Grass carp somatolactin: II. Pharmacological study on postreceptor signaling mechanisms for PACAP-induced somatolactin-α and -β gene expression

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Jiang Q, He M, Wang X, Wong AO. Grass carp somatolactin: II. Pharmacological study on postreceptor signaling mechanisms for PACAP-induced somatolactin-α and -β gene expression. Am J Physiol Endocrinol Metab 295: E477–E490, 2008. First published June 3, 2008; doi:10.1152/ajpendo.90386.2008.—Somatolactin (SL), the latest member of the growth hormone/prolactin family, is a novel pituitary hormone with diverse functions. However, the signal transduction mechanisms responsible for SL expression are still largely unknown. Using grass carp as an animal model, we examined the direct effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on SL gene expression at the pituitary level. In primary cultures of grass carp pituitary cells, SLα and SLβ mRNA levels could be elevated by PACAP via activation of PAC-I receptors. With the use of a pharmacological approach, the AC/cAMP/PKA and PLC/inositol 1,4,5-trisphosphate (IP3)/PKC pathways and subsequent activation of the Ca2+/calmodulin (CaM)/CaMK-II cascades were shown to be involved in PACAP-induced SLα mRNA expression. Apparently, the downstream Ca2+/CaM-dependent cascades were triggered by extracellular Ca2+ ([Ca2+]e) entry via L-type voltage-sensitive Ca2+ channels (VSCC) and Ca2+ release from IP3-sensitive intracellular Ca2+ stores. In addition, the VSCC component could be activated by cAMP/PKA- and PLC/PKC-dependent mechanisms. Similar postreceptor signaling cascades were also observed for PACAP-induced SLβ mRNA expression, except that [Ca2+]e entry through VSCC, PKC coupling to PLC, and subsequent activation of CaMK-II were not involved. These findings, taken together, provide evidence for the first time that PACAP can induce SLα and SLβ gene expression in fish model via PAC-I receptors through differential coupling to overlapping and yet distinct signaling pathways.

pituitary adenylate cyclase-activating polypeptide; grass carp pituitary cells

somatolactin (SL), the latest member of the growth hormone (GH)/prolactin (PRL) family, can be identified only in fish but not in tetrapods (37). Phylogenetic analysis reveals that SL is derived from ancestral GH (16), and gene duplication in bony fish have given rise to two paralogous SL isoforms, namely SLα and SLβ (47). Unlike GH and PRL, which are nonglycosylated and expressed mainly in the anterior pituitary, SL is a glycoprotein secreted by the periodic acid Schiff-positive cells located in the pars intermedia (15). Although the physiological functions of SL have not been fully characterized, its involvement in color presentation (7), body metabolism (8), osmoregulation (18), stress responses (30), and reproductive functions (31) has been proposed by various studies in fish models. Recently, it has been shown that swim bladder development can be inhibited in zebrafish by SLβ but not SLα gene silencing, implying that the two SL isoforms may act differently during the process of embryo organogenesis (46). In rainbow trout, SL release can be suppressed by dopamine but stimulated by corticotropin-releasing hormone and gonadotropin-releasing hormone (GnRH; Ref. 13). In addition, SL mRNA expression can be upregulated by GnRH treatment (e.g., salmon; Ref. 26), which is in agreement with the previous reports (28) that GnRH receptors are expressed in fish pituitary cells with SL immunoreactivity. These findings clearly indicate that SL secretion and synthesis are under the control of hypothalamic factors. However, the postreceptor signaling cascades involved in SL expression are still unknown. To our knowledge, the signal transduction mechanisms for SLα and SLβ gene expression have not been previously examined.

Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide with diverse functions, is widely expressed in various tissues in mammals (5). The protein structure of PACAP is highly homologous to vasoactive intestinal polypeptide (VIP; Ref. 38), and the biological functions of the two peptides are mediated by VPAC receptors, which can bind PACAP and VIP with similar affinity. PACAP can also exert physiological actions independent of VIP via activation of PAC-I receptors, which are specific for PACAP with little/low binding affinity for VIP (10). In mammals, PACAP neurons can be located in the hypothalamus with nerve fibers extending into the median eminence (36). In addition, PACAP immunoreactivity can be detected in hypophysial portal blood (4) and PACAP treatment is known to stimulate pituitary hormone secretion, both in vivo and in vitro (32). The role of PACAP as a hypophysiotropic factor is well conserved in vertebrates, including the fish (42). Although modern-day bony fish do not have a hypophysial portal blood system, their pituitary is directly innervated by the hypothalamus (9) and nerve fibers with PACAP immunoreactivity have been identified in the pituitary of goldfish (41) and eel (23). In representative fish species, PACAP treatment can also induce GH [e.g., common carp (44) and salmon (29)] and LH secretion [e.g., goldfish (2)] through direct actions at the pituitary level.

In fish models, PACAP may also play a role in SL regulation. In stargazer, PACAP nerve fibers are located in the vicinity of SL cells in the pars intermedia (20). With the use of a cell immunoblot assay, the blotting area for SL immunoreactivity in the vicinity of SL cells in the pars intermedia was increased by PACAP treatment, implying that PACAP may induce SL release at the pituitary level (21). This idea is confirmed by our recent studies (12) in grass carp pituitary cells, in which SL release, SL content, and total SL production could be elevated by PACAP stimulation. In the same study, the increase in SL production also occurred...
with parallel rises in SLα and SLβ mRNA levels and these stimulatory actions could be mimicked by the PAC-I receptor agonist Maxadilan but blocked by the PAC-I antagonist M65. These findings suggest that PACAP can not only induce SL expression, grass carp pituitary cells were used as a model to study the receptor signaling events involved in PACAP-induced SLα and SLβ mRNA expression. By direct measurement of second messengers and pharmacological perturbation of signaling cascades, we demonstrate for the first time that PACAP can trigger SLα and SLβ gene expression at the pituitary level by differential coupling of the Ca2+/calmodulin (CaM)-dependent pathway with the AC/cAMP/PKA and PLC/inositol 1,4,5-trisphosphate (IP3)/PKC signaling mechanisms.

MATERIALS AND METHODS

Animals. One-year-old grass carps (Ctenopharyngodon idellus) with a body weight ranging from 1.5 to 2.0 kg were obtained from local markets. After acclimation in the laboratory, the fish were anesthetized in 0.05% MS222 (Sigma, St. Louis, MO) and killed for pituitary cell preparation according to the guidelines for animal use at the University of Hong Kong.

Test substances. Ovine PACAP38 and human VIP were purchased from Bachem Fine Chemicals (La Jolla, CA), dissolved in double-distilled deionized water, and stored frozen at −80°C as 0.1 mM stocks in small aliquots. Forskolin, H89, IBMX, 1,2-dioctanoylglycerol (DiC8), 8-(4-chloro-phenylthio)-cAMP (CPT-cAMP), A23187, Bay K8644, KN62, nifedipine, thapsigargin, 2-aminoethoxydiphenyl borate (2-APB), U73122, GF109203, MDL 12330A, calphostin C, and calmidazolium were obtained from Calbiochem (San Diego, CA). Similar to the peptide hormones, these pharmacological agents were prepared as 10 mM frozen stocks in DMSO. Stock solutions of test substances were diluted with prewarmed (28°C) culture medium to appropriate concentrations 15 min before drug treatment. The final dilutions of DMSO were <0.1% and had no effects on SL gene expression in grass carp pituitary cells.

Measurement of SLα and SLβ mRNA expression. Primary cultures of grass carp pituitary cells were prepared by the trypsin/DNase digestion method as described previously (43). After that, pituitary cells were seeded in 24-well plates at a density of 2.5 × 10^6 cells·ml⁻¹·well⁻¹ and cultured for 15–18 h at 28°C in carp MEM (MEM Eagle Medium supplemented with 26 mM NaHCO₃, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone, pH 7.7) with 5% FBS to recover. On the following day, cell medium was replaced with 0.9 ml HEPES-buffered HBSS (39°C) and allowed to incubate at 28°C for another 48 h. After drug treatment, total RNA was extracted from individual wells using Trizol and subjected to slot blot assays (45) using the DIG-labeled cDNA probes for carp SLα and SLβ mRNA expression. By direct measurement of second messengers and pharmacological perturbation of signaling cascades, we demonstrate for the first time that PACAP can trigger SLα and SLβ gene expression at the pituitary level by differential coupling of the Ca2+/calmodulin (CaM)-dependent pathway with the AC/cAMP/PKA and PLC/inositol 1,4,5-trisphosphate (IP3)/PKC signaling mechanisms.
room temperature. After that, ratiometric measurement of the intracellular Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_{i}\)) was conducted using a PTI Epifluorescence Ca\(^{2+}\) Imaging System (Photon Technology International, Birmingham, NJ) with excitation wavelengths at 340 and 380 nm and emission wavelength at 510 nm. Given that variations in fura-2 loading were noted between different batches of NIL cells, Ca\(^{2+}\) calibration was not performed and the Ca\(^{2+}\) data were simply expressed as a ratio of fluorescence signals with excitation at 340 and 380 nm, respectively (as “F340/380 Ratio”).

Western blot of CaM expression. Grass carp NIL cells (~2.5 × 10^6 cells·mL\(^{-1}\)·well\(^{-1}\)) were incubated at 28°C for 48 h in carp MEM containing increasing concentrations of PACAP. After that, Western blot was performed according to the standard procedures in our laboratory (11). Briefly, NIL cells were rinsed with PBS (pH 7.4) and cell lysate was prepared by three cycles of freezing and thawing in RIPA buffer (50 mM Tris·HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, and 0.25% Na deoxycholate) with 1 mM PMSF and complete protease inhibitor cocktail (Roche). The lysate was resolved in a 10% gel by SDS-PAGE and transblotted onto a PVDF membrane at 65 V for 2.5 h. The membrane was then blocked by 2% nonfat dried milk and incubated overnight at 4°C with an antiserum raised against human CaM (1:1,000; Upstate, Milford, MA). On the following day, HRP-conjugated anti-mouse IgG (1:15,000; Bio-Rad, Hercules, CA) was added and SuperSignal WestPico (Pierce, Rockford, IL) was used as a substrate for signal development. In these studies, Western blot of PKC\(_\alpha\), PKC\(_\beta\), and PKC\(_\gamma\) using a PKC sampler kit (Transduction Laboratories, Lexington, KY) according to the antibody dilutions recommended by the manufacturer.

Data transformation and statistics. For slot blot assays, SL\(_\alpha\) and SL\(_\beta\) mRNA levels were quantified in terms of arbitrary density units and normalized against the amount of 18S RNA expressed in the same sample. Since no significant changes were observed for 18S RNA expression in these experiments, normalized data were simply transformed as a percentage of the mean value in the control group for statistical analysis (referred to as “%Ctrl”). For cAMP measurement, the data for cAMP release and cAMP content were expressed as picomoles cAMP detected per million cells. Data presented, expressed as means ± SE, are the results pooled from separate experiments and were analyzed using Student’s t-test or two-way ANOVA followed by Fisher’s least significant difference test. Differences were considered significant at P < 0.05.

RESULTS

PACAP induction of SL\(_\alpha\) and SL\(_\beta\) mRNA expression. In our recent studies (12), grass carp PACAP was shown to increase SL\(_\alpha\) and SL\(_\beta\) gene expression in the carp pituitary. Since grass carp PACAP is of limited supply, ovine PACAP was used in the present study. To ensure that ovine PACAP behaves similarly in terms of receptor specificity compared with fish PACAP, static incubation experiments were conducted in grass carp pituitary cells with PACAP and VIP of mammalian origin (Fig. 1). In this case, increasing concentrations of ovine PACAP (0.1–100 nM) could upregulate SL\(_\alpha\) and SL\(_\beta\) mRNA levels in a dose-dependent manner. The minimal effective doses for PACAP induction of SL\(_\alpha\) and SL\(_\beta\) gene expression were found to be at 0.1 nM level with maximal responses observed in the 10- to 100-nM dose range. The ED50s for the stimulatory effects of PACAP on SL\(_\alpha\) and SL\(_\beta\) mRNA levels were estimated to be 0.9 and 1.2 nM, respectively. In parallel...
experiments with VIP treatment, increasing doses of human VIP (0.1–100 nM) were not very effective in triggering SL gene expression, and significant rises in SLα and SLβ mRNA levels could be noted only at a 100-nM dose. Apparently, the transcript expression of SLα and SLβ at the pituitary level are more sensitive to PACAP stimulation compared with the corresponding responses caused by VIP and these pharmacological profiles are consistent with that reported for mammalian PAC-I receptors (10).

**cAMP pathway in PACAP-induced SL gene expression.** Since PACAP is known to induce cAMP synthesis via activation of AC activity at the pituitary level in mammals [e.g., in rat (22)], the involvement of the cAMP-dependent pathway in PACAP induction of SL gene expression is suspected. To test the hypothesis, grass carp pituitary cells were challenged with increasing concentrations of the AC activator forskolin (0.001–10 μM; Fig. 2A) or membrane permeant cAMP analog CPT-cAMP (0.1–1,000 μM; Fig. 2B). Similar to the results of PACAP treatment, forskolin and CPT-cAMP were both effective in elevating SLα and SLβ mRNA expression. Apparently, SLα mRNA expression was more sensitive to cAMP activation, as higher doses of forskolin (0.1 μM) and CPT-cAMP (10 μM) were required for inducing significant rises in SLβ mRNA levels (vs. 0.01 and 1 μM for SLα mRNA expression, respectively). In these experiments, a gradual drop in SLα mRNA levels was observed after the peak response at 0.1 μM dose of forskolin, suggesting that SLα gene expression might have been desensitized by cAMP overproduction. To confirm the possible involvement of cAMP in PACAP actions, the effects of PACAP on cAMP production were tested in NIL cells prepared from the carp pituitary. The NIL cells were used in this study as our previous in situ hybridization revealed that pituitary cells with SLα and SLβ gene expression are located exclusively in the pars intermedia of the carp NIL (12). As shown in Fig. 3A, increasing doses of ovine PACAP (0.1–1,000 nM) could elevate cAMP release and cAMP content in grass carp NIL cells. In this dose-response study, “bell-shaped” curves were consistently obtained with minimal effective doses of PACAP in the 0.1- to 1-nM dose range and maximal responses at a 10-nM level. To further evaluate the functional role of the cAMP-dependent pathway in PACAP-induced SL gene expression, grass carp pituitary cells were exposed to ovine PACAP (10 nM) in the presence of the AC inhibitor MDL 12330A (20 μM) and PKA inhibitor H89 (20 μM), respectively (Fig. 3B). In this case, basal levels of SLα and SLβ mRNA were significantly suppressed while PACAP-induced SLα and SLβ transcript expression were markedly reduced or totally abolished.

**PLC/PKC pathway in PACAP-induced SL gene expression.** In mammals, PACAP receptors are also known to couple with the PLC/PKC pathway (19). To test the possible involvement of PLC in PACAP-induced SL gene expression, grass carp pituitary cells were challenged with ovine PACAP (10 nM) with simultaneous treatment of the PLC inhibitor U73122 (20 μM). As shown in Fig. 4A, basal levels of SLα but not SLβ mRNA were reduced by U73122 treatment. Furthermore, the PLC inhibitor also blocked the stimulatory effects of PACAP on SLα and SLβ mRNA expression. Similar to PACAP treatment, SLα and SLβ mRNA levels could be elevated dose dependently by a 6-h incubation with increasing concentrations of the PKC activator TPA (0.1–1,000 nM; Fig. 4B). Since

![Fig. 3. Functional role of cAMP-dependent mechanisms in PACAP-induced SLα and SLβ gene expression.](http://ajpendo.physiology.org/)

- **A:** effects of increasing concentrations of PACAP (0.1–1,000 nM) on cAMP release and cAMP content in neurointermediate lobe (NIL) cells prepared from the grass carp pituitary. *Significant difference compared with the respective control (*P* < 0.05, Student’s *t*-test).
- **B:** effects of MDL12330A and H89 on PACAP-induced SLα and SLβ mRNA expression. Pituitary cells were incubated for 48 h with ovine PACAP (10 nM) in the presence of absence of the AC inhibitor MDL 12330A (MDL; 20 μM) or PKA inhibitor H89 (20 μM). After drug treatment, total RNA was extracted for SLα and SLβ mRNA measurement. In this study, parallel blotting of 18S RNA was used as an internal control. Data presented, expressed as means ± SE, are pooled results from 4 separate experiments. Experimental groups denoted by the same letter represent a similar level of transcript expression (*P* > 0.05, ANOVA followed by Fisher’s LSD test).
prolonged treatment with TPA can also induce PKC desensitization in mammalian cell models via protein degradation of various PKC isoforms (34), it is unclear if the observed stimulation on SL gene expression was the result of PKC activation or desensitization. To test for PKC desensitization after TPA treatment, the Western blot was conducted using the antibodies raised against conventional PKCs, namely PKCα, PKCβ, and PKCγ. In this case, immunoreactivities for PKCβ and PKCγ but not PKCα could be detected in the cell lysate prepared from grass carp pituitary cells (Fig. 4C, left), implying that the β- and γ-isofoms are the major types of PKC expressed in the carp pituitary. In parallel experiments, a 6-h incubation of pituitary cells with TPA (100 nM) did not significantly affect the expression levels of PKCβ and PKCγ (Fig. 4C, right). These results argue against the possibility that TPA-induced SL gene expression was caused by PKC desensitization. This idea was also supported by the studies using DiC8 (10 μM), a nondesensitizing diacylglycerol analog (17) that could mimic TPA stimulation of SLα and SLβ mRNA expression in carp pituitary cells (Fig. 4B, inset). To further
evaluate the functional involvement of PKC activation in PACAP actions, the stimulatory effects of PACAP (10 nM) were tested in the presence of the PKC inhibitors calphostin C (20 μM; Fig. 5A) and GF109203 (20 μM; Fig. 5B), respectively. In these experiments, both basal and PACAP-induced SLα mRNA expression could be reduced by these PKC inhibitors. Similar treatment, however, had no effects on PACAP stimulation of SLβ transcript expression.

**Ca2+/CaM-dependent pathways in PACAP-induced SL gene expression.** To examine the functional coupling of PACAP with Ca2+/CaM-dependent pathways in SL gene expression, [Ca2+]i and CaM expression was monitored in grass carp NIL cells after PACAP treatment. In normal medium with 2.5 mM CaCl2, ovine PACAP (10 nM) consistently triggered a transient rise of [Ca2+]i in NIL cells. However, this Ca2+ response was significantly suppressed by removing extracellular Ca2+ ([Ca2+]e) using a Ca2+-free medium supplemented with 0.1 mM EGTA (Fig. 6A). In parallel experiments, NIL cells were exposed to increasing levels of ovine PACAP (0.1–100 nM) and cellular content of CaM was detected by Western blot using an antiserum for human CaM (Fig. 6B). In recent studies (11), grass carp CaM was cloned and its amino acid sequence was found to be identical to that of human CaM. Furthermore, the antiserum for human CaM has been previously validated for CaM measurement in carp pituitary cells (41). In this study, CaM immunoreactivity detected in NIL cells was increased in a dose-dependent manner without corresponding changes in β-actin levels, implying that PACAP may selectively upregulate CaM expression in SL cells at the protein level. To investigate the functional role of [Ca2+]e entry in SL gene expression, grass carp pituitary cells were treated with increasing doses of the Ca2+ ionophore A23187 (1–50 nM) or voltage-sensitive Ca2+ channel (VSCC) activator Bay K8644 (10–1,000 nM). Interestingly enough, these drug treatments could dose dependently increase basal levels of SLα mRNA but had no effects on SLβ gene expression (Fig. 6C). In parallel experiments, both basal and PACAP (10 nM)-induced SLα mRNA expression could be reduced by removing [Ca2+]e using a Ca2+-free culture medium (Fig. 7A) and blocking L-type VSCC by the dihydropyridine inhibitor nifedipine (5 μM) was also effective in attenuating PACAP induction of SLα gene expression (Fig. 7B). Similar treatment to remove [Ca2+]e or to inhibit VSCC functionality, however, did not alter the stimulatory effect of PACAP on SLβ mRNA expression.

Given that a transient rise in [Ca2+]i with reduced magnitude could be observed after PACAP stimulation in Ca2+-free medium (Fig. 6A), functional coupling of PACAP with Ca2+...
release from intracellular Ca\(^{2+}\) stores was suspected. To test the hypothesis, pituitary cells were challenged with PACAP (10 nM) with pretreatment of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin (50 nM). Depleting intracellular stores of [Ca\(^{2+}\)]\(_i\) by thapsigargin not only suppressed basal but also negated the stimulatory effects of PACAP on SL\(_\alpha\) and SL\(_\beta\) mRNA expression (Fig. 8A). Since PACAP stimulation was shown to be sensitive to PLC inhibition by U73122 (Fig. 4A), the possible involvement of IP3-sensitive Ca\(^{2+}\) stores was also examined using 2-APB, a specific inhibitor for IP3 receptors. Similar to thapsigargin treatment, both basal and PACAP-induced SL\(_\alpha\) and SL\(_\beta\) mRNA expression could be markedly suppressed by blocking [Ca\(^{2+}\)]\(_i\) release via IP3 receptors by 2-APB (100 μM; Fig. 8B). Based on the results of our Western blot, CaM expression in grass carp NIL cells could also be upregulated by PACAP treatment (Fig. 6B), suggesting that Ca\(^{2+}\)/CaM-dependent mechanisms might have been activated. To test the involvement of the Ca\(^{2+}\)/CaM-dependent pathways in PACAP actions, the stimulatory effects of PACAP (10 nM) on SL gene expression were examined with cotreatment of the CaM antagonist calmidazolium (1 μM) or the Ca\(^{2+}\)/CaM-dependent protein kinase II (CaMK-II) inhibitor KN62 (5 μM). In these studies, both basal and PACAP-induced SL\(_\alpha\) and SL\(_\beta\) mRNA expression were reduced by CaM antagonism (Fig. 9A). Similarly, CaMK-II blockade by KN62 also attenuated basal as well as PACAP-induced SL\(_\alpha\) gene expression. KN62 treatment, however, had no effects on SL\(_\beta\) mRNA expression caused by PACAP induction (Fig. 9B). Similar results were also obtained using KN93 (5 μM), another inhibitor for CaMK-II (data not shown).

Coupling of Ca\(^{2+}\)-dependent mechanisms with cAMP and PKC pathways. Since PKA and PKC can induce protein phosphorylation and subsequent activation of VSCC in mammalian cell lines (14), it raises the possibility that functional
coupling of Ca\(^{2+}\)/CaM-dependent mechanisms with the cAMP/PKA and PLC/PKC pathways may contribute to PACAP actions in grass carp pituitary cells. To test the hypothesis, the cAMP analog CPT-cAMP and PKC activator TPA were used as stimulants for PKA- and PKC-dependent pathways, respectively, and their effects on SL\(\alpha\) and SL\(\beta\) mRNA expression were examined with concurrent perturbation of [Ca\(^{2+}\)]\(_{e}\) entry or CaMK-II activation. Similar to our previous results, CPT-cAMP (100 \(\mu\)M) and TPA (100 nM) were both effective in elevating SL\(\alpha\) and SL\(\beta\) mRNA levels in carp pituitary cells. Although cAMP-induced SL\(\alpha\) mRNA expression could be suppressed/totally abolished by removing [Ca\(^{2+}\)]\(_{e}\) with Ca\(^{2+}\)-free medium (Fig. 10A), inactivating L-type VSCC blocker nifedipine (5 \(\mu\)M; Fig. 10B), or blocking CaMK-II activation with KN62 (5 \(\mu\)M; Fig. 10C), similar treatments had no effects on cAMP induction of SL\(\beta\) mRNA expression. In parallel experiments with TPA, however, SL\(\alpha\) and SL\(\beta\) mRNA expression induced by PKC activation was inhibited by treatment with Ca\(^{2+}\)-free medium (Fig. 11A), VSCC blocker nifedipine (5 \(\mu\)M; Fig. 11B) and CaMK-II inhibitor KN62 (5 \(\mu\)M; Fig. 11C).

**DISCUSSION**

PACAP is a member of the glucagon/secretin superfamily and is known to be involved in a wide range of biological functions. Recently, grass carp PACAP has been cloned and structural analysis by NMR techniques reveals that its three-dimensional structures are highly comparable if not identical to that of its mammalian counterparts (35). In grass carp, unlike mammals, PACAP is expressed exclusively in the central nervous system with high levels of transcripts detected in the hypothalamus. These findings are in agreement with the previous reports that PACAP can serve as a hypophysiotropic factor in fish models (42). In salmonid and cyprinid (carp) species, PACAP has been shown to be a potent stimulator for GH secretion (29, 44) and GH gene expression (41). In our recent study, we have cloned the \(\alpha\)- and \(\beta\)-isoforms of grass carp SL, the latest members of the GH/PRL family, and demonstrated that they are expressed in two separate populations of pituitary cells located in the pars intermedia (12). In the same study, PACAP was also found to be effective in stimulating SL release and SL gene expression via activation of pituitary PAC-I receptors. Although SL is a novel pituitary...
hormone in fish models with diverse functions (see introduction), the signal transduction mechanisms responsible for SL gene expression are largely unknown. In this study, SLα and SLβ mRNA expression in grass carp pituitary cells could be upregulated by PACAP in the nanomolar dose range, whereas a 1,000-fold higher concentration was required for VIP to induce similar actions. The differential sensitivity to PACAP stimulation confirms that PAC-I receptors are involved in PACAP induction of SL/H9251 and SL/H9252 gene expression. In NIL cells prepared from the carp pituitary, PACAP treatment could elevate cAMP release and cAMP content in a dose-dependent manner. In parallel experiments with mixed populations of grass carp pituitary cells, PACAP-induced SL/H9251 and SL/H9252 mRNA expression could be mimicked by activating AC with forskolin or increasing functional levels of cAMP using CPT-cAMP. In contrast, both basal and PACAP-induced SLα and SLβ gene expression were markedly reduced by blocking AC with MDL 12330A or inactivating PKA by H89. These results, taken together, indicate that the AC/cAMP/PKA pathway plays a key role in maintaining both basal levels as well as PACAP induction of SLα and SLβ gene expression in the carp pituitary. Our findings are also consistent with the previous reports that the cAMP-dependent cascades are involved in pituitary hormone secretion induced by PACAP in fish pituitary cells, e.g., LH release in goldfish (2) and GH secretion in common carp (44). In addition, expression studies (40) of goldfish PAC-I receptor in CHO cells also confirm that PAC-I receptors of the fish origin are functionally coupled to cAMP production. In mammalian cell models, PACAP not only can trigger cAMP synthesis via Gs coupling with AC (32) but also induce Ca2+ mobilization (1) through Gq/11 coupling with PLC (27). In porcine somatotrophs, PACAP-induced GH release is mediated by cAMP/PKA-, PLC/PKC-, and Ca2+-dependent cascades (19). In grass carp pituitary cells, PACAP can activate GH gene transcription via PAC-I receptors and these stimulatory effects are mediated by the AC/cAMP/PKA pathway coupled with [Ca2+]i entry via L-type VSCC and subsequent activation of CaM/CaMK-II cascades (41). These previous findings have prompted us to speculate that other signaling pathways may be involved in PACAP-stimulated SL gene expression. In this study, PACAP treatment consistently triggered a transient rise in [Ca2+]i and CaM expression in grass carp NIL cells, suggesting that the Ca2+/CaM-dependent mechanisms might have been activated. Since the Ca2+ responses could still be noted with reduced magnitude by removing [Ca2+]i, it would be logical to assume that the Ca2+
responses triggered by PACAP may have two functional components, namely [Ca$^{2+}$]$_e$ entry from extracellular source and [Ca$^{2+}$]$_i$ mobilization of intracellular Ca$^{2+}$ stores. In grass carp pituitary cells, [Ca$^{2+}$]$_e$ entry induced by A23187 or VSCC activation by Bay K8644 could elevate SL$\alpha$ and SL$\beta$ gene expression. In contrast, SL$\alpha$ mRNA expression caused by PACAP was inhibited by removing [Ca$^{2+}$]$_e$ using a Ca$^{2+}$-free medium, blocking L-type VSCC by nifedipine, depleting [Ca$^{2+}$]$_i$ stores with thapsigargin, antagonizing endogenous CaM using calmidazolium, and inactivating CaMK-II by KN62. These results, as a whole, indicate that [Ca$^{2+}$]$_i$ mobilization and [Ca$^{2+}$]$_e$ entry through VSCC followed by activation of CaM/CaMK-II cascades are involved in PACAP induction of SL$\alpha$ gene expression. In the case of SL$\beta$ expression, the Ca$^{2+}$ dependence was found to be similar and yet different from the corresponding responses in SL$\alpha$ gene. Although PACAP-stimulated SL$\beta$ mRNA expression could be negated by [Ca$^{2+}$]$_e$, depletion and CaM antagonism, it was not sensitive to the blockade by [Ca$^{2+}$]$_e$ removal, VSCC inhibition, or CaM-KII inactivation. Apparently, the effects of PACAP on SL$\beta$ gene expression are mediated by [Ca$^{2+}$]$_i$ mobilization and CaM activation but not by [Ca$^{2+}$]$_e$ entry through VSCC. Furthermore, CaM-KII is not involved in the downstream signaling after CaM activation. Given that multiple forms of CaMK have been reported and some of them (e.g., CaMK-IV) are known to be expressed in a tissue-specific manner (24), we do not exclude the possibility that other CaMks may also be involved in PACAP-stimulated SL$\beta$ gene expression.

Apart from Ca$^{2+}$-dependent mechanisms, differential coupling with the PLC/IP$_3$/PKC pathway was also observed for SL$\alpha$ and SL$\beta$ gene expression. In grass carp pituitary cells, PKC activation by TPA and DiC8 was found to increase SL$\alpha$ and SL$\beta$ mRNA levels, whereas inhibiting PLC using U73122, blocking the IP$_3$ receptor with 2-APB, and PKC inactivation by calphostin C and GF10923 suppressed basal as well as PACAP-stimulated SL$\alpha$ mRNA expression. These results indicate that the PLC/IP$_3$/PKC pathway is involved in basal maintenance and PACAP induction of SL$\alpha$ gene expression. Despite the fact that the rise in SL$\beta$ mRNA levels caused by PACAP could be negated by inhibiting PLC and IP$_3$ receptors, this stimulatory action was not affected by PKC blockade, suggesting that the PKC component may be uncoupled from PLC-dependent mechanisms leading to SL$\beta$ gene expression. Since PKC isoforms are expressed in the goldfish pituitary in a cell-type specific manner (17) and the atypical isoforms, e.g., PKC$\xi$ and PKC$\eta$, are known to be insensitive to stimulation by phorbol ester and diacylglycerol (25), it is tempting to speculate that the lack of PKC involvement in PACAP-induced SL$\beta$ gene expression.

**Fig. 9.** Functional role of CaM and CaMK-II in PACAP-induced SL$\alpha$ and SL$\beta$ gene expression. Pituitary cells were incubated for 48 h with PACAP (10 nM) with or without the simultaneous treatment of the CaM antagonist calmidazolium (A: 1 $\mu$M) or the CaM-KII inhibitor KN62 (B: 5 $\mu$M). After drug treatment, total RNA was extracted for SL$\alpha$ and SL$\beta$ mRNA measurement and parallel blotting of 18S RNA was used as an internal control. Data presented are pooled results from 4 separate experiments, and treatment groups denoted by the same letter represent a similar level of transcript expression ($P > 0.05$; ANOVA followed by Fisher’s LSD test).
gene expression may be caused by different complements of PKC isoforms expressed in NIL cells with SL/H9251 and SL/H9252 expression. Since IP3 receptors are Ca2+/H11001 channels responsible for [Ca2+/H11001]i release from IP3-sensitive Ca2+/H11001 stores (6), IP3 produced after PLC activation in grass carp pituitary cells may act as a second messenger to trigger [Ca2+/H11001]i mobilization and contribute to PACAP-induced SL gene expression through Ca2+/H11001/CaM-dependent mechanisms. In mammalian cell lines, the functionality of VSCC can be modulated by PKA and PKC via protein phosphorylation of α1- and β2γ-subunits in the Ca2+ channel (14). These findings raise the possibility that the Ca2+/H11001/CaM-dependent cascades for SL gene expression may be secondarily coupled with the PKA- and PKC-dependent mechanisms. At the pituitary level, SLα mRNA expression caused by activating PKA with CPT-cAMP or stimulating PKC with TPA were sensitive to the inhibition by Ca2+/H11001-free medium, VSCC blockade, and CaMK-II inactivation. Although similar inhibitions were also effective in blocking TPA-induced SLβ mRNA expression, the corresponding stimulation by CPT-cAMP was not affected. These results imply that [Ca2+/H11001]e entry through VSCC and subsequent CaMK-II activation are acting downstream after PKC activation. However, the functional coupling of Ca2+/H11001/CaM-dependent mechanisms with the cAMP/PKA cascades is observed only in SLα but not SLβ gene expression.

In summary, using grass carp pituitary cells as a model, we have confirmed that PACAP can induce SLα and SLβ gene expression via activation of pituitary PAC-I receptors. Our results also demonstrate for the first time that PACAP-induced SLα and SLβ gene expression are mediated by overlapping and yet distinct postreceptor signaling mechanisms (Fig. 12).
activation. In the case of SLβ gene expression, similar involvement of the AC/cAMP/PKA and PLC/IP3 cascades and a subsequent rise in \([\text{Ca}^{2+}]_i\) and CaM activation can be observed after PACAP stimulation. In contrast to SLα gene expression, \([\text{Ca}^{2+}]_i\) influx via VSCC and subsequent CaMK-II activation play no functional role in PACAP action. Although PKC activation of VSCC can still occur in pituitary cells with SLα expression, this PKC component is uncoupled from PLC and the downstream VSCC cannot be activated by the cAMP/PKA pathway. These findings, taken together, provide evidence that activation of PAC-I receptors expressed in two distinct populations of grass carp SL cells can stimulate SLα and SLβ gene expression via differential coupling to different postreceptor signaling cascades. Given that PAC-I receptors with hip/hop inserts in the third intracellular loop (33) and amino acid substitutions in the transmembrane domains (3) are known to differentially activate cAMP-, PLC-, and \([\text{Ca}^{2+}]_i\)-dependent mechanisms, further investigations on the role of PAC-I receptor isoforms in PACAP-induced SL gene expression are clearly warranted.

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**REFERENCES**

**Working Models for PACAP Regulation of SLα and SLβ Gene Expression**

Fig. 12. Working models for PACAP induction of SLα and SLβ gene expression at the pituitary level (see discussion for details). [Ca^{2+}]_{i} in intracellular Ca^{2+}; [Ca^{2+}]_{e}, extracellular Ca^{2+}; CaM-K-II, CaM/CaM-dependent protein kinase II.


