Mechanisms for gonadotropin-releasing hormone potentiation of growth hormone rebound following norepinephrine inhibition in goldfish pituitary cells

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Wong AO, Chuk MC, Chan HC, Lee EK. Mechanisms for gonadotropin-releasing hormone potentiation of growth hormone rebound following norepinephrine inhibition in goldfish pituitary cells. Am J Physiol Endocrinol Metab 292: E203–E214, 2007. First published August 29, 2006; doi:10.1152/ajpendo.00337.2006.—In the goldfish, norepinephrine (NE) inhibits growth hormone (GH) secretion through activation of pituitary α2-adrenergic receptors. Interestingly, a GH rebound is observed after NE withdrawal, which can be markedly enhanced by prior exposure to gonadotropin-releasing hormone (GnRH). Here we examined the mechanisms responsible for GnRH potentiation of this “postinhibition” GH rebound. In goldfish pituitary cells, α2-adrenergic stimulation suppressed both basal and GnRH-induced GH mRNA expression, suggesting that a rise in GH synthesis induced by GnRH did not contribute to its potentiating effect. Using a column perfusion approach, GnRH given during NE treatment consistently enhanced the GH rebound following NE withdrawal. This potentiating effect was mimicked by activation of PKC and adenylyl cyclase (AC) but not by induction of Ca2+ entry through voltage-sensitive Ca2+ channels (VSCC). Furthermore, GnRH-potentiated GH rebound could be alleviated by inactivation of PKC, removal of extracellular Ca2+, blockade of VSCC, and inhibition of Ca2+-calmodulin (CaM)-dependent protein kinase II (CaMKII). Inactivation of AC and PKA, however, was not effective in this regard. These results, as a whole, suggest that GnRH potentiation of GH rebound following NE inhibition is mediated by PKC coupled to Ca2+ entry through VSCC and subsequent activation of CaMKII. Apparently, the Ca2+-dependent cascades are involved in GH secretion during the rebound phase but are not essential for the initiation of GnRH potentiation. Since GnRH has been previously shown to have no effects on cAMP synthesis in goldfish pituitary cells, the involvement of cAMP-dependent mechanisms in GnRH potentiation is rather unlikely.

protein kinase A; protein kinase C; voltage-sensitive Ca2+ channel; Ca2+-calmodulin-dependent protein kinase

IN MAMMALS, EPISODIC RELEASE of growth hormone (GH) is caused by the 180° out-of-phase secretion of GH-releasing hormone (GHRH) and somatostatin (SRIF) from the hypothalamus (54). Recently, ghrelin, the endogenous ligand for GH secretagogues, is also known to be involved in the control of GH pulsatility (53). The functionality of these GH regulators, in turn, is under the modulation of neurotransmitters acting within the central nervous system (CNS). Adrenergic nerve fibers, presumably originated from the A1 to A7 groups in the brain stem (51), innervate the cell bodies of GHRH and SRIF neurons in the medial basal (47) and preoptic hypothalamus (32), respectively. Through activation of α2-adrenoceptors (14), adrenergic stimulation can elevate GH levels by inducing GHRH secretion from the arcuate nucleus (12) with simultaneous inhibition of SRIF release from the periventricular nucleus (31). In the rat, α2-adrenoceptors are located in the nerve terminals of serotonin neurons (18), and an intact serotonergic system is required for α2-induction of GHRH release in the arcuate nucleus (18), suggesting that adrenergic activation of GHRH release may be mediated by serotonin interneurons. In the same animal model, α2-inhibition of SRIF release not only can reduce the inhibitory input to the pituitary but also remove the reciprocal feedback on GHRH neurons by SRIF fibers from the periventricular nucleus (37). Since norepinephrine (NE) (50) and epinephrine (EP) (23) can be detected in hypophyseal portal blood at concentrations higher than that of peripheral blood, adrenergic input acting directly at the pituitary level has been suggested. This idea has been confirmed by the findings that β-adrenoceptors are expressed in somatotrophs (1) and β2-agonists can induce GH release and cAMP production in rat pituitary cells (49). Apparently, α2-adrenoceptors are not involved in GH release at the pituitary level (33). Given that the GH responses induced by β-agonists are much weaker than those of GHRH (15), the pituitary action through β2-adrenoceptors in general is not considered to be a major component for GH regulation.

Unlike mammals, bony fish (or teleosts) do not have a hypophyseal portal blood system, and the pituitary is under the direct innervation of the hypothalamus (16). In the goldfish, a representative model of teleosts, adrenergic neurons can be located in the isthmal tegmentum, periventricular nuclei, and basal hypothalamus and project into the infundibulum through a preoptico-infundibular pathway (20). In general, adrenergic fibers originated from the isthmal tegmentum are considered to be the major source of adrenergic input to the pituitary in fish species (24). Apart from the neural input from the CNS, pituitary cells are also exposed to NE and EP in systemic circulation, which are secreted from chromaffin cells located in the head kidneys and walls of cardinal veins as a part of the stress responses (45). In vivo experiments with the goldfish, intraperitoneal injection of NE suppressed serum GH levels, and this inhibitory effect was not observed by direct injection of NE into the brain (9). These results suggest that the site of action for NE is not within the CNS but located outside the blood-brain barrier. This idea is consistent with our findings that both NE and EP could inhibit GH secretion from goldfish pituitary cells through activation of α2-adrenoceptors (28). These inhibitory actions probably are caused by α2-blocking of the GH-releasing effects mediated by cAMP-, PKC-, and Ca2+-dependent cascades (60). In our previous studies (5), a rebound of GH release was noted following termination of NE...
treatment, which closely resembles the GH rebound observed after SRIF withdrawal in mammalian models. This GH rebound, interestingly, could be markedly enhanced by prior exposure to gonadotropin (GTH)-releasing hormone (GnRH). The potentiation is specific to GnRH and pretreatment with other GH-releasing factors (e.g., dopamine) could not trigger a similar effect (28). Although the phenomenon is unique and has not been reported in other vertebrates, the postreceptor signaling mechanisms responsible for this potentiating effect have not been characterized.

In this study, we sought to elucidate the mechanisms responsible for GnRH potentiation of GH rebound following NE inhibition, using goldfish pituitary cells as a model. Since GnRH is known to stimulate GH synthesis in fish (39), which may contribute to GnRH potentiation of GH rebound, the effects of NE and α2-adrenergic stimulation on basal and GnRH-induced GH gene expression were tested in static cultures of goldfish pituitary cells. Using a column perifusion approach, the signaling mechanisms for GnRH potentiation were studied using pharmacological agents known to modify the functionality of the cAMP-, PKC-, and Ca2+/-calmodulin-dependent protein kinase II (CaMKII).

MATERIALS AND METHODS

Animals. Goldfish (Carassius auratus) with body weight ranging from 30 to 45 g and gonadosomatic index of 1.45 ± 0.37% were obtained from local pet stores and maintained in 500 liters aquaria at 20 ± 2°C under natural photoperiod for ≥2 wk prior to pituitary cell preparation. The fish were fed to satiation daily and killed by spino- sectomy following anesthesia in 0.05% tricane methanesulfonate (Syndel, Vancouver, BC, Canada) according to the regulations of animal use at the University of Hong Kong. Since the fish were in the late stages of gonadal regression and sexual dimorphism was not apparent, goldfish of mixed sexes were used for the preparation of pituitary cell cultures.

Test substances. NE, clonidine, phenylephrine, isoproterenol, and yohimbine were obtained from Sigma (St. Louis, MO). 12-O-tetradecanoylphorbol 13-acetate (TPA), forskolin, Bay K8644, H89, A23187, nifedipine, SQ22536, calphostin C, and KN93 were acquired from Calbiochem (San Diego, CA), whereas (D-Arg6,Pro9-NEt) sGnRHa analog (sGnRHa) was purchased from Peninsula Laboratories (Belmont, CA). Stock solutions of sGnRHa, H89, and SQ22536 were prepared in double-distilled deionized water. Forskolin, TPA, A23187, KN93, calphostin C, Bay K 8644, and nifedipine were dissolved in dimethyl sulfoxide (DMSO). Stock solutions of test substances were stored in small aliquots at −80°C and diluted to appropriate concentrations with culture medium on the day of experi-
Preparation of goldfish pituitary cells. Goldfish pituitary cells were prepared by trypsin-DNase digestion method (6), with minor modifications (28). Briefly, pituitaries were excised from the goldfish and diced in 0.6-mm fragments with a McIlwain tissue chopper (Mickle Lab Eng, Gomshall, UK). After that, pituitary fragments were digested with trypsin (25 mg/ml; GIBCO-BRL, Grand Island, NY) for 35 min at 28°C, followed by treatment with soybean trypsin inhibitor (25 mg/10 ml; Sigma). Pituitary fragments were then washed in Ca2+-free M199 (pH 7.2; Gibco-BRL) supplemented with 0.3% BSA and 0.1 mg/ml DNase II (Sigma) and dispersed by trituration using a DPTP transfer pipet (Bio-Rad, Richmond, CA). Pituitary cells were harvested by filtration through a sterile nylon mesh with 30 μm pore size, followed by centrifugation at 200 g for 10 min at 4°C. The cell pellet was resuspended in Ca2+-free M199, and total cell yield and percentage viability were estimated by cell counting in the presence of trypan blue (GIBCO-BRL). The average cell yield was 0.5–0.7 × 10⁶ cells/pituitary, with a mean viability of 96.2 ± 0.6% (n = 32).

GH release in pituitary cells under column perfusion. After cell counting, goldfish pituitary cells were cultured with Cytodex beads (Amersham Pharmacia, Piscataway, NJ) in Ca2+-containing M199 (pH 7.2) supplemented with 1% (vol/vol) horse serum (GIBCO-BRL). On the following day, Cytodex beads with pituitary cells attached were loaded into 0.5 ml microcolumns (∼2.5 × 10⁶ cells/column) and perfused at a flow rate of 15 ml/h (28°C) in ACUSYST-S Perifusion System (Cellex Biosciences, Minneapolis, MN) with M199 (pH 7.2) containing 0.1% BSA. Before sample collection, pituitary cells were perfused for 4 h to establish a stable baseline for GH release in the absence of exogenous stimulation. After that, test substances were introduced into individual columns from drug reservoirs through a three-way stopcock. Perifuse samples were collected in 5-min fractions and stored frozen until their GH contents were measured by a radioimmunoassay previously validated for goldfish GH (35).

GH mRNA expression in static cultures of pituitary cells. For static incubation experiments, pituitary cells were cultured in 24-well clustered plates precoated with 0.1 mg/ml poly-d-lysine (Roche, Mannheim, Germany) at a density of 2.5 × 10⁶ cells/well in M199 with 1% (vol/vol) horse serum. After overnight incubation, drug treatment was initiated by replacing the old medium with M199 containing 1% BSA and test substances at appropriate concentrations. Unless stated otherwise, the duration of drug treatment was fixed at 48 h. After that, total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA), heat denatured at 70°C, and vacuum-blotted onto a positively charged nylon membrane using a Bio-Dot SF Microfiltration Unit (Bio-Rad). Membrane hybridization was performed as described previously (61) using a digoxigenin (DIG)-labeled GH cDNA probe. The probe was prepared using a DIG High Prime Labeling kit (Roche) with a KpnI/Smal restricted fragment of goldfish GH-I gene (GenBank no. AF069398). Hybridization signals were visualized using a DIG Luminescent Detection kit (Roche) and quantified with IC440 Digital Science Image Station (Eastman Kodak, New Haven, CT). In these experiments, parallel probing of β-actin mRNA was also conducted to serve as an internal control.

Fig. 2. Inhibitory action and receptor selectivity of NE on GH transcription expression in goldfish pituitary cells. A: time course of NE treatment on GH mRNA expression. Pituitary cells were incubated with NE (1 μM) for 12, 24, and 48 h, respectively. Parallel treatment with forskolin (10 μM) was used as a positive control. B: dose dependence of adrenergic stimulation on GH mRNA expression. Pituitary cells were treated for 48 h with increasing doses (1 nM to 10 μM) of NE or the α2-agonist clonidine. C: effects of α1-, α2-, and β-adrenergic stimulation on GH mRNA expression. Pituitary cells were incubated for 48 h with the α1-agonist phenylephrine (5 μM), α2-agonist clonidine (5 μM), and β-agonist isoproterenol (5 μM), respectively. D: effect of α2-agonist on NE inhibition of GH mRNA expression. Pituitary cells were exposed to NE (5 μM) for 48 h in the presence or absence of the α2-antagonist yohimbine (10 μM). In these studies, total RNA was isolated following drug treatment, and GH mRNA was quantified using a slot-blot assay, as described in MATERIALS AND METHODS. Parallel measurement of β-actin mRNA was used as an internal control. Data presented (means ± SE, n = 8) are pooled results from 4 experiments. Treatment groups denoted by different letters represent a significant difference at P < 0.05 [ANOVA followed by Fisher’s least significant difference (LSD) test].
Data transformation and statistics. For pituitary cell perifusion, GH data (in ng/ml) from individual columns were expressed as a percentage of the mean GH contents in the first six fractions collected at the beginning of experiment prior to drug treatment. The transformation (as ”%basal”) was performed to allow for pooling of data from separate columns without distorting the kinetic profile of GH secretion during the course of perifusion. GH responses were quantified by calculating the net change of GH release after the drug treatment (i.e., a net change in area under the curve). In the case of static incubation experiments, GH mRNA in individual wells were measured in terms of arbitrary density units and normalized against \(H_9252\)-actin mRNA expression in the same sample. Since no significant changes could be noted in \(H_9252\)-actin mRNA levels, the normalized data were simply transformed as a percentage of the mean value in the control group (as ”%control”). Data presented were analyzed with Student’s \(t\)-test or ANOVA followed by Fisher’s least significance difference test. Differences between groups were considered significant when \(P < 0.05\).

**RESULTS**

**GnRH potentiation of GH rebound following NE inhibition.**

To study the functional interactions of GnRH and NE on the kinetics of GH release at the pituitary level, goldfish pituitary cells were exposed to continuous perifusion of NE in the presence or absence of the GnRH superagonist sGnRHa (1 \( \mu \)M; A), the AC activator forskolin (5 \( \mu \)M; B), the PKC activator TPA (0.1 \( \mu \)M; C), or the Ca\(^{2+}\) ionophore A23187 (5 \( \mu \)M; D). After drug treatment, total RNA was isolated for the measurement of GH mRNA. Transcript expression of \(\beta\)-actin was also monitored to serve as an internal control. Data presented, expressed as means ± SE (\(n = 8\)), are pooled results from 4 experiments. Treatment groups denoted by different letters represent a significant difference at \(P < 0.05\) (ANOVA followed by Fisher’s LSD test).

Data transformation and statistics. For pituitary cell perifusion, GH data (in ng/ml) from individual columns were expressed as a percentage of the mean GH contents in the first six fractions collected at the beginning of experiment prior to drug treatment. The transformation (as ”%basal”) was performed to allow for pooling of data from separate columns without distorting the kinetic profile of GH secretion during the course of perifusion. GH responses were quantified by calculating the net change of GH release after the drug treatment (i.e., a net change in area under the curve). In the case of static incubation experiments, GH mRNA in individual wells were measured in terms of arbitrary density units and normalized against \(H_9252\)-actin mRNA expression in the same sample. Since no significant changes could be noted in \(H_9252\)-actin mRNA levels, the normalized data were simply transformed as a percentage of the mean value in the control group without drug treatment (as "%control"). Data presented were analyzed with Student’s \(t\)-test or ANOVA followed by Fisher’s least significance difference test. Differences between groups were considered significant when \(P < 0.05\).
reduced after 48-h incubation with NE (1 μM), and shorter duration of drug treatment (12 or 24 h) was not effective in this regard (Fig. 2A). In this experiment, forskolin (10 μM) was used as a positive control, and activation of adenylate cyclase (AC) consistently induced GH mRNA expression in a time-dependent manner.

By fixing the duration of drug treatment at 48 h, increasing levels of NE (1 nM to 10 μM) reduced GH mRNA expression in a dose-related fashion, with effective concentrations in the nanomolar dose range (Fig. 2B). The dose dependence of NE inhibition was mimicked by the α2-agonist clonidine (1 nM to 10 μM), whereas the α1-agonist phenylephrine (5 μM) and β-agonist isoproterenol (5 μM) were not effective in this respect (Fig. 2C). The inhibitory effect of NE (5 μM) could be blocked by the α2-antagonist yohimbine (10 μM; Fig. 2D) but not by a similar dose of the α1-antagonist prazosin or β-antagonist propranolol (data not shown). These results indicate that NE inhibits basal levels of GH gene expression through activation of pituitary α2-adrenoceptors.

To examine the functional interactions between GnRH and NE, GnRH-induced GH gene expression was tested in the presence of the α2-agonist clonidine (Fig. 3A). In goldfish pituitary cells, basal levels of GH transcripts were elevated by 48-h incubation with sGnRHa (1 μM). This stimulatory effect,
however, could be abolished by simultaneous treatment with clonidine (5 μM). To shed light on the signaling mechanisms for α₂-inhibition, pituitary cells were treated with clonidine in the presence of pharmacological agents known to activate the cAMP-, PKC-, and Ca²⁺-dependent cascades (Fig. 3, B–D). In this case, GH mRNA levels were upregulated by 48-h incubation with the AC activator forskolin (5 μM), PKC activator TPA (0.1 μM), and Ca²⁺ ionophore A23187 (5 μM). Similar to the study with sGnRHa, clonidine (5 μM) reduced basal and blocked the stimulatory effects of these pharmacological agents on GH mRNA expression. In our validation studies, GH mRNA levels and total GH production were not affected by short-term incubation with NE (1 μM) and sGnRHa (1 μM) for 1 and 2 h, respectively (data not shown).

Mechanisms to potentiate GH rebound after NE inhibition. To examine the signaling mechanisms that may contribute to the potentiating effect on GH rebound after NE inhibition, pituitary cells were exposed to a 5-min pulse of the activators during the continuous perifusion of NE. In the control group, activation of AC by forskolin (5 μM) induced a rapid rise in GH release (Fig. 4A). This stimulatory effect was inhibited by simultaneous perifusion with NE alone were used as the control groups. Data presented (means ± SE, n = 8) are pooled results from 4 experiments. Drug treatments giving a similar magnitude of GH responses are denoted by the same letter (P > 0.05, Student’s t-test).

Fig. 5. Prior stimulation with A23187 and Bay K8644 on GH rebound following NE inhibition. Pituitary cells were treated with a 5-min pulse of the Ca²⁺ ionophore A23187 (10 μM; A) or L-type voltage-sensitive Ca²⁺ channels (VSCC) activator Bay K8644 (10 μM; B) during the 1.5 h of continuous perifusion with NE (1 μM). In these studies, the 5-min pulse of drug treatment (without NE perifusion) and continuous perfusion with NE alone were used as the control groups. Data presented (means ± SE, n = 8) are pooled results from 4 experiments. Drug treatments giving a similar magnitude of GH responses are denoted by the same letter (P > 0.05, Student’s t-test).
was observed after NE withdrawal with prior exposure to forskolin. Similar to the experiments with forskolin, a 5-min pulse of TPA (50 nM) was also effective in triggering GH release, and this GH response could be reduced by NE perfusion (Fig. 4B). Furthermore, prior exposure to the PKC activator TPA also induced a marked increase in GH rebound (~10.3-fold) after NE inhibition. In parallel experiments to test the possible role of Ca\(^{2+}\)-dependent mechanisms, pituitary cells were challenged with a 5-min pulse of either the Ca\(^{2+}\)-ionophore A23187 (10 \mu M; Fig. 5A) or the L-type VSCC activator Bay K8644 (10 \mu M; Fig. 5B). In both cases, rapid elevations in basal GH release were observed. Interestingly enough, the GH-releasing action of Bay K8644 could be blocked by NE treatment, but the GH responses induced by A23187 were not affected. Apparently, prior exposure to A23187 or Bay K8644 did not alter the GH bound observed following NE withdrawal.

**Mechanisms for GH release during the rebound phase after GnRH potentiation.** To elucidate the signaling mechanisms involved in GH release during the rebound phase after GnRH potentiation, pituitary cells were challenged with a 5-min pulse of sGnRHa (100 nM) during the continuous perfusion with NE (1 \mu M). After that, pharmacological agents known to perturb cAMP-, PKC-, and Ca\(^{2+}\)-dependent cascades were then applied right after NE withdrawal. In these experiments, the kinetics of GH release during the rebound phase were not affected by simultaneous treatment with the AC inhibitor SQ22536 (0.1 mM; Fig. 6A) or PKA inhibitor H89 (10 \mu M; Fig. 6B). However, treatment with Ca\(^{2+}\)-free medium (Fig. 7A) or with normal medium containing the VSCC blocker nifedipine (10 \mu M; Fig. 7B) or CaMKII inhibitor KN93 (4 \mu M; Fig. 7C) could suppress the GH rebound with GnRH potentiation. Similar inhibition was also noted with the PKC inhibitor calphostin C (1 \mu M; Fig. 8A) or by PKC desensitization induced by TPA pretreatment (100 nM; Fig. 8B). The procedures for PKC desensitization have been previously shown to deplete PKC content and suppress GnRH-induced GTH release in goldfish pituitary cells (22). To evaluate the functional relationship between PKC- and Ca\(^{2+}\)-dependent pathways, potentiation of GH rebound was induced by a 5-min pulse of TPA (50 nM) given during continuous perfusion of NE (1 \mu M). After that, GH rebound was tested in the presence of nifedipine (10 \mu M) and KN93 (4 \mu M), respectively. Similar to the studies with sGnRHa, GH rebound with TPA potentiation was significantly reduced by simultaneous treatment with the blocker for VSCC (Fig. 9A) or CaMKII (Fig. 9B).

**DISCUSSION**

Unlike mammals, in which modulation of GH release occurs mainly in the hypothalamus by altering GHRH and SRIF secretion, GH release in bony fish is regulated at the pituitary level by a multitude of neuroendocrine factors via direct innervation of the pars distalis. This phenomenon forms the basis of the “multifactorial” model for GH regulation in teleosts (for a recent review, see Ref. 59). Among the regulators for GH release reported in fish models, GnRH and catecholamines are particularly interesting. In the goldfish, the native forms of GnRH, namely salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), can induce GH secretion both in vivo (34) and in vitro (8). In the same animal model, GnRH-induced GH release also contributes to the parallel rises in GH and GTH-II levels observed during the preovulatory phase of the reproductive cycle (42). Similar to GnRH, catecholamines are the key components of the regulatory mechanisms for GH secretion. Dopamine, the precursor for NE and EP, can stimulate GH release (56) and body growth (57) in the goldfish by acting through pituitary D\(_1\) receptors (55). In contrast, NE and EP suppress GH secretion via \(\alpha_2\)-adrenergic receptors (28), and \(\alpha_2\)-agonists (e.g., clonidine) can block the GH-releasing effects of sGnRH and cGnRH-II, respectively (60). In our previous studies, a GH rebound was noted after NE withdrawal, and this GH rebound could be markedly enhanced by prior exposure to sGnRH (28) and cGnRH-II (60). In this study, potentiation of GH rebound following NE inhibition was established in gold-

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**Fig. 6.** Inactivation of cAMP-dependent mechanisms on GnRH potentiation of GH rebound. Pituitary cells were treated with a 5-min pulse of sGnRHa (100 nM) during 1.5 h of continuous perfusion with NE (1 \mu M). The AC inhibitor SQ22536 (0.1 mM; A) and PKA inhibitor H89 (10 \mu M; B) were applied right after the termination of NE perfusion. In these experiments, continuous perfusion with M199 after NE withdrawal was used as the solvent control. Data presented (means ± SE, \(n = 8\)) are pooled results from 4 experiments. Treatments giving a similar magnitude of GH responses are denoted by the same letter (\(P > 0.05\), Student’s \(t\)-test).
Fish pituitary cells by use of a perifusion approach with the GnRH superagonist sGnRHa as a stimulant. In this case, sGnRHa faithfully mimicked the GH-releasing actions of sGnRH and cGnRH-II. Furthermore, NE perifusion inhibited both basal and sGnRH-induced GH release, whereas the GH rebound was enhanced in a dose-related fashion by sGnRHa treatment. These results confirm that adrenergic input at the pituitary level can interact with GnRH to regulate GH release in a complex manner. Apparently, NE can act as a negative regulator to suppress GnRH stimulation on somatotrophs, whereas GnRH can positively modulate the “off-response” of NE inhibition.

In representative models of bony fish, GnRH receptors can be located in somatotrophs [e.g., goldfish (11)] and functionally coupled to the cAMP- [e.g., tilapia (17)], PKC- [e.g., salmon (2)], and Ca²⁺/CaM-dependent cascades [e.g., goldfish (8)]. To evaluate the possible involvement of these pathways in GnRH potentiation of GH rebound, a pharmacological approach was used in our perifusion studies. In this case, the potentiation effect of sGnRHa was mimicked by activating AC and PKC with forskolin and TPA respectively, whereas Ca²⁺ entry induced by A23187 or VSCC activation using Bay K8644 was not effective in this regard. These results indicate that activation of cAMP- and PKC-dependent cascades during NE inhibition, but not induction of Ca²⁺ entry through VSCC, can trigger potentiation of GH rebound following NE withdrawal. Similar to sGnRHa, the GH responses induced by forskolin, TPA, and Bay K8644 could be suppressed by NE perifusion, suggesting that NE may interfere with the respective pathways to inhibit both basal and stimulated GH secretion induced by GH-releasing factors. Unlike Bay K8644, the GH-releasing action of the Ca²⁺ ionophore A23187 was not affected by NE treatment. These observations raise the possibility that the inhibitory action of NE is acting at the level of VSCC but not on the downstream events after Ca²⁺ entry. In the goldfish, Ca²⁺ currents with properties typical of L-type VSCC have been reported in the pituitary (43). Besides, PKC inactivation (7) and VSCC blockade (21) attenuate the GH-releasing effects of sGnRH and cGnRH-II, whereas PKA inhibition can reduce basal levels of GH secretion (55). At the pituitary level, NE inhibits GH release with a concurrent drop in cAMP production via activation of α²-adrenoreceptors (60). In mammals, AC inactivation and inhibition of VSCC currents are known to be the key elements of the signaling mechanisms for α₂-adrenoreceptors (48). In some model systems, e.g., brain slices of mouse amygdala (13), VSCC inhibition caused by α₂-adrenergic stimulation can be correlated with a rapid activation of inwardly rectifying K⁺ channels, suggesting that membrane hyperpolarization may also play a role in α₂-modulation of VSCC currents.

Fig. 7. Inactivation of Ca²⁺-dependent mechanisms on GnRH potentiation of GH rebound. Pituitary cells were treated with a 5-min pulse of sGnRHa (100 nM) during 1.5 h of continuous perifusion with NE (1 μM). After that, Ca²⁺-free medium with 0.1 mM EGTA (A) or normal medium containing either the L-type VSCC blocker nifedipine (10 μM; B) or CaMKII inhibitor KN93 (4 μM; C) was applied right after the termination of NE perifusion. In these studies, M199 was used as a solvent control for Ca²⁺-free medium, whereas M199 with 0.1% DMSO was used for the experiments with nifedipine and KN93. Data presented (means ± SE, n = 8) are pooled results from 4 experiments. Drug treatments giving a similar magnitude of GH responses are denoted by the same letter (P > 0.05, Student’s t-test).
In the present study, GH release during the rebound phase after sGnRHa potentiation was markedly suppressed by removal of extracellular Ca\(^{2+}\), VSCC blockade by nifedipine, CaMK-II inhibition by KN93, inactivation of PKC with calphostin C, and PKC desensitization induced by TPA pretreatment. The GH rebound, however, was not affected by inhibiting AC and PKA with SQ22536 and H89, respectively. In our previous studies, we have shown that PKA inhibitors (e.g., H89) had no effects on GnRH-induced GH secretion (58), and GnRH did not alter cAMP production in goldfish pituitary cells (10). These results, as a whole, indicate that GH release during the rebound phase does not involve a cAMP/PKA component and is mediated by PKC and Ca\(^{2+}\)/CaMKII-dependent mechanisms. Since the GH rebound with TPA potentiation was also sensitive to inhibition of VSCC and CaMKII, it would be logical to assume that the Ca\(^{2+}\) influx through VSCC and subsequent CaMKII activation are downstream events occur-

Fig. 8. Inactivation of PKC on GnRH potentiation of GH rebound. Pituitary cells were treated with a 5-min pulse of sGnRH\(_{a}\) (100 nM) during 1.5 h of continuous perifusion with NE (1 \(\mu\)M). A: after that, the PKC inhibitor calphostin C (1 \(\mu\)M) was applied following the termination of NE treatment. Perifusion with M199 containing 0.1% DMSO was used as the solvent control for calphostin C. B: in parallel experiments, pituitary cells were incubated with TPA (100 nM) for 4 h to induce PKC desensitization prior to the perifusion experiments with sGnRHa and NE. In this case, the GH rebound was examined with continuous perifusion with M199. Data presented (means ± SE, \(n = 8\)) are pooled results from 4 experiments. Drug treatments giving a similar magnitude of GH responses are denoted by the same letter (\(P > 0.05, \text{ Student's } t\)-test).

In the present study, GH release during the rebound phase after sGnRHa potentiation was markedly suppressed by removal of extracellular Ca\(^{2+}\), VSCC blockade by nifedipine, CaMK-II inhibition by KN93, inactivation of PKC with calphostin C, and PKC desensitization induced by TPA pretreatment. The GH rebound, however, was not affected by inhibiting AC and PKA with SQ22536 and H89, respectively. In our previous studies, we have shown that PKA inhibitors (e.g., H89) had no effects on GnRH-induced GH secretion (58), and

Fig. 9. Inactivation of Ca\(^{2+}\)-dependent mechanisms on TPA potentiation of GH rebound. Cells were treated with a 5-min pulse of TPA (50 nM) during the 1.5 h of continuous perifusion with NE (1 \(\mu\)M). After that, the VSCC blocker nifedipine (10 \(\mu\)M; A) and CaMKII inhibitor KN93 (4 \(\mu\)M; B) were applied right after the termination of NE perifusion. In these experiments, perifusion with M199 containing 0.1% DMSO was used as the solvent control. Data presented (means ± SE, \(n = 8\)) are pooled results from 4 experiments. Treatments giving a similar magnitude of GH responses are denoted by the same letter (\(P > 0.05, \text{ Student's } t\)-test).
ring after PKC activation. It is well-documented that VSCC phosphorylation by protein kinases can serve as an effective mechanism to regulate intracellular Ca\(^{2+}\) signaling (4). In mammalian cell models (e.g., cardiac myocytes), L-type VSCC can be activated by diacylglycerol (19) but inhibited by blocking PKC activity (27). Using a biochemical approach, the \(\alpha_1\) and \(\beta_2\)-subunits of VSCC have been confirmed to be the substrates for PKC phosphorylation (44), and VSCC phosphorylation by PKC can be reverted by protein phosphatase 2C (29). In the goldfish, multiple forms of PKC, including the conventional (\(\alpha\) and \(\beta\)), novel (\(\delta\) and \(\theta\)), and atypical isoforms (\(\zeta\)), are expressed in the anterior pituitary (26). In goldfish pituitary cells, PKC activators (e.g., TPA and DiC8) can enhance the GH-releasing effect of Bay K8644 (58), whereas blockade of VSCC alleviates the GH responses induced by PKC activation (7), confirming that a cross talk between PKC and VSCC also exists in fish model. Using Ca\(^{2+}\) imaging in goldfish pituitary cells, a transient rise in intracellular Ca\(^{2+}\) was also noted in identified somatotrophs after removal of NE treatment, overlapping with the rebound phase of GH secretion (60). These findings, taken together, have prompted us to speculate that PKC activation induced by GnRH treatment, despite its GH-releasing effect, is inhibited by NE perifusion and can enhance Ca\(^{2+}\) influx via VSCC following NE withdrawal, and subsequent CaMKII activation may contribute to the potentiating effect on GH secretion during the “postinhibition” GH rebound.

In mammals, GH rebound following SRIF withdrawal has been reported in the rat (52), dog (46), and human (3). This GH rebound is Ca\(^{2+}\)-dependent (36) and has been proposed to be involved in the maintenance of GH pulsatility in vivo (5). Since SRIF blocks GH release without concurrent inhibition on GH synthesis, the GH rebound has been attributed to a buildup of GH stores in individual somatotrophs during the period of SRIF inhibition (52). In teleosteus, e.g., tilapia (38), salmon (41), and common carp (30), GnRH is known to stimulate GH synthesis by acting at the pituitary level. Recently, upregulation of GH mRNA levels in goldfish pituitary cells has been reported following treatment with sGnRH and cGnRH-II, respectively (25). These findings raise the possibility that GH synthesis induced by GnRH, by increasing pituitary GH contents, may contribute to GnRH potentiation of GH rebound following NE inhibition. This hypothesis, however, is not supported by the results of our static incubation experiments. In this case, basal levels of GH transcripts were suppressed by prolonged incubation (48 h) with NE through activation of pituitary \(\alpha_2\)-adrenoreceptors. Similar to the results of GH secretion, GH mRNA expression induced by sGnRH could be blocked by the \(\alpha_2\)-agonist clonidine. To our knowledge, \(\alpha_2\)-inhibition of GH gene expression has not been reported previously, and the present study represents the first to describe the functional interactions between NE and GnRH acting at the pituitary level to regulate GH synthesis. Since clonidine was also effective in blocking the stimulatory effects of forskolin, TPA, and A23187 on GH mRNA expression, it is conceivable that \(\alpha_2\)-inhibition of GH gene expression is caused by inactivation or perturbations of the cAMP-, PKC-, and Ca\(^{2+}\)/CaM-dependent pathways. In this study, short-tern incubation (1 and 2 h) with NE and sGnRHa did not alter GH mRNA levels in goldfish pituitary cells. These results indicate that GnRH potentiation could not be due to a buildup of pituitary GH stores by stimulating GH gene expression during the period of NE treatment.

In summary, using goldfish as a model for modern-day bony fish, we have demonstrated that adrenergic stimulation can inhibit both basal and GnRH-stimulated GH release and GH gene expression by acting at the pituitary cell level. In accordance with our previous studies, these inhibitory actions are mediated by \(\alpha_2\)-adrenoreceptors, and GnRH can markedly enhance the “postinhibition” GH rebound following removal of NE treatment. The potentiating effect of GnRH is not caused by elevation in GH synthesis. Apparently, PKC activation is involved in the triggering of GnRH potentiation, which may enhance Ca\(^{2+}\) entry via VSCC during NE withdrawal and induce subsequent activation of CaMKII to increase GH exocytosis. The postinhibition GH rebound and its potentiation by GnRH may serve as a novel mechanism to modulate the functional interactions between stimulatory and inhibitory input for GH regulation at the pituitary level. As mentioned earlier, adrenergic input from peripheral tissues (i.e., NE and EP released from chromaffin cells) is a part of the stress responses in vertebrates (45). The present demonstration of adrenergic inhibition of GH synthesis and secretion in goldfish may represent a new facet of the mechanisms responsible for the adverse effects of environmental stress on body growth and metabolism in fish species.

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REFERENCES


