Signaling mechanisms for α2-adrenergic inhibition of PACAP-induced growth hormone secretion and gene expression grass carp pituitary cells

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Wang X, Chu MM, Wong AO. Signaling mechanisms for α2-adrenergic inhibition of PACAP-induced growth hormone secretion and gene expression grass carp pituitary cells. Am J Physiol Endocrinol Metab 292: E1750–E1762, 2007. First published February 20, 2007; doi:10.1152/ajpendo.00001.2007.—Pituitary α2-agonists and the related peptide, phenylethanolamine N-methyltransferase (PACAP), are potent growth hormone (GH)-releasing factors in lower vertebrates. However, its functional interactions with other GH regulators have not been fully characterized. In fish models, norepinephrine (NE) inhibits GH release at the pituitary cell level, but its effects on GH synthesis have yet to be determined. We examined adrenergic inhibition of PACAP-induced secretion and GH gene expression using grass carp pituitary cells as a cell model. Through activation of pituitary α2-adrenoceptors, NE or the α2-agonist clonidine reduced both basal and PACAP-induced GH release and GH mRNA expression. In carp pituitary cells, clonidine also suppressed cAMP production and intracellular Ca2+ levels and blocked PACAP induction of these two second messenger signals. In GH3 cells transfected with a reporter carrying the grass carp GH promoter, PACAP stimulation increased GH promoter activity, and this stimulatory effect could be abolished by NE treatment. In parallel experiments, clonidine reduced GH primary transcript and GH promoter activity without affecting GH mRNA stability, and these inhibitory actions were mimicked by inhibiting adenylate cyclase (AC), blocking protein kinase A (PKA), removing extracellular Ca2+ in the culture medium, or inactivating L-type voltage-sensitive Ca2+ channels (VSCC). Since our recent studies have shown that PACAP can induce GH secretion in carp pituitary cells through cAMP/PKA- and Ca2+/calmodulin-dependent mechanisms, these results, taken together, suggest that α2-adrenergic stimulation in the carp pituitary may inhibit PACAP-induced GH release and gene transcription by blocking the AC/cAMP/PKA pathway and Ca2+/calmodulin entry through L-type VSCC.

In mammals, the central adrenergic system is known to play a role in regulating growth hormone (GH) release from the anterior pituitary (38). Adrenergic nerve fibers, presumably from the A1 to A7 groups in the brain stem (56), innervate the GH-releasing hormone (GHRH) and somatostatin (SRIF) neurons located in the medial basal (46) and preoptic hypothalamus (32), respectively. In representative species, e.g., the rat (6), sheep (55), cattle (27) and human (12), in vivo treatment with the α2-adrenergic agonist clonidine consistently elevates GH levels in systemic circulation. This stimulatory effect is mediated through α2-adrenoceptors expressed in the hypothalamus (12), activation of which can stimulate GHRH secretion from the arcuate nucleus (10) with simultaneous inhibition on SRIF release from the periventricular nucleus (30). In the rat, α2 stimulation of GH release is sexually dimorphic (9), age-dependent (25), and plays a role in regulating GH pulsatility in vivo (28). In the same animal model, norepinephrine (NE) and epinephrine (Ep) can be detected in the hypophyseal portal blood (24, 54), suggesting that adrenergic input may have direct effects at the pituitary level. This idea is supported by the findings that α2-adrenoceptors are expressed in somatotrophs (1) and β2-agonists (e.g., zinterol) can induce GH secretion in rat pituitary cells (51). The GH-releasing effect caused by β2 stimulation at the pituitary level is mediated through cAMP production (16) and elevation in intracellular Ca2+ levels (41). In ovine pituitary cells, β-adrenoceptors (e.g., isoproterenol) can also upregulate GH mRNA expression (49), but the postreceptor signaling mechanisms responsible for this stimulatory action have not been characterized. At the pituitary cell level, α2-adrenoceptors are not involved in GH secretion (17) and GH gene expression (49).

In bony fish (or teleosts), the anterior pituitary is under the direct innervation of the hypothalamus (18), and nerve fibers with tyrosine hydroxylase and dopamine β-hydroxylase immunoreactivities can be detected in the preopticoinfundibular pathway projecting into the proximal pars distalis (20, 26). Given that the expression level of phenylethanolamine N-methyltransferase is either very low or undetectable in the fish brain, NE rather than Ep is considered to be the major neurotransmitter for the central adrenergic system in teleosts (20). Besides the neural input, pituitary cells in bony fish are also exposed to NE and Ep of the humoral origin, which are secreted from chromaffin cells located in the head kidneys and cardinal veins during the acute phase of stress responses (44). In the goldfish, serum GH levels can be reduced by intraperitoneal injection of NE, but similar drug treatment via brain injection has no effects on GH secretion in vivo (5). Apparently, the site of NE action is not within the central nervous system but is located outside the blood-brain barrier. This finding is consistent with the results of previous in vitro studies by our group (29), in which NE and EP were both effective in suppressing GH secretion in goldfish pituitary cells. These inhibitory actions were mediated by α2-adrenoceptors (29), and α2 stimulation at the pituitary cell level also attenuated the GH-releasing effects caused by GH-releasing factors (e.g., dopamine and gonadotropin-releasing hormone) as well as by activation of the cAMP-, PKC-, and Ca2+/calmodulin-dependent cascades (63). Given that α2-adrenergic inhibition of GH release has been reported only in the goldfish, it is still unclear whether the findings represent a common phenomenon in fish models.
Furthermore, the direct effect of adrenergic stimulation on GH gene expression has not been tested at the pituitary level, and the functional role of α2-adrenoceptors in GH synthesis has yet to be determined.

Pituitary adenylyl cyclase-activating polypeptide (PACAP), a member of the glucagon/secretin peptide family, is a potent stimulator of GH secretion in teleosts (60). It is commonly accepted that PACAP and GHRH are both evolved from the glucagon lineage by gene duplication (48) and that PACAP serves as the "ancestral GHRH" in lower vertebrates before the evolution of mammalian GHRH (40). Direct innervation by PACAP nerve fibers in the proximal pars distalis overlapping with the distribution of somatotrophs has been demonstrated in the goldfish (58), grass carp (59), and stargazer (36). In fish pituitary cells, PACAP stimulates GH release in vitro through activation of PAC-1 receptors functionally coupled to the cAMP- and/or Ca2+-dependent mechanisms (58, 62). Recently, using 1-yr-old grass carp as the model for juvenile fish during the "growth-out" phase preceding sexual maturation, we have shown that PACAP was effective in stimulating GH production and GH gene expression by acting directly at the pituitary cell level. In this case, GH mRNA levels could be elevated by PACAP via functional coupling of the Ca2+/calmodulin (CaM) CaM kinase II cascade with the adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway (59). Although the physiological role of PACAP as a novel GH-releasing factor in lower vertebrates has received increasing attention, much information is available regarding its functional interactions with other GH regulators. In this study, using grass carp pituitary cells as a cell model, we examined the functional interactions between NE and PACAP at the pituitary level in regulating GH release and GH gene expression. As a first step, the receptor specificity for NE actions was characterized in grass carp pituitary cells using adrenergic analogs for α1-, α2-, and β-adrenoceptors, respectively. After that, GH release and GH mRNA expression induced by PACAP stimulation were tested with simultaneous treatment of NE or the α2-agonist clonidine. To elucidate the postreceptor signaling mechanisms for NE and PACAP interactions, we also conducted direct measurement of cAMP and intracellular Ca2+ levels ([Ca2+]i). Furthermore, the functional role of cAMP production, PKA activation, and Ca2+ entry via Ca2+ channels in α2-adrenergic regulation of GH mRNA expression and GH gene transcription were evaluated using pharmacological agents to perturb the cAMP- and Ca2+-dependent mechanisms.

**MATERIALS AND METHODS**

**Animals.** One-year-old grass carps (*Ctenopharyngodon idellus*) with body weight ranging from 1.5 to 2.0 kg were purchased from local markets and kept in well-aerated 200-liter aquaria under 12:12-h light-dark photoperiod at 20 ± 2°C. The grass carp at this stage was sexually immature (gonadosomatic index < 0.02%), and sexual dimorphism was not apparent. Therefore, fish of mixed sexes were routinely used for pituitary cell preparation. During the process, the carps were killed by spinosection after anesthesia in 0.05% tricaine methanesulfonate (Syndel, Vancouver, BC, Canada). The protocol for experiments was approved by the Committee of Animal Use for Teaching and Research at the University of Hong Kong.

**Test substances.** Norepinephrine (NE) and adrenergic analogs including phenylephrine, clonidine, isoproterenol, propranolol, prazosin, and yohimbine were obtained from Sigma (St. Louis, MO). Ovine pituitary adenylyl cyclase-activating polypeptide-38 (PACAP) was acquired from Phoenix Pharmaceuticals (Belmont, CA). Other pharmacological agents, including MDL-12330A, H-89, nifedipine, 3-isobutyl-1-methylxanthine (IBMX), and actinomycin D, were purchased from Calbiochem (San Diego, CA). MDL-12330A, H89, nifedipine, IBMX, and various adrenergic analogs were dissolved in dimethyl sulfoxide (DMSO) to form 10 mM stock solutions and were stored frozen in small aliquots at −80°C. Stock solution of PACAP was prepared in a similar manner, except that the peptide was dissolved in double-distilled deionized water. On the day of experiments, frozen stocks of test substances were diluted with prewarmed (28°C) culture medium to appropriate concentrations 15 min before drug treatment. Unlike other test substances, NE was stored under argon gas at −20°C and dissolved freshly in culture medium right before drug administration to avoid oxidation caused by prolonged storage. In these experiments, the final dilutions of DMSO were always <0.1% (vol/vol) and did not affect GH release and GH gene expression in carp pituitary cells.

**Primary cultures of grass carp pituitary cells.** Primary cultures of carp pituitary cells were prepared by trypsin/DNase II digestion as described previously (61). Briefly, pituitaries were excised from grass carps and diced into 0.6-μm fragments using a McIlwain tissue chopper (Brinkmann, Mississauga, ON, Canada). The pituitary fragments obtained were digested with trypsin (2.5 mg/ml) at 28°C for 25 min and dispersed in S-MEM medium (pH 7.7; Sigma) with 26 mM NaHCO3, 25 mM HEPES, 0.1% BSA, and 1% (vol/vol) antibiotics-antimycotic supplemented with DNase II (0.01 mg/ml; Sigma). After that, pituitary cells were filtered through a sterile nylon mesh (20-μm pore size), pelleted by centrifugation at 200 g at 4°C, and resuspended in carp MEM (64) at a density of 1.5 × 106 cells/ml for cell counting with trypan blue. The average cell yield was ~5.7 × 106 cells/pituitary with a mean viability of 96.5 ± 0.5% (n = 12).

**GH secretion in pituitary cells under column perfusion.** After cell counting, grass carp pituitary cells were cultured in carp MEM with 5% FBS and preswollen Cytodex II beads (Pharmacia, Uppsala, Sweden) at 28°C for 15–18 h under 5% CO2 and saturated humidified. On the following day, Cytodex II beads with cells attached were transferred into 0.5-ml microcolumns (3 × 106 cells/column) and perfused with carp MEM at 28°C with 0.1% BSA at a flow rate of 15 ml/h using an ACUSYST-S column perfusion system (Endotronics, Plymouth, MN). The dead volume of the perfusion system was found to be 1.24 ml, which would cause a time delay of ~5 min between the initiation of drug treatment and the onset of hormone responses. Before drug treatment, pituitary cells were perfused for 3 h to establish a stable base line for GH secretion. After that, perfusate was collected in 5-min fractions for 30 min, and test substance was then administered through a three-way stopcock into individual microcolumns for the duration as indicated. Perifusate samples were stored frozen at −20°C until their GH contents were measured using a rat immunoussay previously validated for carp GH (34).

**GH mRNA expression in static cultures of pituitary cells.** Grass carp pituitary cells were seeded at a density of 2.5 × 105 cells·well−1 in 24-well culture plates precoated with 0.1 mg/ml poly-d-lysine (Roche, Mannheim, Germany). After a 15-h static culture in carp MEM with 5% FBS at 28°C, the culture medium was replaced with serum-free carp MEM with appropriate concentrations of test substances. The duration of drug treatment was fixed at 48 h and varied on the other. After drug treatment, pituitary cells were dissolved in Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated according to the instructions of the manufacturer. Slot-blot assay for GH mRNA was performed using a Bio-Dot microfiltration unit (Bio-Rad, Hercules, CA). Membrane hybridization was conducted as described previously (21) using a digoxigenin (DIG)-labeled cDNA probe covering positions 75–444 of grass carp GH gene (GenBank accession no. M27094). Hybridization signals were then visualized using a DIG luminescent detection kit (Roche) and quantified with a Kodak IC440 Image Station (Eastman Kodak, New York).

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Haven, CT). In these experiments, parallel slot-blot assay for 18S RNA was also conducted to serve as an internal control.

Measurement of cellular cAMP content. Pituitary cells were seeded at a density of 2 × 10^5 cells·5 ml^-1 well^-1 in carp MEM with 5% FBS in poly-D-lysine precoated 35-mm petri dishes (Corning). After overnight incubation, culture medium was replaced with 0.9 ml of HHBSA medium (62) supplemented with 0.1 mM IBMX, a phosphodiesterase inhibitor used to prevent cAMP degradation in cell cultures. Following a brief incubation at 28°C for 15 min, drug treatment was initiated by adding 0.1 ml of 10 X solution of test substance prepared in HHBSA medium. The duration of drug treatment was routinely fixed at 20 min. After that, HHBSA medium was removed and cellular cAMP was extracted with 1 ml of ice-cold absolute ethanol. These cAMP samples were vacuum-dried at 45°C using a SpeedVac Plus (Heto Lab, Gydevang, Denmark) and stored at −40°C until their cAMP contents were measured using a Biotrak [3H]-cAMP assay kit (Amersham, Piscataway, NJ).

Measurement of intracellular Ca^2+ levels. After overnight incubation in carp MEM with 5% FBS, pituitary cells were washed briefly and resuspended in prewarmed (28°C) HBS medium (59) with 0.1% BSA. The Ca^2+-sensitive dye indo-1 AM (Molecular Probes, Eugene, OR) was dissolved freshly in DMSO before dye loading. Dye loading was conducted in the dark in 15 × 10^5 cells suspended in 5 ml of HEPES-buffered saline (HBS) medium with 10 μM indo-1 AM for 45 min at 28°C. After that, pituitary cells were washed twice and suspended at a density of 1.5 × 10^5 cells/ml in HBS medium without BSA. Indo-1 fluorescence signals were monitored in a 2-ml cell suspension (~3 × 10^6 cells) in a thermostated (28°C) quartz cuvette using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Indianapolis, IN). Wavelengths for indo-1 excitation and emission were fixed at 329 (5-nm slit width) and 405 nm (10-nm slit width), respectively. Experiments were repeated at least three times with different batches of pituitary cells, and similar trends in Ca^2+ responses were consistently observed. Because variations in fluorescence intensity were also noted between cell preparations, only the representative results are presented for these experiments.

Real-time PCR of GH primary transcript. Pituitary cells were cultured at a density of 3 × 10^5 cells/well in 24-well plates and incubated at 28°C with test substances for 48 h before RNA isolation with Trizol reagent. After that, total RNA was digested with DNase I (Pharmacia) to remove potential contamination of genomic DNA and reverse transcribed with SuperScript II (Invitrogen) using oligo(dT)18 as the primer. The RT samples obtained were then subjected to quantitative PCR for GH primary transcript using a Rotor Gene 2000 real-time amplification system (Corbett Research, Mortlake, NSW, Australia) as described previously (64). Expression of mature GH mRNA was also assayed in these samples by using a similar approach to serve as a parallel control. The primers for mature GH mRNA cover positions 75 to 396 of grass carp GH cDNA (accession no. M27094) flanking the entire intron II, whereas the primers for GH primary transcript cover the junction between exon II and intron II from positions 348 to 557 of grass carp GH gene (accession no. X60419). Based on our validation, the primers for GH primary transcript were ineffective in PCR amplification of mature GH mRNA in the RT samples prepared from grass carp pituitary cells. In these studies, PCR reactions were conducted using a LightCycler SYBR Green I assay kit (Roche), and melting curve analysis was routinely performed to check for the authenticity of PCR products. GH primary transcript, melting temperature (T_m) = 91.2°C; mature GH mRNA, T_m = 92.2°C. The identity of PCR products (210 bp for GH primary transcript; 252 bp for mature GH mRNA) was also confirmed by ethidium bromide staining after electrophoresis in 2% agarose gels. In these assays, serial dilutions of plasmid DNA carrying grass carp GH cDNA and full-length GH gene were used as the standards for real-time PCR of mature GH mRNA and primary transcript, respectively.

Measurement of GH promoter activity. A 986-bp fragment of grass carp GH gene promoter was subcloned into pGL3.Basic (Promega, Madison, WI) to generate the luciferase-expressing reporter pGH(-986).Luc for transfection studies in GH cells. GH3 cells were routinely maintained at 37°C in Ham’s F-10 medium (Invitrogen) with 10% FBS under 5% CO2 and saturated humidity. One day before transfection, GH3 cells (~70~80% confluence) were dispersed and seeded in 24-well culture plates at a density of 0.1 × 10^5 cells·0.5 ml^-1 well^-1. After overnight incubation, transient transfection was performed in individual wells for 6 h with 2 μl of Lipofectamine (Invitrogen) in 400 μl of Opti-MEM (Invitrogen) containing 0.2 μg of pGH(-986).Luc, 0.02 μg of pEGFP-N1 (Clontech, Palo Alto, CA), and 0.18 μg of goldfish α2-adrenergoreceptor-expressing vector pcDNA.gfp2AR or the blank vector pcDNA 3.1 (Invitrogen) without the receptor insert. The green fluorescent protein (GFP)-expressing vector pEGFP-N1 was included in the transfection mixture to serve as an internal control. After transfection, Opti-MEM was replaced with serum-containing Ham’s F-10 medium and the GH3 cells were allowed to recover for 18 h before the initiation of drug treatment. After 24-h static incubation with test substances, the cells were washed and lysed in Reporter lysis buffer (Promega) and luciferase activity in the cell lysate was measured with a firefly luciferase assay kit (Promega) using a Lumat LB9507 luminometer (EG&G, Gaithersburg, MD). GFP expression levels in these samples were also monitored using a CytoFluor 4000 multwell plate reader (Perspective Biosystems, Framingham, MA).

Data transformation and statistical analysis. For perfusion studies, GH data from individual columns were expressed as a percentage of the average GH content of the first six fractions collected at the beginning of the experiment before drug treatment. The transformation (%basal) was conducted to allow for pooling of data from different columns without distorting the kinetic profile of GH release during the course of perfusion (61). In the case of static incubation studies, GH mRNA levels were quantified in terms of arbitrary density units and normalized against 18S RNA expression in the same sample. Because 18S RNA levels did not exhibit significant changes in these experiments, the normalized data were simply transformed as a percentage of the mean value in the control group (%control) for data pooling from separate experiments. The data for cAMP content, mature GH mRNA, GH primary transcript, and GH promoter activity were also transformed in a similar manner before statistical analysis. For GH mRNA clearance analysis, the half-life (T_1/2) of GH transcripts, defined as the time required for GH mRNA to reduce to one-half of its initial level, was deduced by one-phase exponential decay modeling using Graphpad Prism 3.02 (Graphpad, San Diego, CA). For Ca^2+ calibration, fluorescence data were transformed into [Ca^2+]_{free} (nM) using the single-wavelength equation [Ca^2+]_{free} = K_d × (F - F_{min})/(F_{max} - F), where K_d is the dissociation constant for indo-I, F_{min} is the basal fluorescence in the absence of Ca^2+, F_{max} is the maximal fluorescence at saturating Ca^2+, and F is the fluorescence intensity at any unknown Ca^2+ concentration. F_{min} and F_{max} were determined empirically for each dye loading according to the in situ cell lysis method described previously (53). Data presented, except for Ca^2+ measurement, were analyzed with Student’s t-test or ANOVA followed by Fisher’s least significant difference test. Differences were considered significant at P < 0.05.

RESULTS

Adrenergic regulation of GH release in carp pituitary cells. To examine adrenergic regulation of GH release at the pituitary level, we used a perfusion approach to test the direct effects of NE on the kinetics of GH secretion in carp pituitary cells. In this case, increasing concentrations of NE (1 nM–10 μM) administered as 10-min pulses were effective in triggering a dose-dependent decrease in basal GH release (Fig. 1A). The inhibitory effect was rapid and reached its peak within 15~20 min following NE treatment. After termination of NE perfu-
sion, GH secretion gradually returned to basal, and interestingly, a transient rise in GH release was noted during the recovery phase following NE treatment at 1 and 10 μM doses, respectively. To clarify the receptor specificity for NE action, we used a pharmacological approach. The inhibitory effect of NE on GH secretion was mimicked by perifusion with the α2-adrenergic agonist clonidine (1 μM), whereas similar treatment with the α1-agonist phenylephrine (1 μM) or the β-agonist isoproterenol (1 μM) had no effects in this regard (Fig. 1B). In parallel experiments, NE inhibition on GH release was totally abolished by continuous perifusion with the α2-antagonist yohimbine (5 μM), and the antagonist by itself did not modify basal levels of GH secretion (Fig. 1C). Unlike yohimbine, simultaneous treatment with the α1-antagonist prazosin

Fig. 1. Adrenergic regulation of growth hormone (GH) release from perifused grass carp pituitary cells. A: effects of increasing levels of norepinephrine (NE; 1 nM–10 μM) applied as 10-min pulses (filled bars) on basal GH secretion. B: effects of the α1-agonist phenylephrine (1 μM), α2-agonist clonidine (1 μM), and β-agonist isoproterenol (1 μM) on GH release. Duration of drug treatment (filled bars) was fixed at 10 min in these experiments. C: effects of a 1.5-h continuous perifusion of the α1-antagonist prazosin (10 μM), α2-antagonist yohimbine (10 μM), and β-antagonist propranolol (10 μM) on NE inhibition of GH secretion. A 10-min pulse of NE (1 μM; filled bars) was administered 30 min after the initiation of antagonist treatment (open bars). D: effects of a 3-h continuous perifusion with pertussis toxin (50 μg/ml; open bar) on NE inhibition of basal GH release. A 10-min pulse of NE (1 μM; filled bar) was given 30 min after the initiation of pertussis toxin perifusion. E: effects of a 2-h continuous perifusion with NE (1 μM) on pituitary adenylate cyclase-activating polypeptide (PACAP; 1 μM)-induced GH secretion. In these experiments, a 10-min pulse of PACAP (filled bar) was given 30 min after the initiation of NE perifusion (open bar). Parallel treatments with PACAP alone and NE alone were used as the positive and negative controls, respectively. Data presented, expressed as means ± SE (n = 6), are pooled results from 6 separate perifusion experiments. conc, Concentration.
(5 μM) or the β-antagonist propranolol (5 μM) did not affect the inhibitory action on GH release induced by NE perifusion. To evaluate the functional coupling of adrenergic inhibition on GH release with G_{i} protein, we tested the inhibitory effect of NE in the presence of pertussis toxin (Fig. 1D). In grass carp pituitary cells, simultaneous perifusion with pertussis toxin (50 μg/ml) was not able to block NE-inhibited GH secretion. To shed light on the functional role of NE on GH release at the pituitary level, we also tested the GH-releasing effect of PACAP with simultaneous NE perifusion (Fig. 1E). Similar to the dose-response studies, prolonged treatment with NE (1 μM) for 2 h consistently suppressed basal GH secretion. A 10-min pulse of PACAP (1 μM), in contrast, induced a rapid increase in GH release, and this stimulatory action was abrogated by continuous perifusion with NE. Similarly, PACAP-stimulated GH release was also blocked by simultaneous treatment with the α_{2}-agonist clonidine (1 μM; data not shown).

**Adrenergic regulation of basal and PACAP-stimulated GH mRNA expression.** To further examine adrenergic regulation of GH synthesis, we used a static incubation approach to study the effects of α_{2}-adrenergic stimulation on steady-state GH mRNA expression in grass carp pituitary cells. Treatment with NE (1 μM; Fig. 2A) or the α_{2}-agonist clonidine (1 μM; Fig. 2B) was effective in triggering a time-dependent decrease in GH mRNA levels. In both cases, a significant drop in GH mRNA expression could be noted 24 h after the initiation of drug treatment, and the amount of GH transcripts detected attenuated to a lower level after 48 h of incubation. Incubation of pituitary cells with NE (1 μM) and clonidine (1 μM) for a duration shorter than 24 h, however, was not effective in altering GH gene expression. By fixing the duration of drug treatment at 48 h, the expression levels of GH transcripts were reduced in a concentration-related fashion by increasing doses of NE (1 μM).}

**Fig. 2.** Adrenergic regulation of GH mRNA expression in grass carp pituitary cells. For the time-course studies, pituitary cells were incubated with NE (1 μM; A) or the α_{2}-agonist clonidine (1 μM; B) for 12, 24, and 48 h. In the case of dose dependence experiments, pituitary cells were treated for 48 h with increasing concentrations of NE (1 nM–10 μM; C) and clonidine (1 nM–10 μM; D), respectively. After drug treatment, total RNA was isolated from pituitary cells and “steady-state” GH mRNA expression was quantified by slot-blot assay as described in MATERIALS AND METHODS. In these studies, 18S rRNA levels were also measured to serve as the internal control. Data presented, expressed as means ± SE (n = 8), are pooled results from 8 experiments. a,b,cTreatment groups denoted by different letters represent a significant difference at P < 0.05 [ANOVA followed by Fisher’s least significant difference (LSD) test]. Representative slot blots for GH mRNA and 18S rRNA expression are also included in the results for individual experiments. Clon, clonidine; Ctrl, control.
α2-Adrenergic inhibition of cAMP production and intracellular Ca2+ levels. To elucidate the postreceptor signaling mechanisms for α2-adrenergic inhibition of GH release and GH mRNA expression, we conducted direct measurement of cAMP production and [Ca2+]i in grass carp pituitary cells with clonidine treatment. Since PACAP has been previously shown to activate GH release and GH mRNA expression in grass carp through upregulation of cAMP production and Ca2+ influx through voltage-sensitive Ca2+ channels (VSCC) (59), functional interactions between PACAP and clonidine in regulating cAMP and [Ca2+]i levels were also examined. For cAMP production, increasing concentrations of clonidine (0.01 nM–1 μM) were effective in reducing cellular cAMP contents in a dose-dependent manner (Fig. 4A). In parallel experiments, cAMP contents in carp pituitary cells were significantly elevated by PACAP treatment (1 μM), and this stimulatory action could be alleviated by simultaneous incubation with clonidine (1 μM; Fig. 4B). For [Ca2+]i measurement, PACAP (1 μM) consistently induced a rapid rise in [Ca2+]i in carp pituitary cells (Fig. 5A), and this Ca2+ response could be attenuated by sequential application of clonidine (1 μM; Fig. 5B). Prior exposure to clonidine (1 μM) also suppressed [Ca2+]i levels.

Fig. 3. Effects of α2-adrenergic stimulation on PACAP-stimulated GH mRNA expression in grass carp pituitary cells. Pituitary cells were incubated with PACAP (1 μM) for 48 h in the presence or absence of NE (1 μM; A) or the α2-agonist clonidine (1 μM; B). After the drug treatment, total RNA was isolated for measurement of GH mRNA expression. Data presented are expressed as means ± SE (n = 8). abTreatment groups denoted by different letters represent a significant difference at P < 0.05 (ANOVA followed by Fisher’s LSD test).

Fig. 4. Effects of α2-adrenergic stimulation on basal and PACAP-stimulated cAMP production in grass carp pituitary cells. Pituitary cells were incubated for 20 min with increasing concentrations of the α2-agonist clonidine (0.01–1 μM; A) or with PACAP (1 μM) in the presence of absence of NE (1 μM; B). After drug treatment, cAMP was extracted from pituitary cells using ethanol, and cellular cAMP content was determined by radioimmunoassay. Data presented are expressed as means ± SE (n = 6). abcdTreatment groups denoted by different letters represent a significant difference at P < 0.05 (ANOVA followed by Fisher’s LSD test).
and blocked the Ca\textsuperscript{2+} response induced by PACAP (1 \textmu M; Fig. 5C). In these studies, DMSO (0.1\%), the solvent control for clonidine, was not effective in altering Ca\textsuperscript{2+} levels in grass carp pituitary cells (Fig. 5D).

**\( \alpha_2 \)-Adrenergic regulation of GH mRNA stability and primary transcript expression.** Since the steady-state GH mRNA levels measured in the preceding studies represent a dynamic balance of GH gene transcription and GH mRNA degradation, the functional role of GH transcript stability and GH gene transcription in \( \alpha_2 \)-adrenergic inhibition of GH mRNA expression was also examined. Clearance analysis of GH transcript was performed in pituitary cells pretreated with the transcription inhibitor actinomycin D (8 \textmu M). In this case, GH mRNA level was reduced gradually in a time-dependent manner with a \( T_{1/2} \) value of 27 h, and parallel treatment with clonidine (1 \textmu M) did not modify the clearance profile or \( T_{1/2} \) value for GH transcripts (Fig. 6). To test for the possible involvement of GH gene transcription in clonidine’s action, we monitored the expression of GH primary transcript using real-time PCR. Calibration of GH primary transcript levels (fmol/tube) was conducted with a standard curve constructed using increasing amounts of plasmid DNA carrying a full-length grass carp GH gene (Fig. 7A). Melting curve analysis of PCR product routinely produced a single peak at 91.2°C for GH primary transcript. In carp pituitary cells, incubation with increasing levels of the \( \alpha_2 \)-agonist clonidine (0.01–10 \textmu M) suppressed GH primary transcript expression in a dose-related fashion (Fig. 7B, bottom). In these experiments, real-time PCR of mature GH mRNA was also performed to serve as a parallel control. Similar to the results of steady-state GH mRNA, clonidine treatment could dose-dependently attenuate mature GH mRNA levels (Fig. 7B, top). Since \( \alpha_2 \)-adrenergic stimulation was shown to inhibit cAMP and \([\text{Ca}^{2+}]_i\) levels in our preceding studies, the functional role of cAMP- and Ca\textsuperscript{2+}-dependent mechanisms on GH gene expression was also tested using a pharmacological approach. In these studies, increasing levels (0.3–30 \textmu M) of the AC inhibitor MDL-12330A (Fig. 8A) and the PKA inhibitor H-89 (Fig. 8B) could mimic the dose dependence of clonidine inhibition on GH primary transcript expression.

![Fig. 5](http://ajpendo.physiology.org/)

**Fig. 5.** Effects of \( \alpha_2 \)-adrenergic stimulation on basal and PACAP-stimulated intracellular Ca\textsuperscript{2+} levels ([Ca\textsuperscript{2+}]) in grass carp pituitary cells. Pituitary cells were preloaded with the Ca\textsuperscript{2+}-sensitive dye indo-I and challenged with PACAP alone (A), PACAP stimulation followed by clonidine treatment (B), or clonidine treatment followed by PACAP stimulation (C). In these experiments, a 1000X concentrated stock of test substance was injected into the pituitary cell suspension during the process of [Ca\textsuperscript{2+}] measurement, and the final dilutions of PACAP and clonidine were routinely fixed at 1 \textmu M. As a solvent control, parallel treatment with DMSO (0.1\%) was also conducted to confirm that a “stable basal” for [Ca\textsuperscript{2+}] was established in the absence of drug treatment (D).

![Fig. 6](http://ajpendo.physiology.org/)

**Fig. 6.** Effects of \( \alpha_2 \)-adrenergic stimulation on GH transcript stability in grass carp pituitary cells. Pituitary cells were pretreated with the transcription inhibitor actinomycin D (8 \textmu M) and challenged with the \( \alpha_2 \)-agonist clonidine (1 \textmu M) for 0, 12, 36, 48, and 60 h. Total RNA samples were prepared at the respective time points, and GH mRNA clearance curves with and without clonidine treatment were constructed accordingly. The half-life (\( T_{1/2} \)) of GH transcript, defined as the time required for GH mRNA to reduce to 50\% of its original amount, was deduced by GraphPad Prism 3.02 using a 1-phase exponential decay model. Data presented, expressed as means ± SE (n = 4), are pooled results from 4 separate experiments.
transcript and mature GH mRNA expression. Similar inhibitions could also be noted by incubation with a Ca\textsuperscript{2+}-free culture medium (Fig. 9A) or by treatment with the L-type VSCC inhibitor nifedipine (0.3–10 \mu M; Fig. 9B).

\textbf{\(\alpha_2\)-Adrenergic regulation of GH promoter activity.} To further confirm that \(\alpha_2\)-adrenergic stimulation can inhibit GH mRNA expression by acting at the transcriptional level, we performed transfection studies in GH3 cells to test the
effects of NE and clonidine treatment on GH promoter activity. GH3 cells were selected as the host cells for our transfection studies because the cell line has Pit-1 expression to support GH promoter activity (14). Since GH3 cells normally do not express adrenergic receptors (19), cotransfection with pcDNA.gf2AR, an expression vector for goldfish $\alpha_2$-adrenergic receptor, was routinely performed in our promoter studies. In this case, increasing concentrations of NE (0.001–10 μM) were effective in suppressing luciferase activity expression in a dose-dependent manner (Fig. 10A). This inhibitory effect, however, was not observed in the control group transfected with the blank vector pcDNA 3.1. In parallel experiments, the dose dependence of NE inhibition was mimicked by increasing levels of the $\alpha_2$-agonist clonidine (0.001–1 μM; Fig. 10B), the AC inhibitor MDL-12230A (0.3–30 μM; Fig. 11A), or the PKA inhibitor H-89 (0.3–30 μM; Fig. 11B). Similarly, luciferase activity could also be reduced by incubation with Ca$^{2+}$-free medium or by treatment with the L-type VSCC blocker nifedipine (1–10 μM; Fig. 11C). In recent studies in our laboratory (59), PACAP has been shown to stimulate grass carp GH promoter activity. To shed light on the functional interactions between PACAP and NE in GH gene transcription, we tested PACAP-stimulated GH promoter activity in GH3 cells with simultaneous treatment of NE (Fig. 12A) or the $\alpha_2$-agonist clonidine (Fig. 12B). GH3 cells are responsive to PACAP stimulation, because the cell line has endogenous expression of PACAP receptors (43). Treatment with PACAP (1 μM) was effective in elevating basal levels of luciferase activity. Parallel exposure to NE (1 μM) or clonidine (1 μM), in contrast, reduced luciferase activity expression and blocked the stimulatory effect induced by PACAP.

**DISCUSSION**

In mammals, especially in the rat, GH release can be triggered by adrenergic stimulation through direct action at the pituitary level via $\beta$-adrenoreceptors (1) and indirect actions in the hypothalamus via $\alpha_2$-adrenoreceptors (10, 31). Since GH secretion caused by $\beta$-agonists tends to be much weaker than that of GHRH in pituitary cell cultures (16), the pituitary action through $\beta$-adrenoreceptors in general is not considered a major component for GH regulation. Based on the studies in goldfish,
adrenergic stimulation can inhibit GH secretion through activation of pituitary $\alpha_2$-adrenoreceptors (29), and this inhibitory action does not involve a central component (5). These findings are quite different from the reports in mammals, in which $\alpha_2$-adrenergic stimulation via differential actions on GHRH and SRIF neurons in the hypothalamus act as a potent induction signal to trigger GH release in vivo (see introductory remarks). Apparently, the mechanisms for $\alpha_2$-adrenergic regulation of GH secretion have been modified during the course of evolution from fish to mammals. To shed light on the generalization of $\alpha_2$-adrenergic inhibition at the pituitary level in fish models, we tested NE actions on GH release in grass carp pituitary cells and clarified the receptor specificity using a pharmacological approach. In this case, NE treatment could trigger an acute effect to suppress GH secretion, and this inhibitory action was mimicked by the $\alpha_2$-agonist clonidine but not by the $\alpha_1$-agonist phenylephrine or the $\beta$-agonist isoproterenol. Furthermore, NE inhibition on GH release could be abolished by the $\alpha_2$-antagonist yohimbine, whereas the $\alpha_1$-antagonist prazosin and the $\beta$-antagonist propranolol were not effective in this respect. These results confirm that the inhibitory action of NE on GH secretion is mediated by $\alpha_2$-adrenoceptors expressed in the carp pituitary. In general, $\alpha_2$-adrenoceptors are functionally coupled to $G_i$ (23), and their biological actions (e.g., inhibition of AC activity) can be blocked by pertussis toxin through ADP-ribosylation of the $G_\alpha_\text{i}$ subunits (33). In this study, treatment with pertussis toxin was found to have no effects on both basal and NE-inhibited GH secretion, suggesting that the $G$ protein coupling in fish $\alpha_2$-adrenoceptors may be different from that of the mammalian counterparts. Although the sensitivity to pertussis toxin is a common feature for $G_i$ coupled to $\alpha_2$-adrenergic receptors, some members of the $G_\alpha$ family (e.g., $G_\alpha_5$) are known to be resistant to pertussis toxin. Thus, the involvement of $G_\alpha$ family in NE inhibition on GH secretion is expected to be further explored in the future.

**Fig. 11.** Inhibiting cAMP- and Ca$^{2+}$-dependent mechanisms on GH promoter activity expressed in GH3 cells. After transfection with pcDNA.gfo2AR and pGH(-986)Luc, GH3 cells were incubated for 24 h with increasing concentrations (0.3–30 μM) of the AC inhibitor MDL-12330A (A) or PKA inhibitor H-89 (B). In parallel experiments, the cells were also treated with a Ca$^{2+}$-free culture medium or with increasing levels of the L-type Ca$^{2+}$ channel blocker nifedipine (1–10 μM; C). After the drug treatment, cell lysate was prepared for luciferase activity measurement. In these experiments, cotransfection with pEGFP-N1 was conducted to serve as an internal control. Data presented are expressed as means ± SE (n = 8). a,b,c,dTreatment groups denoted by different letters represent a significant difference at P < 0.05 (ANOVA followed by Fisher’s LSD test).

**Fig. 12.** Effects of adrenergic stimulation on PACAP-induced GH promoter activity expressed in GH3 cells. After transfection with pcDNA.gfo2AR and pGH(-986)Luc, GH3 cells were incubated for 24 h with PACAP (1 μM) in the presence or absence of NE (1 μM; A) or the $\alpha_2$-agonist clonidine (1 μM). In these experiments, cotransfection with the GFP-expressing pEGFP-N1 vector was used as an internal control. Data presented are expressed as means ± SE (n = 8). a,b,c,dTreatment groups denoted by different letters represent a significant difference at P < 0.05 (ANOVA followed by Fisher’s LSD test).
to pertussis toxin inhibition (15). The functional role of these “atypical Gi” proteins in the signal transduction of α2-adrenergic receptors has not been fully investigated.

In grass carp pituitary cells, a transient increase in GH release was observed during the recovery phase following high doses of NE treatment. A similar phenomenon was also noted in our previous studies in the goldfish (29). This “off-response” following NE inhibition closely resembles the GH rebound that occurred during SRIF withdrawal in mammals, including the rat (57), dog (45), and human (3). The GH rebound caused by SRIF withdrawal is Ca2+ dependent (37) and may play a role in the maintenance of episodic GH release in vivo (4). Since SRIF reduces GH secretion in pituitary cells without inhibiting GH synthesis, the GH rebound has been attributed to a buildup of GH stores in somatotrophs during the period of SRIF inhibition (57). This idea, however, may not be applicable to the GH rebound caused by NE inhibition. In this study, GH rebound was induced by a 10-min treatment with NE, and it is doubtful whether GH synthesis within such a short period of time could lead to significant changes in GH content in carp pituitary cells. Furthermore, prolonged incubation with NE was effective in reducing steady-state GH mRNA levels, and this inhibitory action could be mimicked by the α2-agonist clonidine. These results indicate that NE can also inhibit GH synthesis through activation of pituitary α2-adrenoceptors. Since clonidine treatment did not alter the T1/2 of GH transcripts, α2 inhibition of steady-state GH mRNA levels could not be due to GH mRNA degradation caused by posttranscriptional modification of GH transcript stability. Similar treatment with clonidine nevertheless suppressed the expression levels of mature GH mRNA and GH primary transcripts in carp pituitary cells. In living cells, primary transcripts in the nucleus are processed rapidly and exported to the cytoplasm as mature mRNA. The expression level of primary transcripts in general can be taken as a faithful index for target gene transcription (2). Therefore, the results based on our real-time PCR studies may imply that α2-adrenergic stimulation can inhibit GH gene transcription by acting directly at the pituitary cell level. This idea is supported by the findings based on our transfection studies, in which the promoter activity of GH gene expressed in GH3 cells could be attenuated by treatment with NE and clonidine, respectively.

To further evaluate the functional role of NE as a GH regulator in the carp species, we also examined its interaction with PACAP in regulating GH release and GH gene expression. PACAP was first identified in ovine hypothalamus by its ability to stimulate AC activity in rat pituitary cells (39), and its molecular structure is highly conserved from fish to mammals (48). Although PACAP is detected in the hypothalamus (52) and hypophyseal portal blood in mammals (13), its GH-releasing effect is either weak or mild [e.g., rat (22), pig (35), and cattle (42)] or even absent in some species [e.g., sheep (47) and human (7)]. In general, it is not considered a major regulator for GH release in higher vertebrates (40). In fish models, however, PACAP serves as a potent GH-releasing factor (60) and can induce GH secretion through activation of pituitary PAC-I receptors (58). In the present study, PACAP stimulated GH release and GH mRNA expression in grass carp pituitary cells, and these stimulatory actions could be blocked by NE or the α2-agonist clonidine. As revealed by our transfection studies, PACAP-induced GH promoter activity was also susceptible to α2-adrenergic inhibition. These results suggest that NE by acting through α2-adrenergic receptors may act as a negative regulator for PACAP induction of GH synthesis and secretion in the carp pituitary. In grass carp pituitary cells, PACAP promotes GH production by enhancing transcript stability of GH mRNA and nuclear expression of GH primary transcript (59). Unlike PACAP, clonidine treatment did not alter GH mRNA stability but suppressed GH promoter activity and GH primary transcript levels. Therefore, it would be logical to postulate that α2-adrenergic inhibition of GH gene expression induced by PACAP is acting mainly at the transcriptional level.

In our recent studies, PACAP was found to induce GH secretion and GH gene expression in the carp species through activation of cAMP production and Ca2+ entry via L-type VSCC (59, 62). In the goldfish, α2-adrenergic stimulation at the pituitary level was shown to be effective in blocking the GH-releasing effects caused by the AC activator forskolin or the Ca2+ ionophore A-23187 (63). These findings have prompted us to speculate that NE may interfere with these signaling pathways to inhibit the stimulatory actions of PACAP on GH synthesis and secretion. This hypothesis was supported by the results of direct measurement of cAMP and Ca2+ levels in grass carp pituitary cells. In this case, clonidine treatment was effective in reducing cAMP production and basal levels of Ca2+. These findings also corroborate with the biochemical properties reported for α2-adrenoceptors. In mammals, AC inactivation and reduction of VSCC currents are known to be the key events occurring during the signal transduction of α2-adrenoceptors (50). Since α2-adrenergic stimulation can also activate inwardly rectifying K+ channels, VSCC inhibition is suspected to be the result of membrane hyperpolarization caused by activation of K+ currents (11). In the present study, PACAP consistently elevated cAMP production and Ca2+ levels in grass carp pituitary cells, and these stimulatory actions could be blocked by simultaneous treatment with the α2-agonist clonidine. Furthermore, clonidine inhibition on mature GH mRNA, GH primary transcript, and GH promoter activity could be mimicked by perturbing the cAMP-dependent pathway with the AC inhibitor MDL-12330A and the PKA inhibitor H-89 by blocking Ca2+ entry with the L-type VSCC inhibitor nifedipine or by removing extracellular Ca2+ using a Ca2+-free culture medium. Similar treatments in carp pituitary cells have been previously shown to suppress basal GH release (62) and block the stimulatory effects of PACAP on GH secretion and GH mRNA expression (59). These results, as a whole, may suggest that α2-adrenergic stimulation can downregulate PACAP-induced GH release and GH gene transcription by inhibiting the AC/cAMP/PKA pathway and Ca2+ entry through L-type VSCC. Given that both our studies on cAMP and Ca2+ measurement were performed in mixed populations of carp pituitary cells and 2) α2-adrenergic stimulation at the pituitary level is known to inhibit prolactin secretion in mammals, e.g., in ovine pituitary cells (8), we do not exclude the possibility that the cAMP and/or Ca2+ responses observed in this study may also be involved in adrenergic regulation of other pituitary hormones in fish models.

In summary, using grass carp as a model for modern-day bony fish, we have demonstrated for the first time that NE can suppress both basal and PACAP-stimulated GH release and...
GH gene expression by acting directly at the pituitary cell level. These inhibitory actions are mediated through α2-adrenoceptors negatively coupled to the cAMP-dependent pathway and Ca2+ entry through L-type VSCCs. Despite our previous findings that PACAP can induce GH synthesis by enhancing GH mRNA stability and GH gene transcription, α2-adrenergic inhibition of GH mRNA expression is mediated by reducing GH promoter activity and does not involve posttranscriptional modification of GH transcript stability. Apparently, α2 inhibition of GH gene transcription represents a major mechanism for NE inhibition of GH synthesis induced by PACAP stimulation. Together with our previous findings in goldfish, the present study provides further evidence to support the hypothesis that adrenergic input at the pituitary level, via activation of α2-adrenoceptors, serves as an integral component of the neuroendocrine control of GH synthesis and secretion in fish models.

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