VIP and PACAP stimulate TSH release from the bullfrog pituitary

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Short Title: VIP and PACAP induce frog TSH release
ABSTRACT

We have recently shown that corticotropin-releasing hormone (CRH) is a major thyrotropin (TSH)-releasing factor in amphibians but we have also found that, besides CRH, other hypothalamic substances stimulate TSH secretion in frog. In order to characterize novel TSH secretagogues, we have investigated the effect of frog (*Rana ridibunda*) vasoactive intestinal polypeptide (VIP) (fVIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) (fPACAP38 and PACAP27) on TSH release from bullfrog (*Rana catesbeiana*) pituitary cells in primary culture. Incubation of pituitary cells for 24 h with graded concentrations of fVIP, fPACAP38 and PACAP27 (10⁻⁹ - 10⁻⁶ M) induced a dose-dependent stimulation of TSH release with minimum effective doses of 10⁻⁹ M for fVIP and 10⁻⁸ M for fPACAP38 and PACAP27. The PAC1-R/VPAC2-R antagonist PACAP6-38 (10⁻⁷ and 10⁻⁶ M) dose-dependently suppressed the stimulatory effects of fVIP and fPACAP38 (10⁻⁷ M each). Likewise, this antagonist (10⁻⁶ and 10⁻⁵ M) dose-dependently attenuated the stimulatory effect of PACAP27 (10⁻⁷ M). On the other hand, the VPAC1-R/VPAC2-R antagonist [D-βCl-Phe⁶, Leu¹⁷]VIP (10⁻⁶ M and 10⁻⁵ M) dose-dependently inhibited the stimulatory effect of fVIP (10⁻⁹ M) and PACAP27 (10⁻⁸ M) but did not affect the response to fPACAP38 (10⁻⁸ M). These data indicate that, in amphibians, the activity of thyrotrophs can be regulated by VIP and PACAP acting likely
through VPAC2-R and PAC1-R.

Key Words: VIP, PACAP, frog TSH, VPAC2-receptor, PAC1-receptor
1. Introduction

Although the pituitary thyroid axis plays a major role in the control of metamorphosis [31], the neuroendocrine mechanisms regulating thyrotropin (TSH) secretion in amphibians have long remained elusive. Indeed, because of the very low concentration of TSH and the relative abundance of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the frog pituitary, highly purified TSH preparations could not be obtained in sufficient amounts to develop a radioimmunoassay (RIA) for amphibian TSH. Recently, cloning of the bullfrog (*Rana catesbeiana*) TSH β-subunit [24] has eventually made it possible to raise antibodies against a C-terminal peptide deduced from the cDNA sequence and to develop a homologous RIA for frog TSH [25]. Using this RIA technique, we have shown that corticotropin-releasing hormone (CRH) is a very potent stimulator of TSH secretion from pituitary cells in both adult and larval bullfrogs while thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH) exhibit weak TSH-releasing activity [25]. However, while extracts of adult and larval bullfrog hypothalami provoked a robust increase in TSH release, the CRH antagonist α-helical CRH9-41 could only reduce by 40-50% the stimulatory effect of the tissue extracts, indicating that hypothalamic factors other than CRH, TRH and GnRH can actually influence the secretion of TSH [26].

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating
polypeptide (PACAP) are two members of a superfamily of structurally related peptides that also include secretin, glucagon, glucagon-like peptide and growth hormone (GH)-releasing hormone [16]. VIP is a 28-amino acid α-amidated peptide, originally isolated from the porcine small intestine by virtue of its vasodilator activity [30], that is also widely expressed in the brain [29]. PACAP is a 38-amino acid α-amidated polypeptide (PACAP38) initially isolated from the ovine hypothalamus, based on its ability to stimulate adenylyl cyclase activity in anterior pituitary cells [20]. PACAP is considered to be a pleiotropic neuropeptide that functions as neurotransmitter, neuromodulator, neurotrophic factor, vasodilator and regulatory factor for pituitary and peripheral hormone release [4, 32]. Alternative processing of the PACAP precursor has the potential to generate a 27-amino acid α-amidated peptide (PACAP27) that exhibits 68% sequence identity with VIP [21]. The biological actions of VIP and PACAP are mediated through three types of receptors: the VPAC1 and VPAC2 receptors (VPAC1-R and VPAC2-R) which bind VIP and PACAP with equal affinity, and the PAC1 receptor (PAC1-R) which shows high affinity for PACAP and a much lower affinity for VIP [32].

The primary structures of VIP and PACAP have been strongly preserved during evolution. In particular, frog VIP only differs from porcine VIP by 4 amino acid substitutions [8] while the sequence of frog PACAP38 exhibits a single conservative
substitution when compared to the mammalian one [2, 7]. The strong evolutionary pressure that has acted to conserve the sequences of VIP and PACAP across vertebrates strongly suggests that these two peptides subserve important functions. Indeed, both VIP and PACAP stimulate the activity of anterior pituitary cells in mammals [28, 32], and VIP acts as a prolactin (PRL)-releasing factor in amphibians [14].

In order to elucidate the possible involvement of hypothalamic peptides other than CRH, TRH and GnRH in the control of thyrotroph activity in amphibians, in the present study, we have investigated the possible effects of VIP and PACAP on TSH release from bullfrog pituitary cells.

2. Materials and methods

2.1. Animals

Adult bullfrogs, weighing approximately 600 g, were supplied from Ohuchi-AAS (Misato, Saitama, Japan) and kept under laboratory conditions for 1 week in plastic containers filled with tap water under a 12L:12D photoperiod and constant temperature at 23°C, being fed with bovine liver. All experiments were approved by the Steering Committee for Animal Experimentation at Waseda University.
2.2. Peptides

Frog (*Rana ridibunda*) VIP (fVIP) [8] was synthesized (0.1-mmol scale) by the solid-phase methodology on a Rink amide 4-methylbenzhydrylamine resin (Biochem, Meudon, France) using a 433A peptide synthesizer (Applied Biosystems, Courtaboeuf, France) and the standard procedure as previously described [15]. The synthetic peptide was purified by reversed phase high-performance liquid chromatography (RP-HPLC) on a 2.2 X 25-cm Vydac 218TP1022 C18 column (Alltech, Templemars, France), using a linear gradient (20-40% over 60 min) of acetonitrile/TFA (99.9:0.1, v/v) in water, at a flow rate of 10 ml/min. Analytical RP-HPLC, performed on a 0.46 X 25-cm Vydac 218TP54 C18 column, showed that the purity of the peptide was greater than 99%. The molecular mass of the peptide was verified by mass spectrometry on a MALDI-TOF Voyager DE-PRO instrument (Applied Biosystems). Frog (*Rana ridibunda*) PACAP38 (fPACAP38) [2, 7] was synthesized by solid-phase methodology as previously described [22]. PACAP27 (identical sequences for mammalian and frog peptides), the PAC1-R/VPAC2-R antagonist PACAP$_{6-38}$ and the VPAC1-R/VPAC2-R antagonist [D-Cl-Phe$^6$, Leu$^{17}$]VIP were purchased from Sigma (St Louis, MO, USA).

2.3. Pituitary cell culture
In order to test the hypothalamic peptides for their hypophysiotropic activity in vitro, enzymatically dispersed anterior lobe cells were used, mainly because they yield homogenous samples in large number as compared with intact lobes. The dispersed cells from adult bullfrogs were prepared as previously described [23]. Briefly, frogs were decapitated and the distal lobes were rapidly dissected out under sterile conditions. The pituitaries were cut in small pieces and transferred into Medium 199 (M199; Nissui Pharmaceutical, Tokyo, Japan) adjusted to *Rana catesbeiana* osmolality (M199/water, 70:30) containing 0.2% collagenase (248 units/mg; Wako Chemicals, Osaka, Japan) and 0.1% DNase I (Sigma). After mechanical and enzymatic dispersion, the suspension was centrifuged at 100 g for 5 min, and the supernatant was removed. Dispersed cells were then resuspended in M199 containing 0.1% BSA (Fraction V; Sigma). An aliquot of the suspension was used to count the cell number and the density of cells was adjusted to 3.5 X 10^5 cells/ml. Two hundred microliters of the suspension (containing 70 000 cells) were plated in each well of 96-multiwell plates (Asahi Techno Glass, Tokyo, Japan) and incubated at 23°C in a humidified atmosphere of 95% air-5% CO2. After preincubation for 24 h, the culture medium was replaced with fresh medium containing fVIP, fPACAP38 or PACAP27 in the absence or presence of PACAP6-38 or [D-pCl-Phe\(^6\), Leu\(^{17}\)]VIP. Incubation was continued for 24 h. After incubation, the medium was collected from each well and
centrifuged, and the supernatant was subjected to RIA for bullfrog TSH [25]. The values, given as mean ± SEM, are expressed as ng/10 000 cells.

2.4. Statistical analysis

The significance of differences between the values obtained in each experiment was assessed by Kruskal–Wallis test followed by Scheffé’s test. A P value lower than 0.05 was considered significant.

3. Results

Incubation of cultured pituitary cells for 24 h with graded concentrations of fVIP (10⁻⁹ to 10⁻⁶ M) provoked a dose-dependent increase (340 to 700% of the control value) in TSH release, the minimum effective concentration being 10⁻⁹ M (Fig. 1A). Incubation of cells with graded concentrations of fPACAP38 or PACAP27 (10⁻⁹ to 10⁻⁶ M) also induced a dose-dependent stimulation of TSH release (140 to 360% of the control value for fPACAP38 and 150 to 520% of the control value for PACAP27), but the minimum effective concentration was 10⁻⁸ M (Fig. 1B,C).

Exposure of cultured pituitary cells to the PAC1-R/VPAC2-R antagonist PACAP₆-₃₈ (10⁻⁷ to 10⁻⁵ M), induced a modest, dose-related stimulation (130 to 190% of the control
value) of TSH secretion, with a significant effect at a concentration of $10^{-6}$ M (Fig. 2A).

Co-incubation of cells with fVIP ($10^{-7}$ M) and PACAP$_{6-38}$ ($10^{-7}$ and $10^{-6}$ M) provoked a concentration-dependent reduction (60 and 35% of the value for the non-additive) of the stimulatory action of fVIP on TSH release (Fig. 2B). Similarly, PACAP$_{6-38}$ ($10^{-7}$ and $10^{-6}$ M) dose-dependently attenuated the stimulatory effect of $10^{-7}$ M fPACAP38 on TSH release (85 and 45% of the value for the non-additive) (Fig. 2C).

fPACAP38 at concentrations of $10^{-6}$ and $10^{-5}$ M suppressed the stimulatory effect of $10^{-7}$ M PACAP27 on TSH release (70 and 50% of the value for the non-additive) (Fig. 2D).

Incubation of pituitary cells with the VPAC1-R/VPAC2-R antagonist [D-pCl-Phe$_6$, Leu$_{17}$]VIP, at concentrations of $10^{-6}$ M and $10^{-5}$ M, did not modify TSH release (Fig. 3A). Co-incubation of cells with fVIP ($10^{-9}$ M) and [D-pCl-Phe$_6$, Leu$_{17}$]VIP ($10^{-6}$ M and $10^{-5}$ M) provoked a dose-dependent decrease (85 and 55% of the value for the non-additive) of fVIP ($10^{-9}$ M)-induced TSH release (Fig. 3B). In contrast, [D-pCl-Phe$_6$, Leu$_{17}$]VIP did not affect fPACAP38 ($10^{-8}$ M)-induced TSH release (Fig. 3C) and only produced a substantial reduction (75% of the value for the non-additive) of PACAP27 ($10^{-8}$ M)-evoked TSH secretion at a concentration of $10^{-5}$ M (Fig. 3D).

### 4. Discussion
Recent studies have shown that the neuroendocrine mechanisms regulating amphibian thyrotrophs are clearly different from those operating in mammals. Thus, in frog (*Rana catesbeiana* and *Rana pipiens*) and toad (*Xenopus laevis*), TRH exerts only a modest effect on TSH secretion whereas CRH is a very potent TSH-releasing factor [6, 10, 25, 26]. The hypophysiotropic effects of VIP and PACAP have been extensively investigated in mammals [28, 32 for review]: VIP triggers the secretion of PRL, GH and LH while PACAP stimulates the secretion of adrenocorticotropic-releasing hormone, GH and LH. However, in mammals, neither VIP [5] nor PACAP [9, 12, 20] have any effect on thyrotroph activity. The present data, showing that VIP and PACAP stimulate TSH release from bullfrog pituitary cells, thus provide additional evidence for differential regulation of thyrotrophs in amphibians and mammals. In order to test VIP and PACAP for their TSH-releasing activity with pituitary cells of *Rana catesbeiana*, we used the peptides from the frog belonging to the same genus but different species (*Rana ridibunda*). Considering that the amino acid sequences of both VIP and PACAP are highly conserved across vertebrates, the results obtained in the present experiments seem to reflect the activity close to that of the homologous peptides, which is yet unavailable.

Consistent with the TSH-releasing activity of VIP and PACAP in bullfrog pituitary cells, an early study had shown that PACAP38 increases cytosolic calcium concentration in
frog (*Rana ridibunda*) thyrotrophs [11]. The occurrence of the mRNAs encoding all three types of VIP/PACAP receptors has been previously reported in the frog pituitary, i.e. the VIP/PACAP mutual receptors VPAC1-R [1] and VPAC2-R [13] and the PACAP-selective receptor PAC1-R [3], but the type of pituitary cells that express each receptor subtype has not been determined. The present data now indicate that thyrotrophs express receptors for VIP and PACAP. The fact that fVIP was more potent than fPACAP38 and PACAP27 to stimulate TSH release strongly suggests the occurrence of VPAC1-R and/or VPAC2-R on frog thyrotrophs. In agreement with this notion, the stimulatory effect of fVIP was dose-dependently attenuated by the VPAC1-R/VPAC2-R antagonist [D-pCl-Phe<sup>6</sup>, Leu<sup>17</sup>]VIP [27]. At the highest concentration tested, this antagonist slightly reduced the TSH-releasing effect of PACAP27 but did not affect that of fPACAP38. This latter observation indicates that the stimulatory effect of fPACAP38, and to some extent PACAP27, are mediated via the PACAP-selective receptor PAC1-R. Concurrently, the fact that the PAC1-R/VPAC2-R antagonist PACAP<sub>6-38</sub> suppressed fVIP-induced TSH release reveals the presence of VPAC2-R on frog thyrotrophs inasmuch as frog PAC1-R, like their mammalian counterparts, have very low affinity for VIP [3]. Taken together, the present data strongly suggest that frog thyrotrophs express both VPAC2-R and PAC1-R.

The quantitative distribution and chromatographic characterization of...
immunoreactive PACAP in the frog brain have been reported by Matsuda et al. [19].

According to them, the concentration of immunoreactive PACAP is highest in the
diencephalon, the predominant form of this peptide being PACAP38.

Immunohistochemical studies have shown that, in both adult [33] and larval frog [18],
PACAP-immunoreactive neurons are located in hypothalamic hypophysiotropic nuclei that
project to the external zone of the median eminence. Although there is no information
regarding the distribution of VIP-producing neurons in the brain of adult amphibians, the
presence of VIP-immunoreactive cell bodies and fibers has been demonstrated in the
hypothalamus and median eminence of tadpoles [17]. These neuroanatomical data provide
strong evidence that VIP and PACAP may act as physiological regulators of TSH secretion
in amphibians, notably during metamorphosis.

In conclusion, the present report has shown that VIP and PACAP stimulate in vitro
the release of TSH from frog pituitary cells in primary culture through activation of
VPAC2-R and PAC1-R. The occurrence of VIP and PACAP fibers in the external zone of
the median eminence indicates that, in frog, the two neuropeptides may actually act as
TSH-releasing factors. Since, in mammals, thyrotrophs are not affected by VIP or PACAP,
the present data provide additional evidence that the neuroendocrine control of TSH
markedly differs between amphibians and mammals.
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Legends to figures

Fig. 1 - Effects of fVIP, fPACAP38 and PACAP27 on TSH release from bullfrog pituitary cells in primary culture. Cells were incubated for 24 h with graded concentrations of each peptide and the culture medium was subjected to RIA for bullfrog TSH. The values, given as means ± SEM, are expressed as ng/10 000 cells (n = 9-12). Blank columns (control) represent the basal amount of TSH release during 24 h of culture. The values with the same superscript do not differ from each other at the 5% level of significance (Scheffe’s test).

Fig. 2 - Effect of the PAC1-R/VPAC2-R antagonist PACAP$_{6-38}$ on fVIP, fPACAP38 and PACAP27-induced TSH release from bullfrog pituitary cells in primary culture. Cells were incubated for 24 h with PACAP$_{6-38}$ (10$^{-7}$ to 10$^{-5}$ M) in the absence (A) or in the presence of 10$^{-7}$ M fVIP (B), 10$^{-7}$ M fPACAP38 (C) or 10$^{-7}$ M PACAP27 (D), and the culture medium was subjected to RIA for bullfrog TSH. The values, given as means ± SEM, are expressed as ng/10 000 cells (n = 6). Blank columns (control) represent the basal amount of TSH release during 24 h of culture. The values with the same superscript do not differ from each other at the 5% level of significance (Scheffe’s test).
**Fig. 3** - Effect of the VPAC1-R/VPAC2-R antagonist [D-pCl-Phe\(^6\), Leu\(^{17}\)]VIP on fVIP-, fPACAP38- and PACAP27-induced TSH release from bullfrog pituitary cells in primary culture. Cells were incubated for 24 h with [D-pCl-Phe\(^6\), Leu\(^{17}\)]VIP (10\(^{-6}\) or 10\(^{-5}\) M) in the absence (A) or presence of 10\(^{-9}\) M fVIP (B), 10\(^{-8}\) M fPACAP38 (C) or 10\(^{-8}\) M PACAP27 (D), and the culture medium was subjected to RIA for bullfrog TSH. The values, given as means ± SEM, are expressed as ng/10 000 cells (n = 6). Blank columns represent the basal amount of TSH released during 24 h of culture. The values with the same superscript do not differ from each other at the 5% level of significance (Scheffe’s test).
Fig. 1

A

B

C

TSH (ng/10,000 cells)

fVIP (M)

fPACAP38 (M)

PACAP27 (M)
Fig. 2

A

![Bar chart showing TSH (ng/10,000 cells) vs PACAP6-38 (M)].

Control

0 10^{-7} 10^{-6} 10^{-5}

B

![Bar chart showing TSH (ng/10,000 cells) vs PACAP5-38 (M)].

Control 10^{-7} M VIP

C

![Bar chart showing TSH (ng/10,000 cells) vs PACAP6-38 (M)].

Control 10^{-7} M fPACAP38

D

![Bar chart showing TSH (ng/10,000 cells) vs PACAP6-38 (M)].

Control 10^{-7} M PACAP27