0013-7227/98/$03.00/0
Endocrinology
Copyright © 1998 by The Endocrine Society
Vol. 139, No. 8
Printed in U.S.A.

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*

A. O. L. WONG, M. Y. LEUNG, W. L. C. SHEA, L. Y. TSE, J. P. CHANG, AND B. K. C. CHOW

Department of Zoology, University of Hong Kong, Hong Kong; and the Department of Biological Sciences, University of Alberta (J.P.C.), Edmonton, Alberta T6G 2E9, Canada

ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the glucagon/secretin peptide family, and its molecular structure is highly conserved in vertebrates. In this study, the functional role of PACAP in regulating GH release in the goldfish was investigated. Using immunohistochemical staining, nerve fibers with PACAP immunoreactivity were identified in the vicinity of goldfish somatotrophs, suggesting that this neuropeptide may influence GH release in the goldfish. The direct regulatory action of PACAP on GH secretion was demonstrated *in vitro* in perifused goldfish pituitary cells. PACAPs (0.01 nM to 1 μM) from different species, including ovine $PACAP_{27}$, ovine $PACAP_{38}$, frog $PACAP_{38}$, zebra fish $PACAP_{27}$, and zebra fish $PACAP_{38}$, were all effective in stimulating GH release with ED_{50} values of 8.9 ± 3.5, 3.3 ± 1.6, 14.4 ± 3.5, 15.4 ± 4.1, and 1.4 ± 0.2 nM, respectively. Similar concentrations of vasoactive intestinal polypeptide (VIP), a peptide related to PACAP, was not effective in this respect. In addition, the GH-releasing action of ovine $PACAP_{38}$ (10 nM) was inhibited by the PACAP antagonist $PACAP_{6-38}$ (10 μM), but not by the VIP antagonist [4-Cl-D-Phe^{6},Leu^{17}]VIP (10 μM). The pharmacology of these GH responses is consistent with the mammalian type I PACAP receptors, suggesting that a similar receptor subtype is present in the goldfish pituitary and mediates the GH-releasing action of PACAP. To establish the structural identity of this goldfish PACAP receptor, a complementary DNA (cDNA) clone sharing a high degree of sequence homology with mammalian type I PACAP receptors was isolated from a goldfish pituitary cDNA library. This cDNA was 5.2 kb in size with a 1.4-kb open reading frame and encoded a 465-amino acid protein with the typical structure of a 7-transmembrane domain-containing, G protein-coupled receptor. Functional expression of this cDNA in COS-7 cells revealed that this fish type I PACAP receptor could be activated by ovine $PACAP_{27}$ and $PACAP_{38}$ to increase cAMP synthesis with ED_{50} values of 2.4 ± 0.8 and 4.2 ± 1.2 nM, respectively. Other structurally related peptides, including VIP (100 nM), GH-releasing hormone (100 nM), glucagon (100 nM), secretin (100 nM), gastric inhibitory polypeptide (100 nM), and PTH (100 nM), were not effective in altering cAMP production. Using Northern blot and RT-PCR, messenger RNA transcripts of this PACAP receptor were identified in the brain, heart, and pituitary of the goldfish. These results, taken together, support the hypothesis that PACAP functions as a novel GH-releasing factor in the goldfish through activation of type I PACAP receptors. (*Endocrinology* **139:** 3465–3479, 1998)

PITUITARY adenylate cyclase-activating polypeptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family (1). In mammals, two biologically active forms of PACAP have been purified, namely $PACAP_{38}$ (2) and $PACAP_{27}$ (3). $PACAP_{38}$ is a 38-amino acid peptide with its N-terminal 28 amino acids sharing 68% sequence identity with VIP, and $PACAP_{27}$ is a truncated form of $PACAP_{38}$ containing only the first 27 amino acids. These 2 peptides are derived from the same precursor prepro-PACAP through posttranslational proteolysis and alternative α-amidation (1). In the rat, immunoreactivity for PACAP has been identified in the brain, intestine, adrenal gland, and testes (4), and $PACAP_{38}$ was the predominant form (5). In general, PACAP is considered a neurotransmitter/neuromodulator in the central nervous system (6), although its roles as a growth factor (7) and vasoregulator (8) have also been suggested.

The molecular structure of PACAP is highly conserved among vertebrates. PACAP complementary DNAs (cDNAs) have been cloned in the rat (9), sheep (10), mouse (11), and human (12), and the deduced amino acid sequences of $PACAP_{38}$ were identical. PACAP has also been isolated from amphibian (13), chicken (14), and teleost fish tissues (15). $PACAP_{38}$ from the frog has only one amino acid substitution at position 35, with isoleucine substituted for valine in rat $PACAP_{38}$ (13). PACAP cDNAs from the salmon (16) and catfish (17) reveal that the fish $PACAP_{38}$ is highly homolo-

Received November 24, 1997.

Address all correspondence and requests for reprints to: Dr. Anderson O. L. Wong, Department of Zoology, University of Hong Kong, Pokfulam Road, Hong Kong.

* Part of the present study was presented in the 10th International Congress of Endocrinology, San Francisco, CA, June 12–15, 1996, and the 2nd International Union of Biological Sciences Symposium on Advances in Molecular Endocrinology of Fish, Toronto, Canada, May 17–19, 1997. This work was supported by Research Grant Council Grants (Hong Kong; to A.O.L.W. and B.K.C.C.) and the Department of Zoology (Hong Kong University; to M.Y.L., L.Y.T., and W.L.C.S.) in the form of teaching assistantship.

gous to the mammalian form and has only three or four amino acid substitutions in the C-terminal. Furthermore, these fish PACAP cDNAs also contain the coding sequence of a GH-releasing hormone (GHRH)-like peptide, and differential expression of PACAP and this GHRH-like peptide as a result of exon skipping has been reported in salmons (18). In general, it is believed that the two separate genes encoding PACAP and GHRH in mammals are the result of gene duplication during evolution from fish to tetrapods (16, 17). Recently, $PACAP_{27}$ has been identified in the neural gland of tunicates (19), suggesting that PACAP may be a neuropeptide in protochordates.

In mammals, the biological actions of PACAP are mediated through two different receptor subtypes, namely type I and type II PACAP receptors (1). Both of them are G protein-coupled receptors with a classical structure of seven transmembrane domains (TMD). Type I PACAP receptors (or PVR1 receptors) exhibit a high binding affinity for $PACAP_{38}$ and $PACAP_{27}$, but not for VIP. Type II PACAP receptors, in contrast, have equal binding affinities for $PACAP_{38}$, $PACAP_{27}$, and VIP. Therefore, this group of PACAP receptors is also referred to as VIP receptors in some studies (20, 21). Type II PACAP receptors can be further subdivided into VIP1 (or PVR2 receptors) and VIP2 receptors (or PVR3 receptors), which are encoded by different genes, respectively (for a recent review on PACAP receptors, see Ref. 22).

In recent years, the role of PACAP as a hypophysiotropic factor in mammals has been proposed based on the findings that PACAP nerve fibers are present in the median eminence (23), PACAP immunoreactivity can be detected in the hypophysial portal blood (24), and PACAP stimulates cAMP production in pituitary cell cultures (2, 3) and under certain conditions induces LH, FSH, GH, and ACTH release from rat pituitary cells (25). Regarding the GH-releasing action, PACAP is a weak (25) or modest stimulator (26) of GH release. In general, it is not considered to be a typical GH-releasing factor but, rather, a modulator of pituitary hormone secretion (22). In the rat, PACAP-stimulated GH release from pituitary cells is more readily observed using a perifusion system (2) or a reverse hemolytic plaque assay (27). In perifused GH_3 cells, a rat pituitary cell line, the stimulatory action of PACAP on GH release is mediated through type II PACAP receptors (28). Results from static incubation studies, however, are more variable; no effects (2) as well as a modest stimulation after a long incubation (25) have been reported. In *in vivo* studies, PACAP increases plasma GH levels in the rat (29), but not in the sheep (30) or human (31), suggesting that the GH-releasing effect of PACAP is species specific. In the ewe, injection of PACAP into the brain induces a paradoxical inhibition of GH release (30). These results indicate that PACAP may also act centrally to regulate GH secretion.

In teleost fishes, the brain-pituitary axis is unique for the lack of a hypophysial portal blood system (32). Unlike that in mammals, the median eminence of teleosts is functionally incorporated into the anterior pituitary, and individual pituitary cells are directly innervated by neuronal fibers from the hypothalamus (33). Neuropeptides (*e.g.* GnRH, TRH, and neuropeptide Y) as well as neurotransmitters (*e.g.* dopamine), besides acting within the hypothalamus, exert their regulatory actions on GH release directly at the pituitary cell level (for a recent review, see Ref. 34). In lower vertebrates, especially in fish, the studies of PACAP have focused mainly on the structural evolution of the peptide, and very little is known about its biological functions. Whether PACAP is a hypophysiotropic factor in teleosts is unclear, as neither the delivery of PACAP nor the presence of PACAP receptors has been demonstrated in the pituitary of fish species. In this study, the role of PACAP as a potential hypophysiotropic factor regulating GH release in goldfish via activation of pituitary type I PACAP receptors was examined.

Materials and Methods

Animals

Goldfish (*Carassius auratus*) of the common or comet variety with body weight ranging from 25–35 g were purchased from local pet stores and maintained in 200 liters aquaria at 18 C under a 12-h light, 12-h dark photoperiod for 2 weeks before experiments. The fish were fed to satiation daily with commercial fish feed. As the fish used in this study were in the early stages of gonadal recrudescence, and sexual dimorphism was not apparent, goldfish of mixed sexes were used for the preparation of pituitary cells and extraction of tissue messenger RNA (mRNA).

Reagents and test substances

$PACAP_{6-38}$, [4-Cl-D-Phe^{6},Leu^{17}]VIP, frog $PACAP_{38}$, ovine $PACAP_{27}$, and $PACAP_{38}$ were obtained from Peninsula Laboratories (Belmont, CA). Zebra fish $PACAP_{27}$ and $PACAP_{38}$ were gifts from Dr. S. Mojsov (Rockefeller University, New York, NY). PACAP-related peptides, including glucagon, secretin, GHRH, PTH, VIP, and gastric inhibitory polypeptide (GIP), were purchased from Bachem Fine Chemicals (La Jolla, CA). All of these peptides were dissolved in doubled distilled water and stored frozen as 1-mM stocks at −20 C. Subsequent dilution to appropriate concentrations with culture medium was performed 15 min before drug treatment. Ionomycin obtained from Calbiochem (La Jolla, CA) and 1-methyl-3-iso-butylxanthine (IBMX) from Research Biochemical International (Natick, MA) were stored as 10-mM and 1-M stocks in dimethylsulfoxide, respectively. The final level of dimethylsulfoxide in culture medium was always 0.1% or less, and it did not alter basal GH release from goldfish pituitary cells.

Column perifusion of goldfish pituitary cells

Dispersed goldfish pituitary cells were prepared by controlled trypsin/deoxyribonuclease digestion as described previously (35). The viability of pituitary cells was always 94% or more, as indicated by trypan blue exclusion test. After dispersion, pituitary cells were resuspended in plating medium (medium 199 with Earle's salts at pH 7.2, containing 26 mM $NaHCO_3$, 25 mM HEPES, 100,000 U penicillin/liter, 100 mg streptomycin/liter, and 1% horse serum) and incubated with preswollen Cytodex beads (Sigma Chemcial Co., St. Louis, MO) at 28 C under 5% CO_2 and saturated humidity. Cell attachment on Cytodex beads was greater than 95% after overnight incubation. Cytodex beads with pituitary cells attached were then transferred into 0.5-ml microcolumns (~2 million cells/column; ACUSYST-S System, Endotronics, Minneapolis, MN) for *in vitro* column perifusion. Pituitary cells were perifused at a flow rate of 15 ml/h with perifusion medium (medium 199 with Hanks' salts at pH 7.2 containing 26 mM $NaHCO_3$, 25 mM HEPES, 100,000 U penicillin/liter, 100 mg streptomycin/liter, and 0.1% BSA). After 3 h of continuous perifusion, GH release from pituitary cells remained relatively stable in the absence of stimulation. Test substances were then applied from a drug reservoir to the perifusion column through a three-way stopcock. Perifusate samples were collected in 5-min fractions and stored frozen at −20 C. GH contents in these samples were assayed using a RIA previously validated for goldfish GH (36).

Immunohistochemical staining of goldfish pituitary sections

Goldfish pituitaries were excised and fixed in Bouin's fixative at 4 C for 48 h. After washing in 70% ethanol to remove picric acid, these fixed

pituitaries were dehydrated with a graded series of ethanol and embedded in paraffin. Pituitary sections, 12 μm thick, were prepared and mounted on gelatin-coated slides. Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with minor modifications. Briefly, pituitary sections were deparaffinized, hydrated, and treated with 0.3% H_2O_2 to inactivate endogenous peroxidase activity. After washing in 10 mM PBS (pH 7.5) for 10 min, pituitary sections were incubated in a blocking agent containing BSA (0.5%), gelatin (0.5%), and normal goat serum (2.5%) for 30 min. After a 10-min wash in PBS, these pituitary sections were exposed to antisera specific for ovine $PACAP_{38}$ (1:2,000; IHC8920, Peninsula Laboratories) and goldfish GH (1:50,000), respectively. Incubation with these antisera was conducted in a humidified chamber for 24 h at 4 C. Pituitary sections were then treated with biotinylated goat antirabbit antibody for 30 min, followed by another 40-min incubation with the freshly prepared avidin-biotin-peroxidase complex reagent. After rinsing briefly in PBS, these pituitary sections were then exposed to diaminobenzidine (1.28 mM) in the presence of nickel ammonium sulfate (0.038 mM) for color development. In this study, the specificity of immunostaining was confirmed by three different approaches. Firstly, pituitary sections were incubated with either PBS or normal rabbit serum without PACAP antiserum. In both cases, no immunostaining was observed in the absence of the primary antibody. Secondly, a serial dilution of PACAP antiserum resulted in a gradual decrease and eventually a complete loss of immunoreactivity. Finally, the specificity of immunostaining was further confirmed by preabsorbing PACAP antiserum (1:2000) with ovine $PACAP_{38}$ (0.1 mM) for 24 h at 4 C. A total loss of PACAP immunoreactivity was observed after preabsorbing the antiserum with ovine $PACAP_{38}$.

Cloning of goldfish type I PACAP receptor cDNA

A partial cDNA clone corresponding to the coding sequence from TMD2 to TMD6 of the goldfish PACAP receptor was obtained using a two-step nested PCR approach. Total RNA was isolated from freshly excised goldfish pituitaries, and polyadenylated [poly(A)$^+$] mRNA was purified using a polyATract mRNA isolation kit (Promega, Madison, WI). First strand cDNA was prepared and used as the template for PCR amplification. The primers for PCR, including G2, G6, and G7, were designed according to the respective consensus regions of TMD2, TMD6, and TMD7 of mammalian PACAP receptors. The nucleotide sequences of G2, G6, and G7 are TGCAG/TTGG/TACA/C/T/GA/CGA/C/T/GAAG/TTAG/TATYCA, AGC/GGGGATC/GAGC/GA/GG/TA/C/T/GAGA/C/T/GGTGGAG/TTT, and TGC/GACCTCA/C/T/GCCA/GTTA/C/T/GAC/GA/GAAA/GCAA/GTA, respectively. The first PCR was performed using G2 and G7 as the primers, and a 1-μl sample of the resulting PCR products was reamplified in a second PCR using the nested primers G2 and G6. PCR products of 500–600 bp in size were purified and subcloned into PUC-18 for subsequent DNA sequencing. PCR amplifications were carried out according to the conditions reported previously (37). The partial cDNA clone of this PACAP receptor was used as a probe to screen a goldfish pituitary cDNA library, which was constructed using a ZAP-Express cDNA library system (Stratagene, Cambridge, UK). A full-length cDNA clone of a goldfish type I PACAP receptor was obtained and excised from the original phagemid to the pBK-cytomegalovirus (CMV) vector for DNA sequencing. This goldfish type I PACAP receptor cDNA was sequenced from both strands using a T7 sequencing kit (Pharmacia, Piscataway, NJ) by primer-walking and subcloning of restriction fragments. The DNA sequences obtained were analyzed using the computer program HIBIO MacDNasis 2.0 (Hitachi, Tokyo, Japan).

Functional expression of goldfish type I PACAP receptor

A 5.2-kb *Bam*HI/*Xho*I fragment of the goldfish PACAP receptor cDNA was subcloned into pBluescript SK$^+$ (Stratagene, La Jolla, CA). From this construct, a 5.2-kb *Not*I/*Apa*I fragment was excised and directionally inserted into the expression vector pRC-CMV (Invitrogen, San Diego, CA). This newly constructed expression vector, pRC-CMV/gf.PACAP.R, was used to transfect the mammalian cell line COS-7 by lipofection. After 2 weeks of G418 (500 μg/ml) selection, a permanent cell line, COS-gf.PACAP.R, with stable transfection of this goldfish PACAP receptor was obtained. To test for the functionality of the expressed PACAP receptors, the effects of PACAPs and its related peptides, including PTH, GIP, glucagon, secretin, GHRH, and VIP on cAMP synthesis in this transformed cell line were examined. These experiments were performed in the presence of 0.2 mM IBMX, a phosphodiesterase inhibitor, to prevent cAMP degradation. The procedures for functional expression of receptors and cAMP RIA have been reported previously (38).

Tissue distribution of goldfish PACAP receptor mRNA

Various tissues of the goldfish, including the heart, brain, pituitary, liver, gall bladder, gills, intestine, gonads, muscle, spleen, and kidney, were freshly excised and homogenized for the preparation of poly(A)$^+$ mRNA. About 3 μg mRNA from individual tissues were size-fractionated by electrophoresis in a denaturing agarose gel (1.2%) with formaldehyde (6.3%), followed by transblotting and UV cross-linking onto a Hybond N$^+$ membrane. A full-length cDNA of the goldfish PACAP receptor was labeled with [α-^{32}P]deoxy-ATP (3 μCi/mmol) using a Mega-primed DNA labeling kit (Amersham, Arlington Heights, IL). After overnight hybridization with the labeled probe at 65 C, the membrane was washed three times under high stringency conditions (0.1 × SSC-0.1% SDS) and exposed to BioMax film (Eastman Kodak, Rochester, NY) for 48 h at −80 C. To serve as an internal control, the membrane was stripped and reprobed with a ^{32}P-labeled partial cDNA (~200 bp) for goldfish β-actin. Tissue distribution of this goldfish PACAP receptor was further confirmed using RT-PCR. First strand cDNAs were prepared from tissue mRNA samples and used as the templates for PCR using specific primers for goldfish β-actin and PACAP receptors. The nucleotide sequences of the primers for goldfish PACAP receptor, gf.PACAP.R1 and gf.PACAP.R2, are AGTGTCGGCAAGGTCGTGGAGGTC and CGCAGGTAGATGCTGGACTCGTTC, respectively. The PCR conditions for this goldfish PACAP receptor were 1 min at 94 C, 40 sec at 68 C, and 1 min at 72 C for a total of 28 cycles. The sequences of primers specific for goldfish β-actin and the respective conditions for PCR have been reported previously (37).

Data transformation and statistics

GH data (nanograms per ml) from individual columns were expressed as a percentage of the mean GH contents of the first six fractions collected at the beginning of perifusion before drug treatment (referred to as % basal). This data transformation was performed to allow pooling of GH data from separate columns without distortion of the profile of GH release. In this study, GH responses were quantitated by calculating the net change in GH release after a pulse of drug treatment (*i.e.* a net change in the area under the curve). cAMP synthesis were measured in terms of picomoles of cAMP produced per ml/well or as the fold increase in cAMP content with respect to the control value. Dose-response curves were analyzed with the Allfit computer program to obtain the respective ED_{50} values and maximal GH responses. The minimal effective concentration was defined as the lowest concentration of peptide tested that induced a significant increase in GH release with respect to the basal value (*i.e.* significantly different from zero GH response). Data for GH release and cAMP contents were subjected to statistical analysis using either Student's t test or ANOVA followed by Fisher's least significant difference (LSD) test. Differences were considered significant at $P < 0.05$.

Results

Effects of ovine $PACAP_{27}$ and $PACAP_{38}$ on GH release from goldfish pituitary cells

Direct actions of PACAP on GH release at the pituitary level were examined in goldfish pituitary cells under column perifusion. Ovine $PACAP_{38}$ (0.01 nM to 1 μM; Fig. 1A) and $PACAP_{27}$ (0.01 nM to 1 μM; Fig. 1B), but not VIP (0.01 nM to 1 μM; Fig. 1C), stimulated GH release from goldfish pituitary cells in a concentration-dependent manner. The kinetics of these GH responses were rapid, with peak hormone release

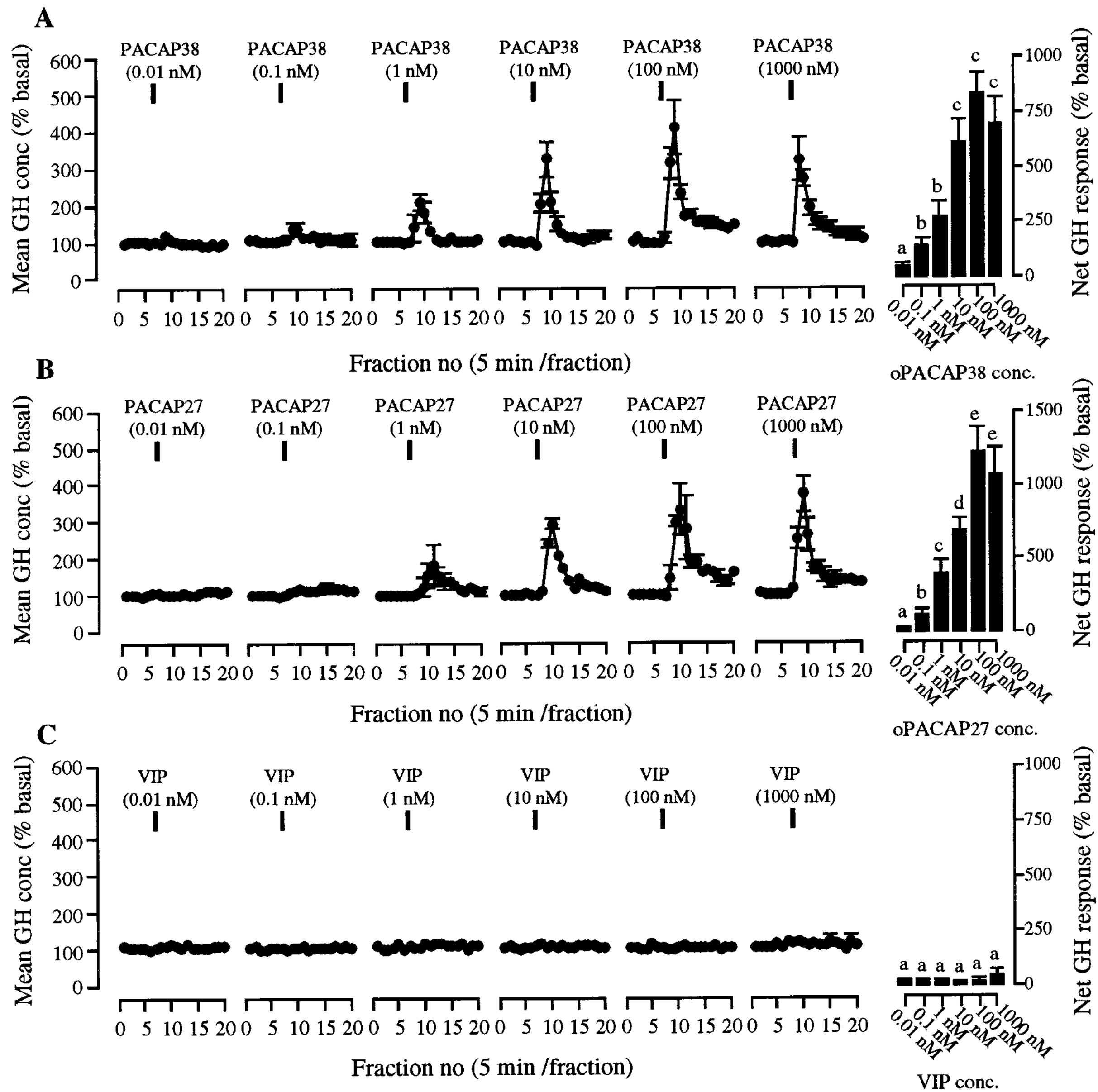


FIG. 1. Effects of ovine $PACAP_{38}$ (A), $PACAP_{27}$ (B), and VIP (C) on basal GH release from perifused goldfish pituitary cells. Increasing concentrations (0.01 nM to 1 μM) of ovine $PACAP_{38}$, $PACAP_{27}$, and VIP were given as 5-min pulses, as indicated by the *vertical bars*. GH data for these peptides, expressed as the mean ± SEM (n = 4–6), were pooled results from at least four independent experiments. In this study, separate columns were used for individual concentrations of peptide tested, and the mean basal GH level was 37.7 ± 3.1 ng GH/ml. Profiles of GH release are presented on the *left*, and the quantitated GH responses are shown on the *right*. GH responses were quantitated as the net change in GH release after a pulse of drug treatment (*i.e.* a net change of area under the curve). A similar magnitude of GH responses is denoted by the same letter (by ANOVA and Fisher's LSD test, $P > 0.05$). ED_{50} values for the GH-releasing effect of ovine $PACAP_{38}$ and $PACAP_{27}$ were 3.3 ± 1.6 nM and 8.9 ± 3.5 nM, respectively.

observed within the first 10 min after drug administration. The ED_{50} values of ovine $PACAP_{38}$ and $PACAP_{27}$ were 3.3 ± 1.6 nM and 8.9 ± 3.5 nM, respectively. The minimal effective concentration of $PACAP_{38}$ (0.1 nM) to induce GH release was 10-fold lower than that of $PACAP_{27}$ (1.0 nM). However, the magnitudes of the maximal GH responses to these two peptides were not significantly different from each other (764 ± 154% basal for $PACAP_{38}$ *vs.* 1149 ± 257% basal for $PACAP_{27}$; $P > 0.05$).

To further confirm the specificity of PACAP's action in the goldfish, the GH-releasing effect of ovine $PACAP_{38}$ (10 nM) was tested in the presence of either the PACAP antagonist $PACAP_{6-38}$ (Fig. 2A) or the VIP antagonist [4-Cl-D-Phe^{6},Leu^{17}]VIP (Fig. 2B). In this case, the GH response to $PACAP_{38}$ (10 nM) was significantly reduced by a 10-μM concentration of $PACAP_{6-38}$ (486 ± 42% basal in the control group *vs.* 321 ± 31% basal with the PACAP antagonist; $P < 0.05$). A similar concentration of [4-Cl-D-Phe^{6},Leu^{17}]VIP was not effective in this regard (475 ± 56% basal in the control *vs.* 446 ± 61% basal with the VIP antagonist; $P > 0.05$).

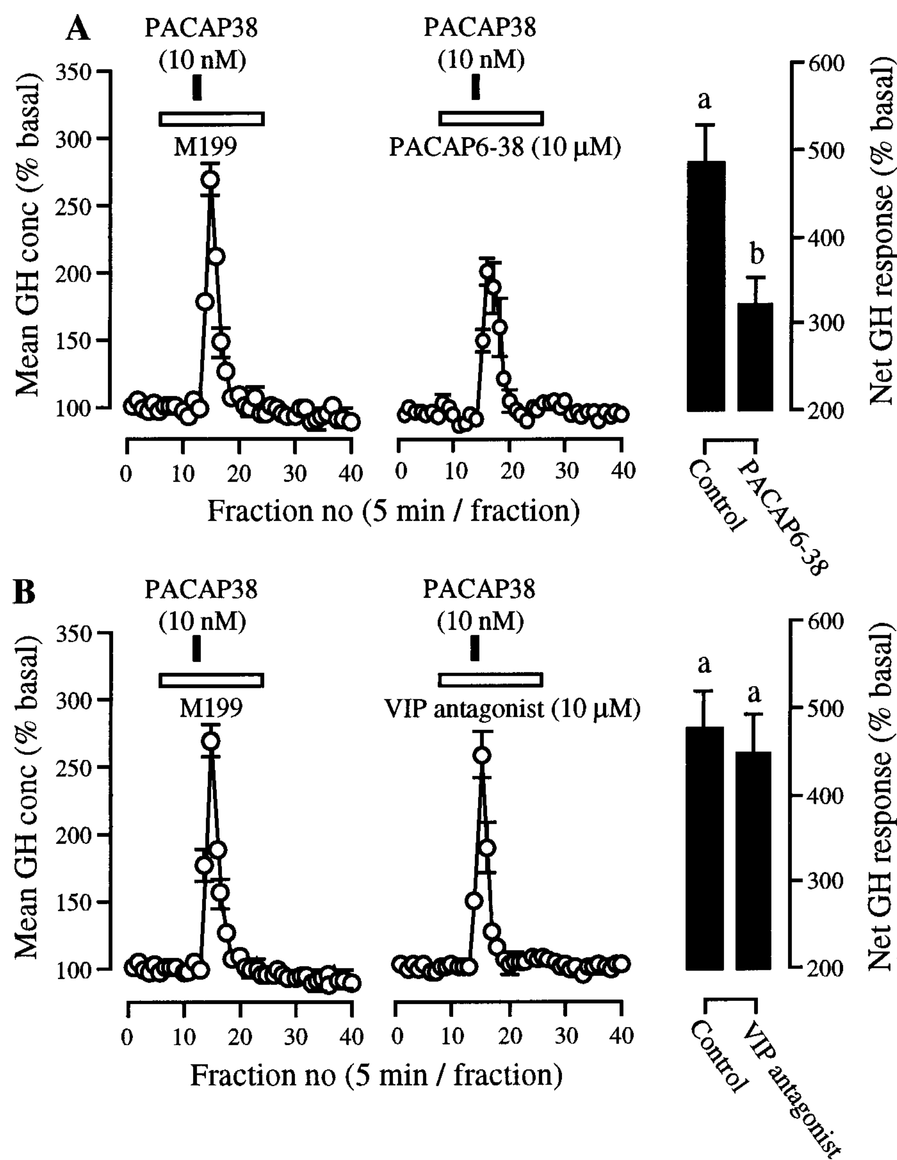


FIG. 2. Effects of the PACAP antagonist $PACAP_{6-38}$ (A) and VIP antagonist [4-Cl-D-Phe[6],Leu[17]]-VIP (B) on ovine $PACAP_{38}$-stimulated GH release from perifused goldfish pituitary cells. A 5-min pulse of ovine $PACAP_{38}$ (10 nM; *vertical bars*) was given during the 1.5-h continuous perifusion (*horizontal bars*) of either the PACAP antagonist $PACAP_{6-38}$ (10 μM) or the VIP antagonist [4-Cl-D-Phe[6],Leu[17]]VIP (10 μM). GH data, expressed as the mean ± SEM (n = 4), were pooled results from four separate experiments, and the mean basal GH level was 34.7 ± 2.5 ng GH/ml. Profiles of GH release are presented on the *left*, and the quantitated GH responses are shown on the *right*. GH responses were quantitated as the net change in GH release after a pulse of drug treatment (*i.e.* a net change in the area under the curve). A similar magnitude of GH responses is denoted by the same letter (by ANOVA and Fisher's LSD test, $P > 0.05$). In this study, PACAP and VIP antagonists were dissolved directly in the perifusion medium, and therefore, the normal culture medium 199 was used as the control treatment.

Effects of nonmammalian PACAPs on GH release from goldfish pituitary cells

To test whether nonmammalian PACAPs also have GH-releasing activity in the goldfish, perifused goldfish pituitary cells were exposed to increasing concentrations of PACAPs from amphibian and teleost fish. Similar to the mammalian counterparts, frog $PACAP_{38}$ (0.01 nM to 1 μM; Fig. 3A), zebra fish $PACAP_{27}$ (0.01–1 μM; Fig. 3B), and zebra fish $PACAP_{38}$ (0.01–1 μM; Fig. 3C) were all effective in stimulating GH release with ED_{50} values of 14.4 ± 3.5, 15.4 ± 4.1, and 1.4 ± 0.2 nM, respectively. The minimal effective concentration of zebra fish $PACAP_{38}$ (0.01 nM) that induced GH release was 100-fold lower than that of zebra fish $PACAP_{27}$ (1.0 nM) and 10-fold lower than that of frog $PACAP_{38}$ (0.1 nM). The magnitudes of maximal GH responses to frog $PACAP_{38}$ (393 ± 89% basal) and zebra fish $PACAP_{27}$ (558 ± 60% basal) were similar ($P > 0.05$), but were significantly smaller ($P < 0.05$) than that to zebra fish $PACAP_{38}$ (1301 ± 174% basal).

Desensitization of PACAP-stimulated GH release in the goldfish

In the preceding studies, individual concentrations of PACAPs were tested in separate perifusion columns for GH-releasing activity. When decreasing concentrations of ovine $PACAP_{38}$ (0.01 nM to 1 μM) were given as consecutive 5-min pulses at 1-h intervals, GH release was observed only after the first pulse of ovine $PACAP_{38}$ (1 μM), but not for subsequent pulses of lower concentrations (100–0.01 nM; data not shown). These results suggest that there may be a down-regulation of GH responses to repeated pulses of PACAP stimulation. To test this hypothesis, increasing concentrations of ovine $PACAP_{38}$ (0.01 nM to 1 μM) were applied to perifused goldfish pituitary cells as three consecutive 5-min pulses at 1-h intervals (Fig. 4). Repeated pulses of the same concentration of $PACAP_{38}$ up to 1 nM induced similar magnitudes of GH responses (Fig. 4, A–C). When the concentrations of $PACAP_{38}$ tested were 10 nM or more, the ability of

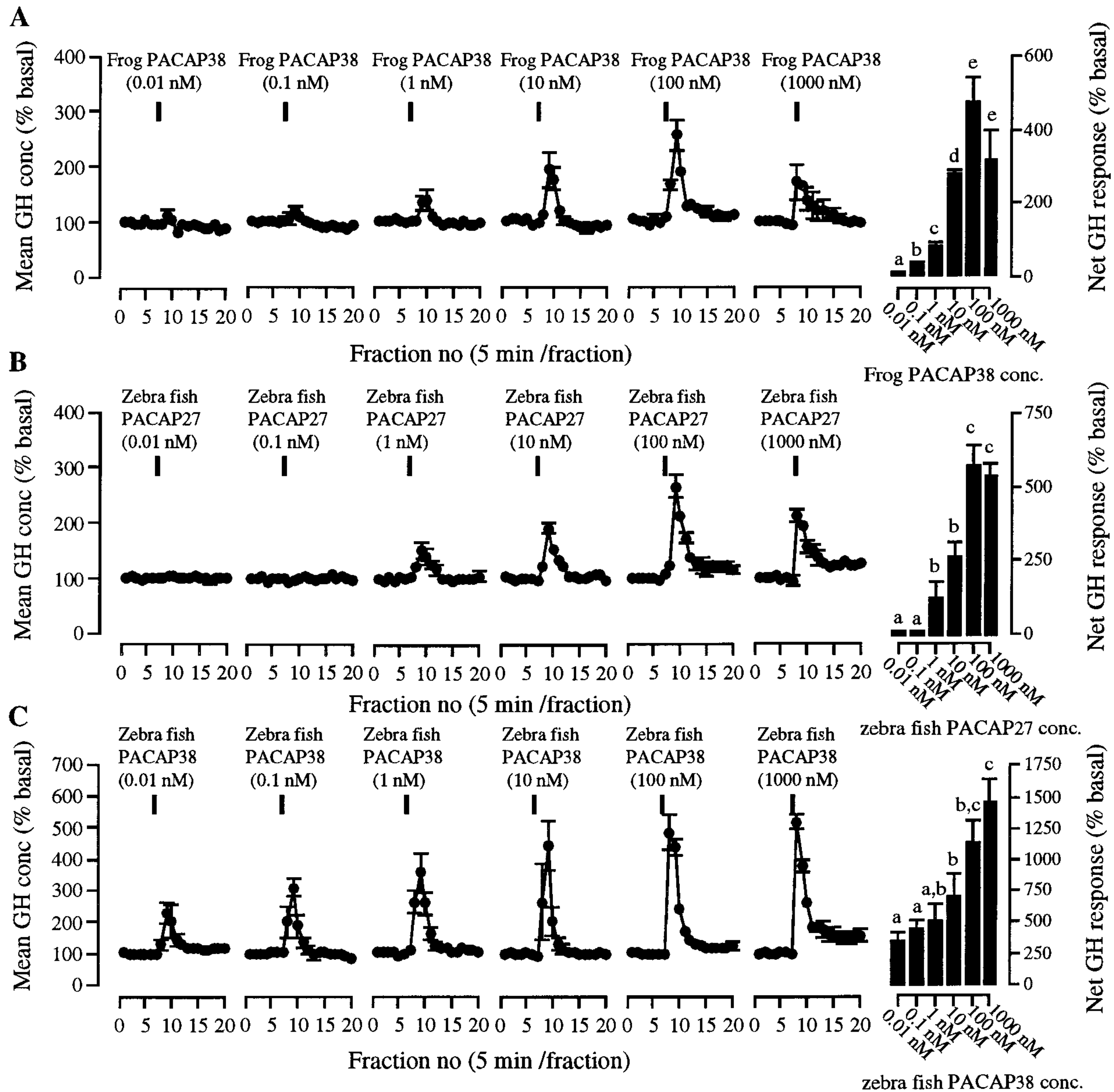


FIG. 3. Effects of frog $PACAP_{38}$ (A), zebra fish $PACAP_{27}$ (B), and zebra fish $PACAP_{38}$ (C) on basal GH release from perifused goldfish pituitary cells. Increasing concentrations (0.01 nM to 1 μM) of frog $PACAP_{38}$, zebra fish $PACAP_{27}$, and zebra fish $PACAP_{38}$ were given as 5-min pulses, as indicated by the *vertical bars*. GH data presented for these peptides, expressed as the mean ± SEM (n = 4–5), were pooled results from at least four independent experiments. In this study, separate columns were used for individual concentrations of peptide tested, and the mean basal GH level was 35.5 ± 4.2 ng GH/ml. Profiles of GH release are presented on the *left*, and the quantitated GH responses are shown on the *right*. GH responses were quantitated as the net change in GH release after a pulse of drug treatment (*i.e.* a net change in the area under the curve). A similar magnitude of GH responses is denoted by the same letter (by ANOVA and Fisher's LSD test, $P > 0.05$). ED_{50} values for the GH-releasing effect of frog $PACAP_{38}$, zebra fish $PACAP_{27}$, and zebra fish $PACAP_{38}$ were 14.4 ± 3.5, 1.4 ± 0.2, and 15.4 ± 4.1 nM, respectively.

the subsequent pulses to induce GH release was diminished (Fig. 4, D–F). At the end of the perifusion experiments, a 5-min pulse of ionomycin (10 μM) was given as a positive control, and ionomycin treatment consistently induced a significant increase in basal GH secretion.

Immunohistochemical staining of PACAP in goldfish pituitary sections

The immunoreactivity of PACAP was demonstrated in the goldfish pituitary sections using antiserum IHC8920 raised against ovine $PACAP_{38}$ (Fig. 5, A and B). PACAP immunostaining was found in nerve fibers located in the pars distalis (PD) and neurointermediate lobe (NIL). These nerve fibers were identified mostly in the periphery of rostral PD (RPD; Fig. 5, A, a), whereas in the proximal PD (PPD; Fig. 5A, b) and NIL (Fig. 5A, c), a random distribution pattern was observed. In the PPD, some of these nerve fibers were located in close proximity to pituitary cells immunoreactive to GH antiserum (Fig. 5B, a–c). Occasionally, pituitary cells with PACAP immunoreactivity were found near some blood vessels in the

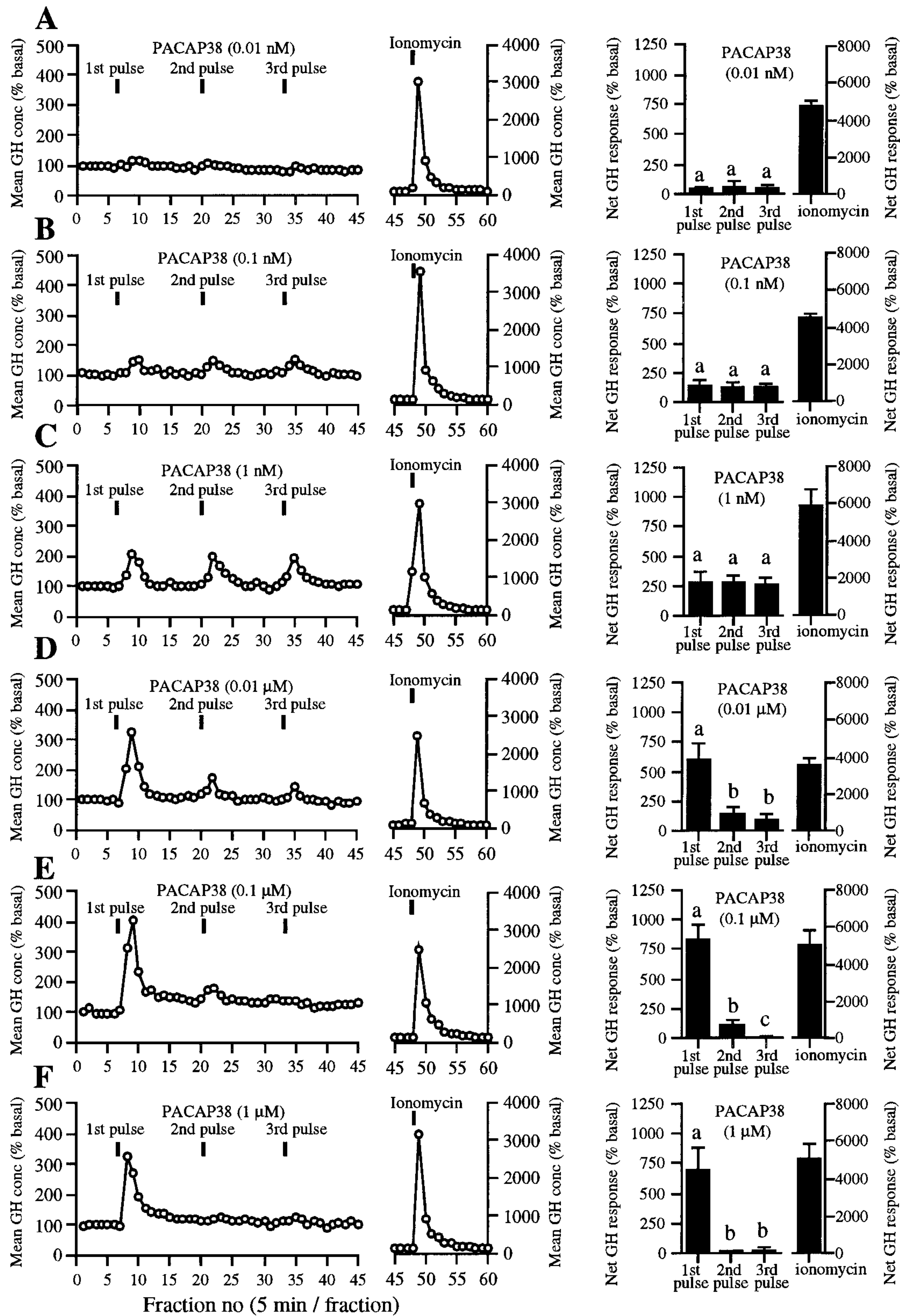
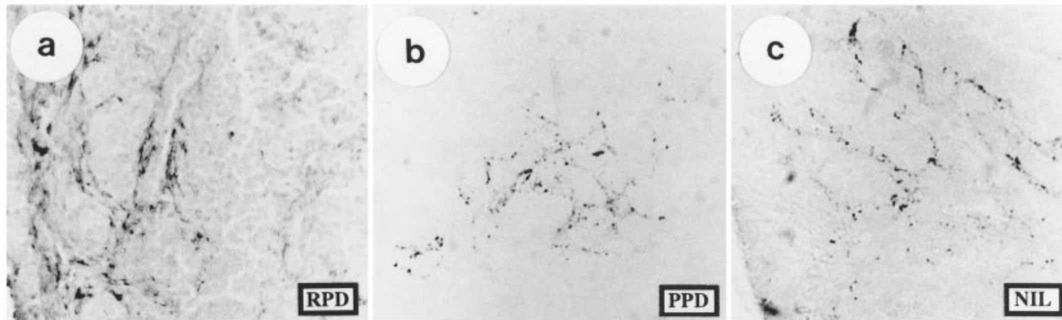


FIG. 4. Effect of repeated exposure to increasing concentrations of ovine $PACAP_{38}$ on basal GH release from perifused goldfish pituitary cells. Ovine $PACAP_{38}$ with concentrations ranging from 0.01 nM to 1 μM (A–F) was given as three consecutive 5-min pulses at 1-h intervals as indicated by the *vertical bars*. As a positive control, a 10-min pulse of ionomycin (50 μM) was given at the end of these perifusion experiments. The kinetics of GH release in response to different concentrations of $PACAP_{38}$ during the course of perifusion are presented in the *left panels* (SEMs are omitted for clarity), whereas the quantitated GH responses, calculated as the net change in the area under the curve, are given in the *right panels*. A similar magnitude of GH responses is denoted by the same letter (by ANOVA followed by Fisher's LSD test, $P > 0.05$). GH data (mean ± SEM) are pooled results from six separate experiments (n = 6), and the mean basal GH release was 36.8 ± 1.3 ng GH/ml.



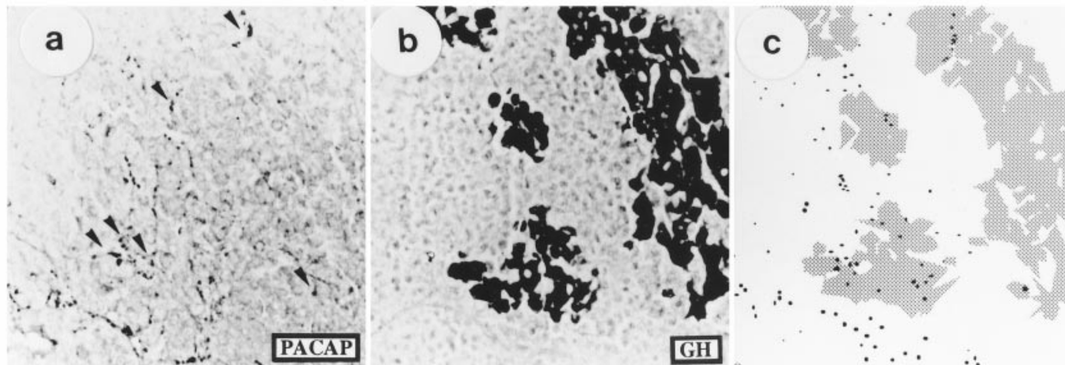


FIG. 5. A, Distribution of PACAP immunoreactivity in the goldfish pituitary. PACAP immunoreactivity was found in branches of nerve bundles located mainly in the periphery of the RPD (a). In the PPD (b) and NIL (c), PACAP immunostaining was identified mainly in nerve fibers of a smaller size. In contrast to that in the RPD, no specific distribution pattern of PACAP immunostaining was observed in the PPD or NIL. B, Immunostaining of PACAP (a) and GH (b) in consecutive goldfish pituitary sections. Pituitary cells with GH immunoreactivity were found only in the PPD, not in the RPD nor NIL. In the PPD, some of the fibers with PACAP immunoreactivity (indicated by *arrows*) were identified in the vicinity of pituitary cells with GH immunostaining. The distribution of PACAP immunostaining (*dots*) and GH cells (*shaded area*) in consecutive pituitary sections was mapped using a computer-generated overlay diagram (c). Brightfield microscopy; magnification, ×100.

NIL (Fig. 6, A–C). In this study, the specificity of PACAP immunostaining was confirmed using normal rabbit serum as the control and preabsorption of IHC8920 with ovine $PACAP_{38}$. In both cases, no immunostaining signals were found in goldfish pituitary sections (data not shown).

Molecular cloning of goldfish type I PACAP receptor cDNA

A partial cDNA clone with nucleotide sequence similar to the coding region from TMD2 to TMD6 of mammalian PACAP receptors was obtained using a nested PCR approach. This partial cDNA clone was used as a probe to screen a goldfish pituitary cDNA library, and a full-length cDNA clone of 5.2 kb in size was isolated (Fig. 7). This full-length cDNA contained a single open reading frame of 1395 bp encoding a 465-amino acid protein. Seven segments of hydrophobic amino acids corresponding to the transmembrane-spanning regions TMD1–6 of G protein-coupled receptors were identified in this receptor protein, as indicated by the results of Kyte-Doolittle hydrophobicity analysis (data not shown). When compared with the human and rat type I PACAP receptors, the deduced amino acid sequence of this goldfish receptor was 85.7% and 85.1% homologous to these mammalian counterparts, respectively (Fig. 8). The intracellular and extracellular loops, TMDs, and the cytoplasmic tail in the C-terminal were largely conserved in this goldfish PACAP receptor. All 15 cysteine residues, aspartic acid at position 75, and proline residues in TMD4–6 reported in mammalian type I PACAP receptors were also present. The N-terminal extracellular domain of this goldfish receptor was more variable; especially, the first putative *N*-linked glycosylation site reported in mammalian PACAP receptors was not found.

Functional expression of goldfish type I PACAP receptors

To establish the functionality of goldfish type I PACAP receptor, the full-length cDNA clone of this receptor was stably transfected into COS-7 cells. This newly transfected cell line, namely COS-gf.PACAP.R, was then exposed to a 100-nM concentration of ovine $PACAP_{27}$ and $PACAP_{38}$, as well as other structurally related peptides, including VIP, GHRH, PTH, GIP, glucagon, and secretin (Fig. 9A). The concentration of peptides tested was fixed at 100 nM, as this concentration of PACAPs was previously shown to induce a maximal GH response in goldfish pituitary cells (Figs. 1 and 3). In this study, a significant increase in cellular cAMP content was observed after stimulating COS-gf.PACAP.R cells with ovine $PACAP_{27}$ and $PACAP_{38}$. Other peptides, including VIP, GHRH, PTH, GIP, glucagon, and secretin,

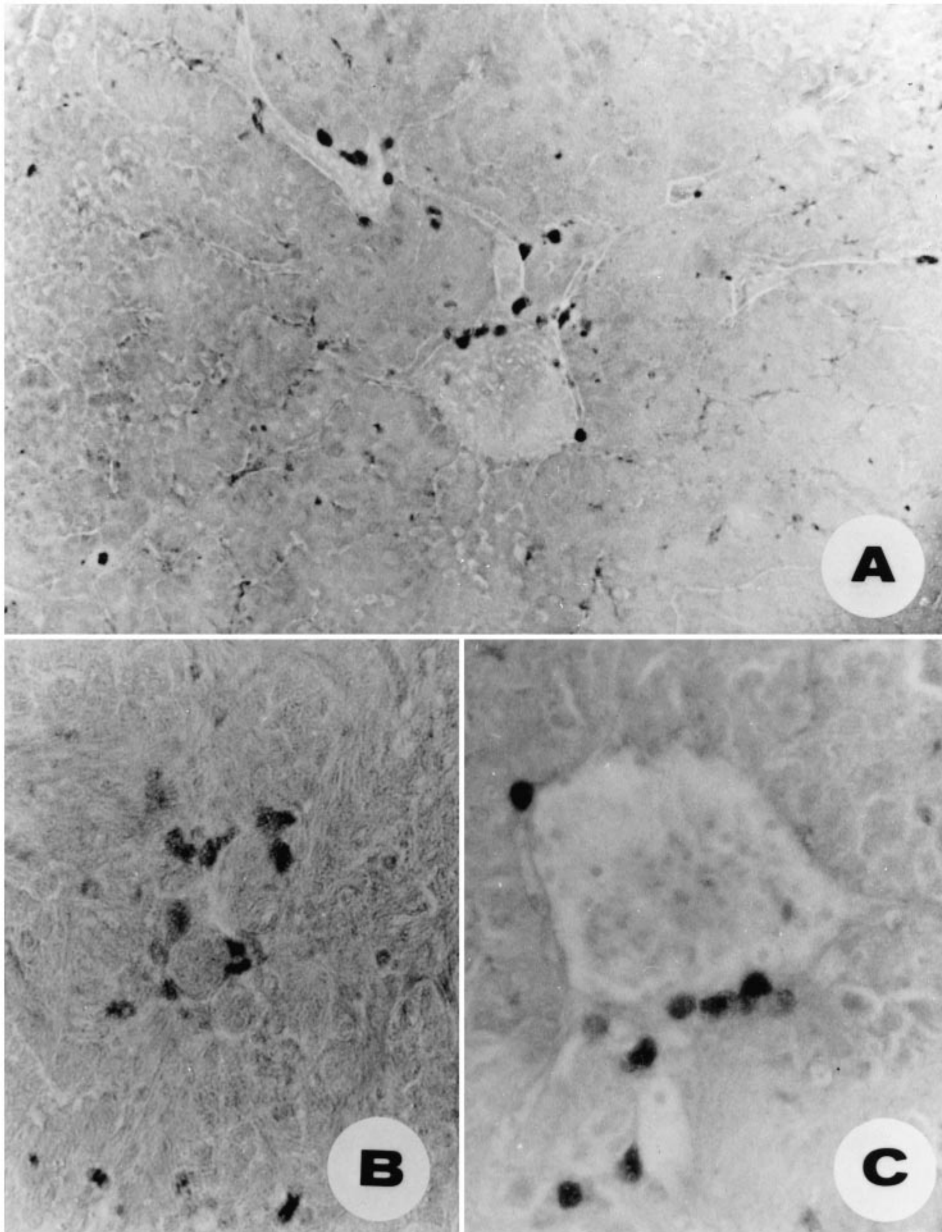


FIG. 6. Pituitary cells with PACAP immunoreactivity in the neurointermediate lobe. A, Pituitary cells with PACAP immunostaining were identified occasionally around the blood vessels in the neurointermediate lobe (brightfield; magnification, ×100). B, PACAP immunoreactivity was found in the cytoplasm but not in the nuclei of these cells (brightfield; magnification, ×200). C, These PACAP-positive pituitary cells do not have the flattened structure typical of the endothelial cells, suggesting that they may not be a part of the blood vessels (brightfield; magnification, ×400).

were not effective in this respect. Furthermore, PACAP-stimulated cAMP production was concentration dependent (Fig. 9B). Ovine $PACAP_{27}$ (0.01 nM to 1 μM) and $PACAP_{38}$ (0.01 nM to 1 μM) increased cellular cAMP contents with ED_{50} values of 2.4 ± 0.8 and 4.2 ± 1.2 nM, respectively. In the same experiment, similar concentrations of GHRH and an inactive fragment of PACAP, $PACAP_{16-38}$, did not alter basal cAMP synthesis.

Tissue distribution of goldfish type I PACAP receptor mRNA

To examine the tissue distribution of goldfish type I PACAP receptors, poly$(A)^+$ mRNAs prepared from various tissues of the goldfish, including the heart, brain, pituitary, liver, gall bladder, gills, intestine, gonads, muscle, spleen, and kidney, were subjected to Northern blot analysis (Fig. 10, A and B). Hybridization signals to a ^{32}P-labeled probe of goldfish type I PACAP receptor were found only in the heart, brain, and pituitary, not in the other tissues tested. Two mRNA transcripts for PACAP receptors of 5.4 and 7.4 kb were consistently identified in these three tissues. The 7.4-kb transcript in the brain was present at a comparable level as the 5.4-kb variant, whereas the predominant form in the heart and pituitary was the one that was 5.4 kb in size. A Northern blot of β-actin transcript was conducted to serve as an internal control for the quality of mRNA prepared. The β-actin transcript 2.3 kb in size was observed in all of the tissues tested, and there was no indication of mRNA degradation (data not shown).

Tissue distribution of type I PACAP receptors in the goldfish was further confirmed using RT-PCR (Fig. 11). The primers specific for goldfish type I PACAP receptor amplified a

```
                                                  GGCACGAGAATCACCGCTCTTCTCTCTCAACGCTCCTCCAGAGCTCTACAGACCTTCATTACTCCGGTCT
GTCGACGGGAGACGCGGATGACAAAGTTGAAATCGCAGGTGCACGAATCGAACCGTCCATTTGCAGACGTTTTAACGAAGAAGAGTGATTATATCGCGGGGTTTTCTGTAATGGCTTT

ATG AGG TTG TGT GTG AGA TCT CTG GGA ACA ATC GTG AGC AGA ATG CAT AGA CAC GCA ACA AGA GAG ACC TTC CTC GTC CTC TTC CTC    275
 M   R   L   C   V   R   S   L   G   T   I   V   S   R   M   H   R   H   A   T   R   E   T   F   L   V   L   F   L

ATC ATC AGC ATG ACA TGG CCC ATA CAT TCA GAG ATC TCC AAC TGC GTC ATC AAA CGT GAG GAG GAG AGA TGC TTG GAG CTA ATT GCC    362
 I   I   S   M   T   W   P   I   H   S   E   I   S   N   C   V   I   K   R   E   E   E   R   C   L   E   L   I   A
                                                         *                                   *

CTG CAT GAC CCT AAT GAT GAC GGA AAG TTT GAG TGT CCG TGG GAG TGG GAC AAT CTG ACC TGC TGG GAG GCG ACC AGT GTC GGC AAG    449
 L   H   D   P   N   D   D   G   K   F   E   C   P   W   E   W   D   N   L   T   C   W   E   A   T   S   V   G   K
                                             *                       #           *

GTC GTG GAG GTC AAC TGT CCC GAG CTC TTC GAT TTC ATG AGT CCA GAG GAA GGG CCT GGG AAG ATC AGC AGA AAC TGT ACA GAG TTC    536
 V   V   E   V   N   C   P   E   L   F   D   F   M   S   P   E   E   G   P   G   K   I   S   R   N   C   T   E   F
                     *                                                                           #   *

GGT TGG TCT GAA TCG TAT CCA CAC TAT GTT GAT GCC TGT ATG ATT GGT GAA AAC ACA ACC AAA CCT GAC ATG TAC TAT GCG TCT GTG    623
 G   W   S   E   S   Y   P   H   Y   V   D   A   C   M   I   G   E   N   T   T   K   P   D   M   Y   Y   A   S   V
                                                 *

AAA GCG CTC TAT ACG GTG GGC TAC AGC ACG TCT CTG GTG TCT CTG ACC ACA GCT ATG GTC ATT CTC TGC CGC TTC CGG AAG CTT CAC    710
 K   A   L   Y   T   V   G   Y   S   T   S   L   V   S   L   T   T   A   M   V   I   L   C   R   F   R   K   L   H
                                             TMD 1                                       *

TGC ACC AGG AAC TTC ATC CAC ATG AAC CTG TTT GTG TCC TTC ATG CTG AGA GCC ATC TCT GTG TTC ATT AAA GAC GGG GTC CTG TAT    797
 C   T   R   N   F   I   H   M   N   L   F   V   S   F   M   L   R   A   I   S   V   F   I   K   D   G   V   L   Y
 *                                                   TMD 2

GCT GAG GAG GAC AGC GAC CAC TGC TTC GTT CAT ACT GTG GGC TGT AAG GCA GTG ATG GTC TTC TTC CAC TAC TGT GTG ATG TCA AAC    884
 A   E   E   D   S   D   H   C   F   V   H   T   V   G   C   K   A   V   M   V   F   F   H   Y   C   V   M   S   N
                             *                       *                                   *  TMD 3

TAC TTC TGG CTC TTC ATC GAA GGT CTT TAT CTC TTC ACA CTT CTG GTG GAA ACC TTC TTT CCT GAA AGA CGC TAC TTC TAC TGG TAC    971
 Y   F   W   L   F   I   E   G   L   Y   L   F   T   L   L   V   E   T   F   F   P   E   R   R   Y   F   Y   W   Y

ACC ATC ATC GGC TGG GGA ACG CCC ACC ATT TGT GTG ACC ATA TGG GCC GTT CTG CGC CTT CAT TTT GAT GAT TCC GGT TGC TGG GAC   1058
 T   I   I   G   W   G   T   P   T   I   C   V   T   I   W   A   V   L   R   L   H   F   D   D   S   G   C   W   D
                                 TMD 4       *                                                       *

ATG AAT GAT AAT ACT GCC CTC TGG TGG GTG ATA AAG GGA CCC GTT GTG GCA TCA ATC ATG ATT AAC TTT GTC CTC TTC ATT GGA ATC   1145
 M   N   D   N   T   A   L   W   W   V   I   K   G   P   V   V   A   S   I   M   I   N   F   V   L   F   I   G   I
                                                                     TMD 5

ATC ATA ATC CTG GTG CAG AAG CTG CAG TCT CCA GAC ATC GGC GGG AAC GAG TCC AGC ATC TAC CTG CGT CTG GCG CGC TCC ACC CTG   1232
 I   I   I   L   V   Q   K   L   Q   S   P   D   I   G   G   N   E   S   S   I   Y   L   R   L   A   R   S   T   L
                                                             #

CTC CTC ATC CCT CTG TTC GGA ATC CAC TAC ACT GTT TTT GCC TTC TCC CCG GAG GAT TTC AGC AAG CGG GAG CGA CTG GTC TTT GAG   1319
 L   L   I   P   L   F   G   I   H   Y   T   V   F   A   F   S   P   E   D   F   S   K   R   E   R   L   V   F   E
                         TMD 6

CTG GGC CTG GGC TCC TTC CAG GGC TTC GTG GTT GCC GTG TTG TAC TGC TTT CTG AAT GGA GAG GTG CAG TCG GAG ATA AAG AGG AAA   1406
 L   G   L   G   S   F   Q   G   F   V   V   A   V   L   Y   C   F   L   N   G   E   V   Q   S   E   I   K   R   K
                                     TMD7                    *

TGG AGG AGT TGG ACG GTG AAC AGG TAC TTT GCT GTG GAC CTG AAG CAG CAG CGT CAC CCT TCG CTA GCG AGC AGT GGG GTG AAC GGG   1493
 W   R   S   W   T   V   N   R   Y   F   A   V   D   L   K   Q   Q   R   H   P   S   L   A   S   S   G   V   N   G

GGG ACG CAG CTG TCC ATC CTC AGC AAG AGC AGC TCA CAG ATA CGC ATG TCC AGC CCT CTG GCC GAG ACG GTC AAC CTC AAC CTG CCC   1580
 G   T   Q   L   S   I   L   S   K   S   S   S   Q   I   R   M   S   S   P   L   A   E   T   V   N   L   N   L   P

ACC    1583
 T

TGAGCGCCTGATTGGCCAGGCCGTCCATAAGCCCCGCCCTCCAACTCACCCTCTTCACCTGCAGTAGGGCCGACCAATCACAATTAGTTAAGAACTAGTAATAATTCATTAAATCGCT
GGATAGTATTTAAAGATTCCAAACAAGATCCGGAAAGTGTCACCCAACAATTCGTCCAGGGAAATATTTAAAGTGCCAAAACTATTTAAAGCTTAAAAGTTGTAATTCAGGTTAGGAA
GTTCTTTGACCATGACGCAACTTTTTGAAGGCTTTCTAATTAATCTAATAAGTACATAACAATACAAGCATAAAAGAATGTTATTTTTAATCAATCATAATGCAGAACAGGGGCTGTA
TTATTCACACCGCAATGCCAAAATACGATTGCGGCGGAAATTAGGGGGAATTTTCATATCGCGTTTTGAGCCATGGAGAATGTGAACCATTTGTGGTTCGAATGTAGTCAGAGAAACA
TCAATCCATCCAACAGCACATTGCTTTCTAGTTTAACTGTAAATCAGATATCAGAGAGAGAGAGAGAAGACGGGTTACATTTCAGTGTTATGATCAAATATAATAATATAAAGTGCAA
AACATGAATATTTCAGTATTGGTTTATCTGCAAGCTACGGTGTTTATCAGAAGTACACTATAAAAGAAATGTACAGACTTGGTGTTGCTCGTTCAGATGCTGTGTATTTATTAATTAA
ATGTTCTCCACCGAGAGATTGTAAAACATATTTTTTTACAATCAGCCGTAAGTACAGAGTATGTCAACAATTTAAAGACTTATGAATGACTCATGAATTTAGATTATATGTAAATGTT
GTTATGGTTTTGTGATTTAGCTTTGGGCCAGTGTTATTTTATTGTTATGTATGTGAATGGTTTGTAACTTCACAGAGCCAATATCAGCAAATCAAGGATTGTCAAAAAATTGTTCTGCAAAC
GATTCCCTGCTCTGAAGTGCCTGATGGTCACAAAAATAGCTTTGATGAATCTACTCTAAACAACTAGTGTCATGCAGTGTTGATTTAATTATTAGTCATAGTGTAAGATTAACAGATT
CAGTGTTCAGGTTTGCTTTTAAAATCTTTCATTTCTTCATAGTAGGAATATATTAATTTTTACGCCGTATTAGAGTGCATACAGTATAAACATTTTTAGAGCTTTGAGATGCGTAGAT
CAATGTCATTACGTTTCTATCAGGTGCATTATAAATGCTTTTAGGGCTGACATTTGAACTGTGAGAGAAATGAGACATTTGAGTGAGATGTGAAAGTGTTAAAAGATATTTCAACCAA
AAATGCTGATTCTGTCATAATGTACTCGCTTCATGTCATTCTAAACCTTATACAATTTTTTTATTTTTATGTTTGTACTCTTAGAATGATAAAGTAAAGAATGGTAAGCCCCAAAAT
GACTAAAAGCATAATTTTAGTACCATAAAATATCATATTGAATGACTTTTATGTTGTTGTTCTTCACATTAAGAGCTTGACAGCTGGGTGATCAATAATATGTTGCATGTTAACAAGT
AAAGTATGCTAGTTTTGAAATGGCATGAAGATGAGTAAATGATGATAGGGTTTGATTCATTTTTGAGAGAATGTCTTTTAATACTTGAGAGACAGGCAACCCCCCCAACCCTTTACAAGT
ACAATATCTGATGTCAATCTATGAATCCAACCAAATTGACTGCATCACCACTGACTGACAATCATAATTAGTTCTCATCACTCACATTTTACATCAGTGTGCAAAGTGGCAAGAGTCT
CTGCTAAAAGTGTTGTTTATATCCAGTACAGTATTATTCTTTGCCATGAGTGTAAATTCTCTATATGTTAAAGATGCACATGTGCCAGTAAATGATGGATGTACCAGCACTCTCTCTT
TATTTTGAAATGCAACATGCTTTGTTGCAGCTCATAGTTGTTGGTGAATCACATGGCAGAGTCGAGGTGATTATTGAAATGTACAGTAAAGTGATGGTTGTGTAGATCGCACAGATCT
TTTAAAAAAATGTGGGGAAGATGTAAACGTTTTCCTTTGGTTGTTGTTTTGAAGTTGAAGTTGTGTAATATGTGTGCTACTATCGTCATCAGCAAATGAACATGCAAAAATAATGACA
TAAGGTTTCGAATGCTCCTCATCTTTCATTGGCCAGATTAAACATAGCCCCGCCCCCTCACGCAATTAGTTGAGAAAGAGTGTAGCTTTTCTTTAAAAAAAAAATTTCTGATGTAAAAT
ACTGGTTCTGTGTTTAATATGCAGAGATGAGTTTAAAATAGTGCTGTCAAAAATAAATCGCATCCGAAATAAAAGTTTTGTTTGCGTAATATATGTGTGTGTGCTGTGTATATTTCT
TTTGTGTGTATATATATATATATATATAAAACACACATTCATGCATGTATATATTTCAGAAAAATATGTTTATATATTACATATATTTATTTTAATGTAAATTATGTGAATAGAAATA
TAAACATAAAGACATTTTCAAAATATATACTCTATGTGTGAATTTATTATATATATATATATATATGCATGACAATTATACATAGAACACACACATTGTACAAATGAAGACTTGTTTT
GGATGCAATTTATTGTGATTAATCTTTGGACAGCACTACTATAAAATAATTTGTAGCTGGATTTTTCTAAAAAAAAATATACTTATAAACACTGTGGCTCTTTTATGCTAAAGCTTTT
TTTTTATTGCTTTGAGCATTGTGAACTGCAACATTAGCTCAAACAATTGCGTGAGGTTGGAGTGTTTTTTTGTTTTGTTGTTTGTTTAGTTTTTTTGGCCAATGGCAAACTAGGAGAG
TCATTATTTTTGCAAATCTGCTCGGTGGGGGGGGGGGAATTTAACACTAAAATTCTCACCATTGATGATAAAAAAAGAACTTTTTTTCTTTTTTTCTTATCGTCTTTATTTTTCAAC
CTGTAAATATTTATGATCTTGAAAAAGCTCTCAAGTGATAGTGAACTTGTCTGTAGAAATAAATTGTGAAATCCTAACAGAGAGCTCCAGATGTTGCTTGGATCATGTGACCTTACAG
CTTCATATGTGTTTGGGTATATGAGGGGCCGAGCTCCCTCTGTGGCTCTTTCTAGATATATCTGCAATGAAAAAAAAAAAAGACAAAAAGGAGACAGTTTTTGCTGCTTTCTATGTTCT
TGACACCAGCCGTCATTCTGAGTGTGAGCTCAGAAGGTAACGACCATGTCTCAAAGCTGTTACAGACGTGTTTTCTTGTGCTTGTAACACATCTGCCTGCCAGTAGGTAATGCTTTTT
ATTTGATTGTGTAGGCTATTTGAGCACTGTACAGTATATTTGAGGAATGAGGAGATAAGTGGGAGATTTGAGTGTCTCTCGTGTAGGTCTGACATGTTTCATTGTGTCTTTGAGATG
TCTTCATCTTGTGGACTAACTCATTTCGATCTGAATGTAACACTCAGACAACACTTGTATCGTACATTCAGCTGCTCTGTAAAGCTGCCTGCTGTTTCTTCTGCTTTGATGTATAGAA
ATGATGTCCATCTTTGACCTGTGAACATTTTTGTACGTGTCTGAATTCCATTCTTGACAAACAAAACAAGTTTCTGTTTTATGTAAAAAAAAAAAAAAAAAA  5225
```

FIG. 7. The cDNA and deduced amino acid sequences of the goldfish type I PACAP receptor. The seven transmembrane domains (TMD1–7) of this goldfish receptor are *double underlined* and labeled accordingly. The conserved cysteine residues and putative *N*-linked glycosylation sites are in **bold** and labeled * and #, respectively. The proline residues in TMD4–6, which are highly conserved among G protein-coupled receptors, are in ***bold italic***. The consensus sequence RLAR in the third intracellular loop for the coupling with $G_s\alpha$ is in **bold**, and the polyadenylation signals AATAAA/AACAAA in the 3′-end are *underlined*.

FIG. 8. Amino acid sequence alignment of human, rat, and goldfish type I PACAP receptor. The *horizontal arrows* indicate the seven putative transmembrane domains (TMD1–7) of the receptor. The amino acid residues that are identical or conserved among these PACAP receptors are *boxed*. The conserved sequences of *N*-linked glycosylation sites, cysteine residues, and aspartic acid at position 75 are labeled #, *, and +, respectively.

PCR product of 700 bp in the brain, heart, and pituitary. In other tissues, including the gall bladder, gills, gonads, intestine, kidney, liver, muscle, and spleen, a faint band of the same size was also observed, suggesting that type I PACAP receptors are expressed at low levels in these tissues. The authenticity of PCR products was confirmed by Southern blotting using a ^{32}P-labeled full-length cDNA probe. In the same study, RT-PCR of β-actin was performed to serve as an internal control for the integrity of first strand cDNAs. Except for the negative control (*i.e.* PCR without a template), a 200-bp PCR product specific for goldfish β-actin was consistently observed.

Discussion

In mammals, such as the rat, PACAP perikarya are located in the paraventricular and supraoptic nuclei of the hypothalamus (39), PACAP nerve terminals are present in the median eminence (23), and PACAP immunoreactivity can be detected in the hypophysial portal blood (24). These findings support the idea that PACAP is produced in the hypothalamus and delivered to the anterior pituitary to serve as a hypophysiotropic factor. Teleosts, unlike mammals, do not have a hypophysial portal blood system, and their anterior pituitaries are directly innervated by the hypothalamus (32). In this study, direct evidence has been presented for the first time to demonstrate the presence of PACAP immunoreactivity in the pituitary of a fish species. In the goldfish, nerve fibers with PACAP immunostaining were identified in the periphery of RPD, but were randomly distributed in the PPD and NIL. Some of these nerve fibers were localized in the vicinity of somatotrophs in the PPD, suggesting that PACAP may influence GH release in the goldfish. Occasionally, pituitary cells with PACAP immunostaining were found near the blood vessels in the NIL. Although the identity of these cells is still unknown, they may represent the sites of local production of PACAP at the pituitary level. Alternatively, PACAP may be taken up from circulation and concentrated in these cells. In the rat, a detectable level of PACAP immunoreactivity has been identified in some gonadotrophs and lactotrophs (40), and these two cell types are known to be target cells for PACAP (41). Therefore, we do not exclude the possibility that PACAP may also function as an autocrine/paracrine factor in the goldfish pituitary.

Consistent with our hypothesis that PACAP may function as a GH regulator in the goldfish, PACAPs from different species, including mammalian and nonmammalian variants, concentration dependently stimulated GH release from perifused goldfish pituitary cells. The rank order of ED_{50} of these peptides was: zebra fish $PACAP_{38} \leq$ ovine $PACAP_{38} <$ ovine $PACAP_{27} \approx$ zebra fish $PACAP_{27} \approx$ frog $PACAP_{38} \ll$ mam-

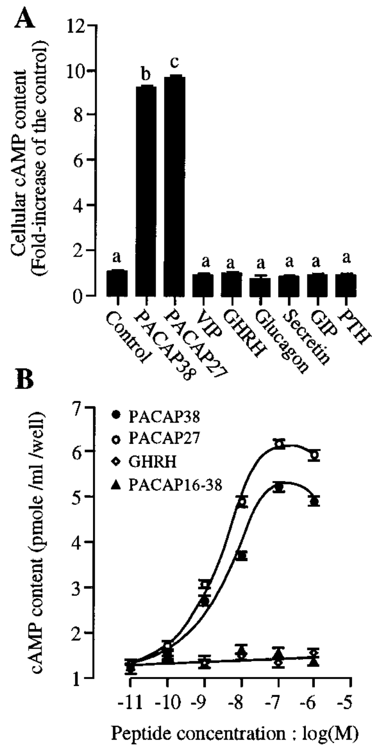


FIG. 9. Functional expression of goldfish type I receptor in COS-7 cells. A, Effects of ovine $PACAP_{27}$, $PACAP_{38}$, and related peptides, including VIP, GHRH, glucagon, secretin, GIP, and PTH, on cellular cAMP contents in COS-7 cells with stable expression of goldfish type I PACAP receptors. The concentration of peptide tested was fixed at 100 nM, and cAMP data were transformed into the fold increase in cAMP content in the control group (without drug treatment). B, Effect of increasing concentrations (0.01 nM-1 μM) of ovine $PACAP_{27}$ and $PACAP_{38}$ on cAMP synthesis in COS-7 cells with goldfish type I PACAP receptors. Similar concentrations of GHRH (0.01 nM to 1 μM) and $PACAP_{16-38}$ (0.01 nM to 1 μM), an inactive analog of $PACAP_{38}$, were used as the negative control. In these experiments, cAMP data were simply expressed as picomoles of cAMP per ml/well, and the estimated ED_{50} values for ovine $PACAP_{27}$ and $PACAP_{38}$ were 2.4 ± 0.8 and 4.2 ± 1.2 nM, respectively. In this study, the duration of drug treatment was fixed at 45 min in the presence of 0.2 mM IBMX.

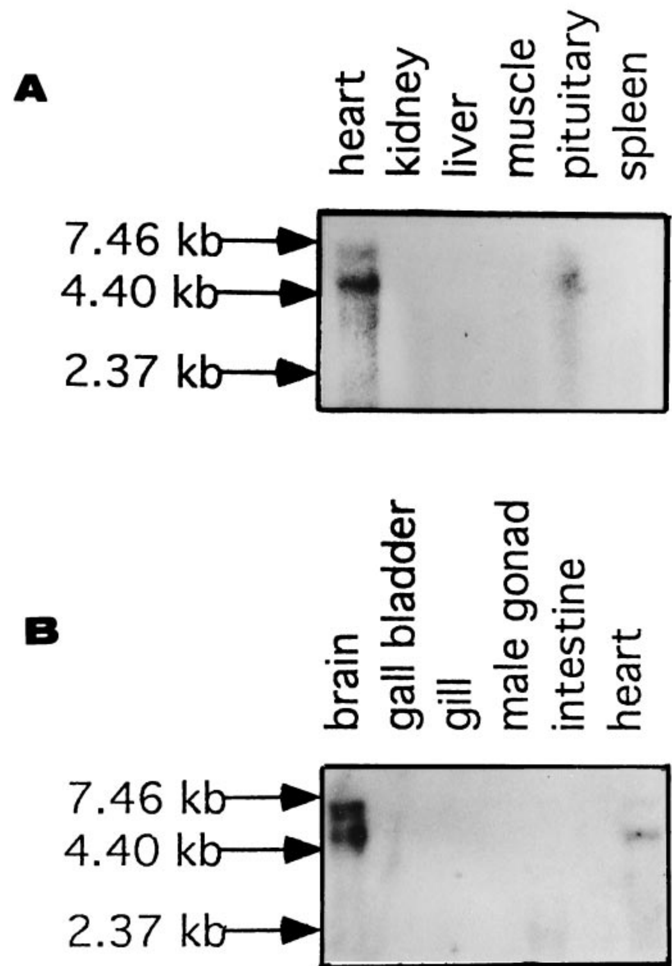


FIG. 10. Northern blot analysis of the goldfish type I PACAP receptor. Poly(A)$^+$ RNA was prepared from various tissues of the goldfish, including A) heart, kidney, liver, muscle, pituitary, and spleen; and B) brain, gall bladder, gill, gonad, intestine, and heart. These samples (3 μg) were electrophoresed in a 1.2% agarose gel with formaldehyde and transblotted onto a Hybond N$^+$ nylon membrane. Northern hybridization was performed using a full-length goldfish PACAP receptor cDNA probe. Transcripts of 7.4 and 5.4 kb were detected at high levels in the brain and heart and at a modest level in the pituitary, but were not detectable in other tissues. In this study, a Northern blot of goldfish β-actin was also performed to control for the quality of mRNA prepared (results not shown), and there was no indication of mRNA degradation.

malian VIP. Similarly, the rank order of sensitivity of goldfish pituitary cells to PACAP stimulation (as indicated by the minimal effective concentration of PACAP to induce GH release) was: zebra fish $PACAP_{38}$ < ovine $PACAP_{38} \approx$ frog $PACAP_{38}$ < zebra fish $PACAP_{27} \approx$ ovine $PACAP_{27}$. Apparently, zebra fish $PACAP_{38}$ is the most potent PACAP analog tested in this study. Comparison with other known GH secretagogues in the goldfish tested under a similar, if not identical, column perifusion system showed that the potency of zebra fish $PACAP_{38}$ (ED_{50} = 1.4 ± 0.2 nM) is higher than those of chicken GnRH-II (ED_{50} = 19.2 ± 1.3 nM) (42) and dopamine (ED_{50} = 0.26 ± 0.06 μM) (43), similar to those of TRH (ED_{50} = 5.7 ± 3.1 nM) (44) and salmon GnRH (ED_{50} = 2.5 ± 1.4 nM) (45), but lower than that of mammalian neuropeptide Y (ED_{50} = 0.5 ± 0.2 nM) (46) and common carp GHRH (ED_{50} = 0.1 ± 0.1 nM) (47). The maximal GH responses induced by these GH secretagogues, *e.g.* dopamine (184% of basal) (43), salmon GnRH (150% of basal) (45), TRH (125% of basal) (44), mammalian neuropeptide Y (150% of basal) (46), and common carp GHRH (175% of basal) (47), were lower than that of zebra fish $PACAP_{38}$ (1301 ± 174% of basal). These findings suggest that, unlike mammals, PACAP is not only a modulator of pituitary hormone secretion, but may also function as a novel GH-releasing factor in the goldfish.

Stimulatory effects of PACAP on GH release have been demonstrated *in vitro* in rat somatotrophs (27) and clonal pituitary cell lines (26, 28). In perifusion studies with GH_3 cells, PACAP-stimulated GH release can be mimicked by the structurally related peptide VIP and abolished by simultaneous treatment with a VIP antagonist (28). The pharmacology of these GH responses suggests that PACAP stimulates GH release from GH_3 cells via activation of type II PACAP receptors. This is in agreement with the findings that type II PACAP receptors, in particular the helodermin-preferring

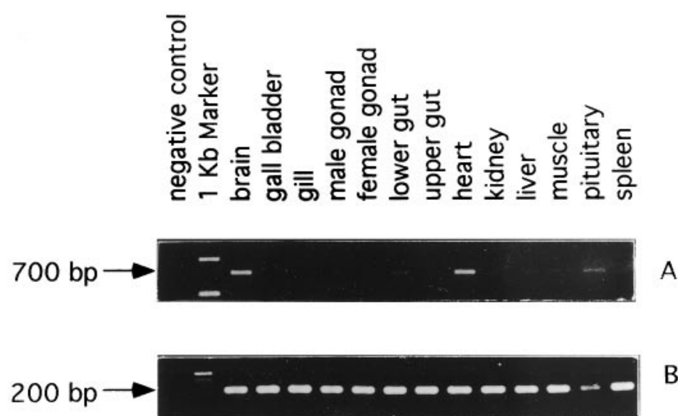


FIG. 11. Tissue distribution of goldfish type I PACAP receptors as revealed by RT-PCR. Poly(A)$^+$ RNA was prepared from various tissues of the goldfish, including the brain, gall bladder, gill, gonad, intestine, heart, kidney, liver, muscle, pituitary, and spleen. RT-PCR was performed using the specific primers for goldfish type I PACAP receptor, and a 700-bp PCR product was observed in the brain, heart, and pituitary (A). In other tissues, a faint band of the same size was also observed. As an internal control, RT-PCR for goldfish β-actin was performed (B). In these tissue samples, except for the negative control (*i.e.* PCR without a template), a 200-bp PCR product specific for goldfish β-actin was consistently observed.

VIP2 (PVR3) receptors, are the major form of PACAP receptors expressed in GH_4C_1 cells (48). In this clonal GH cell line, type I PACAP receptors are not expressed at a significant level. In the present study, VIP was unable to stimulate GH release from perifused goldfish pituitary cells. In addition, the GH-releasing action of ovine $PACAP_{38}$ was blunted by the PACAP antagonist $PACAP_{6-38}$, and the VIP antagonist [4-Cl-D-Phe^6,Leu^{17}]VIP was not effective in this regard. These results indicate that PACAP-stimulated GH release in the goldfish is mediated through pituitary receptors resembling the mammalian type I PACAP receptors.

Given that the structure of type I PACAP receptors is virtually unknown in lower vertebrates, molecular cloning of the goldfish type I PACAP receptor was performed to establish its structural identity and functional relevance in regulating GH release in the goldfish. A full-length cDNA clone of goldfish type I PACAP receptor was isolated, and the deduced amino acid sequence revealed that it is a G protein-coupled receptor with 7 transmembrane domains. At the amino acid sequence level, this goldfish receptor is highly homologous to that of the rat (85.7%) (49) and human (85.1%) (50) type I PACAP receptors. The 15 cysteine residues reported in these mammalian counterparts together with the proline residues found in TMD4, TMD5, and TMD6 are all conserved in this fish receptor. Cysteine residues, by forming intramolecular disulfide bonds, determine the 3-dimensional structure of receptors (51), whereas proline residues are known to introduce kinks in the α-helixes of TMDs and are involved in the formation of a ligand binding pocket in some receptors, *e.g.* dopamine receptors (52). Based on this structural information, it would be expected that the goldfish PACAP receptor should have a tertiary structure similar to that of the mammalian receptor. This idea is in agreement with the present findings that ovine $PACAP_{27}$ and $PACAP_{38}$ were effective in stimulating GH release from goldfish pituitary cells, suggesting that this fish type I PACAP receptor can cross-react with mammalian PACAPs.

The aspartic acid residue at position 75 of the N-terminal, which is a common feature of the secretin receptor family, is also found in this goldfish type I PACAP receptor. A point mutation that altered this aspartic acid to glycine has been reported in the GHRH receptor of the little (*lit*) mouse model (53), and this mutated receptor is defective in transducing signals through the cAMP-dependent pathway. In this goldfish type I PACAP receptor, a consensus sequence, RLAR, has been identified in the third intracellular loop. A similar motif reported in β-adrenergic receptors is known to be essential for the coupling to $G_s\alpha$ and the activation of adenylate cyclase (54). These structural characteristics suggest that the biological actions of goldfish PACAP receptor are mediated through the cAMP-dependent pathway. To test this hypothesis, functional expression of goldfish type I PACAP receptors was conducted in COS-7 cells. Ovine $PACAP_{27}$ and $PACAP_{38}$ stimulated cAMP production in these COS-7 cells in a concentration-dependent manner. Other related peptides, including VIP, GHRH, GIP, PTH, glucagon, and secretin, were not effective in this respect. The lack of a stimulatory action of VIP on cAMP synthesis further confirms that this newly cloned goldfish receptor is indeed a type I PACAP receptor. The ED_{50} values for $PACAP_{27}$- and $PACAP_{38}$-stimulated cAMP production were 2.4 ± 0.8 and 4.2 ± 1.2 nM, respectively. These ED_{50} values are in the same concentration range as that for PACAP-stimulated GH release from goldfish pituitary cells. In our previous studies, the adenylate cyclase activator forskolin and membrane- permeant cAMP analogs were effective in stimulating GH release from goldfish pituitary cells (55). Therefore, it is conceivable that the cAMP signaling pathway is involved in PACAP-stimulated GH release in goldfish via activation of type I PACAP receptors.

To examine the tissue distribution of this goldfish type I PACAP receptor, Northern blot using mRNAs prepared from various tissues of the goldfish was performed. Transcripts of this goldfish receptor were identified in the brain, heart, and pituitary, but not in other tissues, including the liver, kidney, gonads, spleen, intestine, gills, muscle, and gall bladder. The presence of type I PACAP receptors in the brain is consistent with the roles of PACAP as a neurotransmitter/neuromodulator (6) and nerve growth factor (7). Although PACAP is also proposed to be a vasoregulator (8), the expression of PACAP receptors in the heart has not been previously reported. In the goldfish, the heart contains a high level of mRNA for type I PACAP receptors. Therefore, it is logical to speculate that PACAP may play a role in the control of cardiac functions in this fish species. Compared with those in the brain and heart, the level of expression of goldfish PACAP receptors was lower in the pituitary. Nevertheless, the presence of type I PACAP receptors in the goldfish pituitary is consistent with our findings that PACAP stimulates GH release from goldfish pituitary cells through activation of type I PACAP receptors. Together with the identification of PACAP nerve fibers in the goldfish pituitary, it is highly possible that PACAP may function as a classical hypophy-

siotropic factor in teleost fishes. In this study, the tissue distribution of this goldfish type I PACAP receptor was further confirmed using RT-PCR. Using this more sensitive approach, besides the predominant signals found in the brain, heart, and pituitary, a faint band of the same size was demonstrated in other tissues as well (*e.g.* gall bladder, gills, gonads, intestine, kidney, liver, muscle, and spleen). The authenticity of these PCR signals was confirmed by Southern blot, suggesting that type I PACAP receptors are also expressed in these tissues at low levels. At present, the biological relevance of such a low level of receptor expression in the goldfish is unclear.

In this study, direct actions of PACAP on GH release from goldfish pituitary cells were tested using a column perifusion system. Static incubation of goldfish pituitary cells were deliberately avoided. In mammals, such as the rat, PACAP-stimulated GH release is not a consistent observation using static incubation of pituitary cells; both no effects (2) and a weak stimulation after a long incubation has been reported (25, 29). In those studies with a stimulatory effect, an indirect action of PACAP by inducing the release of autocrine/paracrine factors has been suggested (29). This idea is supported by the findings that PACAP stimulates interleukin-6 release from folliculo-stellate cells in the rat pituitary (56), and interleukin-6 is known to have GH-releasing activity (57). In the goldfish, the rapidity of GH responses to PACAP stimulation ($<$5 min) argues against the possibility of an indirect action of PACAP through autocrine/paracrine factors. Furthermore, indirect actions of PACAP in this study were avoided using a perifusion system that removes the secreted products from goldfish pituitary cells and prevents any accumulation of autocrine/paracrine factors. Besides, in this study it has been shown that repeated exposure to PACAP dose dependently reduced the GH-releasing action of PACAP in perifused goldfish pituitary cells. This desensitization of GH-releasing action may explain why some of the static incubation studies with PACAP did not exhibit a consistent GH-releasing effect. In the goldfish, this loss of responsiveness could not be due to a depletion of cellular GH stores, as the positive control ionomycin was still effective in stimulating GH release at the end of these experiments. At present, the mechanism(s) for this down-regulation of PACAP-stimulated GH release is unknown, but similar observations in other receptor systems have been attributed to a decrease in receptor capacity [*e.g.* GnRH receptors, (58)] and/or an uncoupling of signaling pathways from membrane receptors [*e.g.* β-adrenergic receptors (59)].

In summary, we have demonstrated that PACAP nerve fibers are present in the goldfish pituitary, and PACAPs from different species, including the mammalian and nonmammalian variants, are effective in stimulating GH release from perifused goldfish pituitary cells. The potency of these PACAPs on GH release is in the nanomolar concentration range, and the efficacy of their GH-releasing actions, especially for zebra fish $PACAP_{38}$, is higher than that of other known GH-releasing factors reported in the goldfish. The GH-releasing effect of PACAP can be desensitized by repeated exposure to increasing concentrations of ovine $PACAP_{38}$. In this study, the pharmacology of PACAP-stimulated GH release reveals that this stimulatory action is mediated through pituitary type I PACAP receptors. This idea is supported by the molecular cloning and subsequent tissue distribution studies of goldfish type I PACAP receptor. This newly cloned goldfish PACAP receptor has the classical structure of a G protein-coupled receptor with seven transmembrane domains and is highly homologous to the mammalian type I PACAP receptors. Functional expression of this goldfish receptor indicates that it is functionally coupled to the cAMP-dependent pathway and exhibits a similar pattern of ligand selectivity as that of type I PACAP receptors. In the goldfish, this type I PACAP receptor is expressed at a high level in the brain and heart and to a modest level in the pituitary, but to a low level in other tissues, including the liver, kidney, gonads, spleen, gill, intestine, muscle, and gall bladder. These results, as a whole, suggest that PACAP, through activation of pituitary type I PACAP receptors, may function as a novel GH-releasing factor in the goldfish.

Acknowledgments

Special thanks are given to Dr. R. E. Peter (University of Alberta, Edmonton, Canada) for the supply of GH standard and antiserum for GH RIA, Dr. S. Mojsov (Rockefeller University, New York, NY) for providing us with zebra fish $PACAP_{27}$ and $PACAP_{38}$, and Mr. E. K. Y. Lee (Hong Kong University) for his help with PACAP immunohistochemical staining. We are also grateful to Drs. K. L. Yu, D. K. O. Chan, F. C. Leung, and S. M. Chan for their support throughout the research project.

References

1. **Rawlings SR** 1994 PACAP, PACAP receptors, and intracellular signalling. Mol Cell Endocrinol 101:C5–C9
2. **Miyata A, Arimura A, Dahl DH, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH** 1989 Isolation of a novel 38 residue hypothalamic peptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 164:567–574
3. **Miyata A, Dah DH, Jiang L, Kitada K, Fujino M, Minamino N, Arimura A** 1990 Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues ($PACAP_{38}$). Biochem Biophys Res Commun 170:643–648
4. **Arimura A, Shioda S** 1995 Pituitary adenylate cyclase polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. Front Neuroendocrinol 16:53–88
5. **Arimura A, Somogyvari-Vigh A, Miyata A, Mizuno K, Coy DH, Kitada C** 1991 Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testes. Endocrinology 129:2787–2789
6. **Masuo Y, Suzuki N, Matsumoto H, Tokito F, Matsumoto Y, Tsuda M, Fujino M** 1993 Regional distribution of pituitary adenylate cyclase activating polypeptide (PACAP) in the rat central nervous system as determined by sandwich-enzyme immunoassay. Brain Res 602:57–63
7. **Chang JY, Korolev VV, Wang JZ** 1996 Cyclic AMP and pituitary adenylate cyclase-activating polypeptide (PACAP) prevent programmed cell death of cultured rat cerebellar granule cells. Neurosci Lett 206:181–184
8. **Carlsson PO, Ostenson CG, Efendic S, Langel U, Jansson L** 1996 Pituitary adenylate cyclase activating polypeptide (PACAP) redistributes the blood within the pancreas of anesthetized rats. Regul Pept 63:123–128
9. **Ogi K, Kimura C, Onda H, Arimura A, Fujino M** 1990 Molecular cloning and characterization of the cDNA for the precursor of rat pituitary adenylate cyclase-activating polypeptide (PACAP). Biochem Biophys Res Commun 173:1271–1279
10. **Kimura C, Ohkubo S, Ogi K, Hosoya M, Itoh Y, Onda H, Miyata A, Jiang L, Dahl RR, Stibbs HH, Arimura A, Fujino M** 1990 A novel peptide which stimulates adenylate cyclase: molecular cloning and characterization of the ovine and human cDNAs. Biochem Biophys Res Commun 166:81–89
11. **Okazaki K, Itoh Y, Ogi K, Ohkubo S, Onda H** 1995 Characterization of murine PACAP mRNA. Peptide 16:1295–1299
12. **Ohkubo S, Kimura C, Ogi K, Okazaki K, Hosaya M, Onda H, Miyata A, Arimura A, Fujino M** 1992 Primary structure and characterization of the precursor to human pituitary adenylate cyclase activating polypeptide. DNA Cell Biol 11:21–30
13. **Chartrel N, Tonon MC, Vaudry H, Conlon JM** 1991 Primary structure of frog pituitary adenylate cyclase-activating polypeptide (PACAP) and effects of ovine PACAP on frog pituitary. Endocrinology 129:3367–3371

14. **Yasuhara T, Mizuno K, Somogyvari-Vigh A, Komaki G, Arimura A** 1992 Isolation and primary structure of chicken PACAP. Regul Pept 37:326 (Abstract)
15. **Montero M, Yon L, Rousseau K, Arimura A, Fournier A, Dufour S, Vaudry H** 1997 Distribution, characterization, and hypophysiotropic activity of PACAP in the European eel *Anguilla anguilla.* Regul Pept 71:123 (Abstract)
16. **Parker DB, Coe IR, Dixon GH, Sherwood NM** 1993 Two salmon neuropeptides encoded by one brain cDNA are structurally related to members of the glucagon superfamily. Eur J Biochem 215:439–448
17. **McRory JE, Parker DB, Ngamvongchon S, Sherwood NM** 1995 Sequence and expression of cDNA for pituitary adenylate cyclase activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH)-like peptide in catfish. Mol Cell Endocrinol 108:169–177
18. **Parker DB, Power ME, Swanson P, Rivier J, Sherwood NM** 1997 Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. Endocrinology 138:414–423
19. **McRory J, Sherwood NM** 1997 Two protochordate genes encode pituitary adenylate cyclase-activating polypeptide and related family members. Endocrinology 138:2380–2390
20. **Convineau A, Rouyer-Fessard C, Darmoul D, Maoret JJ, Carrero I, Ogier-Denis E, Laburthe M** 1994 Human intestinal VIP receptor: cloning and functional expression of two cDNA encoding proteins with different N-terminal domains. Biochem Biophys Res Commun 200:769–776
21. **Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G, Harmar AJ** 1993 The VIP2 receptor: molecular characterization of a cDNA encoding a novel receptor for VIP. FEBS Lett 334:3–8
22. **Rawlings SR, Hezareh M** 1996 Pituiaty adenylate cyclase activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. Endocr Rev 17:4–29
23. **Koves K, Arimura A, Somogyvari-Vigh A, Vigah S, Miller J** 1990 Immunohisto-chemical demonstration of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, in the ovine hypothalamus. Endocrinology 127:264–271
24. **Dow RC, Bennie J, Fink G** 1994 Pituitary adenylate cyclase-activating polypeptide-38 (PACACP38) is released into hypophysial portal blood in the male and female rat. J Endocrinol 142:R1–R4
25. **Hart GR, Gowing H, Burrin JM** 1992 Effects of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, on pituitary hormone release in rats. J Endocrinol 134:33–41
26. **Propato-Mussafiri R, Kanse SM, Ghatei MA, Bloom SR** 1992 Pituitary adenylate cyclase activating polypeptide releases 7B2, adenocorticotropin, growth hormone, and prolactin from the mouse and rat clonal pituitary cell lines AtT-20 and GH_3. J Endocrinol 132:107–113
27. **Goth MI, Lyons CE, Canny BJ, Thorner MO** 1992 Pituitary adenylate cyclase activating polypeptide, growth hormone (GH)-releasing peptide and GH-releasing hormone stimulate GH release through distinct pituitary receptors. Endocrinology 130:939–944
28. **Murakami Y, Koshimura K, Yamauchi K, Nishiki M, Tanaka J, Furuya H, Miyake T, Kato Y** 1995 Pituitary adenylate cyclase activating polypeptide (PACAP) stimulates growth hormone release from GH3 cells through type II PACAP receptor. Regul Pept 56:35–40
29. **Jarry H, Leonhardt S, Schmidt WE, Creutzfeldt W, Wuttke W** 1992 Contrasting effects of pituitary adenylate cyclase activating polypeptide (PACAP) on *in vivo* and *in vitro* prolactin and growth hormone release in male rats. Life Sci 51:823–830
30. **Sawangjaroen K, Curlewis JD** 1994 Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) on prolactin, luteinizing hormone and growth hormone secretion in the ewe. J Neuroendocrinol 6:549–555
31. **Chiodera P, Volpi R, Capretti L, Caffarri G, Magotti MG, Coiro V** 1996 Effects of intravenously infused pituitary adenylate cyclase-activating polypeptide on adenohypo-physeal hormone secretion in normal men. Neuroendocrinology 64:242–246
32. **Kah O, Anglade I, Lepretre E, Dubourg P, De Monbrison D** 1993 The reproductive brain in fish. Fish Physiol Biochem 11:85–98
33. **Peter RE, Yu KL, Marchant TA, Rosenblum PM** 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool [Suppl] 4:84–98
34. **Peng C, Peter RE** 1997 Neuroendocrine regulation of growth hormone secretion and growth in fish. Zool Stud 36:79–89
35. **Chang JP, Cook H, Freeman 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
36. **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
37. **Chow BKC** 1997 The goldfish vasoactive intestinal polypeptide receptor: functional studies and tissue distribution. Fish Physiol Biochem 17:213–222
38. **Chow BKC** 1995 Molecular cloning and functional characterization of a human secretin receptor. Biochem Biophys Res Commun 212:204–211
39. **Takahashi K, Totsune K, Murakami O, Satoh F, Sone M, Ohneda M, Sasano H, Mouri T** 1994 Pituitary adenylate cyclase activating polypeptide (PACAP)-like immunoreactivity in human hypothalamus: co-localization with arginine vasopressin. Regul Pept 50:267–275
40. **Koves K, Molnar J, Kantor O, Gorcs TJ, Lakatos A, Arimura A** 1995 New aspects of the neuroendocrine role of PACAP. Ann NY Acad Sci 805:648–654
41. **Vigh S, Arimura A, Gottschall PE, Kitada C, Somogyvari-Vigh A, Childs GV** 1993 Cytochemical characterization of anterior pituitary target cells for the neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP) using biotinylated ligands. Peptides 14:59–65
42. **Habibi HR, Peter RE, Nahorniak CS, De L Milton RC, Millar RP** 1992 Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul Pept 37:271–284
43. **Wong AOL, Chang JP, Peter RE** 1992 Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamone D1 receptors. Endocrinology 130:1201–1210
44. **Trudeau VL, Somoza GM, Nahorniak CS, Peter RE** 1992 Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinoloy 56:483–490
45. **Marchant TA, Chang JP, Nahorniak CS, Peter RE** 1989 Evidence that gonadotropin-releasing hormone functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124:2509–2518
46. **Peng C, Huang YP, Peter RE** 1990 Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro.* Neuroendocrinology 52:28–34
47. **Vaughan JM, Rivier J, Spiess J, Peng C, Chang JP, Peter RE, Vale W** 1992 Isolation and characterization of hypothalamic growth hormone-releasing factor from common carp, *Cyprinus carpio*. Neuroendocrinology 56:539–549
48. **Rawlings SR, Piuz I, Schlegel W, Bockaert J, Journot L** 1995 Differential expression of pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptor subtypes in clonal pituitary somatotrophs and gonadotrophs. Endocrinology 136:2088–2098
49. **Pisegna JR, Wank SA** 1993 Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. Proc Natl Acad Sci USA 90:6345–6349
50. **Ogi K, Miyamoto Y, Masuda Y, Habata Y, Hosoya M, Ohtaki T, Masuo Y, Onda H, Fujion M** 1993 Molecular cloning and functional expression of a cDNA encoding the human pituitary adenylate cyclase activating polypeptide receptor. Biochem Biophys Res Commun 196:1511–1521
51. **Sprengel R, Braum T, Nikolics K, Segalooff DL, Seeburg PH** 1990 The testicular receptor for follicle-stimulating hormone: structure and functional expression of cloned cDNA. Mol Endocrinol 4:525–530
52. **Dahl SG, Edvardsen O, Style I** 1991 Molecular dynamics of dopamine at the D2 receptor. Proc Natl Acad Sci USA 88:8111–8115
53. **Godfrey PG, Rahal JO, Beamer WG, Copeland NG, Jenkins NA, Mayo KE** 1993 GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor function. Nat Genet 4:227–232
54. **Okamoto T, Murayama Y, Hayashi Y. Inagaki M, Ogata E, Nishimoto I** 1991 Identification of a Gs activator region of the β2-adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation. Cell 67:723–730
55. **Wong AOL, Van der Kraak G, Chang JP** 1994 Cyclic 3′,5′-adenosine monophosphate mediates dopamine D1-stimulated growth hormone release from goldfish pituitary cells. Neuroendocrinoloy 60:410–417
56. **Tatsuno I, Somogyvari-Vigh A, Mizuno K, Gottschall PE, Hidaka H, Arimura A** 1991 Neuropeptide regulation of interleukin-6 production from the pituitary: stimulation by pituitary adenylate cyclase activating polypeptide and calcitonin gene-related peptide. Endocrinology 129:1797–1804
57. **Artz E, Buric R, Stelzer G, Stalla J, Sauer J, Renner U, Stalla GK** 1993 Interleukin involvement inanterior pituitary cell growth regulation: effects of IL-2 and IL-6. Endocrinology 132:459–467
58. **Habibi HR** 1991 Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: effects of native GnRH peptides and a synthetic GnRH antagonist. Biol Reprod 44:275–283
59. **Benovic JL, Strasser RH, Daniel JL, Lefkowitz RJ** 1986 Beta adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. Proc Natl Acad Sci USA 83: 2797–2801