Mechanisms for PACAP-induced prolactin gene expression in grass carp pituitary cells

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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 PAC1 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 Ca²⁺ ([Ca²⁺]_i) by Ca²⁺ ionophore/voltage-sensitive Ca²⁺ channel (VSCC) activator. PACAP-induced *prl* gene expression, however, was attenuated/abolished by suppressing cAMP production, inhibiting PKA activity, blocking [Ca²⁺]_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 PAC1 receptor expressed in carp lactotrophs could induce PRL synthesis and secretion, and (ii) PRL production induced by PACAP was mediated by upregulation of *prl* gene expression, presumably via functional coupling of cAMP/PKA-, Ca²⁺/CaM-, and MAPK-dependent cascades.

Key Words

- ▶ prolactin
- ▶ PACAP
- ▶ PAC-1 receptor
- ▶ signal transduction
- ▶ pituitary
- ▶ grass carp

Journal of Endocrinology
(2017) **233**, 37–51

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 PACAP₃₈ and PACAP₂₇ (Miyata *et al.* 1989, 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 levels 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 PAC1, VPAC1 and VPAC2 receptors, functionally coupled to cAMP/PKA-, PLC/PKC-, and Ca²⁺-dependent cascades (Harmar *et al.* 2012). In mammals, the PAC1 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 PAC1 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 example, PACAP was shown to upregulate 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 freshwater adaptation (Shu *et al.* 2016, Watanabe *et al.* 2016). Except for a single

report on 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 growth hormone (GH) secretion and gene expression via cAMP/PKA- and Ca²⁺/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 PAC1 and VPAC receptors. The signal transduction involved in PACAP regulation of *prl* gene expression were also elucidated using specific blockers for cAMP/PKA, Ca²⁺/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 PAC1 receptor in carp lactotrophs could induce PRL synthesis and secretion and (ii) the stimulatory effect of PACAP on Prl production was mediated by upregulation of *prl* gene expression, presumably via functional coupling of cAMP/PKA-, Ca²⁺/CaM-, and MAPK-dependent cascades.

Materials and methods

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-h L:12-h D photoperiod at 18±2°C. The fish were sacrificed under anesthesia with 0.05% tricaine methanesulfonate (Sigma) followed by spinosectomy according to protocol approved by the

Committee for Animal Use in Research and Teaching at University of Hong Kong (Hong Kong).

Reagents and test substances

Minimum essential medium (MEM), Ca²⁺-free MEM, TRIzol reagent, fetal bovine serum (FBS), type II trypsin, DNase I/II, and antibiotic–antimycotic stock solution were purchased from Invitrogen. Ovine PACAP₃₈, human VIP, cod VIP, ovine PACAP_{6–38}, and (4-Cl-D-Phe⁶, Leu¹⁷)VIP were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA), whereas grass carp PACAP₃₈ (purity: 93%) was synthesized by HSC Biotechnology Services Center (University of Toronto, Canada). Forskolin, H89, MDL12330A, 3-isobutyl-1-methylxanthine (IBMX), 8-(4-chloro phenylthio)-cAMP (cpt-cAMP), A23187, Bay K8644, nifedipine, verapamil, 2-aminoethoxydiphenyl borate (2-APB), xestospongine C (XeC), U73122, KN62/93, calmidazolium, TPA, GF109203, U0126, PD169316, Ly294002, (L)-JNK1, and SP600125 were obtained from Calbiochem. Peptide hormones were dissolved in double-distilled deionized water and frozen stored 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.

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 precoated 24-well cluster plate at a density of 2.5×10^6 cells/well, and incubated overnight (~15 h) 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.

Immunostaining and LCM capture of grass carp lactotrophs

Immunostaining was performed in Cytospin preparation of grass carp pituitary cells seeded at a density of $\sim 5 \times 10^4$ cells/slide using a Vectastain ABC Kit (Vector Lab, Burlingame, CA, USA) with the antiserum for carp PRL (1:10,000) and GH (1:50,000), respectively (Lin *et al.* 2015). After signal development, immunoidentified 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, USA). 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 μ m. 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 PAC1R (Accession No: EU305549, F2: 5'-CGTGTGTTGAAGTGGTCTTGG-3'; R1: 5'-GAGCTGCTCTTGCTGAGGAT-3'). PCR was conducted for 40 cycles with denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. 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.

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 2.5×10^6 cells/mL/well and incubated with test substances for 48 h at 28°C under 5% CO₂ and saturated humidity. After drug treatment, the culture medium was collected and centrifuged at 1500g. 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 μ L/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 10,000g 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 PVDF membranes by low-current electrotransfer at 50 mA for 1 h at RT using a Hoefer TE70 Semi-Dry Transfer Unit (Pharmacia). 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). In these studies, Western blot for β-actin was used as internal control with the antibody for mouse β-actin (1:15,000; Oncogen, Cambridge, MA, USA).

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'-CTCAGCACCTCTCTACCAATGACC-3' and PRL-2: 5'-GCGGAAGCAGGACAACAGAAAATG-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 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. 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.

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 $\sim 2 \times 10^6$ cells/2 mL/dish in poly-D-lysine precoated 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 I]cAMP RIA Kit (Amersham). 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.

Measurement of intracellular Ca^{2+} levels

Carp pituitary cells were seeded at a density of 1×10^4 cells/coverlip 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, and 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+} ($[\text{Ca}^{2+}]_i$) signals was conducted at room temperature ($\sim 22^\circ\text{C}$) 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-s 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.

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 fmol/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 (<https://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 $[Ca^{2+}]_i$ 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 340nm and 380nm was used as index for $[Ca^{2+}]_i$ levels (expressed as 'F340/F380 Ratio'). The data presented (as mean $\pm$ S.E.M.) were analyzed by Student's *t*-test or ANOVA followed by Fisher's least significance difference test. Differences were considered significant at $P < 0.05$.

Results

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 h. As shown in Fig. 1A and B, increasing

levels of ovine and grass carp PACAP (0.1–1000nM) 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 1–10nM dose range while the maximal Prl responses were noted in the 10–100nM dose range. In parallel experiments, increasing concentrations of human and cod VIP (0.1–100nM) were not effective in altering basal levels of PRL release, PRL cell content, and total Prl production (Fig. 1C and D). Nevertheless, a rise in Prl secretion was detected with VIP treatment at 1000nM 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 (0.1–1000nM) and total RNA harvested was used for *prl* mRNA measurement (Fig. 2A and B). 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 1nM with maximal response observed at 10nM 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 0.1–100nM (Fig. 2A and B).

Given that the differential effects of PACAP and VIP on Prl production and gene expression were in agreement with the pharmacological profile of PAC1 but not VPAC receptors, the possible involvement of PAC1 receptor in *prl* regulation by PACAP was also examined. As a first

Figure 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 PACAP₃₈ (A), ovine PACAP₃₈ (B), cod VIP (C), or human VIP (D) for 48 h. 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 β -actin expression was used as internal control. Data presented are expressed as mean $\pm$ S.E.M. ($N=4$) and are pooled results of four separate experiments. The asterisk (*) represents a significant difference compared to the respective control (Student's *t*-test, $P < 0.05$).

Figure 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 h with increasing doses of (A) grass carp PACAP or cod VIP and (B) ovine PACAP₃₈ 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 ($\pm$ 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 PACAP₃₈ (10 nM, 48 h) in the presence or absence of (D) the PACAP antagonist PACAP₆₋₃₈ (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 $\pm$ s.e.m. ($N=4$) 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).

step, RT-PCR for PAC1 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 PAC1 receptor was consistently detected in grass carp lactotrophs (Fig. 2C). To further confirm the involvement of PAC1 receptor in PACAP-induced *prl* gene expression, carp pituitary cells were treated with ovine PACAP₃₈ (10 nM) with/without simultaneous treatment with the PACAP antagonist PACAP₆₋₃₈ (100 nM, Fig. 2D) or VIP antagonist (4-Cl-D-Phe⁶, Leu¹⁷)VIP (100 nM, Fig. 2E). In this case, PACAP-induced *prl* transcript expression were blocked by the co-treatment with the PACAP antagonist PACAP₆₋₃₈, but not the VIP antagonist (4-Cl-D-Phe⁶, Leu¹⁷ VIP.

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 which 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 upregulated in a dose-dependent manner with increasing levels of ovine PACAP₃₈ (0.1–1000 nM) (Fig. 3A). In parallel studies, the membrane-permeable cAMP analog cpt-cAMP (10–1000 μ M, 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 μ M) or PKA inhibitor H89 (20 μ M) (Fig. 3D). Treatment with the two inhibitors alone also suppressed basal expression of *prl* mRNA in carp pituitary cells.

Involvement of Ca²⁺/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

Figure 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 and methods' section and challenged for 15 min with increasing doses of ovine PACAP₃₈ (0.1–1000 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 h 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 h) in the presence of the AC inhibitor MDL12330A (30 μM) or PKA blocker H89 (20 μM) (D). Data presented are expressed as mean ± S.E.M. (N=4) and different letters denote a significant difference at $P < 0.05$ (ANOVA followed by Fisher's LSD test).

Ca²⁺/CaM-dependent cascades (Wong *et al.* 2005). To evaluate the possible role of Ca²⁺-dependent mechanisms in PACAP-induced *prl* gene expression, carp pituitary cells were preloaded with the Ca²⁺-sensitive dye Fura-2 to check for the effect of PACAP on [Ca²⁺]_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 [Ca²⁺]_i in carp pituitary cells. Using immunostaining with PRL antiserum, some of the cells with Ca²⁺ responses were confirmed to be carp lactotrophs.

To unveil the functional role of [Ca²⁺]_i in *prl* gene expression, carp pituitary cells were treated with increasing doses of the Ca²⁺ ionophore A23187 (1–100 nM, Fig. 4B) and VSCC activator Bay K8644 (1–100 nM, Fig. 4C), which are known to increase [Ca²⁺]_i via Ca²⁺ 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 [Ca²⁺]_e using a Ca²⁺-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 that occurs after Ca²⁺ 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

Figure 4

Functional role of Ca²⁺-dependent pathway in PACAP-induced *prl* mRNA expression in carp pituitary cells. (A) Effect of PACAP on intracellular Ca²⁺ ([Ca²⁺]_i) signals in carp lactotrophs ('Prl cells'). Pituitary cells were preloaded with the Ca²⁺-sensitive dye Fura-2 and challenged with ovine PACAP₃₈ (1 μM) as described in 'Materials and methods' section. 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 Ca²⁺ signals after PACAP treatment. Effect of increasing [Ca²⁺]_i on *prl* mRNA expression at pituitary level. Pituitary cells were treated for 48 h with increasing doses of (B) Ca²⁺ ionophore AZ3187 or (C) L-type VSCC activator Bay K8644. Inhibiting extracellular Ca²⁺ entry on *prl* mRNA expression in carp pituitary cells. In this study, pituitary cells were treated with ovine PACAP₃₈ (1 μM, 48 h) in (D) Ca²⁺-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 ± S.E.M. (N=4) and different letters denote a significant difference at P<0.05 (ANOVA followed by Fisher's LSD test).

CaMK-II inhibitors KN62 (5 μM, Fig. 5C) and KN93 (5 μM, Fig. 5D), respectively.

In mammals, PAC1 receptor activation can also increase [Ca²⁺]_i by mobilization of intracellular Ca²⁺ stores via the PLC/IP₃/PKC pathway (Vaudry *et al.* 2009). In carp pituitary cells, both basal and PACAP (100 nM)-induced *prl* mRNA expression were downregulated 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 IP₃ receptor inhibitor 2-APB (100 μM, Fig. 5F) or the IP₃ 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 (Supplementary Fig. 1A, see section on supplementary data given at the end of this article), but co-treatment with the PKC inhibitor GF109203 (1 μM, Supplementary Fig. 1B) or Calphostin C (10 μM, data not shown) was not effective in blocking the PACAP induction of *prl* gene expression.

Functional coupling of Ca²⁺-dependent and cAMP-dependent pathways in PRL gene expression

Given that PKA is known to trigger protein phosphorylation and subsequent activation of Ca²⁺ channels (Kamp & Hell 2000), it raises the possibility that Ca²⁺/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 Ca²⁺ 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 [Ca²⁺]_e with a Ca²⁺-free medium (Fig. 6A), CaM antagonist with calmidazolium (1 μM, Fig. 6C), or inactivating CaMK-II

Figure 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 and methods' section and challenged for 48 h 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 h) 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 h 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 Xestospingon 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 ± S.E.M. (N=4) and different letters denote a significant difference at $P < 0.05$ (ANOVA followed by Fisher's LSD test).

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 immunoreactivity 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).

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, for example, 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 MEK_{1/2} inhibitor U0126 (10 μM), P₃₈^{MAPK} inhibitor PD 169316 (10 μM), or PI3K inhibitor Ly294002 (10 μM) (Supplementary 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.

Figure 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 h) 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 h) 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 ± S.E.M. (N=4) and different letters denote a significant difference at *P* < 0.05 (ANOVA followed by Fisher's LSD test).

Discussion

Within the CNS, PACAP can not only act as a neurotransmitter (Fahrenkrug 2006) and neurotrophic factor (Vallejo & Vallejo 2002, Botia *et al.* 2007), but also serve 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 (Hannibal 2002, Durr *et al.* 2007); (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, for example, Lh, Gh and Prl (Murakami *et al.* 2001, Kanasaki *et al.* 2013). 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

Figure 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 h) 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 h) 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 ± S.E.M. (N=4) and different letters denote a significant difference at *P* < 0.05 (ANOVA followed by Fisher's LSD test).

and fish origin could induce Prl release, Prl production and *prl* mRNA expression via direct action 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 PAC1 receptor, which is known to have a high affinity for PACAP but not VIP binding (Murakami *et al.* 2001). Of note, expression of PAC1 receptor has been reported in the pituitary of fish models, for example, in goldfish (Kwok *et al.* 2006), and mediates the effects of PACAP on GH and somatolactin secretion/gene expression (Wong *et al.* 2005, Azuma *et al.* 2013). In grass carp pituitary cells, PACAP-induced *prl* mRNA expression could be blocked by the PACAP antagonist PACAP_{6–38} but not VIP antagonist (4-Cl-D-Phe⁶, Leu¹⁷)VIP. In parallel study with RT-PCR coupled to LCM isolation of immuno-identified pituitary cells, PAC1 receptor was also detected in carp lactotrophs. These findings, taken together, indicate that PAC1 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 upregulation of *prl* gene expression through activation of pituitary PAC1 receptors.

In mammals, functional coupling of PAC1 receptor with cAMP- and Ca²⁺-dependent signaling mechanisms is well-documented (Dickson & Finlayson 2009). In lactotroph cell lines, for example, GH₃ 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 PAC1 receptor activation in lactotrophs. In our previous studies in carp pituitary cells, PACAP was shown to elevate [Ca²⁺]_i signals, presumably by activation of VSCC and/or mobilization of intracellular Ca²⁺ stores (Wong *et al.* 2005). Given that

induction of Ca²⁺ entry in pituitary lactotrophs is known to induce PRL release, for example, in rat (Andric *et al.* 2005), it is possible that Ca²⁺-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 [Ca²⁺]_i in carp lactotrophs and *prl* gene expression induced by PACAP was mimicked by inducing [Ca²⁺]_e entry with Ca²⁺ ionophore A23187 or activating VSCC with Bay K8644. Furthermore, PACAP-induced *prl* mRNA expression in carp pituitary cells was totally abolished by removing [Ca²⁺]_e using Ca²⁺-free medium or inhibiting VSCC by nifedipine and verapamil. In fish models, for example, goldfish (Huo *et al.* 2004), protein expression of CaM can be upregulated 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 Ca²⁺ entry via VSCC and subsequent activation of CaM and CaMK-II may be involved in PACAP induction of *prl* gene expression.

Of note, the Ca²⁺-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 Ca²⁺-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 Ca²⁺-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 [Ca²⁺]_e entry at pituitary cell level. Previous studies have shown that both L-type and T-type VSCC are expressed in PRL-secreting pituitary cells, for example, in rat (Lewis *et al.* 1988). Therefore, we do not exclude the possibility that Ca²⁺ channels other than L-type VSCC (i.e., target for nifedipine) may be involved in PAC1 activation. It is worth mentioning that treatment with Ca²⁺-free medium not only can remove [Ca²⁺]_e but also deplete intracellular Ca²⁺ stores in cell culture, the inhibitory effect of Ca²⁺-free medium on PACAP or

cpt-cAMP induction may also involve an intracellular Ca^{2+} component. In carp pituitary cells, PAC1 receptor activation can induce somatotactin gene expression via the PLC/ IP_3 /PKC pathway (Jiang *et al.* 2008a,b). 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 Ca^{2+} channels responsible for 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 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 PAC1/VPAC2 receptors has also been reported, for example, in rat schwannoma cells (Castorina *et al.* 2014). In grass carp pituitary cells, however, PACAP-induced *prl* mRNA expression was not sensitive to $\text{MEK}_{1/2}$ inhibition by U0126, $\text{P}_{38}^{\text{MAPK}}$ 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 $\text{P}_{38}^{\text{MAPK}}$ or $\text{MEK}_{1/2}$ /ERK $_{1/2}$) 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 cross talk 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 PAC1 receptor activation. This stimulatory effect is mediated through functional coupling of cAMP-, Ca^{2+} /CaM-,

Figure 8

Working model for signal transduction mechanisms involved in PACAP stimulation of *prl* gene expression via PAC1 receptor in grass carp lactotrophs.

and MAPK-dependent cascades, presumably within the carp lactotrophs. In our working model (Fig. 8), PACAP activation of PAC1 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, PAC1 receptor activation can also trigger $[\text{Ca}^{2+}]_i$ signals via two separate mechanisms, including (i) Ca^{2+} influx through activation of L-type VSCC and (ii) $[\text{Ca}^{2+}]_i$ mobilization from intracellular stores by activation of the PLC/ IP_3 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 $[\text{Ca}^{2+}]_i$ and CaM expression presumably can trigger *prl* gene expression through CaMK-II activation. Other than the functional coupling with Ca^{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 PAC1 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.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0433>.

Declaration of interest

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

Funding

The project was supported by GRF grants (17128215, 781113, and 780312) and NSFC/RGC joint grant (N_HKU 732/12) from Research Grant Council (HK), HMRG Grant (13142591) from Food and Health Bureau (HKSAR) (to A O L W), and NSFC grant (31660335) from National Natural Science Foundation of China (to C L). Financial support to C L and J X from the School of Biological Sciences (University of Hong Kong) is also acknowledged.

Author contribution statement

C L and J X were responsible for grass carp pituitary cell preparation and pharmacological studies to decipher the receptor specificity and signal transduction mechanisms involved in PRL regulation by PACAP; M H, L Z, and T H took care of Ca^{2+} imaging, cAMP experiments, and LCM capture of carp lactotrophs for RT-PCR of PAC-I receptor; and A O L W was the PI and grant holder, and worked with C L and Z B on manuscript preparation and revision.

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Received in final form 6 January 2017

Accepted 27 January 2017

Accepted Preprint published online 27 January 2017