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# Effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on prolactin and somatolactin release from the goldfish pituitary in vitro

Kouhei Matsuda [a,*], Yumika Nejigaki [a], Misuzu Satoh [a], Chika Shimaura [a], Mio Tanaka [a], Keiichi Kawamoto [a], Minoru Uchiyama [a], Hiroshi Kawauchi [b], Seiji Shioda [c], Akiyoshi Takahashi [b]

[a] Laboratory of Regulatory Biology, Graduate School of Science and Engineering, University of Toyama, Toyama 930-8555, Japan
[b] Laboratory of Molecular Endocrinology, School of Fisheries Sciences, Kitasato University, Iwate 022-0101, Japan
[c] Department of Anatomy, Showa University School of Medicine, Tokyo 142-8555, Japan

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**Abstract**

Pituitary adenylate cyclase-activating polypeptide (PACAP) plays a role in mediating growth hormone and gonadotropin release in the teleost pituitary. In the present study, we examined the immunohistochemical relationship between PACAP nerve fibers and prolactin (PRL)- and somatolactin (SL)-producing cells in the goldfish pituitary. Nerve fibers with PACAP-like immunoreactivity (PACAP-LI) were identified in the neurohypophysis in close proximity to cells containing PRL-LI or SL-LI. Several cells with PRL-LI or SL-LI showed PACAP receptor ($PAC_1R$)-LI. The cell immunoblot assay method was used to examine the effect of PACAP on PRL and SL release from dispersed goldfish pituitary cells. Treatment with PACAP increased the immunoblot area for PRL- and SL-LI from individual pituitary cells in a dose-dependent manner. The effect of PACAP on the expression of mRNAs for PRL and SL in cultured pituitary cells was also tested. Semiquantitative analysis revealed that the expression of SL mRNA, but not PRL mRNA, was increased significantly by the treatment with PACAP. The effect of PACAP on intracellular calcium mobilization in isolated pituitary cells was also investigated using confocal laser-scanning microscopy. The amplitude of $Ca^{2+}$ mobilization in individual cells showing PRL- or SL-LI was increased significantly following exposure of cells to PACAP. These results indicate that PACAP can potentially function as a hypophysiotropic factor mediating PRL and SL release in the goldfish pituitary.


*Keywords:* PACAP; Goldfish; Pituitary; Prolactin; Somatolactin

## 1. Introduction

The teleost pituitary is directly attached to the hypothalamus by a short stalk posterior to the optic chiasma. The neurohypophysis interdigitates with all regions of the adenohypophysis – the rostral pars distalis, proximal pars distalis, caudal pars distalis and pars intermedia – and adenohypophysial cells are directly innervated from the hypothalamic neurons. In teleost fish, several hypothalamic neuropeptides have been identified as hypophysiotropic factors [1]. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide, which was first isolated from ovine hypothalami during an attempt to isolate a novel hypophysiotropic peptide that could activate adenylate cyclase in cultured rat pituitary cells [2]. The primary structure of PACAP is highly conserved among vertebrates [3]. PACAP is widely distributed in the central nervous system and peripheral tissues, and is involved in many physiological processes, such as cell proliferation, differentiation, cell death, neurotransmission and neuroprotection in mammals [4–6].

Several reports have also described the distribution and physiological function of PACAP in teleosts [5,7,8]. Neuronal cell bodies with PACAP-like immunoreactivity (PACAP-LI) are distributed mainly in the diencephalon, and their fibers project into the adenohypophysis of teleosts such as the goldfish [9], stargazer [10], and European eel [11]. PACAP is able to stimulate growth hormone (GH) and gonadotropin release from cultured pituitary cells of teleosts, such as the goldfish, European eel and salmon, in vitro [9,11–16]. PACAP receptors such as $PAC_1$ receptor (PACAP type I receptor) and VPAC receptor

* Corresponding author. Laboratory of Regulatory Biology, Graduate School of Science and Engineering, University of Toyama, 3190-Gofuku, Toyama 930-8555, Japan. Tel.: +81 76 445 6638; fax: +81 76 445 6549.
*E-mail address:* kmatsuda@sci.u-toyama.ac.jp (K. Matsuda).

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were cloned and characterized from the goldfish pituitary and brain [9,17,18]. It is well known that the role of PRL as a FW-adapting pituitary hormone, whereas the function of SL is under way in fish. We have reported that PACAP nerve fibers can be identified in the neurohypophysis in close proximity to pituitary cells containing GH, prolactin (PRL) and somatolactin (SL) in a teleost, the stargazer [19,20]. The aim of the present study was thus to use the goldfish, a fish model commonly employed to study the neuronal actions of PACAP, to examine the functional relationship between PACAP and PRL or SL in the teleost pituitary. To this end, immunohistochemical studies were carried out to examine the nature of relationship between PACAP nerve fibers and PRL- or SL-producing cells in the goldfish pituitary. The effect of PACAP on PRL and SL release in dispersed and cultured pituitary cells was studied using the cell immunoblot assay method, while the effect of PACAP on the expression of mRNAs for PRL and SL in the cultured pituitary cells was also tested. PACAP is known to modulate the adenylate cyclase/cAMP/protein kinase A pathway in teleost pituitary cells [12,14–16], and thereby increased $[Ca^{2+}]_i$ leading to GH and gonadotropin release. Given that a similar mechanism might take place in relation to PRL- and SL-containing pituitary cells, we used confocal laser-scanning microscopy to examine if PACAP increases $[Ca^{2+}]_i$ in these cells.

## 2. Materials and methods

### 2.1. Animals

Goldfish (*Carassius auratus*, body weight [BW] 10–25 g) of both sexes were purchased from a commercial supplier, and kept for 2 weeks under controlled light/dark conditions (12L/12D) with the water temperature maintained at 20–24 °C. The fish were fed once per day at 12:00 noon with a uniform granular diet (containing 32% protein, 5% dietary fat, 2% dietary fiber, 6% minerals and 8% water, Tetragold, Tetra GmbH, Melle, Germany) until used in experiments. All animal experiments were conducted in accordance with the University of Toyama guidelines for the care and use of animals.

### 2.2. Immunohistochemistry for PACAP, PRL, SL and $PAC_1R$

For immunostaining of PACAP and adenohypophysial hormones, we used rabbit primary antisera raised against stargazer PACAP27 (anti-PACAP serum, diluted 1:1000; [10,21]), salmon prolactin (anti-PRL serum, diluted 1:4000; [22]) and salmon somatolactin (anti-SL serum, diluted 1:8000; [23]). Previous preabsorption tests of these antisera resulted in no specific staining; the specificity of each antiserum has been well demonstrated in the teleost pituitary, and checked by immunoblotting analysis, enzyme immunoassay and radioimmunoassay [21–24]. The double-immunostaining for PACAP and PRL or SL was used for the simultaneous localization of multiple tissue antigens, in which the elution and the indirect immunohistochemical procedure were repeated each time with a different substrate [25]. Six goldfish of both sexes were used. In brief, fish were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, MO, USA), and the brain of each fish was removed immediately, immersed in Bouin's fixative at 4 °C for 48 h, trimmed, dehydrated, and embedded in paraffin. Sagittal sections (thickness 8-μm) were then cut. Several sections containing the hypothalamo-pituitary region were treated with normal swine serum (diluted 1:50, DAKO A/S, Glostrup, Denmark), then stained with anti-PACAP serum at room temperature for 24 h. Following a 1.5-h incubation with biotinylated swine anti-rabbit immunoglobulin (diluted 1:200, DAKO), the sections were treated with avidin-biotinylated peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA) for 1.5 h and then incubated with 10 mg 3,3′-diaminobenzidine-4HCl (DAB, Dojindo Laboratory, Kumamoto, Japan) and 0.03% $H_2O_2$ in 15 ml 0.05 M Tris–HCl buffer (pH 7.6) containing 150 mM NaCl. After immunostaining for PACAP, anti-PACAP serum was removed from its antigen in the following manner. Sections were reacted with 0.1 M glysine HCl buffer (pH 2.0) for 2 h. After washing with 0.01 M phosphate-buffered saline (PBS) (pH 7.4), sections were immunostained with anti-PRL serum or anti-SL serum at room temperature for 24 h, treated with alkaline phosphatase-labeled swine anti-rabbit immunoglobulin (diluted 1:100, DAKO) for 1 h, and finally treated with 450 μg nitro blue tetrazolium (NBT, Sigma) and 175 μg 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) in 1 ml 0.1 M Tris–HCl buffer (pH 9.5) containing 100 mM NaCl and 5 mM $MgCl_2$. After all immunostaining steps had been completed, the sections were dehydrated, cleared in xylene, and mounted. The specificity of each immunoreaction in the method was checked by substitution of the respective antiserum with PBS or replacement of each antiserum with non-immune rabbit serum (diluted 1:500, Dako). In all cases, objective immunoreactions were negative, and there was no overlap of the color developments by DAB and NBT/BCIP. Immunostained sections were observed with the aid of a light microscope (BH40, Olympus, Tokyo, Japan), and digital images were recorded using a digital camera (CoolPix 995, Nikon, Tokyo, Japan).

For immunostaining of the PACAP receptor ($PAC_1R$), and PRL or SL, we used rabbit primary antiserum raised against mouse $PAC_1R$ (anti-$PAC_1R$ serum, diluted 1:600; [26]), anti-PRL serum and anti-SL serum. Previous preabsorption tests of anti-$PAC_1R$ serum resulted in no specific staining; the specificity of an antiserum has been well demonstrated, and checked by immunoblotting analysis [26]. We also tested the specificity of an anti-$PAC_1R$ serum, and its immunoreaction was negative when preabsorbed with synthetic antigen peptide. Several sections were immunostained with anti-$PAC_1R$ serum, treated with biotinylated swine anti-rabbit immunoglobulin, and subsequently with avidin-biotinylated peroxidase complex. Each neighboring section, which was a mirror image of the former, was also immunostained with anti-PRL serum or anti-SL serum as described above. After treatment, sections were reacted with 10 mg DAB and 0.03% $H_2O_2$ in 15 ml 0.05 M Tris–HCl buffer (pH 7.6) containing 150 mM NaCl. The specificity of each immunoreaction in the method was checked by substitution of the respective antiserum with PBS or replacement of each antiserum with nonimmune rabbit serum. In all cases, objective immunoreactions were negative. Immunostained sections were