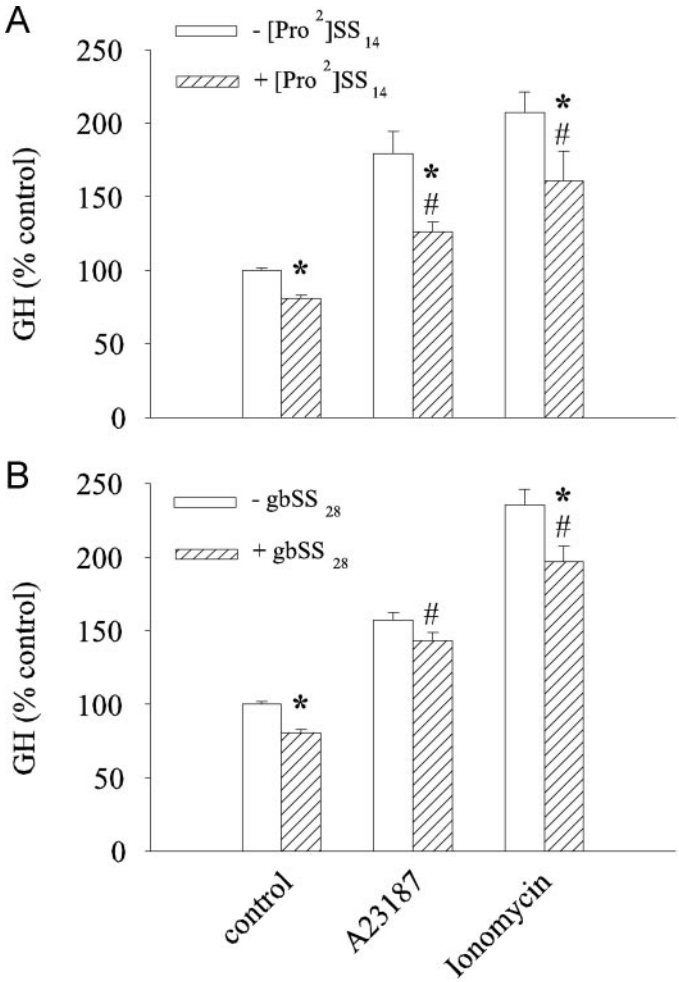

FIG. 6. Effects of $[Pro^2]SS_{14}$ (A) and $gbSS_{28}$ (B) on Ca^{2+} ionophore-stimulated GH release. An *asterisk* (*) represents a significant reduction in GH release, compared with the non-SS-exposed column of the pair. A *number sign* (#) represents a significant difference, compared with the SS-treated control.

$[Pro^2]SS_{14}$ and $gbSS_{28}$ actions on Ca^{2+}-stimulated GH secretion

Ca^{2+} mobilization, from both intracellular and extracellular sources, is a component of both GnRH- and DA-stimulated GH release (37, 44). Previously, we have shown that SS_{14} is capable of inhibiting Ca^{2+}-ionophore-stimulated GH release (45) (Table 1). Here we compared the abilities of three goldfish SSs to affect GH responses to elevations of $[Ca^{2+}]_i$. This was achieved through the use of the Ca^{2+} ionophores A23187 (10 μM) and ionomycin (10 μM), both of which increased $[Ca^{2+}]_i$ in goldfish pituitary cells in previous experiments (46) and significantly increased GH secretion in this study (Fig. 6). Coincubation with $[Pro^2]SS_{14}$ significantly inhibited both A23187- and ionomycin-stimulated GH release (Fig. 6A). In contrast, $gbSS_{28}$ significantly inhibited ionomycin-evoked GH release but did not affect A23817-stimulated GH release (Fig. 6B). Treatment with a depolarizing concentration of KCl (30 mM) has been shown to increase $[Ca^{2+}]_i$ (47). Here this treatment significantly increased GH secretion (Fig. 7). All three goldfish SSs completely abolished 30 mM KCl-stimulated GH release (Fig. 7). These data show that apart from one exception (*i.e.* $gbSS_{28}$ on A23187) all three SSs are able to affect GH responses to elevated $[Ca^{2+}]_i$.

K^+ channel involvement in mediating SS actions on basal GH release

Results from ovine somatotropes have demonstrated the importance of inwardly rectifying K^+ channels (K_{ir}) in regulating GH secretion (48). Furthermore, in rat somatotropes, SS_{14} has been shown to activate K_{ir} as a means of reducing basal GH secretion (49). Because K_{ir} channels display a particularly high affinity for monovalent and divalent cations (50), we examined K_{ir} involvement in SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ inhibition of basal GH secretion using 5 mM extracellular CsCl. Application of CsCl had no effect on basal GH release and did not affect the ability of either SS_{14} or $[Pro^2]SS_{14}$ to significantly inhibit GH release (Fig. 8). However, $gbSS_{28}$ was unable to inhibit GH secretion in the presence of CsCl (Fig. 8). These data suggest that SS_{28}, but not SS_{14} and $[Pro^2]SS_{14}$, rely on K_{ir} channels to inhibit GH release in this system.

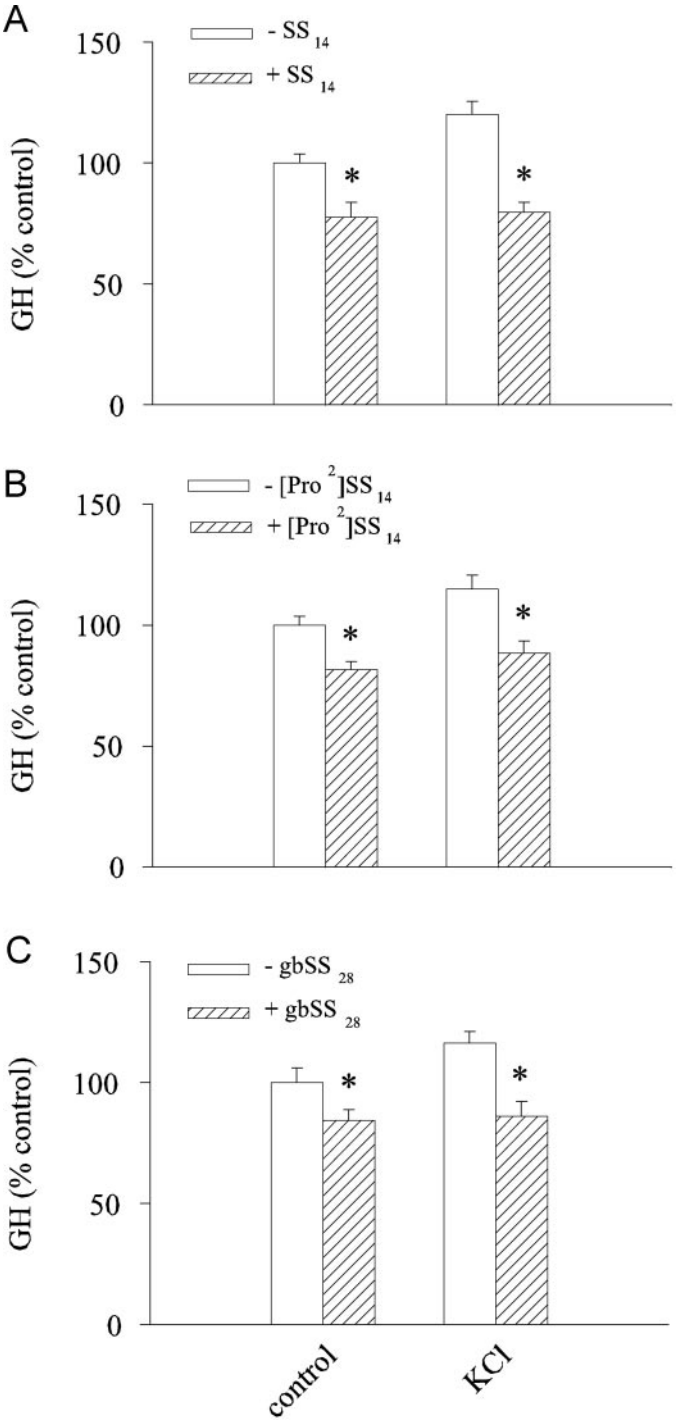

FIG. 7. Effects of SS_{14} (A), $[Pro^2]SS_{14}$ (B), and $gbSS_{28}$ (C) on 30 mM KCl -stimulated GH release. An *asterisk* (*) represents a significant reduction in GH release, compared with the non-SS-exposed column of the pair.

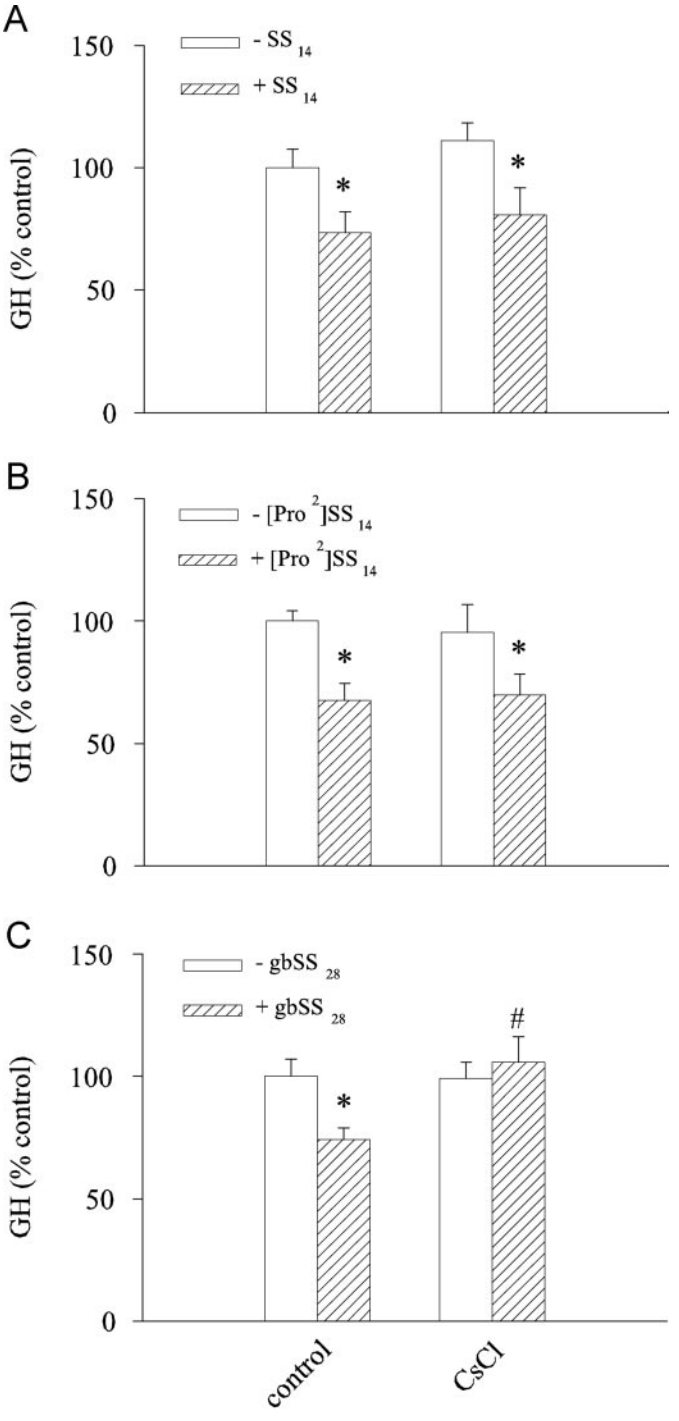

FIG. 8. Effects of 5 mM extracellular CsCl on SS_{14} (A), $[Pro^2]SS_{14}$ (B), and $gbSS_{28}$ (C) inhibition of basal GH secretion. An *asterisk* (*) represents a significant reduction in GH release, compared with the non-SS-exposed column of the pair. A *number sign* (#) represents a significant difference, compared with the SS-treated control.

Mammalian SS_{28} differs from $gbSS_{28}$ in its ability to inhibit GH release

The ability of mSS_{28} to inhibit GH secretion in goldfish has been previously shown (34). However, whether the mechanisms employed by mSS_{28} to modulate GH release differ from those activated by $gbSS_{28}$ has not been considered. Using mSS_{28} at the same concentration as $gbSS_{28}$ (10 nM) and the secretagogues at the same concentrations as above, we examined the ability of mSS_{28} to act on both the PACAP/DA and GnRH signaling cascades. The mSS_{28} abolished the GH responses to PACAP, SKF-38393, sGnRH, cGnRH-II, and SNP and reduced those induced by forskolin, 8Br-cAMP,

DiC8, TPA, and AA (Fig. 9). This profile of inhibition differed noticeably from that of $gbSS_{28}$ (Table 1), particularly in terms of effects on forskolin-, 8Br-cAMP-, SNP-, AA-, and TPA-induced secretion. These results demonstrate that despite identical concentrations, $gbSS_{28}$ and mSS_{28} differ in the mechanisms underlying their inhibition of somatotrope GH release.

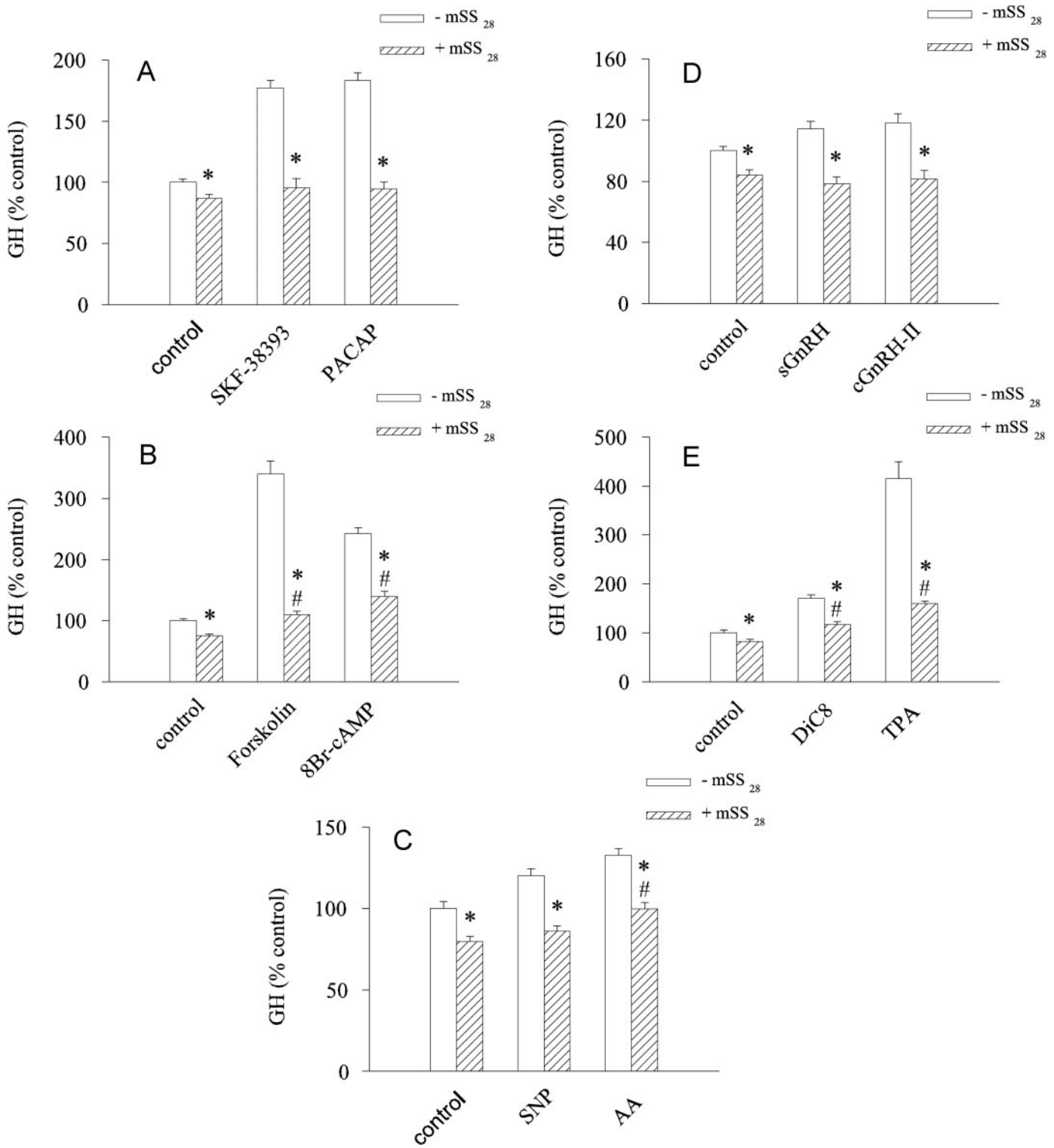

FIG. 9. Effects of mSS_{28} on GH release evoked by SKF-38393 and $mPACAP_{38}$ (A), forskolin and 8Br-cAMP (B), SNP and AA (C), sGnRH and cGnRH-II (D), and DiC8 and TPA (E). An *asterisk* (*) represents a significant reduction in GH release, compared with the non-SS-exposed column of the pair. A *number sign* (#) represents a significant difference, compared with the SS-treated control.

Discussion

In this study, we provide evidence that, in goldfish, pituitary cells may be exposed to multiple SS isoforms. Furthermore, we demonstrate that three different hypothalamic SSs, SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$, differentially regulate GH secretion. Lastly, we establish that the goldfish somatotrope is capable of differentiating between endogenous $gbSS_{28}$ and mSS_{28}.

Exposure of pituitary cells to multiple SS isoforms

As had been previously shown in goldfish of unspecified sexual states (4), we demonstrate in this study the existence of three different PSS mRNAs in hypothalami obtained from sexually regressed goldfish. Therefore, it is conceivable that the mature peptides, SS_{14}, $gbSS_{28}$, and $[Pro^2]SS_{14}$, are participating in the neuroendocrine regulation of goldfish pituitary function. In addition, we also identified mRNA for PSS-I and PSS-II within preparations of dispersed pituitary cells. This suggests that SS_{14} and $gbSS_{28}$ may also be produced locally at the level of the pituitary. Synthesis of hypothalamic neuropeptides within the pituitary has been well documented (reviewed in Ref. 51). Immunoreactivity and/or mRNA for vasoactive intestinal polypeptide (52, 53), GnRH (54, 55), TRH (54, 56), and SS (57, 58) have all been found within mammalian anterior pituitary tissues. In addition, $[Pro^2, Met^{13}]SS_{14}$ synthesis has been localized to melanotropes within the intermediate lobe of the frog pituitary (59), and $[Pro^2]SS_{14}$ has been purified from the pituitary of the Russian sturgeon (9). Although we have not localized the PSS mRNA to a specific pituitary lobe or cell type, the occurrence of PSS mRNA within dispersed cells suggests that local, pituitary level peptide production may be occurring. In combination with the observed expression of PSS-I, -II, and -III mRNA in the hypothalamus, it is plausible that the different SS peptides participate in the neuroendocrine, as well as paracrine and/or autocrine, regulation of goldfish pituitary physiology.

However, if hypophyseal SS synthesis were occurring, PSS mRNA should have also been detected within pituitary fragments. Although it is conceivable that the presence of other tissues within the pituitary fragments diluted the level of PSS mRNA transcripts, at least two other explanations present themselves as more likely alternatives. PSS-I, -II, and -III mRNA levels in the forebrain have been shown to vary seasonally and to differ between males and females (4). Furthermore, studies have demonstrated that, in the forebrains of both male and female fish, PSS-I and PSS-III expression is increased by estradiol (60). In the present study, pituitary fragment cDNA was made from pooled tissues collected from sexually regressed males and females (July) although dispersed pituitary cell cDNA was made from pooled tissues collected from sexually recrudescing males and females (November). As such, it is plausible that the absence of PSS mRNA transcripts within the pituitary fragments was the result of low steroid levels. Interestingly, in a previous study, PSS-I mRNA, but not PSS-II or -III mRNA, was detected within goldfish pituitary fragments by Northern blot analysis (4). Unfortunately, the gonadal stage of the fish used was not reported. Nevertheless, the hypothesis that SS peptides are being synthesized within pituitary cells and undergoing seasonal, sex steroid-dependent regulation, is consistent with this previous report.

It is also conceivable that the reason PSS mRNA was detected in cultured pituitary cells, and not freshly excised pituitaries fragments, was because the PSS-I and -II genes were transcribed only after the pituitary cells were deprived of hypothalamic influences. It should be noted that PSS-I and -II mRNA within cultured pituitary cells could not be visualized by ethidium bromide staining, even after 30 cycles of PCR. This indicates that the PSS mRNA levels within the pituitary cells were quite low. Such a finding is consistent with the possibility that PSS gene transcription commenced during overnight culture. The possibility that the removal of hypothalamic influences up-regulates the transcription of genes encoding for hypothalamic neuropeptides within pituitary cells in culture is currently being investigated in our laboratory. However, PSS-I mRNA has been previously detected within freshly excised pituitary fragments (4). Thus, it seems unlikely that PSS transcription following removal of hypothalamic innervation is solely responsible for the disparity between the results obtained from dispersed cells and pituitary fragments in the current study. Regardless of whether steroid and/or removal of hypothalamic influences modulate pituitary PSS mRNA expression, the data presented here are strongly suggestive of *in vivo* pituitary level peptide production. Future studies on SS release by, and/or immunocytochemical localization of SS in, dispersed pituitary cells would be an interesting test of this hypothesis.

SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ differentially affect GH secretion

In this study we demonstrate that three different hypothalamic SSs differ in their ability to alter GH release (Table 1). Of the SSs, $gbSS_{28}$ was a more potent inhibitor of basal GH secretion than either SS_{14} or $[Pro^2]SS_{14}$. In addition, results with CsCl suggest that K_{ir} channels participate in mediating $gbSS_{28}$, but not SS_{14} and $[Pro^2]SS_{14}$, inhibition of basal GH secretion. The $gbSS_{28}$ also differs from SS_{14} and $[Pro^2]SS_{14}$ in its ability to alter stimulated GH secretion. For example, the differential ability of these three goldfish SSs to inhibit forskolin-, 8Br-cAMP-, and AA-induced GH secretion suggests that the 14-amino-acid SSs are able to act subsequent to cAMP formation to inhibit GH release, but $gbSS_{28}$ does not. Furthermore, $gbSS_{28}$ differed from SS_{14} and $[Pro^2]SS_{14}$ in that it was not as effective at inhibiting GH release resulting from the activation of PKC or liberation of NO. The ability of $gbSS_{28}$ to affect Ca^{2+}-ionophore-induced, as well as DA-D1- and PACAP-stimulated, GH release also differed from that of SS_{14} and $[Pro^2]SS_{14}$. Overall, SS_{14} and $[Pro^2]SS_{14}$ are very similar in terms of their spectrum of activity; however, their activity differs markedly from that of $gbSS_{28}$. Although we cannot yet conclude from these findings that the SSs are differentially regulating GH secretion or any other cellular functions in a physiologically relevant manner, differences in intracellular signaling, such as these, would be requisite.

It is also apparent that the goldfish GH secretion system not only differentiates among the three endogenous hypothalamic SSs, but also mSS_{28}. Unlike $gbSS_{28}$, mSS_{28} was able to inhibit forskolin-, 8Br-cAMP-, AA-, SNP-, and

TPA-induced GH secretion, as well as abolish GnRH-evoked responses, when used at the same dose. These characteristics of mSS_{28} action resemble those of SS_{14} and $[Pro^2]SS_{14}$. This is not surprising given that the C terminus of mSS_{28} is identical to that of SS_{14}.

Consistent with a previous study in frogs demonstrating the ability of SS_{14} and $[Pro^2, Met^{13}]SS_{14}$ to regulate basal cAMP formation (61), all three goldfish SS peptides suppressed basal cAMP production. However, our results also demonstrate that whereas $gbSS_{28}$ was the most potent inhibitor of GH release, it was the least effective at lowering cellular cAMP levels. This provides further evidence to support the idea that SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ differentially couple to intracellular effector systems. Although we cannot exclude the possibility that the sensitivity of the cAMP assay did not allow us to properly examine the relationships between declining cAMP levels and a reduction in basal GH release, our data suggest that these two events are not tightly coupled. This is consistent with results in rat showing that SS_{14} lowers basal GH release without altering intracellular cAMP levels and blocks GHRH-stimulated GH release while only partially attenuating cAMP production (62).

The cellular mechanisms responsible for the differences in SS function presented here are not known. However, it seems likely that the different sst subtypes are involved. In mammals, each sst subtype couples to a distinct set of intracellular signaling pathways, and although all five receptor subtypes bind SS_{14} and mSS_{28} with high affinity, sst_5 does exhibit selectivity for mSS_{28} (reviewed in Ref. 19). Similarly, characterization of goldfish sst_{5A} revealed that although it binds all three endogenous goldfish brain SS ligands, it displays selectivity for the 28-amino-acid SSs (22). In addition, $gfsst_2$ can be differentially activated by these three goldfish SSs. In COS-7 cells expressing $gfsst_2$, SS_{14} and $[Pro^2]SS_{14}$, but not $gbSS_{28}$, are able to inhibit forskolin-stimulated cAMP formation (24). Interestingly, $gfsst_2$ and $gfsst_5$ mRNA are predominantly expressed in the pituitary, compared with other brain regions (22, 24), and mammalian studies have shown that sst_2 and sst_5 are the primary regulators of somatotrope function (20). Thus, it is conceivable that $gbSS_{28}$ is acting mainly through $gfsst_5$, whereas SS_{14} and $[Pro^2]SS_{14}$ are acting more through $gfsst_2$. We have begun to test this hypothesis and are currently evaluating gfsst subtype-specific actions in primary cultures of goldfish pituitary cells using nonpeptidyl sst-selective agonists.

However, this hypothesis cannot explain the differences in mSS_{28} and $gbSS_{28}$ activity observed in the present study. Because $gfsst_5$ binds with, and can be activated by, both mSS_{28} and $gbSS_{28}$ with similar affinity (63), differences in the ability of these two 28-amino-acid SSs to affect GH secretion may be mediated through sst_2. This remains a speculation at present because nothing is known regarding the ability of mSS_{28} to bind to and activate $gfsst_2$. Nevertheless, the presence of the SS_{14} sequence in the C terminus of mSS_{28} is consistent with such a hypothesis.

Summary

Regardless of the mechanisms responsible for the differences in SS action presented, these differences have some very interesting physiological implications. In goldfish, GH secretion is regulated by a variety of neuropeptides and hypothalamic factors, some of which, stimulate GH release through different intracellular mechanisms. For example, sGnRH and cGnRH-II signaling is mediated by intracellular Ca^{2+} stores, extracellular Ca^{2+} entry, and PKC (64). In contrast, DA and PACAP stimulate GH secretion through AC/cAMP/PKA-sensitive mechanisms (reviewed in Ref. 25). Furthermore, our laboratory has also shown that, in pituitary cells, the Ca^{2+} stores regulating hormone mRNA levels as well as hormone secretion, storage, and production are different (65, 66). The result is a system wherein ligands employ distinct signaling cascades to affect not only GH release but also the steps involved in hormone synthesis. Given the differences in SS_{14}, $[Pro^2]SS_{14}$, and $gbSS_{28}$ action presented here (Table 1), it seems likely that, *in vivo*, they are responsible for regulating different aspects of cell function.

Acknowledgments

Received April 8, 2003. Accepted May 23, 2003.

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This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) operating grants (to R.E.P. and J.P.C.) and NIH Grant DK50124 (to J.E.R.). W.K.Y. was supported by an NSERC Post-Graduate Studentship and an Alberta Heritage Foundation for Medical Research (AHFMR) MD/PhD studentship. P.J.D. was supported by an AHFMR summer studentship.

References

1. **Tannenbaum GS, Epelbaum J** 1999 Somatostatin. In: Kostyo JL, Goodman HM, eds. Handbook of physiology. Section 7: The endocrine system, V. Hormonal control of growth. New York: Oxford University Press; 221–265
2. **Trabucchi M, Tostivint H, Lihrmann I, Sollars C, Vallarino M, Dores RM, Vaudry H** 2002 Polygenic expression of somatostatin in the sturgeon Acipenser transmontanus: molecular cloning and distribution of the mRNAs encoding two somatostatin precursors. J Comp Neurol 443:332–345
3. **Lin X-W, Otto CJ, Peter RE** 1998 Evolution of neuroendocrine peptide systems: gonadotropin-releasing hormone and somatostatin. Comp Biochem Physiol C 119:375–388
4. **Lin X-W, Otto CJ, Peter RE** 1999 Expression of three distinct somatostatin messenger ribonucleic acids (mRNA) in goldfish brain: characterization of the complementary deoxyribonucleic acids, distribution and seasonal variation of the mRNAs, and action of a somatostatin-14 variant. Endocrinology 140:2089–2099
5. **Morel A, Chang JY, Cohen P** 1984 The complete amino-acid sequence of anglerfish somatostatin-28 II. A new octacosapeptide containing the (Tyr^7, Gly^{10}) derivative of somatostatin-14 I. FEBS Lett 175:21–24
6. **Noe BD, Andrews PC, Dixon JE, Spiess J** 1986 Cotranslational and post-translational proteolytic processing of preprosomatostatin-I in intact islet tissue. J Cell Biol 103:1205–1211
7. **Andrews PC, Nichols R, Dixon JE** 1987 Post-translational processing of preprosomatostatin-II examined using fast atom bombardment mass spectrometry. J Biol Chem 262:12692–12699
8. **Andrews PC, Dixon JE** 1987 Isolation of products and intermediates of pancreatic prosomatostatin processing: use of fast atom bombardment mass spectrometry as an aid in analysis of prohormone processing. Biochemistry 26:4853–4861
9. **Nishii M, Movérus B, Bukovskaya OS, Takahashi A, Kawauchi H** 1995 Isolation and characterization of $[Pro^2]$somatostatin-14 and melanotropins from Russian sturgeon, *Acipenser gueldenstaedti* Brandt. Gen Comp Endocrinol 99:6–12
10. **Lin X, Otto CJ, Peter RE** 2000 Somatostatin family of peptides and its receptors in fish. Can J Physiol Pharmacol 78:1053–1066
11. **Patel YC** 1999 Somatostatin and its receptor family. Front Neuroendocrinol 20:157–198
12. **Müller EE, Locatelli V, Cocchi D** 1999 Neuroendocrine control of growth hormone secretion. Physiol Rev 79:511–607
13. **Patel YC, Wheatley T, Ning C** 1981 Multiple forms of immunoreactive somatostatin: comparison of distribution in neural and nonneural tissues and portal plasma of the rat. Endocrinology 109:1943–1949
14. **De Lecea L, del Rio JA, Criado JR, Alcantara S, Morales M, Danielson PE, Henriksen SJ, Soriano E, Sutcliffe JG** 1997 Cortistatin is expressed in a distinct subset of cortical interneurons. J Neurosci 17:5868–5880

15. **De Lecea L, Ruiz-Lozano P, Danielson PE, Peelle-Kirley J, Foye PE, Frankel WN, Sutcliffe JG** 1997 Cloning, mRNA expression, and chromosomal mapping of mouse and human preprocortistatin. Genomics 42:499–506
16. **Tostivint H, Lihrmann I, Bucharles C, Vieau D, Coulouarn Y, Fournier A, Conlon JM, Vaudry H** 1996 Occurrence of two somatostatin variants in the frog brain: Characterization of the cDNAs, distribution of the mRNAs, and receptor-binding affinities of the peptides. Proc Natl Acad Sci USA 93:12605–12610
17. **Nozaki M, Miyata K, Oota Y, Gorbman A, Plisetskaya EM** 1988 Different cellular distributions of two somatostatins in brain and pancreas of salmonids, and their associations with insulin- and glucagon-secreting cells. Gen Comp Endocrinol 69:267–280
18. **Moore CA, Kittilson JD, Ehrman MM, Sheridan MA** 1999 Rainbow trout (*Oncorhynchus mykiss*) possess two somatostatin mRNAs that are differentially expressed. Am J Physiol 277:R1553–R1561
19. **Csaba Z, Dournaud P** 2001 Cellular biology of somatostatin receptors. Neuropeptides 35:1–23
20. **Parmar RM, Chan WW, Dashkevicz M, Hayes EC, Rohrer SP, Smith RG, Schaeffer JM, Blake AD** 1999 Nonpeptidyl somatostatin agonists demonstrate that sst_2 and sst_5 inhibit stimulated growth hormone secretion from rat anterior pituitary cells. Biochem Biophys Res Commun 263:276–280
21. **Lin X, Janovick JA, Brothers S, Conn PM, Peter RE** 1999 Molecular cloning and expression of two type one somatostatin receptors in goldfish brain. Endocrinology 140:5211–5219
22. **Lin X, Nunn C, Hoyer D, Rivier J, Peter RE** 2002 Identification and characterization of a type five-like somatostatin receptor in goldfish pituitary. Mol Cell Endocrinol 189:105–116
23. **Lin X, Peter RE** 2003 Somatostatin-like receptors in goldfish: cloning of four new receptors. Peptides 24:53–63
24. **Lin X, Janovick JA, Cardenas R, Conn PM, Peter RE** 2000 Molecular cloning and expression of a type-two somatostatin receptor in goldfish brain and pituitary. Mol Cell Endocrinol 166:75–87
25. **Chang JP, Johnson JD, Van Goor F, Wong CJH, Yunker WK, Uretsky AD, Taylor D, Jobin RM, Wong AOL, Goldberg JI** 2000 Signal transduction mechanisms mediating secretion in goldfish gonadotropes and somatotropes. Biochem Cell Biol 78:139–153
26. **Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, De Leeuw R, Peter RE** 1990 Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus* I. Initial morphological, static, and cell column perifusion studies. Gen Comp Endocrinol 77:256–273
27. **Kwong P, Chang JP** 1997 Somatostatin inhibition of growth hormone release in goldfish: possible targets of intracellular mechanisms of action. Gen Comp Endocrinol 108:446–456
28. **Wong AOL, Chang JP, Peter RE** 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology 130:1201–1210
29. **Yunker WK, Lee EKY, Wong AOL, Chang JP** 2000 Norepinephrine regulation of growth hormone release from goldfish pituitary cells. II. Intracellular sites of action. J Neuroendocrinol 12:323–333
30. **Van Goor F, Goldberg JI, Chang JP** 1997 Extracellular sodium dependence of GnRH-stimulated growth hormone release in goldfish pituitary cells. J Neuroendocrinol 9:207–216
31. **Peyon P, Lin XW, Himick BA, Peter RE** 1998 Molecular cloning and expression of cDNA encoding brain preprocholecystokinin in goldfish. Peptides 19:199–210
32. **Cook AF, Wilson SW, Peter RE** 1983 Development and validation of a carp growth hormone radioimmunoassay. Gen Comp Endocrinol 50:335–347
33. **Peter RE, Yu KL, Marchant TA, Rosenblum PM** 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool 4:84–89
34. **Marchant TA, Fraser RA, Andrews PC, Peter RE** 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus* L.) pituitary fragments *in vitro*. Regul Pept 17:41–52
35. **Frohman LA** 1996 Cellular physiology of growth hormone releasing hormone. In: Bercu BB, Walker RF, eds. Growth hormone secretagogues. New York: Springer-Verlag; 137–146
36. **Wong AOL, Van Goor F, Jobin RM, Neumann CM, Chang JP** 1994 Interactions of cyclic adenosine 3′, 5′-monophosphate, protein kinase-C, and calcium in dopamine- and gonadotropin-releasing hormone-stimulated growth hormone release in the goldfish. Endocrinology 135:1593–1604
37. **Wong CJH, Johnson JD, Yunker WK, Chang JP** 2001 Caffeine stores and dopamine differentially require Ca^{2+} channels in goldfish somatotropes. Am J Physiol 280:R494–R503
38. **Wirachowsky NR, Kwong P, Yunker WK, Johnson JD, Chang JP** 2001 Mechanisms of action of pituitary adenylate cyclase-activating peptide (PACAP) on growth hormone release from dispersed goldfish pituitary cells. Fish Physiol Biochem 23:201–214
39. **Wong AOL, Leung MY, Shea WLC, Tse LY, Chang JP, Chow BKC** 1998 Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: Immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. Endocrinology 139:3465–3479
40. **Chang JP, Abele JT, Van Goor F, Wong AOL, Neumann CM** 1996 Role of arachidonic acid and calmodulin in mediating dopamine D1-and GnRH-stimulated growth hormone release in goldfish pituitary cells. Gen Comp Endocrinol 102:88–101
41. **Chang JP, Jobin RM, De Leeuw R** 1991 Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen Comp Endocrinol 81:447–463
42. **Uretsky AD, Chang JP** 2000 Evidence that nitric oxide is involved in the regulation of growth hormone secretion in goldfish. Gen Comp Endocrinol 118:461–470
43. **Uretsky AD, Weiss BL, Yunker WK, Chang JP** 2003 NO produced by a novel NO synthase isoform is necessary for gonadotropin-releasing hormone-induced GH secretion via a cGMP-dependent mechanism. J Neuroendocrinol 15:667–676
44. **Johnson JD, Chang JP** 2000 Novel, thapsigargin-insensitive intracellular Ca^{2+} stores control growth hormone release from goldfish pituitary cells. Mol Cell Endocrinol 165:139–150
45. **Cullter L, Glaum SR, Collins BA, Miller RJ** 1992 Calcium signalling in single growth hormone-releasing-factor-responsive pituitary cells. Endocrinology 130:945–953
46. **Jobin RM, Chang JP** 1992 Actions of two native GnRHs and protein kinase C modulators on goldfish pituitary cells. Cell Calcium 13:531–540
47. **Yunker WK, Chang JP** 2001 Somatostatin actions on a protein kinase C-dependent growth hormone secretagogue cascade. Mol Cell Endocrinol 175:193–204
48. **Xu R, Zhao Y, Chen C** 2002 Growth hormone-releasing peptide-2 reduces inward rectifying K^+ currents via a PKA-cAMP-mediated signalling pathway in ovine somatotropes. J Physiol 545:421–433
49. **Sims SM, Lussier BT, Kracier J** 1991 Somatostatin activates an inwardly rectifying K^+ conductance in freshly dispersed rat somatotrophs. J Physiol 441:615–637
50. **Kubo Y, Baldwin TJ, Jan YN, Jan LY** 1993 Primary structure and functional expression of a mouse inward rectifier potassium channel. Nature 362:127–133
51. **Peillon F, Le Dafniet M, Pagesy P, Croissandeau G, Schussler N, Joubert D, Li JY** 1997 Paracrine regulation of anterior pituitary hormones by neuropeptides. Ann Endocrinol (Paris) 58:31–38
52. **Arnaout MA, Garthwaite TL, Martinson DR, Hagen TC** 1986 Vasoactive intestinal polypeptide is synthesized in anterior pituitary tissue. Endocrinology 119:2052–2057
53. **Byrne JM, Jones PM, Hill SF, Bennet WM, Ghatei MA, Bloom SR** 1992 Expression of messenger ribonucleic acids encoding neuropeptide-Y, substance-P, and vasoactive intestinal polypeptide in human pituitary. J Clin Endocrinol Metab 75:983–987
54. **May V, Wilber JF, U'Prichard DC, Childs GV** 1987 Persistence of immunoreactive TRH and GnRH in long-term primary anterior pituitary cultures. Peptides 8:543–558
55. **Pagesy P, Li JY, Berthet M, Peillon F** 1992 Evidence of gonadotropin-releasing hormone mRNA in the rat anterior pituitary. Mol Endocrinol 6:523–528
56. **Pagesy P, Croissandeau G, Le Dafniet M, Peillon F, Li JY** 1992 Detection of thyrotropin-releasing hormone (TRH) mRNA by the reverse transcription-polymerase chain reaction in the human normal and tumoral anterior pituitary. Biochem Biophys Res Commun 182:182–187
57. **Mesguich P, Benoit R, Dubois PM, Morel G** 1988 Somatostatin-28- and somatostatin-14-like immunoreactivities in the rat pituitary gland. Cell Tissue Res 252:419–427
58. **Pagesy P, Li JY, Rentier-Delrue F, Le Bouc Y, Martial JA, Peillon F** 1989 Evidence of pre-prosomatostatin mRNA in human normal and tumoral anterior pituitary gland. Mol Endocrinol 3:1289–1294
59. **Tostivint H, Vieau D, Chartrel N, Boutelet I, Galas L, Fournier A, Lihrmann I, Vaudry H** 2002 Expression and processing of the [Pro^2, Met^{13}]somatostatin-14 precursor in the intermediate lobe of the frog pituitary. Endocrinology 143:3472–3481
60. **Canosa LF, Lin X, Peter RE** 2002 Regulation of expression of somatostatin genes by sex steroid hormones in goldfish forebrain. Neuroendocrinology 76:8–17
61. **Jeandel L, Okuno A, Kobayashi T, Kikuyama S, Tostivint H, Lihrmann I, Chartrel N, Conlon JM, Fournier A, Tonon MC, Vaudry H** 1998 Effects of the two somatostatin variants somatostatin-14 and [Pro^2, Met^{13}]somatostatin-14 on receptor binding, adenylyl cyclase activity and growth hormone release from the frog pituitary. J Neuroendocrinol 10:187–192
62. **Bilezikjian LM, Vale W** 1983 Stimulation of adenosine 3′, 5′-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in anterior pituitary cells *in vitro*. Endocrinology 113:1726–1731
63. **Nunn C, Feuerbach D, Lin X, Peter R, Hoyer D** 2002 Pharmacological characterisation of the goldfish somatostatin sst_5 receptor. Eur J Pharmacol 436:173–186
64. **Johnson JD, Chang JP** 2002 Agonist-specific and sexual stage-dependent inhibition of gonadotropin-releasing hormone-stimulated gonadotropin and growth hormone release by ryanodine: relationship to sexual stage-dependent caffeine-sensitive hormone release. J Neuroendocrinol 14:144–155
65. **Johnson JD, Klausen C, Habibi H, Chang JP** 2003 A gonadotropin-releasing hormone insensitive, thapsigargin-sensitive Ca^{2+} store reduces Basal gonadotropin exocytosis and gene expression: comparison with agonist-sensitive Ca^{2+} stores. J Neuroendocrinol 15:204–214
66. **Johnson JD, Klausen C, Habibi HR, Chang JP** 2002 Function-specific calcium stores selectively regulate growth hormone secretion, storage, and mRNA level. Am J Physiol 282:E810–E819