Distribution, Characterization, and Growth Hormone-Releasing Activity of Pituitary Adenylate Cyclase-Activating Polypeptide in the European Eel, Anguilla anguilla*

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ABSTRACT

The complementary DNA encoding pituitary adenylate cyclase-activating polypeptide (PACAP) has been cloned from two species of teleost fishes, the Sockeye salmon and the Thai catfish, and the amino acid sequence of PACAP has been determined in another teleost, the stargazer. However, to date, the detailed distribution of PACAP immunoreactivity has never been investigated in the fish brain. In the present study, we have determined the localization of PACAP-immunoreactive neurons in the central nervous system of a primitive teleost fish, the European eel *Anguilla anguilla*, using an antiserum raised against PACAP27. PACAP-positive perikarya were exclusively observed in the diencephalon, *i.e.* in the preoptic nucleus of the hypothalamus and in the dorsal and ventral nuclei of the thalamus. PACAP-immunoreactive fibers were detected in various areas of the brain, notably in the ventral telencephalon, the diencephalon, the mesencephalon, the cerebellar valvula, and the medulla oblongata. In addition, a dense accumulation of PACAP-containing nerve terminals was found in the pars distalis of the pituitary. The PACAP-like immunoreactivity contained in the eel brain was characterized by HPLC analysis combined with RIA quantification. The major form of PACAP-immunoreactive material coeluted with mammalian PACAP38. Molecular cloning of the PACAP precursor has previously shown that in fish, PACAP and GH-releasing hormone (GHRH) originate from the same precursor. We have thus investigated the effects of PACAP and GHRH on GH secretion from eel pituitary cells in primary culture. Dose-response experiments revealed that PACAP27 and PACAP38 possessed the same efficacy, but PACAP38 was 12 times more potent than PACAP27 in stimulating GH release (ED_{50} = 4.3×10^{-10} and 3.5×10^{-9} M, respectively). In contrast, GHRH, even at a high concentration (10^{-6} M), had no effect on GH release. Taken together, these data indicate that in the eel, PACAP may play a significant role in the regulation of somatotrope cells: 1) PACAP-immunoreactive neurons are exclusively located in the diencephalon and send numerous projections in the pars distalis; and 2) PACAP, but not GHRH, dose dependently stimulates GH secretion from cultured eel pituitary cells. (*Endocrinology* **139:** 4300–4310, 1998)

PITUITARY adenylate cyclase-activating polypeptide (PACAP) was first isolated from the ovine hypothalamus on the basis of its ability to stimulate adenylyl cyclase activity in rat pituitary cells (1). The peptide was found to exist in two amidated forms with 38 (PACAP38) and 27 (PACAP27) amino acid residues (2). The N-terminal portion of PACAP shows 68% identity with vasoactive intestinal polypeptide (VIP), identifying PACAP as a member of the VIP/secretin/glucagon/GH-releasing hormone (GHRH) superfamily. The sequence of PACAP has been highly conserved during evolution from protochordates to mammals. In particular, the primary structure of PACAP38 is identical in all mammalian species studied to date (3), whereas tunicate, fish, amphibian, and avian PACAP27 exhibit more than 95% sequence similarity with their mammalian counterpart (4–9) (Table 1).

The distribution of PACAP has been investigated in the central nervous system of human and monkey (10), sheep (11), rat (12), frog (13), and newt (14). In both mammals and amphibians, PACAP-immunoreactive neurons are particularly abundant in hypothalamic hypophysiotropic nuclei, and a dense network of PACAP-containing fibers innervates the external zone of the median eminence. Concurrently, functional studies have shown that PACAP stimulates the secretory activity of adenohypophysial cells (15) and increases the intracellular calcium concentration in various types of pituitary cells in mammals and amphibians (16, 17).

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TABLE 1. A comparison of the amino acid sequences of PACAP from different species

PACAP38	Mammals	HSDGI	FTDSY	SRYRK	QMAVK	KYLAA	VLGKR	YKQRV	KNK_NH_2
	Chicken	-I---	-----	-----	-----	-----	-----	-----	---
	Frog	-----	-----	-----	-----	-----	-----	----I	---
	Salmon	-----	-----	-----	-----	-----	-----	-R--Y	R--
	Stargazer	-----	-----	-----	----Q	-----	---R-	-R---	R--
	Catfish	-----	-----	-----	-----	-----	---R-	-R--F	R--
	$Tunicate_1$	-----	-----	----N	-----	-----	-----	----	
	$Tunicate_2$	-----	-----	----N	-----	--IN-	L----	----	
PACAP27	Mammals	-----	-----	-----	-----	-----	--_NH_2		

–, denotes residue identity; residues that are *underlined* indicate a cleavage-amidation recognition sequence.

In mammalian vertebrates, PACAP and GHRH originate from two distinct precursors (18, 19). In contrast, the recent cloning of the complementary DNA encoding the PACAP precursor in salmon (5), catfish (6), chicken (7), and tunicate (8) has revealed that in all of these species, PACAP and GHRH originate from a single common precursor. Although the functional significance of the cosynthesis of PACAP and GHRH is still a matter of speculation, it has been reported that PACAP actually stimulates GH secretion by salmon (20), frog (21), rat (22), and cow (23) somatotrophs as well as by human tumor cells (24).

In teleost fish, GH plays an important role not only in the stimulation of growth (25, 26), but also in the regulation of hydromineral homeostasis (27–29) and reproductive functions (30–33). In the eel, it has been recently shown that animals submitted to starvation exhibit a differential pattern of GH regulation depending on their physiological stage (34), suggesting that GH may be involved in the metabolic adaptation during the long period of fasting associated with the reproductive migration of this animal.

In the present study, we have determined the anatomical distribution of PACAP-containing neurons in the brain and pituitary of the eel. The molecular forms of PACAP were characterized by combining HPLC analysis with RIA quantification. Finally, the effect of PACAP on GH secretion by cultured pituitary cells was investigated.

Materials and Methods

Animals

Juvenile female European eel *Anguilla anguilla* (100–200 g BW) were net-caught in ponds in the north and west of France. The animals were transferred to the laboratory at the National Museum of Natural History (Paris, France) where they were kept in running oxygenated freshwater (12 ± 2 C) for at least 1 week. Animal manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators.

Reagents and test substances

Medium 199 (Earle's salts with sodium bicarbonate), penicillin, streptomycin, and fungizone were purchased from Life Technologies (Cergy-Pontoise, France). BSA was obtained from Boehringer Mannheim (Mannheim, Germany), and $Na^{125}I$ was obtained from Amersham (Les Ulis, France). Mammalian PACAP38 was synthesized by the solid phase methodology as previously described (13), and its identity was confirmed by amino acid analysis and mass spectrometry. PACAP27 was purchased from Bachem (Voisins-le-Bretonneux, France). Chicken VIP (cVIP), human GHRH (hGHRH), 3-aminobenzoic acid ethyl ester, gelatin, trifluoroacetic acid (TFA), polyethylene glycol, and polylysine were supplied by Sigma (St. Quentin Fallavier, France). Acetonitrile was obtained from Carlo Erba (Milan, Italy).

Immunohistochemical procedure

Eels were anesthetized by immersion in a solution of 0.1% of 3-aminobenzoic acid ethyl ester in water. The animals were perfused via the aortic bulb with a 0.9% NaCl solution. The perfusion was continued with 150 ml of a freshly prepared fixative solution consisting of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). The brains with the attached pituitaries were carefully removed and post-fixed overnight at 4 C in the same fixative solution. The tissues were cryoprotected by immersion in 15% sucrose in phosphate buffer overnight (4 C) and then transferred into 30% sucrose. Brains were embedded in Tissue-Tek (Jung, Nussloch, Germany) and frozen on dry ice. Frontal or parasagittal sections (10 μm thick) were cut in a cryostat (Frigocut 2800E, Reichert-Jung, Nussloch, Germany) and mounted on glass slides coated with 0.5% gelatin, 5% chrom-alum, and 50 μg/ml polylysine. Slices were processed for the indirect immunofluorescence technique, as previously described (13), with an antiserum raised against PACAP27 (11). Briefly, tissue sections were incubated overnight at 4 C with the PACAP antiserum diluted 1:100 in 0.1 M Coons buffer (sodium diethylmalonylurea; pH 7.4) containing 0.3% Triton X-100 and 1% BSA. The sections were rinsed in Coons buffer for 30 min and incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat antirabbit γ-globulins (Caltag Laboratories, San Francisco, CA) diluted 1:100. Finally, the slices were rinsed in Coons buffer, mounted with buffer/glycerol (1:1), coverslipped, and observed on a Leitz Orthoplan microscope (Leitz, Heidelberg, Germany) equipped with a Vario-Orthomat photographic system. Selected slices were also analyzed using a confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with a Diaplan optical system and an argon/krypton ion laser (excitation wavelengths, 488/568/610 nm).

Several types of controls were performed to verify the specificity of the immunostaining: 1) substitution of the primary antiserum with Coons buffer or 2) nonimmune rabbit serum, and 3) preincubation of the PACAP antibodies (diluted 1:100) with synthetic PACAP27, mammalian PACAP38 (mPACAP38), or cVIP (10^{-6} M each).

The nomenclature of eel brain areas was based on the atlas of Peter and Gill (35) and reports by Fremberg *et al.* (36), Kah *et al.* (37), and Medina *et al.* (38, 39).

Tissue extraction

Eels were decapitated, and the brains were dissected in three regions: olfactory bulbs and telencephalon, diencephalon and mesencephalon, and metencephalon and myelencephalon, as previously described (40). Each brain region was frozen and kept at −20 C until extraction. The tissues were immersed for 10 min in a cooled solution of ethanol-hydrochloric acid-water (75:18:7, vol/vol/vol) and sonicated. The homogenates were centrifuged (13,000 × *g*, 4 C) for 30 min. The supernatants were collected, dried by vacuum centrifugation (Speed-Vac concentrator, AES 2000, Savant, Hicksville, NY) and kept in a dry atmosphere until direct RIA or chromatographic analysis. The protein concentration in the pellet was determined by the Lowry method (41).

Characterization of PACAP in eel brain extracts

Dried samples were reconstituted in 2 ml of a water-TFA-acetonitrile solution (89.9:0.1:10, vol/vol/vol) and loaded onto three Sep-Pak C_{18} cartridges (Alltech Europe, Eke, Belgium) connected in series. The car-

tridges were rinsed with 20 ml of the same solution, and the bound material was eluted with 10 ml of a water-TFA-acetonitrile solution (43.9:0.1:56, vol/vol/vol). The solvent was evaporated in a Speed-Vac concentrator. The samples were reconstituted in 500 μl of a water-TFA-acetonitrile solution (89.9:0.1:10, vol/vol/vol) and centrifuged (13,000 × *g*, 4 C). The supernatant was injected onto a 0.46 × 25-cm Vydac C_{18} column (Hesperia, CA) equilibrated with a solution of 20% acetonitrile and 0.1% TFA at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 40% over 20 min using a linear gradient. HPLC standards consisted of synthetic PACAP27 and mPACAP38 (1 μg each). Fractions of 0.75 ml were collected, evaporated, and kept dry until RIA.

PACAP RIA

The concentrations of PACAP-like material in the eel brain were measured by RIA. Iodination of synthetic PACAP27 was performed by means of the lactoperoxidase technique as previously described (42). The radioligand was purified by reverse phase HPLC on a 0.46 × 25-cm Vydac C_{18} column using a mobile phase of acetonitrile in 0.1% TFA. Monoiodinated PACAP27 eluted at 36% acetonitrile. The RIA was performed in veronal buffer (0.02 M; pH 9.1) containing 0.1% Triton X-100 and 3% BSA, using an antiserum against PACAP38 at a dilution of 1:20,000 (43). After a 2-day incubation at 4 C, the antibody-bound fraction was immunoprecipitated by addition of 200 μl goat antirabbit γ-globulins (1:30), 200 μl normal rabbit serum (1:150), and 500 μl 5% polyethylene glycol 8000. After a 2-h incubation at room temperature, the mixture was centrifuged, and the pellet containing the bound fraction was counted on a γ-counter (LKB-Wallac, Rockville, MD). The standard curves were set up with synthetic PACAP27 and mPACAP38 at concentrations ranging from 0.1–333 ng/tube.

Primary culture of eel pituitary cells

Dispersion of eel pituitary cells was performed using an enzymatic and mechanical procedure as previously described (44). Forty to 100 animals were used for each experiment. Cells were cultured at a density of 62,500 cells/250 μl in each well on poly-L-lysine-precoated 96-well plates (Costar, Brumath, France) in serum-free culture medium (medium 199 containing Earle's salts with 1.25 g/liter sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone) (45). Cells were incubated at 18 C in a humidified incubator (Napco, Tualotin, OR) with an atmosphere of CO_2-air (3:97). After 1 day of culture, the medium was renewed, and the cells were cultured for 2 more days. The effect of PACAP-related peptides on GH secretion was then studied during a 24-h incubation.

Stock solutions of synthetic PACAP27, mPACAP38, cVIP, and hGHRH were diluted in distilled water at a concentration of 10^{-4} M. Final dilutions were prepared in culture medium just before addition to the wells. Six-well replicates were used for each peptide treatment. After a 24-h incubation, the culture medium was collected and kept frozen until RIA.

GH RIA

The concentration of GH secreted in the culture medium by cultured pituitary cells was measured in duplicate using a homologous RIA for eel GH, as previously described (34). Briefly, the antiserum generated against eel GH was used at a dilution of 1:20,000. Purified eel GH was radioiodinated by the chloramine-T method and purified on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden). Results are expressed as nanograms of GH per 62,500 cells.

Statistical analysis

The effect of each neuropeptide on GH secretion was tested in six wells, and the results were expressed as the mean ± SEM. The experiments were repeated three times independently. The significance of the differences between control and treated cells was assessed by ANOVA (GraphPad Software, Inc., San Diego, CA) followed by a *post-hoc* Tukey's test.

Results

Distribution of PACAP-immunoreactive structures in the eel brain

The distribution pattern of PACAP-like immunoreactivity in the central nervous system of the eel is schematically represented in Figs. 1 and 2. Table 2 summarizes the location and relative density of immunoreactive cell bodies and fibers in the eel brain.

PACAP-immunoreactive perikarya were observed in the ventral part of the diencephalon, *i.e.* in the anterior and posterior parvocellular preoptic nucleus and in the magnocellular preoptic nucleus (Fig. 2, B–E). Parasagittal sections at the level of the hypothalamus revealed the presence of clusters of immunoreactive cell bodies along the rostrocaudal axis of the preoptic nucleus (Figs. 1 and 3A). Groups of immunoreactive perikarya were also found in the thalamus within the ventral and dorsal thalamic nuclei (Fig. 2, E and F). The olfactory bulbs, telencephalon, mesencephalon, metencephalon, and myelencephalon were devoid of PACAP-positive cell bodies.

Scattered PACAP-immunoreactive fibers were detected in the ventral telencephalon (Fig. 2, A and B). These processes probably originated from perikaria located in the preoptic region of the diencephalon. In this latter area, a very dense network of PACAP-positive fibers was observed (Fig. 2, C and D), and some of these immunoreactive elements established contacts with the cerebrospinal fluid. Bundles of labeled fibers were seen ascending dorsally within the thalamus along the ependyma of the third ventricle (Fig. 2, E and F). Another bundle of nerve processes was observed in the ventral part of the diencephalon, oriented toward the pituitary (Fig. 2, E and F). At the level of the pituitary stalk, a dense tract of PACAP-positive fibers entered the neurohypophysial digitations (Figs. 2F and 3B). Parasagittal sections revealed that these PACAP-immunoreactive fibers were located in the digitations invading the anterior part of the

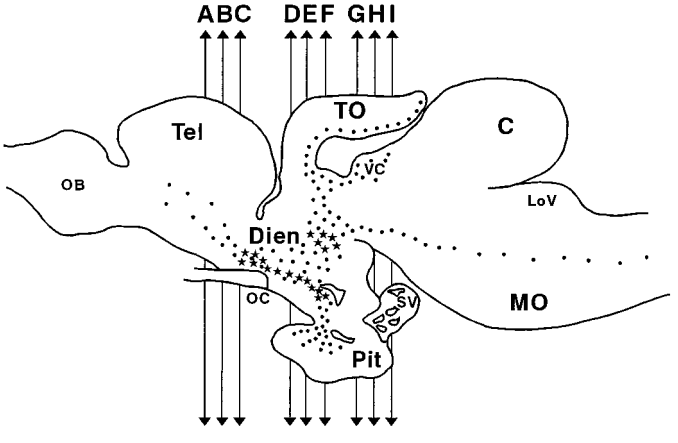

FIG. 1. Schematic parasagittal section through the eel brain depicting the distribution of PACAP-immunoreactive cell bodies (*stars*) and fibers (*dots*). The anteroposterior position of the frontal sections studied is mentioned (*arrows*). The relative density of the symbols is meant to be proportional to the density of the immunoreactive elements. C, Cerebellum; Dien, diencephalon; LoV, vagal lobe; MO, medulla oblongata; OB, olfactory bulbs; OC, optic chiasma; Pit, pituitary; SV, saccus vasculosus; Tel, telencephalon; TO, optic tectum; VC, cerebellar valvula.

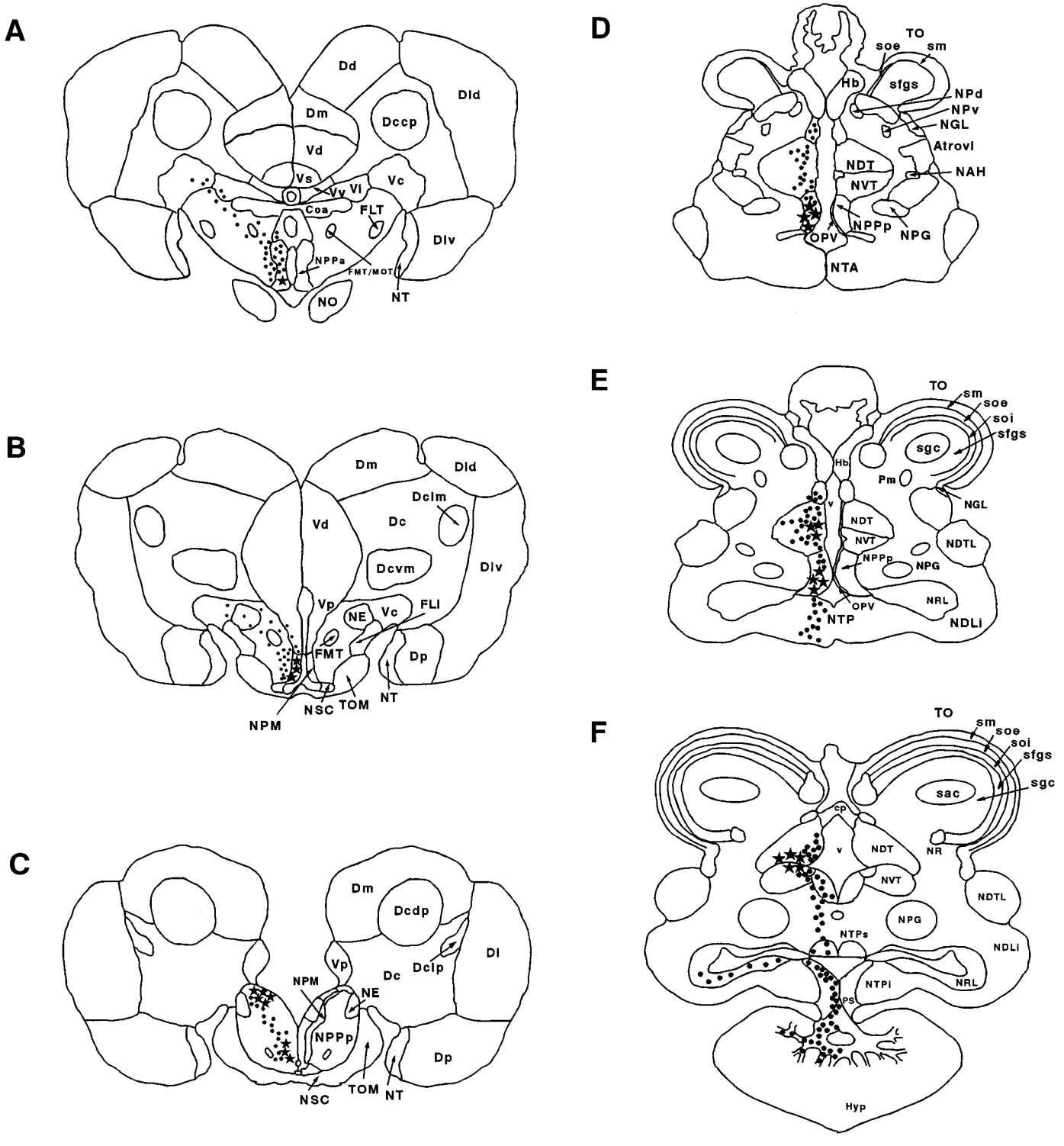

FIG. 2. Schematic frontal sections through the eel brain depicting the distribution of PACAP-immunoreactive cell bodies (*stars*) and fibers (*dots*). The localization of anatomical structures is indicated on the *right* hemisection. The capital letters (A–I) refer to the rostrocaudal levels of the sections as indicated in Fig. 1. For abbreviations, see Table 2. Figures are taken from the atlas of Medina *et al.* (39).

pituitary (Fig. 3C), whereas no immunoreactivity was detected in the posterior part (Fig. 1). In the mesencephalon, ascending PACAP-containing fibers innervated the torus semicircularis and the stratum album centrale of the optic tectum (Fig. 2, G and H). The locus coeruleus and the lateral parts of the mesencephalon exhibited a moderate density of immunoreactive processes (Fig. 2, H and I). Scattered PACAP-positive fibers were also found in the cerebellar valvula and in the granular eminence of the cerebellum (Fig. 2I). In the ventral zone of the medulla oblongata, PACAP-

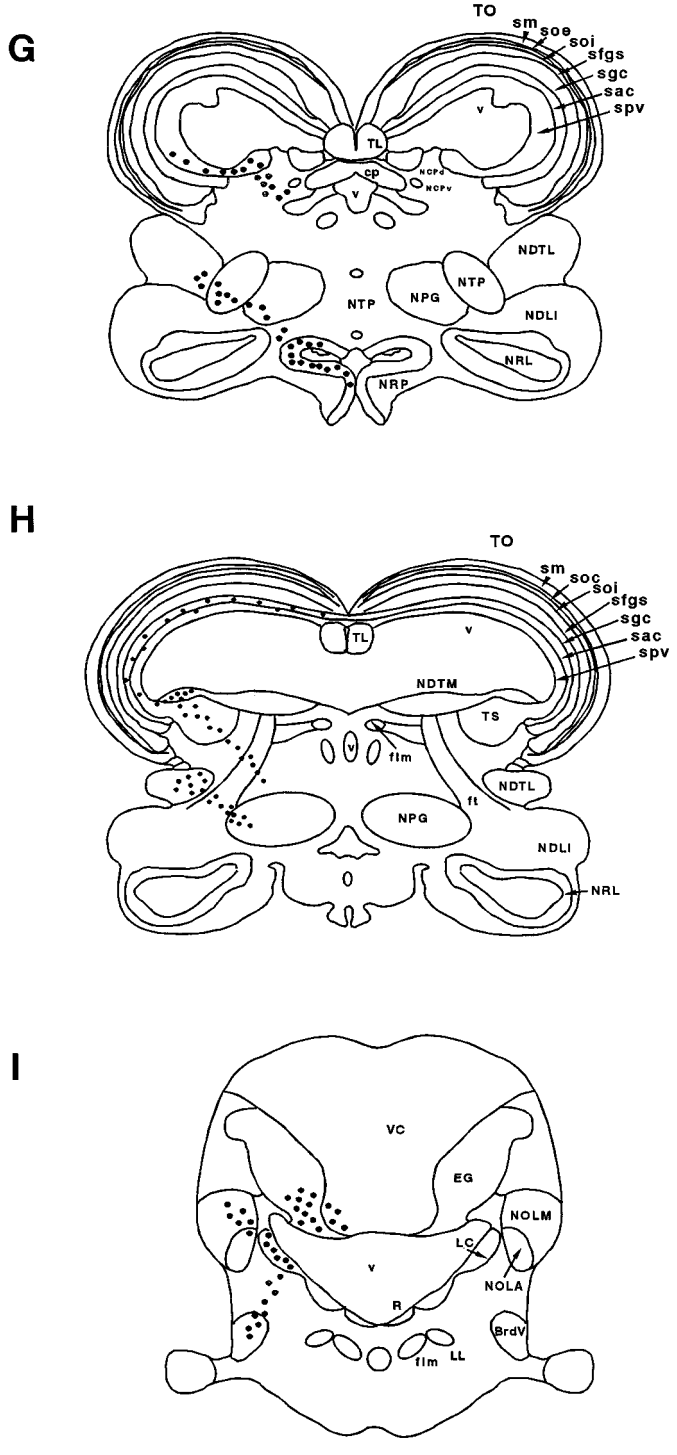

FIG. 2. Continued

immunoreactive fibers were seen running ventrally to the central canal (Figs. 1 and 3D).

No labeling was observed when the PACAP antiserum was replaced with Coons buffer or nonimmune rabbit serum. Similarly, preincubation of the primary antiserum with synthetic PACAP27 or mPACAP38 totally abolished immunostaining. In contrast, preincubation of the antiserum with VIP did not modify the distribution or the intensity of the immunostaining.

Characterization of PACAP-like material in eel brain extracts

Synthetic PACAP27 and mPACAP38 displaced antibody-bound [^{125}I]PACAP27 with similar efficacies (Fig. 4). In contrast, cVIP did not displace binding of the tracer. Serial dilutions of extracts from the three different regions of the eel brain generated displacement curves that were parallel to those obtained with synthetic PACAP27 or mPACAP38 (Fig. 4). The apparent concentration of PACAP-immunoreactive material contained in the tissue homogenates was 2272 pg/mg protein in the olfactory bulb-telencephalon region, 4054 pg/mg protein in the diencephalon-mesencephalon region, and 1640 pg/mg protein in the metencephalon-myelencephalon region. The elution profiles of synthetic standards and eel diencephalon-mesencephalon extract are shown in Fig. 5. The retention times of mPACAP38 and PACAP27 were 12 and 14.6 min, respectively, as determined by their optical absorbance at 215 nm (Fig. 5A). HPLC analysis of an eel brain extract resolved a major peak of PACAP-immunoreactive material that coeluted with synthetic mPACAP38 (Fig. 5B).

Effects of PACAP and GHRH on GH secretion

The effects of graded doses of PACAP27 and mPACAP38 on GH secretion are shown in Fig. 6. Both peptides induced a dose-dependent stimulation of GH release. PACAP38 appeared to be more potent than PACAP27 (ED_{50} = 4.3 ± 0.23 × 10^{-10} and 3.5 ± 0.8 × 10^{-9} M, respectively), although the two peptides exhibited similar efficacies (2.7- and 3.2-fold over control values, respectively). In contrast, cVIP only induced a significant stimulation of GH release at a concentration of 10^{-6} M (data not shown).

At a concentration of 10^{-6} M, both PACAP27 and PACAP38 induced a significant stimulation of GH release ($P < 0.001$). In contrast, at the same concentration, hGHRH had no effect on GH secretion (Fig. 7). In addition, concomitant administration of PACAP27 and hGHRH did not modify the stimulatory effect of PACAP27 on GH release (Fig. 7).

Discussion

The present study has provided the first detailed description of PACAP-immunoreactive structures in the central nervous system of a fish. The antisera against mammalian PACAP employed in this study have been successfully used previously to localize PACAP-immunoreactive neurons in the frog brain (13, 43). In the central nervous system of the eel, PACAP-containing cell bodies were exclusively observed in the diencephalon, specifically in the preoptic nucleus of the hypothalamus and in the dorsal and ventral nuclei of the thalamus. The occurrence of a dense plexus of PACAP-immunoreactive fibers in the rostral and proximal pars distalis suggested that these fibers, in much the same way as GnRH nerve terminals (46), originate from the preoptic nucleus. In support of this hypothesis, retrograde tracing experiments have shown that in the eel, perikarya located

TABLE 2. Localization and relative abundance of PACAP-immunoreactive perikarya and fibers in the eel brain

Structure	Perikarya	Fibers
Telencephalon		
Commissure anterior (Coa)	−	−
Central portion of the area dorsalis (Dc)	−	−
Central posterior portion of the central zone of the area dorsalis (Dccp)	−	−
Dorsal posterior portion of the central zone of the area dorsalis (Dcdp)	−	−
Lateral medial portion of the central zone of the area dorsalis (Dclm)	−	−
Lateral posterior portion of the central zone of the area dorsalis (Dclp)	−	−
Ventral medial portion of the central zone of the area dorsalis (Dcvm)	−	−
Dorsal zone of the area dorsalis (Dd)	−	−
Lateral zone of the area dorsalis (Dl)	−	−
Dorsal portion of the lateral zone of the area dorsalis (Dld)	−	−
Ventral portion of the lateral zone of the area dorsalis (Dlv)	−	−
Medial zone of the area dorsalis (Dm)	−	−
Posterior zone of the area dorsalis (Dp)	−	−
Fasciculus lateralis telencephalis (FLT)	−	−
Fasciculus medialis telencephalis (FMT)	−	−
Medial olfactory tract (MOT)	−	−
Nucleus entopeduncularis (NE)	−	−
Nucleus taenia (NT)	−	−
Olfactorius bulbus (OB)	−	−
Central nucleus of the area ventralis (Vc)	−	+
Dorsal nucleus of the area ventralis (Vd)	−	−
Lateral nucleus of the area ventralis (Vl)	−	+
Postcommissuralis nucleus of the area ventralis (Vp)	−	+
Supracommissuralis nucleus of the area ventralis (Vs)	−	−
Ventral nucleus of the area ventralis (Vv)	−	−
Diencephalon		
Commissure posterior (cp)	−	−
Nucleus habenularis (Hb)	−	−
Nucleus anterior hypothalami (NAH)	−	−
Nucleus commissurae posterioris pars dorsalis (NCPd)	−	−
Nucleus commissurae posterioris pars ventralis (NCPv)	−	−
Nucleus diffusus lobi inferioris (NDLi)	−	−
Nucleus dorsalis thalami (NDT)	+++	+++
Nucleus diffusus tori lateralis (NDTL)	−	++
Nucleus preglomerulosus (NPG)	−	−
Nucleus preopticus magnocellularis (NPM)	+++	+++
Nucleus preopticus parvocellularis anterior (NPPa)	++	+++
Nucleus preopticus parvocellularis posterior (NPPp)	+++	+++
Nucleus rotondus (NR)	−	−
Nucleus recessus lateralis (NRL)	−	+++
Nucleus recessus posterioris (NRP)	−	+++
Nucleus suprachiasmaticus (NSC)	−	−
Nucleus tuberis anterior (NTA)	−	−
Nucleus tuberis posterior (NTP)	−	+++
Nucleus tuberis posterior pars inferior (NTPi)	−	+++
Nucleus tuberis posterior pars superior (NTPs)	−	+++
Nucleus ventralis thalami (NVT)	+++	+++
Paraventricular organ (OPV)	−	++
Tractus opticus marginalis (TOM)	−	−
Mesencephalon		
Area optica tractus optici ventrolateralis (Atrovl)	−	−
Fasciculus longitudinalis medialis (flm)	−	−
Fasciculus transversalis (ft)	−	+
Locus coeruleus (LC)	−	+++
Nucleus dorsalis tegmenti mesencephali (NDTM)	−	+
Nucleus geniculatus lateralis (NGL)	−	−
Nucleus octavolateralis anterior (NOLA)	−	+
Nucleus octavolateralis medialis (NOLM)	−	+
Nucleus pretectalis dorsalis (NPd)	−	−
Nucleus pretectalis ventralis (NPv)	−	−
Nucleus pretectalis medialis (Pm)	−	−
Stratum album centrale (sac)	−	+++
Stratum fibrosum griseum superficiale (sfgs)	−	−
Stratum griseum centrale (sgc)	−	−
Stratum marginale (sm)	−	−
Stratum opticum pars externa (soe)	−	−
Stratum opticum pars interna (soi)	−	−
Stratum periventriculare (spv)	−	++
Torus longitudinalis (TL)	−	−
Torus semicircularis (TS)	−	++

TABLE 2. Continued

Structure	Perikarya	Fibers
Metencephalon		
Eminentia granularis (EG)	−	++
Valvula cerebellaris (VC)	−	+
Myelencephalon		
Descending tract of the trigeminal nerve (BrdV)	−	++
Lateral lemniscus (LL)	−	−
Raphe nucleus (R)	−	++
Hypophysis		
Hypophysis cerebri (Hyp)	−	−
Pituitary stalk (ps)	−	+++
Proximal pars distalis	−	+++
Rostral pars distalis	−	+++
Pars proximalis	−	−

+, Sparse; ++, moderately dense; +++, highly dense; −, no immunoreactive perikarya or fibers. Abbreviations according to Peter and Gill (35), Fremberg *et al.* (36), Kah *et al.* (37), and Medina *et al.* (38, 39).

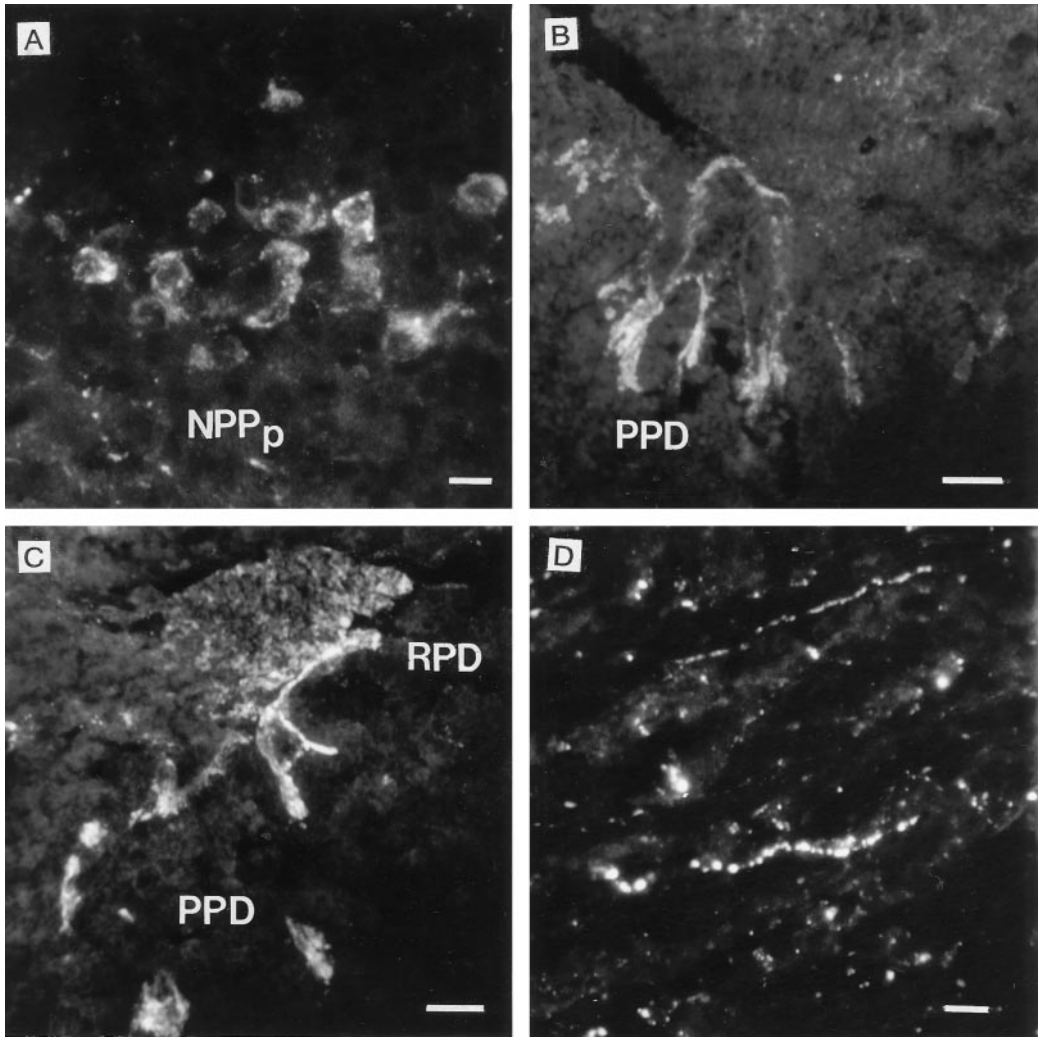

FIG. 3. Confocal laser scanning microscope microphotographs illustrating the localization of PACAP-immunoreactive elements in the eel brain. A, Parasagittal section at the level of the diencephalon showing the presence of numerous PACAP-positive cell bodies in the preoptic nucleus (NPPp). *Scale bar* = 10 μm. B, Frontal section through the proximal pars distalis of the pituitary (PPD) showing the presence of a dense network of PACAP-immunoreactive fibers in the neurohypophysial digitations. *Scale bar* = 50 μm. C, Parasagittal section through the pituitary showing a dense accumulation of PACAP-immunoreactive processes in the rostral pars distalis (RPD). *Scale bar* = 50 μm. D, Parasagittal section through the medulla oblongata showing the presence of PACAP-immunoreactive fibers running ventrally to the central canal. *Scale bar* = 10 μm.

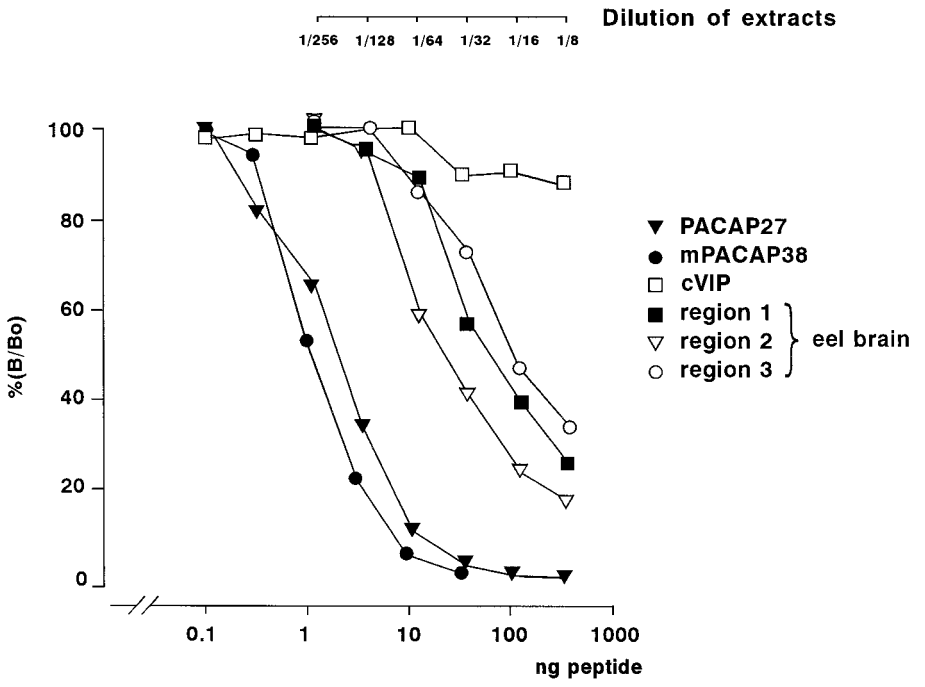

FIG. 4. Semilogarithmic plots comparing competitive inhibition of antibody-bound ^{125}I-labeled PACAP27 by synthetic PACAP27 (▼), mPACAP38 (●), cVIP (□), and serial dilutions of eel brain extracts. Region 1 corresponds to the olfactory bulbs and telencephalon (■), region 2 corresponds to the diencephalon and mesencephalon (▽), and region 3 corresponds to the metencephalon and myelencephalon (○). Each tissue extract contained the equivalent of 16 brain regions.

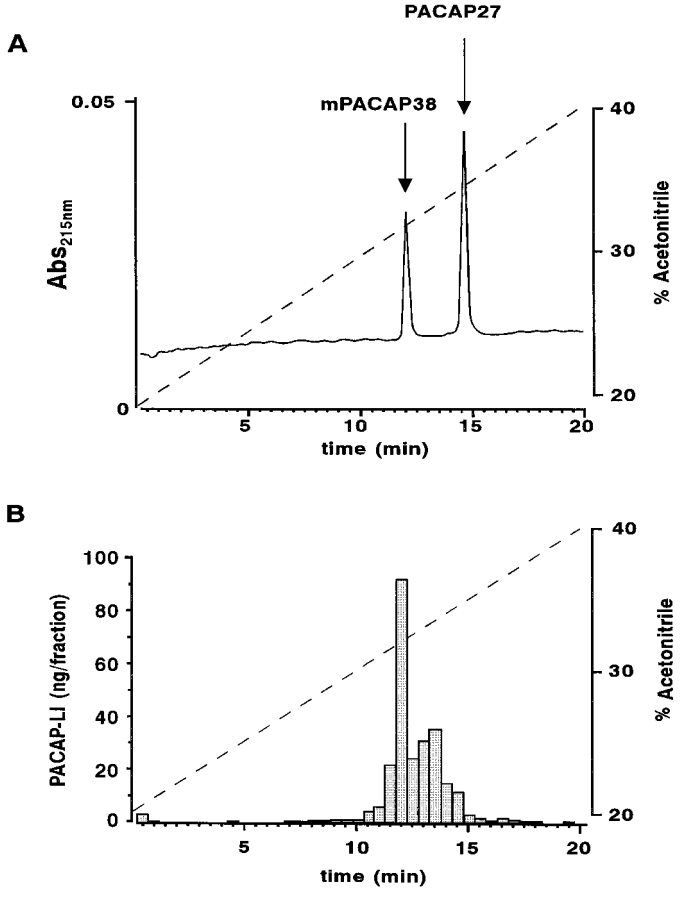

FIG. 5. Reverse phase HPLC analysis of a Sep-Pak-prepurified eel diencephalon-mesencephalon extract. A, Elution profiles of synthetic PACAP27 and mPACAP38 detected by their absorbance at 215 nm. B, RIA quantification of PACAP-like material in the elution fractions from the brain extract. The *dashed line* shows the concentration of acetonitrile in the eluting solvent.

in the preoptic area directly project into the pars distalis (Dufour, S., unpublished data). Concurrently, PACAP-immunoreactive fibers were widely distributed outside neuroendocrine territories, suggesting that in the eel, as in other vertebrate species, PACAP may be involved in various neurotransmitter and/or neuromodulator functions.

In agreement with the immunohistochemical distribution of PACAP-positive elements, quantification of PACAP-like immunoreactivity in crude brain extracts has shown that the diencephalon-mesencephalon region contains the highest concentration of peptide. HPLC analysis combined with RIA detection has revealed that the major immunoreactive component coeluted with mPACAP38. Consistent with this observation, it has been previously shown that PACAP38 is the predominant molecular form in the brain of mammals (47).

The occurrence of a dense network of PACAP-immunoreactive terminals in the pars distalis indicated that in the eel, PACAP could act as a hypophysiotropic neurohormone. In fact, cloning of the complementary DNA encoding the PACAP precursor has shown that in all submammalian vertebrate species studied to date, GHRH and PACAP originate from the same precursor (5–7). The observation that in the eel, PACAP-immunoreactive perikarya are located in the parvo- and magnocellular portions of the preoptic nucleus where GHRH-positive cell bodies have been previously detected (48) supports the view that in *A. anguilla*, the two peptides may also originate from a common precursor. These data prompted us to investigate the effects of PACAP and GHRH on GH secretion. The present study has demonstrated that PACAP27 and PACAP38 both induce a dose-dependent stimulation of GH release from cultured eel pituitary cells. These findings are consistent with previous reports showing that PACAP stimulates GH secretion from bovine (23), sheep (49), porcine (50), rat (51), chicken (52), frog (21), and salmon (20) pituitary cells. PACAP was also found to increase cy-

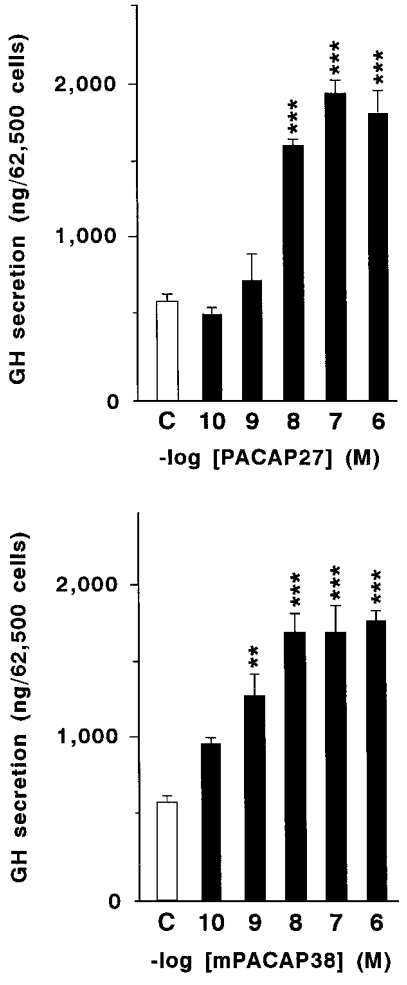

FIG. 6. Effects of graded doses of PACAP27 and mPACAP38 on GH secretion by cultured eel pituitary cells. The concentration of GH was measured in the culture medium after 24 h of incubation. Data are the mean ± SEM of six separate determinations from one representative experiment. The experiment was repeated three times, and similar results were obtained. **, $P < 0.01$; ***, $P < 0.001$ (*vs.* control).

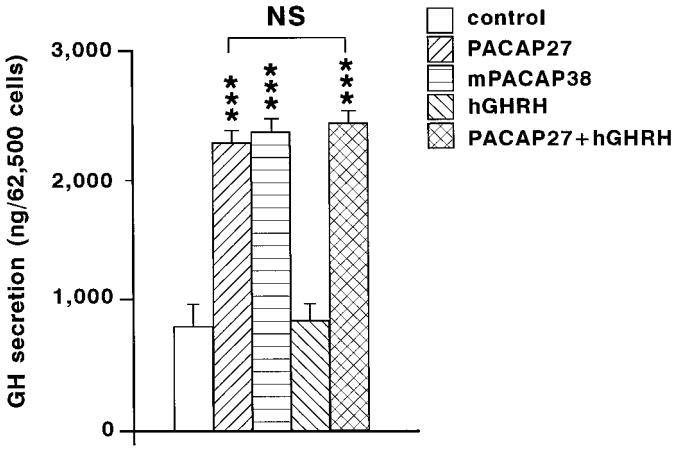

FIG. 7. Effects of PACAP and GHRH on GH secretion by cultured eel pituitary cells. Cells were cultured for 24 h in the absence (control) or presence of 10^{-6} M PACAP27, mPACAP38, or hGHRH alone or PACAP27 and hGHRH together. GH secretion into the culture medium was measured using a homologous RIA. The results represent the mean ± SEM of six separate determinations from one representative experiment. The experiment was repeated three times, and similar results were obtained. ***, $P < 0.001$ (*vs.* control).

tosolic calcium concentrations in GH cells from the rat (53, 54) and frog (17) pituitary. In the eel, PACAP38 was approximately 12 times more potent than PACAP27, but the two peptides exhibited the same efficacy to stimulate GH secretion. In contrast, VIP had no effect at doses ranging from 10^{-10}-10^{-7} M and stimulated GH secretion only at a concentration of 10^{-6} M, indicating that the action of PACAP on eel somatotrope cells was mediated through a receptor exhibiting the pharmacological profile of type I mPACAP receptor (55). In agreement with this finding, it has been recently shown that GH-enriched rat pituitary cells express exclusively type I PACAP receptors (56).

It has been previously reported that GHRH stimulates GH secretion in various species of teleosts, including the Sockeye salmon (20), the rainbow trout (57), and the goldfish (58). In contrast, the present data have shown that in the eel, hGHRH has no effect, even at a high concentration, on GH release. Moreover, GHRH did not modify the response of eel somatotrope cells to PACAP27. These observations indicate that in the primitive teleost fish, PACAP rather than GHRH plays a role in the regulation of GH secretion. Consistent with this finding, it has been reported that in the salmon, PACAP is far more efficient than native GHRH in stimulating GH release (20). Alternatively, the lack of effect of hGHRH on GH secretion could be ascribed to species specificity, inasmuch as the structure of GHRH has been less conserved than that of other hypophysiotropic neuropeptides. In particular, catfish GHRH (6) has only 58% identity to salmon GHRH (5) and 60% identity to carp GHRH (58). Molecular cloning of the eel PACAP precursor is clearly required to determine whether it possesses a GHRH-like sequence and, if so, whether this peptide has any GH-releasing activity.

In conclusion, the present study has provided the first detailed mapping of PACAP-immunoreactive neurons in a fish. The presence of a population of PACAP-containing perikarya in the preoptic area, the dense accumulation of PACAP-immunoreactive fibers in the distal lobe of the pituitary, and the stimulatory effect of PACAP on GH secretion *in vitro* indicate that PACAP may act as a physiological GH-releasing factor in the eel. The lack of effect of GHRH on GH secretion suggests that in this primitive teleost fish, PACAP may play a crucial role in the control of somatotrope cell activity.

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