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Mechanisms for PACAP-induced Prolactin Gene Expression in Grass Carp Pituitary Cells

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Abbreviations: PRL, Prolactin; PACAP, Pituitary Adenylate Cyclase-Activating Polypeptide; AC,
Adenylyl Cyclase; PKA, Protein Kinase A; PLC, Phospholipase C; IP₃, Inositol 1,4,5-
Triphosphate; PKC, Protein Kinase C; VSCC, Voltage-Sensitive Calcium Channel;
CaM, Calmodulin; CaMK-II, Ca²⁺/CaM-dependent Protein Kinase II.

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Abstract

In mammals, pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic hormone with diverse functions but its role in prolactin (PRL) regulation is highly controversial. To shed light on PRL regulation by PACAP in fish model, grass carp pituitary cells was used as a model to examine the receptor specificity and signal transduction for PACAP modulation of PRL gene expression in the carp pituitary. Using RT-PCR, PACAP-selective PAC-I receptor was detected in carp lactotrophs. In carp pituitary cells, nanomolar doses of PACAP, but not VIP, could elevate PRL secretion and protein production with concurrent rise in PRL mRNA and these stimulatory effects were blocked by PACAP antagonist but not VIP antagonist. PACAP-induced PRL mRNA expression could be mimicked by activating adenylate cyclase (AC), increasing cAMP level by cAMP analog, or increasing intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) by Ca\(^{2+}\) ionophore/voltage-sensitive Ca\(^{2+}\) channel (VSCC) activator. PACAP-induced PRL gene expression, however, was attenuated/abolished by suppressing cAMP production, inhibiting PKA activity, blocking [Ca\(^{2+}\)]\(_i\) mobilization and VSCC activation, calmodulin (CaM) antagonism, and inactivation of JNK and CaM Kinase-II (CaMK-II). Similar sensitivity to CaM, JNK and CaMK-II blockade was also noted by substituting cAMP analog for PACAP as the stimulant for PRL mRNA expression. These results, as a whole, provide evidence for the first time that (i) PACAP activation of PAC-I receptor expressed in carp lactotrophs could induce PRL synthesis and secretion, and (ii) PRL production induced by PACAP was mediated by up-regulation of PRL gene expression, presumably via functional coupling of cAMP/PKA-, Ca\(^{2+}\)/CaM- and MAPK-dependent cascades.

(248 words)
1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon family, is a 38-amino acid (a.a.) peptide isolated from ovine hypothalamus with stimulatory activity on cAMP production in rat pituitary cells (Miyata et al. 1989). In mammals, two forms of PACAP have been reported, namely PACAP$_{38}$ and PACAP$_{27}$ (Miyata et al. 1989; Miyata et al. 1990). The a.a. sequence of PACAP is highly conserved from fish to mammals, especially in the N-terminal 27-a.a. region (Xu et al. 2012), and with high level of structural homology to the related peptide vasoactive intestinal peptide (VIP) (Moody et al. 2011). The biological functions of PACAP are mediated by three subtypes of PACAP receptors, including PAC-I, VPAC-I and VPAC-II receptors, functionally coupled to cAMP/PKA-, PLC/PKC- and Ca$^{2+}$-dependent cascades (Harmar et al. 2012). In mammals, the PAC-I receptor is specific for PACAP and with high affinity for PACAP but not VIP, whereas the VPAC receptors can bind PACAP and VIP with similar affinity (Vaudry et al. 2009).

In mammals, VIP is well-documented as a stimulator for prolactin (PRL) release (Christian et al. 2007) but the functional role of PACAP in PRL regulation is controversial and the data published are highly variable and contradictory to one another. For examples, the central effect via ICV injection of PACAP, which is inhibitory to PRL release in vivo (Tohei et al. 2009), is different from the results of systemic administration (e.g., IV infusion) with either no effect (Sawangjaroen & Curlewis 1994) or stimulation on plasma PRL level (Jarry et al. 1992). At pituitary level (e.g., in rat pituitary cells), the direct effect of PACAP on PRL regulation is also not consistent, as stimulatory (Benter et al. 1995),
inhibitory (Jarry et al, 1992) and no effect has been reported (Hart et al. 1992). Although PAC-I and VPAC receptors can be detected at the pituitary level (Vertongen et al. 1996), no consensus has been reached regarding the receptor specificity for PRL regulation by PACAP. For the signal transduction of PACAP, the previous studies on PRL regulation were largely based on pituitary cell lines/tumor cells with little information for primary cell culture. Besides, the results obtained are fragmentary and with inconsistency among different reports. For examples, PACAP was shown to up-regulate PRL promoter activity via cAMP and MEK/ERK pathways in GH3 cells (Mijiddorj et al. 2011). In another study with the same cell model, cAMP was found to be not involved in PACAP-induced PRL gene transcription (Coleman & Bancroft 1993). Recently, inhibition of PRL promoter activity in GH3 cells by prolonged treatment with PACAP, probably via cAMP- and MAPK-dependent mechanisms, has also been reported (Mijiddorj et al. 2013). To date, a comprehensive model for the post-receptor signaling of PRL regulation by PACAP is still lacking and a systematic study on signal transduction based on primary culture of pituitary cells/purified lactotrophs is clearly warranted.

In fish models, PRL is a pleiotropic hormone from the pituitary with diverse functions, especially for osmoregulation and ion transport in fresh water adaptation (Shu et al. 2016, Watanabe et al. 2016). Except for a single report in goldfish with PRL release induced by PACAP but with no effect on PRL mRNA expression (Matsuda et al. 2008), no information is available regarding the receptor specificity or signal transduction for PRL regulation by PACAP in lower vertebrates, including fish species. In our recent studies, grass carp PRL has been cloned and its expression in the pars distalis of the carp pituitary has been confirmed (Lin, et al. 2015). Besides, nerve fibers with PACAP immunoreactivity
could be located in the pars distalis of carp pituitary overlapping with the distribution of somatotrophs and lactotrophs (Wong et al. 2005), and in parallel study with carp pituitary cells, PACAP was shown to induce GH secretion and gene expression via cAMP/PKA- and Ca\(^{2+}/\text{CaM}\)-dependent mechanisms (Sze, et al. 2007). Given that PACAP nerve fibers were located in the vicinity of carp lactotrophs, this anatomical finding has prompted us to speculate that PACAP may play a role in PRL regulation in carp pituitary. To test the hypothesis, static incubation of grass carp pituitary cells was conducted to examine the effects of PACAP treatment on PRL secretion, PRL production and PRL transcript expression via direct actions acting at the pituitary level. Receptor specificity for the pituitary actions of PACAP was investigated using a pharmacological approach with antagonists for PAC-I and VPAC receptors. The signal transduction involved in PACAP regulation of PRL gene expression were also elucidated using specific blockers for cAMP/PKA, Ca\(^{2+}/\text{CaM}\), PLC/PKC and MAPK cascades. The results obtained were further confirmed by direct measurement of second messengers produced during the process of PACAP induction. Our findings for the first time provide evidence that (i) PACAP activation of PAC-I receptor in carp lactotrophs could induce PRL synthesis and secretion, and (ii) the stimulatory effect of PACAP on PRL production was mediated by up-regulation of PRL gene expression, presumably via functional coupling of cAMP/PKA-, Ca\(^{2+}/\text{CaM}\)- and MAPK-dependent cascades.

2. Materials and Methods
2.1 Animals

One-year-old Chinese grass carp (*Ctenopharyngodon idellus*) with body weights ranging from 1.5 to 2.0 kg were purchased from local markets and kept in a well-aerated 200-L aquaria under a 12-hr L:12-hr D photoperiod at 18 ± 2°C. The fish were sacrificed under anesthesia with 0.05% tricaine methane sulphonate (Sigma, St. Louis, MO, USA) followed by spinosectomy according to protocol approved by the Committee for Animal Use in Research and Teaching at University of Hong Kong (Hong Kong).

2.2 Reagents and Test Substances

Minimum essential medium (MEM), Ca^{2+}-free MEM, TRIzol reagent, fetal bovine serum (FBS), type II trypsin, DNase I/II, and antibiotic-antimycotic stock solution were purchased from Invitrogen (Grand Island, NY, USA). Ovine PACAP_{38}, human VIP, cod VIP, ovine PACAP_{6-38}, and (4-CI-D-Phe6, Leu17)VIP were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA), whereas grass carp PACAP_{38} (purity: 93%) was synthesized by HSC Biotechnology Services Center (University of Toronto, Canada). Forskolin, H89, MDL12330A, 3-isobutyl-1-methylxanthine (IBMX), 8-(4-chlorophenylthio)-cAMP (cpt-cAMP), A23187, Bay K8644, nifedipine, verapamil, 2-aminoethoxydiphenyl borate (2-APB), xestospongic C (XeC), U73122, KN62/93, calmidazolium, TPA, GF109203, U0126, PD169316, Ly294002, (L)-JNKI 1, and SP600125 were obtained from Calbiochem (San Diego, CA, USA). Peptide hormones were dissolved in double-distilled deionized water and stored frozen as 0.1
mM stocks in small aliquots at -80°C. The pharmacological agents were prepared in similar manner except that the stock solutions were dissolved in dimethyl sulfoxide (DMSO). On the day of the experiment, stock solutions of test substances were diluted with pre-warmed (28°C) culture medium to appropriate concentrations 15 min prior to drug treatment. The final dilutions of DMSO were always less than 0.1% and had no effects on PRL release and PRL mRNA expression in grass carp pituitary cells.

2.3 Grass Carp Pituitary Cell Culture

Primary culture of grass carp pituitary cells were prepared by trypsin/DNase II digestion method as described previously (Wong, et al. 1998), cultured in poly-D-lysine pre-coated 24-well cluster plate at a density of 2.5 × 10^6 cells/well, and incubated overnight (~15 hr) in plating medium [MEM with 26 mM NaHCO₃, 25 mM HEPES, and 1% antibiotic-antimycotic; pH 7.7] with 5% FBS at 28°C under 5% CO₂ and saturated humidity. On the following day, the culture medium was replaced with serum-free testing medium [MEM with 26 mM NaHCO₃, 25 mM HEPES, and 1% antibiotic-antimycotic; pH 7.7] with 1% BSA and drug treatment was initiated for the duration as indicated in individual experiments.

2.4 Immunostaining and LCM Capture of Grass Carp Lactotrophs
Immunostaining was performed in Cytospin™ preparation of grass carp pituitary cells seeded at a density of ~5 × 10⁴ cells/slide using a Vectastain ABC Kit (Vector Lab, Burlingame, CA) with the antiserum for carp PRL (1:10,000) and growth hormone (GH, 1:50,000) respectively (Lin et al. 2015). After signal development, immuno-identified lactotrophs and somatotrophs were isolated from mixed populations of pituitary cells by laser-capture microdissection (LCM) using a PixCell-II Cell Isolation Workstation (Arcturus, Mountain View, CA). During cell capturing, the infrared laser was set at 65 mW with pulse duration at 0.8-1.2 ms and beam diameter at 7.5 µm. A total of 50 cells were captured for individual cell types and dissolved in TRIzol for RNA isolation. After DNase I digestion and reverse transcription with SuperScript III (Invitrogen), the RT samples were subjected to PCR with primers for grass carp PAC-1R (Accession No: EU305549, F2: 5'-CGTGGTTGAACCTGTCTTGGA-3'; R1: 5'-GAGCTGCTTTGCTGTAGG-3'). PCR reactions were conducted for 40 cycles with denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec. PCR products obtained were size-fractionated in a 1% agarose gel and visualized by ethidium bromide staining. In this study, RT-PCR of GAPDH was used as the internal control.

2.5 Western Blot for PRL expression in Carp Pituitary Cells

Carp pituitary cells were seeded in poly-D-lysine coated 24-well culture plates at a density of 2.5 × 10⁶ cells/ml/well and incubated with test substances for 48 hr at 28°C under 5% CO₂ and saturated humidity. After drug treatment, the culture medium was collected and centrifuged at 1,500 × g. The supernatant obtained was used as the samples for PRL release. The cells remaining in individual
wells were lysed for 30 min on ice with frequent agitations in 100 µl/well RIPA medium (50 mM Tris-HCl, 1% Nonidet-P40, 0.25% sodium deoxycholate, 1 mM EDTA and 150 mM NaCl) supplemented with protease and phosphatase inhibitor cocktail (Roche). The lysate was harvested and cell debris was removed by centrifugation at 10,000 × g at 4°C for 10 min. The supernatant was collected and used as samples for PRL cell content. Samples for total PRL production were reconstituted for individual wells by pro rata pooling of the protein samples for PRL release and cell content. Prior to Western blot, the protein content of the cell lysate and/or culture medium was determined using a BCA protein assay kit (Thermo Fisher). After that, protein samples (20 µg/lane) was resolved in 10% gel by SDS-PAGE and transblotted onto a PVDF membranes by low-current electrotransfer at 50 mA for 1 hr at RT using a Hoefer TE70 Semi-Dry Transfer Unit (Pharmacia, San Francisco, CA). PRL signals were detected with the antiserum for carp PRL (1:20,000) according to the standard procedures for Western blot (Lin et al. 2015). A similar approach was also used to probe CaM expression using the antibody for human CaM (1:1000; Upstate, Milford, MA). In these studies, Western blot for β-actin was used as internal control with the antibody for mouse β-actin (1:15000; Oncogen, Cambridge, MA).

2.6 Real-time PCR for PRL mRNA measurement

Pituitary cells were seeded in 24-well plates at a density of 2.5 × 10^6 cells/ml/well. After drug treatment, culture medium was aspirated and pituitary cells were dissolved in TRIzol reagent. Total RNA was extracted, digested with DNase I, and reversely transcribed with Superscript III according
to standard protocols. RT samples obtained were then subjected to real-time PCR for PRL mRNA measurement with primers for carp PRL (PRL-1: 5’-CTCAGCACCTCTCTCACCAATGACC-3’ and PRL-2: 5’-GCGGAAGCAGGACAACAGAAAATGA-3’) using a LightCycler SYBR Green Master kit (Roche) as described previously (Lin et al. 2015). PCR was initiated by a denaturing step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Melting curve analysis was performed to verify the PCR product for PRL mRNA (with \( T_m \) of 93.6°C). In these experiments, serial dilutions of plasmid DNA carrying the coding sequence of carp PRL were used as the standards. Parallel real-time PCR for 18S RNA was also performed to serve as the internal control.

2.7 Measurement of cAMP Production

The rostral pars distalis (RPD) of carp pituitaries were manually dissected under a stereomicroscope and pituitary cells (referred to as “RPD cells”) were prepared as previously described (Zhou, et al. 2004). The RPD cells were then seeded at a density of \(~2 \times 10^6\) cells/2 ml/dish in poly-D-lysine pre-coated 35-mm dishes and cultured overnight at 28°C in carp MEM with 5% FBS. On the following day, carp MEM was replaced with 0.9 mL of HEPES-buffered Hanks balanced salt solution (Wang, et al. 2007) with 0.1% BSA and 0.1 mM IBMX. IBMX, a phosphodiesterase inhibitor, was included to prevent cAMP degradation in the cell culture. Drug treatment was initiated by adding 0.1 mL of the 10× stocks of PACAP prepared at appropriate concentrations. The cells were then incubated at 28°C for 15 min. After that, culture medium was harvested and used for measurement
of cAMP secretion, whereas cellular cAMP content was extracted from RPD cells using 1 ml ice-cold absolute ethanol. The samples collected were freeze-dried and stored at -20°C until their cAMP contents quantified by a BioTrak \[^{125}\text{I}]\text{cAMP RIA Kit (Amersham, Piscataway, NJ). In these studies, total cAMP production was defined as the sum of cellular cAMP content and the amount of cAMP released into the culture medium in individual culture dishes.}

2.8 Measurement of Intracellular Ca\(^{2+}\) Levels

Carp pituitary cells were seeded at a density of \(1 \times 10^4\) cells/coverslip on poly-D-lysine precoated Cell-locate Cover Glass (Thermo Fisher Scientific) and cultured in plating medium with 10% FBS. After overnight incubation, the culture medium was replaced with Krebs-Ringer (K-R) Buffer (120 mM NaCl, 4.7 mM KCl, 0.7 mM MgSO\(_4\), 1.2 mM CaCl\(_2\), 10 mM glucose & 15 mM HEPES, pH 7.4). For single-cell Ca\(^{2+}\) imaging, the cells were preloaded for 45 min at room temperature with the Ca\(^{2+}\)-sensitive dye Fura-2/AM (5 µM, Molecular Probes). After that, the cells were rinsed with K-R buffer and incubated for 10-15 min to allow for cytoplasmic de-esterification of the Fura-2/AM dye. Detection of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]i) signals was conducted at room temperature (~22°C) using a epifluorescence microscopy with a PTI DeltaScan System (Photon Technology, West Sussex, UK). Emission signals were monitored at 510 nm with excitation wavelength alternating between 340 and 380 nm at 1-sec interval. Test substances were introduced gently into the imaging chamber with the cells attached to Cell-locate coverslip by hand pipetting. For the studies under Ca\(^{2+}\)-free condition, old medium was replaced with K-R buffer prepared without CaCl\(_2\) and supplemented with 1 mM
EGTA. After Ca\(^{2+}\) imaging, lactotrophs were identified by immunohistochemical staining using the antiserum for PRL as described in the preceding section.

2.9 Data Transformation and Statistical Analysis

“Steady-state” PRL mRNA was quantified by real-time PCR using standard curve calibration as described previously (Lin et al. 2015). Since no significant changes were noted for 18S RNA in our studies, the raw data for PRL mRNA (in fmole/tube) were simply transformed as a percentage of the mean value in the control group (as “%Ctrl”). For Western blot, the protein levels were quantified in terms of “arbitrary density unit” after densitometric scanning using ImageJ (http://imagej.nih.gov/ij/) and transformed as a percentage of the control group (%Ctrl) without drug treatment in a similar way. For cAMP experiments, cAMP levels were measured in terms of “picomole cAMP/well” for cAMP release and cellular cAMP content, respectively. These data were then used to calculate total cAMP production for individual wells and normalized as a function of cell number (expressed as “picomole cAMP produced/1 × 10^6 cells”). For [Ca\(^{2+}\)]\text{\textsubscript{i}} measurements, calibration of Ca\(^{2+}\) concentration was not performed mainly due to variations in dye loading between individual experiments. In our study, the ratio of Fura-2 emission signals detected under excitation wavelength at 340 nm and 380 nm was used as index for [Ca\(^{2+}\)]\text{\textsubscript{i}} levels (expressed as “F340/F380 Ratio”). The data presented (as mean ± SEM) were analyzed by Student’s t-test or ANOVA followed by Fisher’s least significance difference (LSD) test. Differences were considered significant at P < 0.05.
3. Results

3.1 Effects of PACAP on PRL Secretion and Production

To study the functional role of PACAP in regulating PRL synthesis and secretion at the pituitary level, static incubation was conducted in primary culture of grass carp pituitary cells with PACAP and VIP of mammalian and fish origin for 48 hr. As shown in Fig.1A and 1B, increasing levels of ovine and grass carp PACAP (0.1–1000 nM) could stimulate PRL release and PRL production in a dose-dependent manner without notable effects on PRL cell content. The minimal effective concentration for PACAP stimulation was within the 1–10 nM dose range while the maximal PRL responses were noted in the 10–100 nM dose range. In parallel experiments, increasing concentrations of human and cod VIP (0.1–100 nM) were not effective in altering basal levels of PRL release, PRL cell content and total PRL production (Fig.1C & 1D). Nevertheless, a rise in PRL secretion was detected with VIP treatment at 1,000 nM dose but with no significant changes in PRL cell content and total production.

To test if the effect of PACAP on PRL production was caused by stimulation of PRL gene expression, pituitary cells were treated with increasing doses of PACAP (0.1–1000 nM) and total RNA harvested was used for PRL mRNA measurement (Fig.2A & 2B). In this case, grass carp and ovine PACAP were both effective in triggering a dose-dependent increase in PRL mRNA expression at the pituitary level. The minimal effective dose for PACAP induction was 1 nM with maximal response observed at 10 nM level. In parallel studies with human and cod VIP, despite the trend of a gradual rise in
basal levels, no significant increase in PRL gene expression was observed in carp pituitary cells with VIP treatment within the doses of 0.1-100 nM (Fig. 2A & B).

Given that the differential effects of PACAP and VIP on PRL production and gene expression were in agreement with the pharmacological profile of PAC-I but not VPAC receptors, the possible involvement of PAC-I receptor in PRL regulation by PACAP was also examined. As a first step, RT-PCR for PAC-I receptor was performed in LCM-captured immuno-identified grass carp lactotrophs. Similar to the results with mixed populations of pituitary cells and immuno-identified somatotrophs (as positive control of the experiment), the 216-bp PCR product for PAC-I receptor was consistently detected in grass carp lactotrophs (Fig. 2C). To further confirm the involvement of PAC-I receptor in PACAP-induced PRL gene expression, carp pituitary cells were treated with ovine PACAP$_{38}$ (10 nM) with/without simultaneous treatment with the PACAP antagonist PACAP$_{6,38}$ (100 nM, Fig. 2D) or VIP antagonist (4-Cl-D-Phe$^6$, Leu$^{17}$)VIP (100 nM, Fig. 2E). In this case, PACAP-induced PRL transcript expression were blocked by the co-treatment with the PACAP antagonist PACAP$_{6,38}$, but not the VIP antagonist (4-Cl-D-Phe$^6$, Leu$^{17}$)VIP.

3.2 Involvement of cAMP/PKA Pathway in PACAP-induced PRL Gene Expression

To evaluate the possible involvement of cAMP-dependent mechanisms in PACAP induction of PRL gene expression in the carp pituitary, the effects of PACAP on cAMP synthesis were tested in grass carp RPD cells. RPD cells were used as lactotrophs are known to be concentrated in the RPD...
region of the carp pituitary (Wong et al. 1998). In this experiment, cAMP release, cAMP cell content, and total cAMP production were up-regulated in a dose-dependent manner with increasing levels of ovine PACAP\textsubscript{38} (0.1–1000 nM) (Fig.3A). In parallel studies, the membrane permeable cAMP analog cpt-cAMP (10–1,000 µM, Fig.3B) and AC activator forskolin (100–1000 nM, Fig.3C) were found to mimic the stimulatory effect of PACAP on PRL mRNA expression in carp pituitary cells. In contrast, PACAP (10 nM)-induced PRL mRNA expression was totally abolished by co-treatment with the AC inhibitor MDL12330A (20 µM) or PKA inhibitor H89 (20 µM) (Fig.3D). Treatment with the two inhibitors alone also suppressed basal expression of PRL mRNA in carp pituitary cells.

3.3 Involvement of Ca\textsuperscript{2+}/CaM-dependent Pathways in PACAP-induced PRL Gene Expression

In carp pituitary cells, PACAP can induce GH secretion and gene expression via activation of the Ca\textsuperscript{2+}/CaM-dependent cascades (Wong et al. 2005). To evaluate the possible role of Ca\textsuperscript{2+}-dependent mechanisms in PACAP-induced PRL gene expression, carp pituitary cells were preloaded with the Ca\textsuperscript{2+}-sensitive dye Fura-2 to check for the effect of PACAP on [Ca\textsuperscript{2+}]\textsubscript{i} levels in grass carp lactotrophs. As shown in Fig.4A, ovine PACAP (10 nM) was effective in triggering a rapid and transient increase in [Ca\textsuperscript{2+}]\textsubscript{i} in carp pituitary cells. Using immunostaining with PRL antiserum, some of the cells with Ca\textsuperscript{2+} responses were confirmed to be carp lactotrophs.

To unveil the functional role of [Ca\textsuperscript{2+}]\textsubscript{i} in PRL gene expression, carp pituitary cells were treated with increasing doses of the Ca\textsuperscript{2+} ionophore A23187 (1–100 nM, Fig.4B) and VSCC activator Bay
K8644 (1–100 nM, Fig.4C), which are known to increase \([Ca^{2+}]_i\) via \(Ca^{2+}\) influx in live cells. In both cases, PRL mRNA levels were found to be elevated in a dose-dependent manner after drug treatment. In contrast, both basal and PACAP-induced PRL mRNA expression could be suppressed by removing \([Ca^{2+}]_e\) using a \(Ca^{2+}\)-free culture medium (Fig.4D). Furthermore, the stimulatory effect of PACAP on PRL gene expression was also blocked by inactivating L-type VSCC using the inhibitor nifedipine (10 µM) or verapamil (5 µM) (Fig.4E).

To shed light on the downstream signaling occurs after \(Ca^{2+}\) rise, the role of CaM/CaMK cascade in PACAP-induced PRL gene expression was also tested. As shown in Fig.5A, treatment with ovine PACAP (0.1–100 nM) could elevate the cellular content of CaM in carp RPD cells in a dose-related fashion without corresponding changes in β-actin level. In parallel experiments with carp pituitary cells, both basal as well as PACAP (100 nM)-induced PRL mRNA expression could be suppressed by co-treatment with the CaM antagonist calmidazolium (1 µM, Fig.5B). Similarly, the stimulatory effect of PACAP on PRL gene expression was also sensitive to the blockade with the CaMK-II inhibitors KN62 (5 µM, Fig.5C) and KN93 (5 µM, Fig.5D), respectively.

In mammals, PAC-1 receptor activation can also increase \([Ca^{2+}]_i\) by mobilization of intracellular \(Ca^{2+}\) stores via the PLC/IP\(_3\)/PKC pathway (Vaudry et al. 2009). In carp pituitary cells, both basal and PACAP (100 nM)-induced PRL mRNA expression were down-regulated by co-treatment with the PLC inhibitor U73122 (10 µM, Fig.5E). Furthermore, the inhibitory effect of U73122 on PACAP-induced PRL mRNA expression was also mimicked by the IP\(_3\) receptor inhibitor 2-APB (100 µM,
Fig. 5F) or the IP$_3$ antagonist XeC (3 µM, Fig.5F). In parallel experiments, the PKC activator TPA (10-1000 nM) could elevate PRL mRNA levels in carp pituitary cells (Supplemental Fig.1A), but co-treatment with the PKC inhibitor GF109203 (1 µM, Supplemental Fig.1B) or Calphostin C (10 µM, data not shown) was not effective in blocking the PACAP induction of PRL gene expression.

### 3.4 Functional Coupling of Ca$^{2+}$-dependent and cAMP-dependent Pathways in PRL Gene Expression

Given that PKA is known to trigger protein phosphorylation and subsequent activation of Ca$^{2+}$ channels (Kamp & Hell 2000), it raises the possibility that Ca$^{2+}$/CaM-dependent mechanisms may be acting downstream of cAMP/PKA pathway to mediate PACAP-induced PRL gene expression. To test the hypothesis, the membrane permeable cAMP analog cpt-cAMP was substituted for PACAP as the stimulant for cAMP-dependent pathway, and its effect on PRL gene expression was examined with concurrent perturbation of Ca$^{2+}$ entry or CaM/CaMK-II activation. Similar to our preceding studies, cpt-cAMP (100 µM) treatment consistently elevated basal levels of PRL mRNA in grass carp pituitary cells. This stimulatory action, however, could be inhibited/negated by removing [Ca$^{2+}$]$_e$ with a Ca$^{2+}$-free medium (Fig.6A), CaM antagonism with calmidazolium (1 µM, Fig.6C), or inactivating CaMK-II with KN62 (5 µM, Fig.6D). Interestingly, inhibiting VSCC with nifedipine (10 µM, Fig.6B) was not effective in blocking cpt-cAMP-induced PRL mRNA expression. In parallel study, CaM immuno-reactivity in RPD cells could be elevated by treatment with the AC activator forskolin (100 nM) but not the PKC activator TPA (as negative control of the experiment) and this stimulatory effect could be blocked by inactivating PKA using the inhibitor H89 (20 µM, Fig.6E).
3.5 Functional Role of PI3K and MAPK cascades in PACAP-induced PRL Gene Expression

In mammals, PACAP activation of PI3K and MAPK cascades have been reported, e.g., in human neuroblastoma cells (Kojro et al. 2006). Therefore, the functional role of these pathways in PACAP-induced PRL gene expression was also examined. In carp pituitary cells, PACAP induction (100 nM) consistently elevated basal level of PRL mRNA and this stimulating effect was not affected by co-treatment with the MEK1/2 inhibitor U0126 (10 µM), P38 MAPK inhibitor PD 169316 (10 µM), or PI3K inhibitor Ly294002 (10 µM) (Supplemental Fig.2). In parallel experiments, however, PACAP (100 nM)-induced PRL mRNA expression was totally abolished by simultaneous treatment with the JNK inhibitor (L)-JNKI 1 (10 µM, Fig.7A) or SP600125 (10 µM, Fig.7B). To test the functional coupling of JNK- and cAMP-dependent mechanisms in PACAP-induced PRL gene expression, PACAP was replaced by cpt-cAMP (100 µM) as the stimulant for PRL gene expression and the stimulatory effect of cpt-cAMP was examined in the presence of JNK inhibitors. In this case, PRL mRNA expression induced by cpt-cAMP was partially blocked by the JNK inhibitor (L)-JNKI 1 (10 µM, Fig.7C) and SP600125 (10 µM, Fig.7D), respectively.

4. Discussion
Within the CNS, PACAP not only can act as a neurotransmitter (Fahrenkrug 2006) and neurotrophic factor (Botia et al. 2007; Vallejo & Vallejo 2002), but also serves as a hypophysiotropic factor with pituitary functions (Wong et al. 2000). This idea is supported by the findings that (1) PACAP cell bodies are located within the hypothalamus (Durr, et al. 2007; Hannibal 2002), (2) PACAP nerve fibers can be identified in the external zone of the median eminence (Piggins, et al. 1996), (3) PACAP immunoreactivity can be detected in the hypophyseal portal blood (Dow, et al. 1994), and (4) under certain conditions, PACAP can induce pituitary hormone secretion, e.g., LH, GH and PRL (Kanasaki et al. 2013; Murakami et al. 2001). In grass carp, PACAP nerve fibers can be located in the anterior pituitary overlapping with the distribution of somatotrophs and lactotrophs (Wong et al. 2005). The close proximity of PACAP fibers with carp lactotrophs also provides the anatomical substrate for PRL regulation by PACAP in the carp pituitary. In the present study, the functional role of PACAP in PRL regulation in grass carp was confirmed by the demonstration that PACAP of both mammalian and fish origin could induce PRL release, PRL production and PRL mRNA expression via direct action acting at pituitary cell level. The stimulatory actions of PACAP were noted in the nanomolar dose range and similar levels of VIP were not effective in mimicking PACAP stimulation on PRL expression. The specificity for PACAP over VIP in terms of efficacy/potency of PRL induction was consistent with the pharmacological properties of mammalian PAC-I receptor, which is known to have a high affinity for PACAP but not VIP binding (Murakami et al. 2001). Of note, expression of PAC-I receptor has been reported in the pituitary of fish models, e.g., in goldfish (Kwok et al. 2006), and mediates the effects of PACAP on GH and somatolactin secretion/gene expression (Azuma et al. 2013, Wong et al. 2005). In grass carp pituitary cells, PACAPA-induced PRL mRNA expression could be blocked by the
PACAP antagonist PACAP$_{6-38}$ but not VIP antagonist (4-Ci-D-Phe$^6$, Leu$^{17}$)VIP. In parallel study with RT-PCR coupled to LCM isolation of immuno-identified pituitary cells, PAC-I receptor was also detected in carp lactotrophs. These findings, taken together, indicate that PAC-I receptor is involved in PRL regulation by PACAP in the carp pituitary. To our knowledge, our study represents the first report to provide evidence that PACAP can induce PRL production via up-regulation of PRL gene expression through activation of pituitary PAC-I receptors.

In mammals, functional coupling of PAC-I receptor with cAMP- and Ca$^{2+}$-dependent signaling mechanisms is well documented (Dickson & Finlayson 2009). In lactotroph cell lines, e.g., GH$_3$ cells, PACAP has been previously shown to trigger PRL gene expression via cAMP-dependent (Mijiddorj et al. 2011) and cAMP-independent signaling pathways (Coleman & Bancroft 1993). Apparently, the cAMP/PKA pathway is also involved in PACAP-induced PRL gene expression in the carp pituitary, as PACAP treatment could trigger cAMP production in RPD cells enriched with carp lactotrophs. Furthermore, PACAP-induced PRL mRNA expression could be mimicked by activating AC activity with forskolin or increasing the functional level of cAMP with cpt-cAMP, whereas the stimulatory action of PACAP was blocked by inhibiting AC with MDL12330A and inactivating PKA with H89, respectively. These results strongly suggest that the AC/cAMP/PKA pathway is involved in PACAP-induced PRL gene expression, probably via PAC-I receptor activation in lactotrophs. In our previous studies in carp pituitary cells, PACAP was shown to elevate [Ca$^{2+}$]$_i$ signals, presumably by activation of VSCC and/or mobilization of intracellular Ca$^{2+}$ stores (Wong et al. 2005). Given that induction of Ca$^{2+}$ entry in pituitary lactotrophs is known to induce PRL release, e.g., in rat (Andric et al. 2005), it
is possible that Ca\textsuperscript{2+}-dependent mechanisms are also involved in PACAP-induced PRL gene expression in the carp model. This hypothesis is supported by the findings that PACAP induction could trigger a rapid and transient rise in [Ca\textsuperscript{2+}]i in carp lactotrophs and PRL gene expression induced by PACAP was mimicked by inducing [Ca\textsuperscript{2+}]e entry with Ca\textsuperscript{2+} ionophore A23187 or activating VSCC with Bay K8644. Furthermore, PACAP-induced PRL mRNA expression in carp pituitary cells was totally abolished by removing [Ca\textsuperscript{2+}]e using Ca\textsuperscript{2+}-free medium or inhibiting VSCC by nifedipine and verapamil. In fish models, e.g., goldfish (Huo et al. 2004), protein expression of CaM can be up-regulated at pituitary level by PACAP treatment, and in our study, similar finding was also observed in grass carp RPD cells enriched with lactotrophs. In addition, PACAP-induced PRL mRNA expression in carp pituitary cells could be blocked by CaM antagonism with calmidazolium or CaMK-II inactivation by KN62 / KN93. These results imply that Ca\textsuperscript{2+} entry via VSCC and subsequent activation of CaM and CaMK-II may be involved in PACAP induction of PRL gene expression.

Of note, the Ca\textsuperscript{2+}-dependent mechanisms may be functionally coupled with the cAMP pathway in PRL regulation in carp model, as the stimulatory effect of cAMP analog cpt-cAMP on PRL mRNA expression in carp pituitary cells was also sensitive to the inhibition by Ca\textsuperscript{2+}-free medium, CaM antagonism and CaMK-II inactivation. Meanwhile, forskolin treatment could mimic PACAP induction of CaM expression and this stimulatory effect was blocked by PKA inactivation with H89. Presumably, the coupling between Ca\textsuperscript{2+}-dependent CaMK-II activation and cAMP-dependent pathway is mediated by PKA-dependent activation of CaM expression. However, cpt-cAMP-induced PRL gene
expression was not affected by VSCC blockade with nifedipine, suggesting that PACAP may act through cAMP-independent mechanisms to trigger VSCC activation for \( [\text{Ca}^{2+}]_e \) entry at pituitary cell level. Previous studies have shown that both L-type and T-type VSCC are expressed in prolactin-secreting pituitary cells, e.g., in rat (Lewis et al. 1988). Therefore, we do not exclude the possibility that \( \text{Ca}^{2+} \) channels other than L-type VSCC (i.e., target for nifedipine) may be involved in PAC-I activation. It is worth mentioning that treatment with \( \text{Ca}^{2+} \)-free medium not only can remove \( [\text{Ca}^{2+}]_e \) but also deplete intra-cellular \( \text{Ca}^{2+} \) stores in cell culture, the inhibitory effect of \( \text{Ca}^{2+} \)-free medium on PACAP or cAMP induction may also involve an intracellular \( \text{Ca}^{2+} \) component. In carp pituitary cells, PAC-I receptor activation can induce somatolactin gene expression via the PLC/IP_3/PKC pathway (Jiang et al. 2008a, Jiang et al. 2008b). In the present study, PACAP-induced PRL mRNA expression was blocked by inhibiting PLC with U73122, IP_3 antagonism with XeC, or inactivating IP_3 receptor using 2-APB. Although PKC activation by TPA could mimic PACAP induction of PRL gene expression, PKC inactivation by GF109203 or Calphostin C did not alter the stimulatory effect of PACAP. These findings indicate that the PLC/IP_3 pathway (but without the PKC component) is involved in PACAP stimulation of PRL gene expression in the carp pituitary. Given that IP_3 receptors are intracellular \( \text{Ca}^{2+} \) channels responsible for \( \text{Ca}^{2+} \) release from IP_3-sensitive [\( \text{Ca}^{2+} \)]_i stores (Foskett et al. 2007), the inhibitory effects observed during IP_3 antagonism/IP_3 receptor blockade may imply that IP_3-mediated [\( \text{Ca}^{2+} \)]_i mobilization may also contribute to PACAP-induced PRL gene expression. To our knowledge, the functional coupling of cAMP with CaM expression and \( \text{Ca}^{2+} \) signaling in PRL gene expression has not been reported previously in mammalian models.
In mammals, PAC-I receptors are known to couple with MAPK signaling via cAMP-dependent (Monaghan et al. 2008) and/or independent pathways (Hamelink et al. 2002, Vaudry et al. 2003). In addition, PACAP activation of the PI3K/Akt pathway via the PAC-I/VPAC2 receptors has also been reported, e.g., in rat schwannoma cells (Castorina et al. 2014). In grass carp pituitary cells, however, PACAP-induced PRL mRNA expression was not sensitive to MEK1/2 inhibition by U0126, P38MAPK blockade with PD16316, or PI3K inactivation using Ly294002. Interestingly, the stimulatory effect of PACAP was abolished using the JNK inhibitors (L)-JNKI 1 and SP600125, implying that JNK (not P38MAPK or MEK1/2/ERK1/2) of the MAPK cascades, but not PI3K/Akt pathway, is involved in PACAP-induced PRL gene expression. Since PRL mRNA expression in carp pituitary cells induced by cpt-cAMP was also sensitive to the blockade by (L)-JNKI 1 and SP600125, it is likely that a functional crosstalk between cAMP-dependent cascade and MAPK pathway (via JNK activation) also plays a role in PRL regulation by PACAP in carp lactotrophs.

In summary, using grass carp pituitary cells as a model, we have demonstrated for the first time that PACAP stimulates PRL production at the pituitary level via upregulation of PRL gene expression through PAC-I receptor activation. This stimulatory effect is mediated through functional coupling of cAMP-, Ca2+/CaM- and MAPK-dependent cascades, presumably within the carp lactotrophs. In our working model (Fig.8), PACAP activation of PAC-I receptor expressed in lactotrophs can activate PRL gene expression via the AC/cAMP/PKA pathway, which may also play a role in elevating CaM expression at cellular level. Meanwhile, PAC-I receptor activation can also trigger [Ca2+]i signals via two separate mechanisms, including (i) Ca2+ influx through activation of L-type VSCC and (ii) [Ca2+]i
mobilization from intracellular stores by activation of the PLC/IP3 pathway. Although PKC
activation can also induce PRL gene expression, it is not involved in PRL regulation by PACAP. In
our cell model, the parallel rises in [Ca\textsuperscript{2+}]i and CaM expression presumably can trigger PRL gene
expression through CaMK-II activation. Other than the functional coupling with Ca\textsuperscript{2+}/CaM-
dependent pathway, cAMP production caused by PACAP can also induce a “cross-activation” of
JNK, which may also contribute to the stimulation on PRL gene expression via PAC-I receptor. Our
study, taken together, provides evidence for a comprehensive model with new information on receptor
specificity and signal transduction for PRL regulation by PACAP in a fish model. Our findings also
shed light on the role of PACAP as a hypophysiotropic factor with stimulatory actions on PRL
synthesis and secretion at the pituitary level in carp species.

Declaration of interest

All the authors declare that there is no conflict of interest that could be perceived as prejudicing the
impartiality of the research reported.

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Author contribution statement

CL and JX were responsible for grass carp pituitary cell preparation and pharmacological studies to decipher the receptor specificity & signal transduction mechanisms involved in PRL regulation by PACAP; MH, LZ and TH took care of Ca²⁺ imaging, cAMP experiments and LCM capture of carp lactotrophs for RT-PCR of PAC-I receptor; AOLW was the PI and grant holder, and worked with CL and ZB on manuscript preparation and revision.
References

Andric SA, Zivadinovic D, Gonzalez-Iglesias AE, Lachowicz A, Tomic M & Stojilkovic SS 2005
Endothelin-induced, long lasting, and Ca2+ influx-independent blockade of intrinsic secretion in

polypeptide (PACAP) stimulates release of somatolactin (SL)-alpha and SL-beta from cultured
goldfish pituitary cells via the PAC(1) receptor-signaling pathway, and affects the expression of

Benter S, Leonhardt S, Wuttke W & Jarry H 1995 Paracrine cell to cell interactions determine the
effects of pituitary adenylate cyclase activating polypeptide (PACAP) on in vitro prolactin release

Botia B, Basille M, Allais A, Raoult E, Falluel-Morel A, Galas L, Jolivel V, Wurtz O, Komuro H,
1746-1752.

Castorina A, Scuderi S, D'Amico AG, Drago F & D'Agata V 2014 PACAP and VIP increase the
expression of myelin-related proteins in rat schwannoma cells: Involvement of PAC1/VPAC2

Christian HC, Chapman LP & Morris JF 2007 Thyrotrophin-releasing hormone, vasoactive intestinal
peptide, prolactin-releasing peptide and dopamine regulation of prolactin secretion by different


Figure Legends

Fig.1. Effects of PACAP and VIP on PRL secretion, PRL cell content, and total PRL production in grass carp pituitary cells. Pituitary cells were treated with grass carp PACAP$_{38}$ (A), ovine PACAP$_{38}$ (B), cod VIP (C), or human VIP (D) for 48 hr. After drug treatment, culture medium was collected to monitor PRL release and cell lysates were prepared for detection of PRL cell content. Samples from the same well for PRL release and PRL cell content were pooled pro rata to reconstitute the protein sample for total PRL production. In these studies, parallel probing of $\beta$-actin expression was used as internal control. Data presented are expressed as mean ± SEM (N = 4) and are pooled results of four separate experiments. The asterisk (*) represents a significant difference compared to the respective control (Student’s t-test, P < 0.05).

Fig.2. Receptor specificity of PACAP-induced PRL mRNA expression in grass carp pituitary cells. Differential effects of PACAP and VIP on PRL mRNA expression. Pituitary cells were treated for 48 hr with increasing doses of (A) grass carp PACAP or cod VIP, and (B) ovine PACAP$_{38}$ or human VIP. Involvement of PAC-I receptor in PACAP induction of PRL gene expression at pituitary level. (C) Detection of PAC-I receptor expression in grass carp lactotrophs. Lactotrophs identified by PRL immunostaining (“PRL cells”) were isolated from mixed populations of carp pituitary cells by laser capture microdissection and subjected to RT-PCR using primers for carp PAC-I receptor. Parallel capture of immuno-identified somatotrophs (“GH cells”) was used as a positive control. RT-PCR in RNA samples with/without reverse transcription (± RT) was conducted to check for genomic DNA
contamination while parallel RT-PCR for GAPDH mRNA was used as the internal control. Effects of PACAP and VIP antagonists on PACAP-induced PRL mRNA expression. In these experiments, pituitary cells were challenged with ovine PACAP (10 nM, 48 hr) in the presence or absence of (D) the PACAP antagonist PACAP (100 nM) or (E) VIP antagonist (4-Cl-D-Phe6, Leu17)VIP (“VIP-R antagonist”, 100 nM). After drug treatment, total RNA was isolated and subjected to real-time PCR for PRL mRNA measurement. Except for the gel pictures for RT-PCR, data presented are expressed as mean ± SEM (N = 4) and are pooled results of four separate experiments. For dose-response studies with PACAP/VIP treatment, the asterisk (*) represents a significant difference compared to the respective control (Student’s t-test, P < 0.05). For the corresponding studies with PACAP/VIP antagonist, different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher’s LSD Test).

Fig.3. Functional role of AC/cAMP/PKA pathway in PACAP-induced PRL mRNA expression in carp pituitary cells. (A) Effects of PACAP treatment on cAMP release, cAMP cell content, and total cAMP production in carp RPD cells. RPD cells enriched with lactotrophs were prepared from the carp pituitary as described in Materials & Methods and challenged for 15 min with increasing doses of ovine PACAP (0.1–1,000 nM). Culture medium and cellular contents were collected for cAMP measurements using a cAMP RIA. Samples from individual wells for cAMP release and cAMP cell content were pooled together to reconstitute the samples for total cAMP production. Activation of cAMP-dependent pathway on PRL mRNA expression at pituitary level. Pituitary cells were treated for 48 hr with increasing doses of (B) the cAMP analog cpt-cAMP and (C) the adenylate cyclase (AC)
activator forskolin, respectively. Inhibition of cAMP-dependent pathway on PACAP-induced PRL mRNA expression in carp pituitary cells. In this study, pituitary cells were challenged with ovine PACAP38 (100 nM, 48 hr) in the presence of the AC inhibitor MDL12330A (30 µM) or PKA blocker H89 (20 µM) (D). Data presented are expressed as mean ± SEM (N = 4) and different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher's LSD Test).

Fig.4. Functional role of Ca\(^{2+}\)-dependent pathway in PACAP-induced PRL mRNA expression in carp pituitary cells. (A) Effect of PACAP on intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]) signals in carp lactotrophs (“PRL cells”). Pituitary cells were preloaded with the Ca\(^{2+}\)-sensitive dye Fura-2 and challenged with ovine PACAP38 (1 µM) as described in Materials & Methods. After that, post facto identification of PRL cells on Cell-locate coverslip was conducted by immunostaining with PRL antiserum and mapped with the responsive cells with Ca\(^{2+}\) signals after PACAP treatment. Effect of increasing [Ca\(^{2+}\)] on PRL mRNA expression at pituitary level. Pituitary cells were treated for 48 hr with increasing doses of (B) Ca\(^{2+}\) ionophore A23187 or (C) L-type VSCC activator Bay K8644. Inhibiting extracellular Ca\(^{2+}\) entry on PRL mRNA expression in carp pituitary cells. In this study, pituitary cells were treated with ovine PACAP38 (1 µM, 48 hr) in (D) Ca\(^{2+}\)-free medium (with 0.1 mM EGTA) or (E) with the L-type VSCC blocker nifedipine (10 mM) or verapamil (5 mM). After treatment, total RNA was isolated and subjected to real-time PCR for PRL mRNA measurement. Data presented are expressed as mean ± SEM (N = 4) and different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher's LSD test).
Fig. 5. Functional role of CaM/CaMK-II cascades in PACAP-induced PRL mRNA expression in carp pituitary cells. (A) Effect of PACAP on calmodulin (CaM) expression in grass carp RPD cells. RPD cells enriched with lactotrophs were prepared from the carp pituitary as described in Materials & Methods and challenged for 48 hr with increasing doses of ovine PACAP38. After that, cell lysate was prepared and used for Western blot detection of CaM immunoreactivity (CaM IR) using the antiserum for human CaM. Parallel blotting of β-actin expression was used as the internal control.

Blockade of CaM/ CaMK-II cascades on PACAP-induced PRL mRNA expression at the pituitary level. Carp pituitary cells were challenged with ovine PACAP38 (100 nM, 48 hr) in the presence of the CaM antagonist Calmidazolium (1 mM, B) or CaMK-II inhibitors KN62 (5 mM, C) and KN93 (5 mM, D), respectively. Functional role of PLC/IP₃ cascade in PACAP-induced PRL gene expression in carp pituitary cells. Pituitary cells were challenged with ovine PACAP38 (100 nM) for 48 hr with co-treatment of (E) the PLC inhibitor U73122 (10 mM) or (F) the inhibitors for IP₃ action, including the IP₃ receptor blocker 2-APB (100 mM) and IP₃ antagonist Xestospongin C (XeC, 3 mM). After treatment, total RNA was isolated and subjected to real-time PCR for PRL mRNA measurement. Data presented are expressed as mean ± SEM (N = 4) and different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher's LSD test).

Fig. 6. Functional coupling of cAMP- and Ca²⁺/CaM-dependent pathways in PRL mRNA expression in carp pituitary cells. Pituitary cells were treated with the cAMP analog cpt-cAMP (100 µM, 48 hr) in (A) Ca²⁺-free medium (with 0.1 mM EGTA) or with co-treatment of (B) VSCC blocker nifedipine (10 µM), (C) CaM antagonist calmidazolium (10 µM), or (D) CaMK-II inhibitor KN62 (100 µM).
In parallel experiments, the effect of the AC activator forskolin (100 nM, 48 hr) on CaM expression was tested in grass carp RPD cells in the presence or absence of the PKA inhibitor H89 (20 µM, E). Parallel treatment with the PKC activator TPA (1 µM) was used as the negative control for the study. After treatment, cell lysate was prepared for Western blot detection of CaM immunoreactivity (CaM IR). Except for the results of Western blot, data presented are expressed as mean ± SEM (N = 4) and different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher's LSD test).

Fig. 7. Functional role of JNK in PACAP-induced PRL mRNA expression in carp pituitary cells. Pituitary cells were challenged with ovine PACAP38 (100 nM, 48 hr) with co-treatment of the JNK inhibitor (L)-JNKI 1 (10 µM, A) or SP600125 (10 µM, B). To examine the functional coupling of JNK with cAMP pathway, the effect of cpt-cAMP (100 µM, 48 hr) on PRL mRNA expression was also tested in parallel experiments in the presence of (L)-JNKI 1 (10 µM, C) and SP600125 (10 µM, D), respectively. After drug treatment, total RNA was isolated and subjected to real-time PCR for PRL mRNA measurement. Data presented are expressed as mean ± SEM (N = 4) and different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher's LSD test).

Fig. 8. Working model for signal transduction mechanisms involved in PACAP stimulation of PRL gene expression via PAC-I receptor in grass carp lactotrophs.
Fig. 2

A. Carp PACAP (●) and Cod VIP (○) at different concentrations (nM).
B. Ovine PACAP (●) and Human VIP (○) at different concentrations (nM).

C. PAC-1 Receptor control (216 bp) and GAPDH control (251 bp).

D. Bar graph showing PRL mRNA levels (% Ctrl).

E. Bar graph showing PRL mRNA levels (% Ctrl) with VIP-R antagonist.
**Fig. 4**

**A**

![Immunostaining with PRL antiserum](image)

- Ca\(^{2+}\) image at 25 second (a)
- Ca\(^{2+}\) image at 75 second (b)
- Ca\(^{2+}\) image at 130 second (c)

**B**

![Bar chart](image)

- PRL mRNA (% Ctrl) vs. A23187 conc (nM)

**C**

![Bar chart](image)

- PRL mRNA (% Ctrl) vs. Bay K8644 conc (nM)

**D**

![Bar chart](image)

- PRL mRNA (% Ctrl) vs. PACAP and Ca\(^{2+}\)-free Medium

**E**

![Bar chart](image)

- PRL mRNA (% Ctrl) vs. PACAP, Nifedipine, and Verapamil
Fig. 6

A

B

C

D

E

CaM IR

β actin

CaM IR

β actin

Ctrl  FSK  H89  FSK + H89

Ctrl  TPA
Fig. 7

A

PRL mRNA (% Ctrl)

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PACAP

PAC1 Receptor

AC

PLC

L-type VSCC

cAMP

IP$_3$

IP$_3$R

[Ca$^{2+}$]e Influx

IP$_3$

[Ca$^{2+}$]i Mobilization

JNK

PKA

Calmodulin

CaM Kinase II

Cytosolic Ca$^{2+}$

PRL gene expression

Page 46 of 48
Supplemental Fig. 1  Functional role of PKC in PACAP-induced PRL gene expression in carp pituitary cells. (A) Effect of PKC activation on PRL mRNA expression. Pituitary cells were treated for 48 hr with increasing doses of the PKC activator TPA. (B) PKC inactivation on PACAP-induced PRL mRNA expression. Pituitary cells were treated with ovine PACAP38 (100 nM, 48 hr) in the presence of the PKC inhibitor GF109203 (1 μM). After drug treatment, total RNA was isolated, reversely transcribed, and subjected to real-time PCR for PRL mRNA measurement. Data presented are expressed as mean ± SEM (N = 4) and different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher's LSD test).
Supplemental Fig. 2

Functional role of MEK\textsubscript{1/2}, P\textsubscript{38}MAPK and PI3K in PACAP-induced PRL gene expression in carp pituitary cells. Pituitary cells were challenged with ovine PACAP38 (100 nM, 48 hr) with co-treatment of the MEK\textsubscript{1/2} inhibitor U0126 (10 μM, A), P\textsubscript{38}MAPK inhibitor PD169316 (10 μM, B), and PI3K inhibitor Ly294002 (10 μM, C). After drug treatment, total RNA was isolated, reversely transcribed, and subjected to real-time PCR for PRL mRNA measurement. Data presented are expressed as mean ± SEM (N = 4) and different letters denote a significant difference at p < 0.05 (ANOVA followed by Fisher's LSD test).