Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Regulates the Expression of PACAP in Cultured Tilapia Astrocytes

Lo Chi-Wei, Shang-Lin Chang, and Ching-Feng Weng[1]

Institute of Biotechnology, National Dong Hwa University, Hualien 974, Taiwan

Neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic hormone that is involved in numerous physiologic functions. The present study examines the presence and the functional significance of PACAP and its receptor in the brain and astrocytes of tilapia (*Oreochromis mossambicus*). This is the first demonstration of the full-length nucleotide sequence of tPACAP gene in tilapia pituitary, brain, and cultured astrocytes. Two cDNA variants of the growth hormone–releasing hormone (GHRH)–PACAP gene were identified in tilapia pituitary, brain, and cultured astrocytes as a result of exon skipping with a long form (271 bp) encoding both $tPACAP_{38}$ and tGHRH and a short form (166 bp) encoding only $tPACAP_{38}$. The short form was found to be more abundant in astrocytes. Addition of ovine $PACAP_{38}$ (1 nM) to cultured astrocytes significantly stimulated the expression of $tPACAP_{38}$ at 4 hrs, but the effect dropped after 8 hrs of treatment. By contrast, the expression of PACAP type I receptor (PAC_1-R) mRNA in the astrocytes was not responsive to $PACAP_{38}$ treatment. The $tPACAP_{38}$ expression also was activated by the cAMP analog, dibutyryl-cAMP, in a dose-dependent manner. Adding high salinity (170 mM NaCl, 500 mOsm/kg osmolarity) to cultured medium substantially increased astroglial $tPACAP_{38}$ expression over 4 hrs to a level that was maintained for 16 hrs. This observation was not found when mannitol (270 mM) was supplemented as an osmolarity-enhancing agent (500 mOsm/kg). Taken together, tPACAP expression in tilapia astrocytes was well regulated by exogenous PACAP, cAMP, and salinity and might be involved in the adaptation to high salinity when the fish is in a seawater environment. Exp Biol Med 232:262–276, 2007

Key words: PACAP; PACAP type I receptor; autocrine/paracrine; astrocyte; salinity; tilapia; *Oreochromis mossambicus*

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that was originally purified from ovine hypothalamus tissues because of its capacity to stimulate cAMP production in cultured rat anterior pituitary cells (1). Based on its high amino acid sequence homology with vasoactive intestinal peptide (VIP), this peptide is one of the glucagons/secretin/growth hormone–releasing hormone (GHRH) superfamily. PACAP is present in two biologically active forms with 38 and 27 amino acid residues, designated $PACAP_{38}$ and $PACAP_{27}$, respectively (1, 2). GHRH is encoded in the same gene with PACAP in protochordates (3), fish (4–8), amphibians (9, 10), and birds (11, 12). At some point before or during the emergence of mammals, separate genes encoded PACAP and GHRH. The biologic functions of PACAP in mammals have been widely studied (14). The diverse functions of PACAP include the regulation of cell proliferation, differentiation, and apoptosis and the regulation of metabolism and the cardiovascular, endocrine, and immune systems. However, the physiologic events that coordinate PACAP responses have not yet been identified (15). PACAP is widely dispersed in the central nervous system (CNS) and may act as a hypophysiotropic factor in adenohypophysial hormone release and a neurotransmitter and neuromodulator in the CNS (13). Three PACAP receptors (PAC_1-R, $VPAC_1$-R, and $VPAC_2$-R) have been identified. All of them are G protein–coupled receptors with a classical structure of seven transmembrane domains (16). PACAP type I receptor (PAC_1-R) has a high binding affinity for $PACAP_{38}$ and $PACAP_{27}$ but a much lower affinity toward VIP, whereas PACAP type II receptors ($VPAC_1$-R and $VPAC_2$-R) have similar binding affinities toward $PACAP_{27}$, $PACAP_{38}$, and VIP (20–22). All three receptors signal by stimulating adenylate cyclase activity, increasing levels of intracellular cAMP (20–22). Astrocytes, the major macroglial cells in the CNS, are crucially involved in maintaining homeostasis. PACAP stimulates the proliferation of astrocytes at a physiologic concentration of 10^{-12} M (23). The presence of specific PACAP binding sites on rat astrocytes has been demon-

Table 1. Primer Sequence and Expected Lengths of Fragments in RT-PCR Analysis of Selected Genes in Tilapia and β-Actin[a]

Gene	Primer sequence	Expected size (bp)	GenBank accession no.
EtPACAP$_{38}$			
Forward	5′-CGCCATCCTTAAACGACGAC-3′	271	AY522580
Reverse	5′-AGGTATTTCTGCACGGCCATCTG-3′	166	—
tPACAP$_{38}$			
Forward	5′-CAGATGGCCGTGCAGAAATACCT-3′	199	AY522580
Reverse	5′-AAAGAACACAGGCGCAATCCACT-3′	—	—
PAC$_1$-R			
Forward	5′-CTCAGGAAG ACAGCGACCATTGC-3′	446	AY830121
Reverse	5′-CATTCGTATCCCAGCATCCAGTG-3′	—	—
Reverse[b]	5′-CATTCGTATCCCAGCATCCAGTG-3′	267	—
β-actin			
Forward	5′-ACCACCACAGCCGAGAGGGA-3′	571	AB037865
Reverse	5′-TTGTTGGGCGTTTGGTTGGG-3′	—	—

[a] Two RT-PCR bands were amplified to identify EtPACAP$_{38}$ (where E and t indicate exon skipping and tilapia, respectively). The large band (271 bp) encodes tGHRH and tPACAP$_{38}$, and the small band (166 bp) encodes tPACAP$_{38}$ only. However, the specific primers of tPACAP$_{38}$ (199 bp), amplifying only the region of PACAP$_{38}$, were used to perform the semi-quantitative RT-PCR in the experiments and to prevent the dimerization of PCR and the formation of a hairpin with β-actin primers. The primers of PAC$_1$-R were used to identify tilapia PAC$_1$-R.
[b] Degenerate primer of PAC$_1$-R.

strated (24, 25). Pharmacologic studies reveal that astrocytes predominantly express the PAC$_1$-R that mediates cAMP accumulation by PACAP 10^3 times more effectively than VIP (17, 23, 24). PACAP also stimulates intracellular calcium mobilization in astrocytes, indicating that astrocytes might be the main target cells for PACAP in the CNS (23, 24, 26, 27).

The actions of PACAP in fish are more interesting, especially when PACAP serves as a hypophysiotropic agent (8). It effectively stimulates the secretion of GH (8, 28–32) and gonadotropin (33); it functions in the gut by affecting contractions of the intestines (34, 35); it can trigger catecholamine secretion from chromaffin cells (36); and it controls the differentiation and proliferation of cells during zebrafish ontogeny (37). PACAP was presented recently as a potential ovarian factor for mediating the activity of gonadotropin in the manner of paracrine/autocrine in zebrafish follicle cells (38). Mossambic tilapia, a euryhaline teleost, can adapt to various salinities of sea water (SW). Studies have shown that the physiologic responses involve the coexistence of two isoforms of creatine kinase in gills (39). Studies have demonstrated that creatine kinase provides energy in gills (40, 41) and the brain (42) during the transfer of fresh water (FW) to 25 parts per thousand (ppt) SW in tilapia. However, the underlying mechanism of tilapia brain in response to osmolarity challenge has not yet been examined. Astrocytes, the dominant component of glial cells in brain, are known to support a normal CNS by optimizing ion and pH homeostasis. Previous studies have demonstrated that cultured astrocytes under various osmotic conditions exhibit various biochemical and physiologic responses, including the augmentation of mRNA and protein expression of water channel (43), osmolytes, synthetic enzymes, and transporter expression (44). Astrocytes respond to hyperosmotic stress by activation of p38 kinase (45) and change in the regulated volume through the chloride channel (46). By contrast, astrocytes trigger a calcium response (47) in response to a hypo-osmotic challenge. Our preliminary study has found that the mRNA level of tPACAP$_{38}$ in tilapia brain was significantly stimulated at 8 hrs after a transfer from FW to SW. However, the presence and involvement of PACAP and its receptor in tilapia brain, especially in specific types of cells, such as astrocytes, that are under osmotic stress, remains unknown. Therefore, the present study examined the effects of PACAP in cultured tilapia astrocytes. We isolated a new GHRH-PACAP gene from tilapia brain (designated tGHRH-tPACAP$_{38}$, where t denotes tilapia). The tGHRH-tPACAP$_{38}$ cDNA expressed in tilapia pituitary and astrocytes also were found. The role of tPACAP$_{38}$ in astrocytes was investigated by considering (i) the expression of tPACAP$_{38}$ and PAC$_1$-R in tilapia astrocytes, (ii) the regulation of tPACAP$_{38}$ expression by exogenous ovine PACAP$_{38}$ and the signaling mechanism involved, and (iii) the effect of hyperosmolarity (NaCl and mannitol) on the expression of tPACAP$_{38}$ and PAC$_1$-R.

Materials and Methods

Chemicals. Hydrocortisone and dibutyryl cyclic AMP (db-cAMP) were purchased from Sigma (St. Louis, MO), and ovine PACAP$_{38}$ was purchased from Calbiochem (La Jolla, CA). NaCl and mannitol were purchased from Sigma. First, db-cAMP and ovine PACAP$_{38}$ were dissolved in water and then diluted with L-15 medium to the desired

concentrations before use. NaCl (170 mM) and mannitol (270 mM) were added directly to L-15 medium (300–310 mOsm/kg osmolarity) with 10% fetal bovine serum (FBS) to make hyperosmolarity at 500 mOsm/kg (according to previous studies, the osmolarity of cerebrospinal fluid is 450–500 mOsm/kg at 4–8 hrs following exposure to 25 ppt seawater) before use. Rabbit anti-human (ovine, rat) PACAP$_{38}$ polyclonal antibody (dilution 1:500) was purchased from Phoenix Pharmaceuticals (Belmont, CA), rabbit anti-human PAC$_1$-R polyclonal antibody (dilution 1:500) was purchased from LifeSpan Biosciences (Seattle, WA), and rabbit anti-human glial fibrillary acidic protein (GFAP) polyclonal antibody (dilution 1:1000) was purchased from Chemicon (Temecula, CA).

Animals. Tilapia (*Oreochromis mossambicus*) fish were obtained originally from the Tainan Fish Culture Station of the Taiwan Fisheries Research Institute. All fish were maintained in a freshwater recirculating tank at 25°C–28°C on a 12:12-hr light:dark photoperiod and fed daily at the Institute of Biotechnology, National Dong Hwa University (Hualien, Taiwan). Euryhaline tilapia are able to live in both sea water (SW) and fresh water (FW).

Primary Culture of Astrocytes. Brains of adult tilapia were used for primary astrocyte culture. Tilapia 40–60 g body wt were put on ice, immersed briefly (30 secs) in 70% alcohol (sterile), and decapitated. Brains were dissected out into 35-mm culture dishes containing ice-cold phosphate-buffered saline (PBS) with 10 mM glucose and were rinsed twice with PBS. They were digested with 0.5% (v/v) trypsin in L-15 medium (Gibco, Gaithersburg, MD) for 15 mins at 28°C, followed by mechanical dispersion with 25-G needles in L-15 medium supplemented with 10% FBS and filtration with a 40-μM Falcon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove cell debris. After centrifugation at 500 g for 10 mins, the supernatant was discarded, and approximately 2×10^6 cells were seeded in 25-T flask at 28°C without CO_2 supplement. The medium was changed twice per week. Experiments were conducted after third-passage cultures, which showed greater than 95% positive staining for GFAP. These cultures were not immunoreactive to other major CNS cell markers, including microglial markers (GSA-IB4 and ED-1), neuronal markers (MAP-2 and NF-L), and oligodendrocyte (MBP and GalC).

Total RNA Isolation. Male or female tilapia (80–100 g body wt) were put on ice and decapitated. The brains and pituitaries were removed and immediately homogenized for RNA isolation. Total RNA was isolated from the brains, pituitaries, or cultured astrocytes using RNA-Bee RNA isolation reagent (Tel-Test, Friendswood, TX) according to the manufacturer's instructions.

Cloning of PACAP and PAC$_1$-R Fragments from Tilapia Brain and Astrocytes. The sequences of gene-specific primers in PACAP and PAC$_1$-R were designed from EST clone of our tilapia brain cDNA library and VIP receptor of *Takifugu rubripes* (accession no. AJ408877),

respectively. The cDNA was synthesized using a reverse transcriptase–polymerase chain reaction (RT-PCR) kit (Invitrogen Co., Carlsbad, CA). Five micrograms of total RNA from the brain was dissolved in 9 µl diethyl pyrocarbonate (DEPC) water with 1 µl oligo (dT)$_{12–18}$ (0.5 µg/µl), and 1 µl dinucleoside triphosphate (dNTP) mix (10 mM) was denatured at 65°C for 5 mins and then chilled on ice immediately for 1 min. The denatured RNA was mixed with 4 µl 5× first-strand buffer, 2 µl of 0.1 M 1,4-dithiothreitol (DTT), and 1 µl RNAse Out (40 U) and denatured at 42°C for 2 mins. Subsequently, 1 µl random hexamer (50 ng/µl) and 1 µl Superscript II transcriptase (200 U) were added and incubated at 25°C for 2 min. The condition was set at one cycle at 25°C for 10 mins, one cycle at 42°C for 90 mins, and 70°C for 15 mins. After reverse transcription (RT), the product was incubated with 1 µl (2 U) *Escherichia coli* RNAse H at 37°C for 20 mins and stored at −20°C until analysis. The PCR products were purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid Co., Bade City, Taiwan) and cloned into the pGEM-T Easy vector (Promega, Madison, WI) for sequencing. The same gene-specific primers of PACAP (EtPA-CAP$_{38}$; Table 1) also were used to investigate the characteristics of exon skipping on the brain, pituitary, and astrocytes, whereas the gene-specific primers of PAC$_1$-R (Table 1) were newly designed according to the data after sequencing in this study.

5′ RACE and 3′ RACE. The gene-specific primers of EtPACAP$_{38}$ (Table 1) were used for 5′ and 3′ rapid amplification of cDNA ends (RACE) to amplify the 5′ and 3′ cDNA ends using the SMART-RACE cDNA amplification kit (Clontech, Palo Alto, CA) with the same protocol as previously described (39). The amplified cDNA ends were cloned into the pGEM-T Easy vector for sequencing to complete the full length of tilapia GHRH/PACAP gene.

Validation of Semiquantitative RT-PCR Assays for tPACAP$_{38}$ and β-Actin. The gene-specific primers for tPACAP$_{38}$ and β-actin are listed in Table 1. One microliter of cDNA solution (1:1 diluted with sterile water from stock) and two sets of primers for tPACAP$_{38}$ and β-actin were used for PCR amplification simultaneously in 50 µl of PCR reaction samples. The parameters of the PCR reaction were 35 cycles at 95°C for 30 secs, 55°C for 30 secs, and 72°C for 1 min, and then a single cycle at 72°C for 7 mins. PCR products were separated in 1.2% agarose gel. After ethidium bromide (EtBr, 0.5 µg/mL) staining and photographing, the signals were analyzed by Phoretix ID standard software (PHORETIX, 61397/28052 MemoHASP1; Feng Jin Biomedical & Instruments Co., Ltd,, Taipei, Taiwan). Data were expressed as the mRNA ratio of PACAP or PAC1 receptor to β-actin following the measurement of the optical density for each cDNA fragment detected. The ratio to control presented the comparisons between control and treatments after β-actin (as an internal control) normalization. For validation of semiquantitative RT-PCR assays, the amplified cycle numbers of PCR were determined by checking the linear
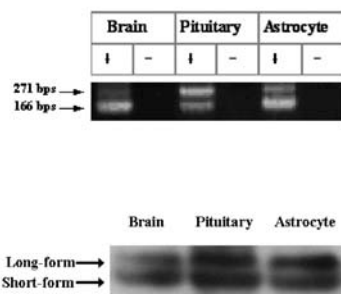
(A) Nucleotide and deduced amino acid sequence

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gcacacacgcaaacacgcactgccacagccgcgcccggatcacaacctacagcacagcct       60
cctctctgctttcctgctatagc                                          83
                     atggccagttcgagtaaagcgactttaatcttgctca       120
                     M  A  S  S  S  K  A  T  L  I  L  L         12
tctacgggatctttatgcactacagcgtcttctgcacacctcttggactaagttacccgtcc   180
I  Y  G  I  F  M  H  Y  S  V  F  C  T  P  L  G  L  S  Y  P       32
agatgagacttgaaaacgacgcattcgatgaggacgcgggaattcgttatccgacatgggtt   240
K  I  R  L  E  N  D  A  F  D  E  D  G  N  S  L  S  D  M  G       52
ttgatagtgatcagattgctatacgaagcccgccatccttaaacgacgacgcatacaccc    300
F  D  S  D  Q  I  A  I  R  S  P  P  S  L  N  D  D  A  Y  T       72
tatactacccacaggagaagaaccagaaagcatgctgaggaagaattagatagagagcct     360
L  Y  Y  P  Q  E  K  R  F  E  R  H  A  E  E  E  L  D  R  A       92
tgagggagatcctgggtcagttaacagcgagacattatctgcattctctgatgacaattc    420
L  R  E  I  L  G  Q  L  T  A  R  H  Y  L  H  S  L  M  T  I       112
gtgcaggtgacgacaacagcatggaggaagaatcagagcccttatccaaaagacactcag    480
R  A  G  D  N  S  M  E  E  E  S  E  P  L  S  K  R  H  S          132
atgggatcttcactgacagctacagtcgctatagaaagcagatggccgtgcagaaatacc    540
D  G  I  F  T  D  S  Y  S  R  Y  R  K  Q  M  A  V  Q  K  Y       152
tggcagcggttgtgggtagaaggtacagacagagagttaggaacaaagagacgccggcttg    600
L  A  A  V  L  G  R  R  Y  R  Q  R  V  R  N  K  G  R  R  L       172
cctatttgtag                                                      611
A  Y  L  *                                                       175
           cgctgttaaggcgctcaaactgccctcctgtgtatatacatccagtcgt    660
taaatcgaagtcattcagatatatctgaccaaccagtggattgcgcctgtgttctttcaa    720
catgtatttatgtatgaagtaaagccattaaaatgaatatttaataacaaaaaaaaaaa    780
aaaaaaaaaaaagaaaaaaa                                             798
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(B) PCR and Southern blot

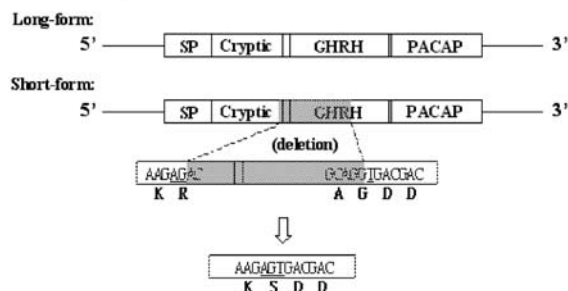**(C) Structure of long and short forms**

Figure 1. Tilapia GHRH/PACAP cDNA. (A) Full-length nucleotide sequence (GenBank accession no. AY522580) and deduced amino acid sequence. The deduced amino acids constitute the regions that encode tGHRH (double underlined) and $tPACAP_{38}$ (bold underlined). The gray background area in the coding region is the sequence deleted from the short spliced form of the transcript. The poly-adenylation signal (attaaa) in the 3′ untranslational region is underlined with a wavy line, and an asterisk marks the terminal codon. (B) Distribution of $tPACAP_{38}$ in tilapia brain, pituitary, and astrocyte. $EtPACAP_{38}$ primers were used to perform RT-PCR, yielding two bands. The large band (271 bp) encodes tGHRH and $tPACAP_{38}$, and the small band (166 bp) encodes only $tPACAP_{38}$. +, RT; -, RNA alone, without RT. The PCR-amplified DNA was separated and transferred to a nylon membrane hybridized with a

(A) Time course
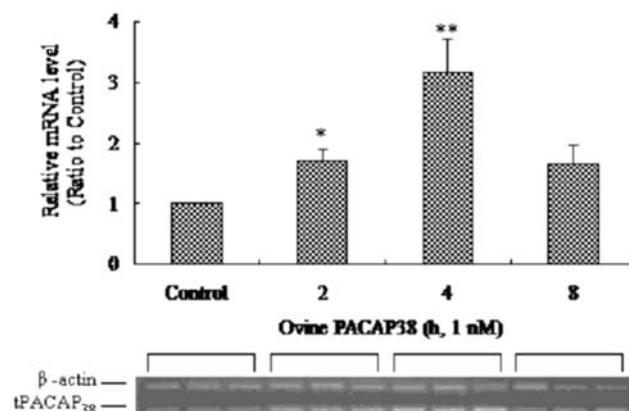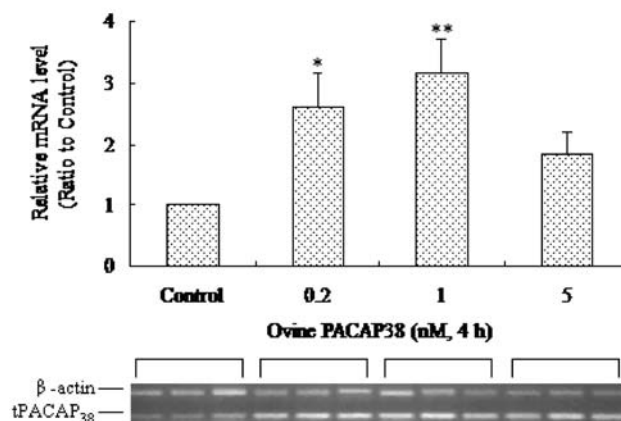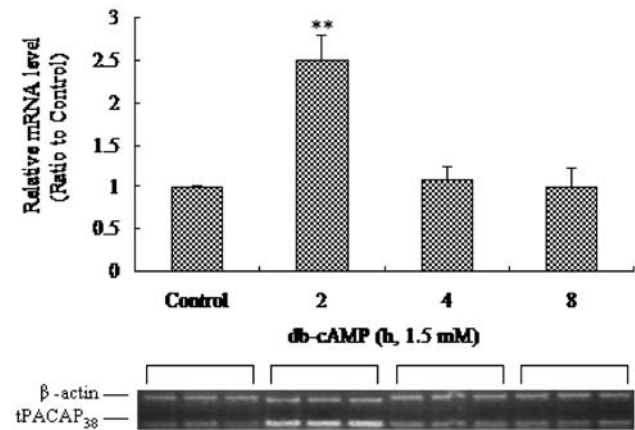

(B) Concentration-dependent

Figure 2. Effects of exogenous ovine $PACAP_{38}$ on the expression of endogenous $tPACAP_{38}$ in cultured astrocyte of tilapia brain. (A) In the time course experiment, exogenous ovine $PACAP_{38}$ (1 nM) was applied to the cultured astrocyte for various periods (0–8 hrs) before RNA was extracted. (B) In the concentration-response experiment, the cells were treated for 4 hrs with various concentrations of exogenous ovine $PACAP_{38}$ (0–5 nM). The data obtained after β-actin normalization were presented as ratios with the data for the control. Three independent experiments were performed in triplicates. Asterisks (* and **) represent significant differences ($P < 0.05$ and $P < 0.01$) among treatments.

intensity of amplified product in various cycles, and the ratio of two set of primers for $tPACAP_{38}$ and β-actin used in one reaction was measured the same way. In the meantime, inserted $tPACAP_{38}$ plasmid as a template was used to generate a standard curve for the semiquantitative of $tPACAP_{38}$ to do comparisons.

Southern Hybridization. The PCR-amplified DNA (50 ng) was separated in a 1.2% agarose gel and transferred

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←

cDNA clone of $tGHRH$-$tPACAP_{38}$ to form the probe. (C) Structures of long and short forms. The long form comprises GHRH and PACAP, and the short form comprises PACAP alone. From the shadow region 105 nucleotides were deleted, including most of the GHRH, during exon skipping. SP and Cryptic are abbreviations for the signal peptide and the cryptic peptide, respectively.

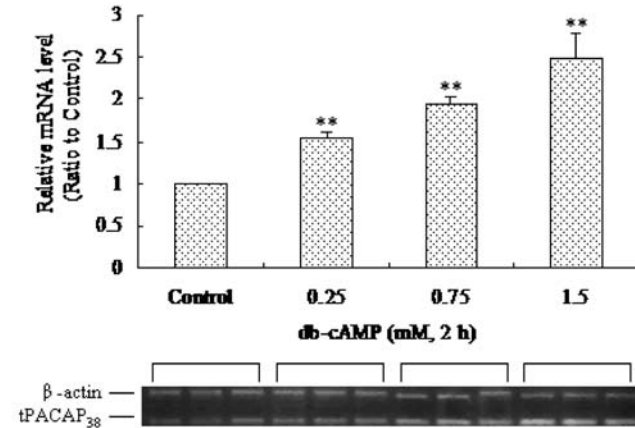## (A) Time course

(B) Concentration-dependent

Figure 3. Effects of db-cAMP on the expression of endogenous tPACAP$_{38}$ in cultured astrocyte of tilapia brain. (A) In the time course experiment, the cells were treated by db-cAMP (1.5 mM) for various periods (0–8 hrs). (B) In the concentration-response experiment, the cells were treated for 2 hrs with various concentrations of db-cAMP. The data obtained after β-actin normalization were presented as ratios with the data for the control. **A significant difference ($P <$ 0.01) among treatments.

to a nylon membrane (NEN Life Science Products, Boston, MA), then analyzed by Southern hybridization as described previously (75). Gel-purified inserts of cDNA clones of tGHRH-tPACAP$_{38}$ were used as probes (DIG Southern Starter Kit; Roche, Mannheim, Germany). The PCR product of RNA without RT was concurrently performed as a negative control.

Immunocytochemistry. The experiment was performed using the VECTASTAIN ABC system kit (avidin and biotinylated horseradish peroxidase macromolecular complex; Vector, Burlingame, CA). Astrocytes grown on 12-well tissue culture plates that had flat bottoms and low evaporation lids (Falcon, Bedford, MA) were washed three

(A) Partial nucleotide sequence of PAC$_1$-R

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ctcaggaagacagcgaccattgcttcgtcggacacagtggcatgtaaagcagtgatggttt      60
tcttccattactgtgtcatgtccaactatttctggctgttcattgaaggtctgtatctct     120
tcactctgctggtggagactttctttcccgagagacgctacttctactggtataccattg     180
ttgggtggggaaccccacaatttgtgtgactgtctgggctgttctgaggctccattttc     240
atgacactggatgctgggatacgaatgagaacactgccctctggtgggtgatcaaaggac     300
ctgtggtggcatctataatgataaatttcgtcctcttcattggaataatcgtgatcctgg     360
tccagaagctgcagtcgcctgatatcggaggaaatgaatcgagcatttacctgcgtctgg     420
cacgctccaccctcctcctcatccct                                       446
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(B) Distribution

	Brain		Pituitary		Astrocyte	
	+	−	+	−	+	−

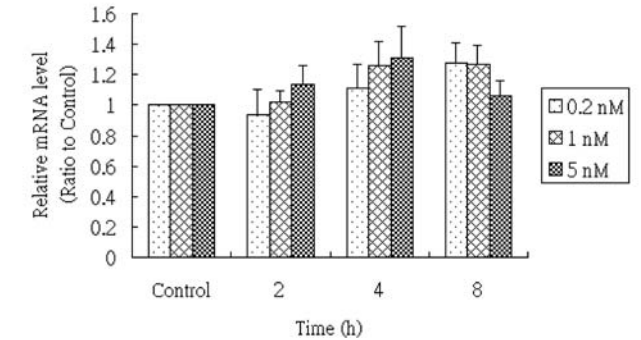267 bps →
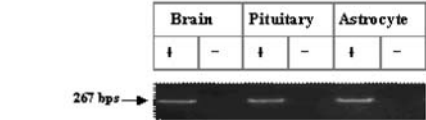
(C) Exogenous ovine PACAP treatment

Figure 4. (A) Partial nucleotide sequence (446 bp) of PAC$_1$-R. (B) Presence of PAC$_1$-R in tilapia brain, pituitary, and astrocyte. (C) Effect of exogenous ovine PACAP$_{38}$ on the expression of PAC$_1$-R in cultured astrocyte. The data obtained after β-actin normalization were presented as ratios with the data for the control. Three independent experiments were performed in triplicate.

times with 10 mM PBS for 3 mins and fixed in ice-cold methanol for 5 mins before being washed three more times with PBS for 3 mins. The fixed astrocytes were blocked at nonspecific binding sites with 1%–2% normal mouse serum (blocking serum) in PBS for 1 hr at 37°C. Astrocytes were identified with antibodies against GFAP (1:1000). The tPACAP$_{38}$ and PAC$_1$-R were identified using antibodies against PACAP$_{38}$ (1:500) and PAC$_1$-R (1:500), respectively. Astrocytes were incubated with primary antibodies or rabbit serum (as a negative control) at 4°C overnight and washed three times with PBS, followed by incubation with biotinylated secondary antibody at 37°C for 1 hr. After washing, the astrocytes were visualized by the addition of VECTASTAIN ABC. The cells were examined by phase-contrast microscopy.

Western Blotting. Brain or cell homogenate (total protein 40 μg) was mixed with 6× electrophoresis sample buffer containing DTT (Sigma). The proteins were separated by electrophoresis on a 4%–12% gradient polyacrylamide slab gel and electrophoretically transferred to a polyvi-

nylidene fluoride membrane (NEN Life Science Products). The blots were incubated for 2 hrs in blocking buffer (3% bovine serum albumin in TBST buffer) and were washed twice with TBST buffer (0.8% NaCl, 0.02% KCl, 0.3% Tris base, pH 7.5, 0.024% KH_2PO_4, and 0.2% Tween-20). Two membranes were incubated overnight at 4°C with rabbit anti-human $PACAP_{38}$ (1:500) and rabbit anti-human PAC_1-R (1:500) and internal control β-actin antibody (1:4000; Sigma), respectively. After incubation with secondary antibody (alkaline phosphatase–conjugated goat anti-rabbit antibody, 1:4000), the protein bands were developed by BCIP/NBT staining. Bands were quantified using computer software (PHORETIX, 61397/28052 MemoHASP1).

Statistical Analysis. The experimental results were presented as mean ± standard error (SE). The significant difference ($P < 0.05$) between treatment and control was compared by Duncan multiple range test after one-way analysis of variance (ANOVA).

Results

Identifying PACAP ($tPACAP_{38}$) Expressed in the Tilapia Astrocyte. The full-length cDNAs that encode tGHRH and $tPACAP_{38}$ in the brain of tilapia were constructed from overlapping 5′ and 3′ RACE. The cDNA was 798 bp, encoding 175 amino acids (Fig. 1A). The result demonstrates that the nucleotide sequence of tGHRH starts at 333, ends at 467, and encodes 45 amino acids, and $tPACAP_{38}$ starts at 474, ends at 587, and encodes 38 amino acids. In this study, the exon skipping of GHRH/PACAP is evident in tilapia brain and pituitary, including astrocytes (Fig. 1B). This result was verified by Southern blot. The structural scheme elucidates the difference between the long and short forms and the characteristics of exon skipping (Fig. 1C). Based on the cloning and sequencing of various-size PCR products, the nucleotide sequence reveals that the long form (271 bp) comprises both tGHRH and $tPACAP_{38}$. However, the short form (166 bp) consists of $tPACAP_{38}$. Comparing these two forms indicates that the small product has a complete region of coding of $tPACAP_{38}$ and a region that encodes 13 amino acids of tGHRH at the C terminus, but the region that encodes most of tGHRH mRNA, from nucleotide 323 to nucleotide 427 (105 bp), was absent. Additionally, viewing from the deduced amino acid sequence revealed that the long form, KRFERHAEEEL-DRALREILGQLTARHYLHSLMTIRAGDD, was substituted by the short form, KSDD, and 35 amino acids were absent (Fig. 1C). To investigate the underlying mechanism of PACAP regulation, the short form was designed to explore the alterations of $tPACAP_{38}$ following various treatments in subsequent experiments.

Exogenous Ovine $PACAP_{38}$ and db-cAMP Regulation of $tPACAP_{38}$ in Cultured Astrocytes. Whether exogenous PACAP regulates the expression of endogenous PACAP ($tPACAP_{38}$) in the astrocytes of tilapia brain was investigated. Experiments were conducted using semi-

quantitative RT to elucidate whether $tPACAP_{38}$ is expressed in tilapia astrocytes in response to exogenous ovine $PACAP_{38}$ PCR. The RT-PCR assays were validated carefully by determining the number of amplifying cycles and from the ratio of the two sets of primers for $tPACAP_{38}$ and β-actin used in a single reaction. Meanwhile, $tPACAP_{38}$ plasmid was inserted as a template to generate a standard curve to ensure the semiquantitative accuracy of $tPACAP_{38}$ (data not shown). The cultured astrocytes were treated with exogenous ovine $PACAP_{38}$ (1 nM) for various periods. The data showed that the expression of $tPACAP_{38}$ by treated astrocytes increased significantly for 2 hrs, reaching a peak at 4 hrs (Fig. 2A). The stimulation of tPACAP38 expression in astrocytes by exogenous ovine PACAP38 depended on concentration (Fig. 2B). However, the effect of exogenous ovine PACAP38 at 5 nM on tPACAP38 expression was significant, reaching a peak at 2 hrs and then declining to 4 hrs (data not shown). Cyclic AMP is well known to be the main second messenger that is involved in exogenous PACAP stimulation through the protein kinase A (PKA) signaling pathway (27), so experiments were designed to determine whether intracellular cAMP level affects the expression of $tPACAP_{38}$. Treatment of cultured astrocytes with 1.5 mM db-cAMP, a cAMP analog, was associated with strong stimulation of $tPACAP_{38}$ for 2 hrs, after which the response declined (Fig. 3A). Stimulation with db-cAMP for 2 hrs significantly promoted the expression of $tPACAP_{38}$ in a concentration-dependent manner (Fig. 3B).

Expression of PAC_1-R in the Tilapia Astrocyte. Primary cultures were used to explore whether the PACAP receptor was expressed in the tilapia astrocyte and thus to determine whether PACAP is a potential paracrine/autocrine factor in astrocytes. First, *T. rubripes* PAC_1-R gene (accession no. AJ408877) primers were designed for use in RT-PCR of tilapia brain, and a 510-bp partial sequence of PAC_1-R from tilapia brain was cloned (data not shown). Subsequently, based on the tilapia PAC_1-R sequence, gene-specific primers of tilapia PAC_1-R (446 bp) and one more nested primer of tilapia PAC_1-R (267 bp) were designed to analyze and verify the existence of PAC_1-R in various tissues of tilapia. The partial nucleotide sequence of PAC_1-R (446 bp) was obtained (Fig. 4A). PAC_1-R was present in tilapia brain, pituitary, and astrocytes, as determined using RT-PCR with tilapia PAC_1-R primer (Fig. 4B). Adding exogenous ovine PA-CAP_{38} to the cultured tilapia astrocyte did not affect the expression level of PAC_1-R mRNA (Fig. 4C). The downstream activation of PACAP may involve the cAMP-PAK signaling pathway to promote astrocytic PACAP expression.

Colocalization of $tPACAP_{38}$ and PAC_1-R in Astrocyte. In the present work, cultured astrocytes were subcultured with at least three passages and were used to perform experiments. The light field view and anti-GFAP immunostaining of the cultures demonstrated that more than 95% of the cells were positive stained for astroglial marker
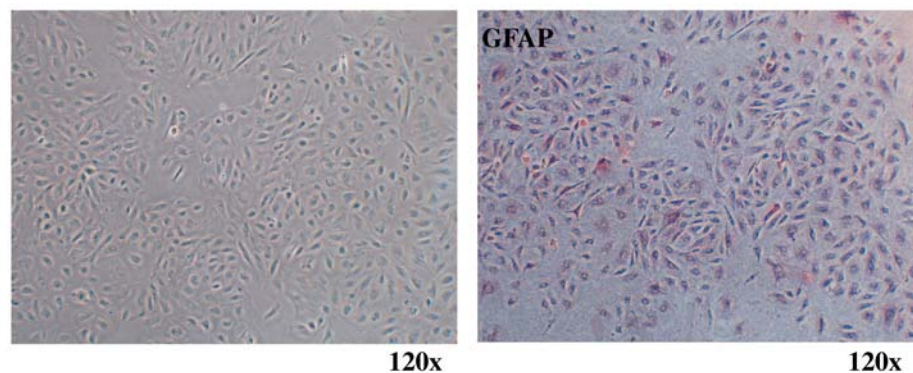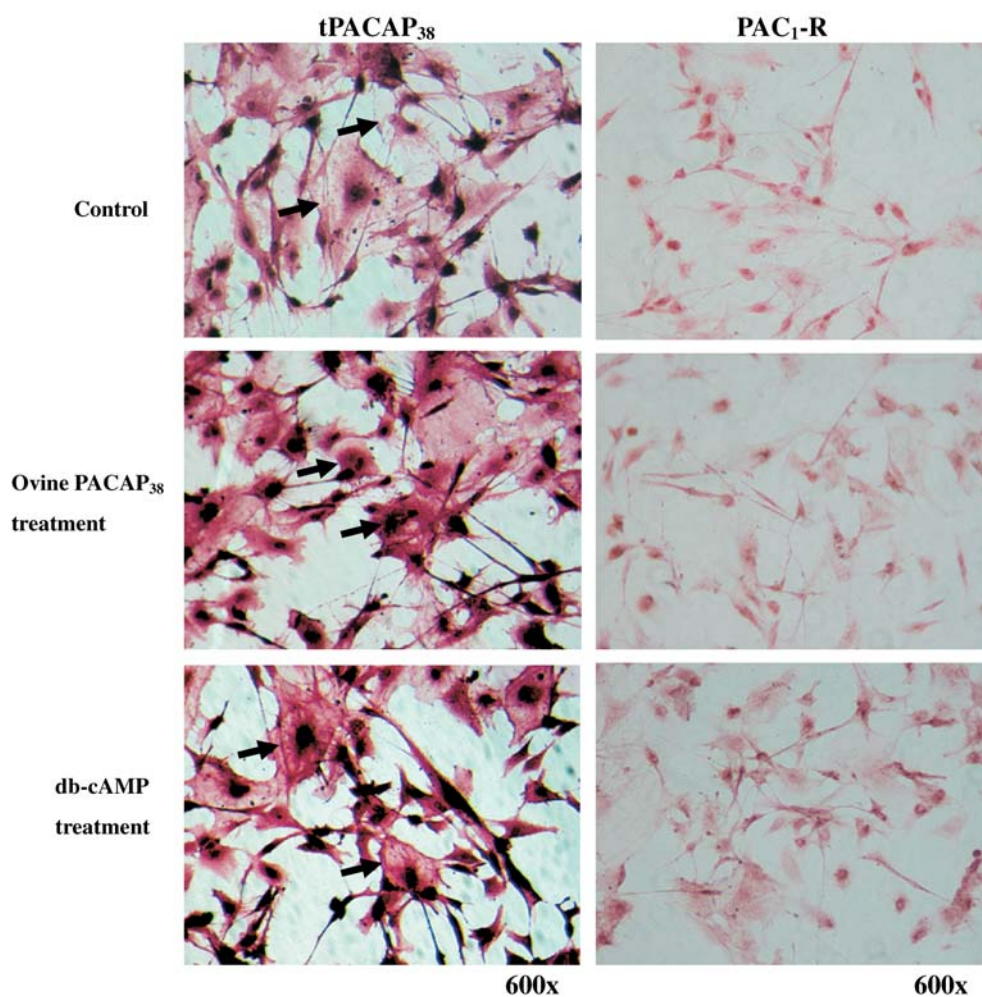
(A) Morphology

120x   120x

(B) tPACAP and PAC$_1$-R immunocytochemistry

600x   600x

Figure 5. (A) Morphology of astrocyte in light field with GFAP staining. Magnifications: ×300 and ×600. (B) Immunocytochemistry of PACAP and PAC$_1$-R in cultured astrocytes treated with exogenous ovine PACAP$_{38}$ (1 nM, 4 hrs) and db-cAMP (1.5 mM, 2 hrs). Magnification: ×600. The arrows indicate signals with various densities. (C) Western blot analysis of PACAP (3.8 kDa) and PAC$_1$-R (50 kDa) in cultured astrocytes treated with exogenous ovine PACAP$_{38}$ (1 nM) and db-cAMP (1.5 mM) for various periods (0, 2, 4, or 8 hrs). Three independent experiments were performed in triplicate. Asterisks (* and **) represent significant differences ($P < 0.05$ and $P < 0.01$) among treatments. Color version of this figure is available in the on-line journal.
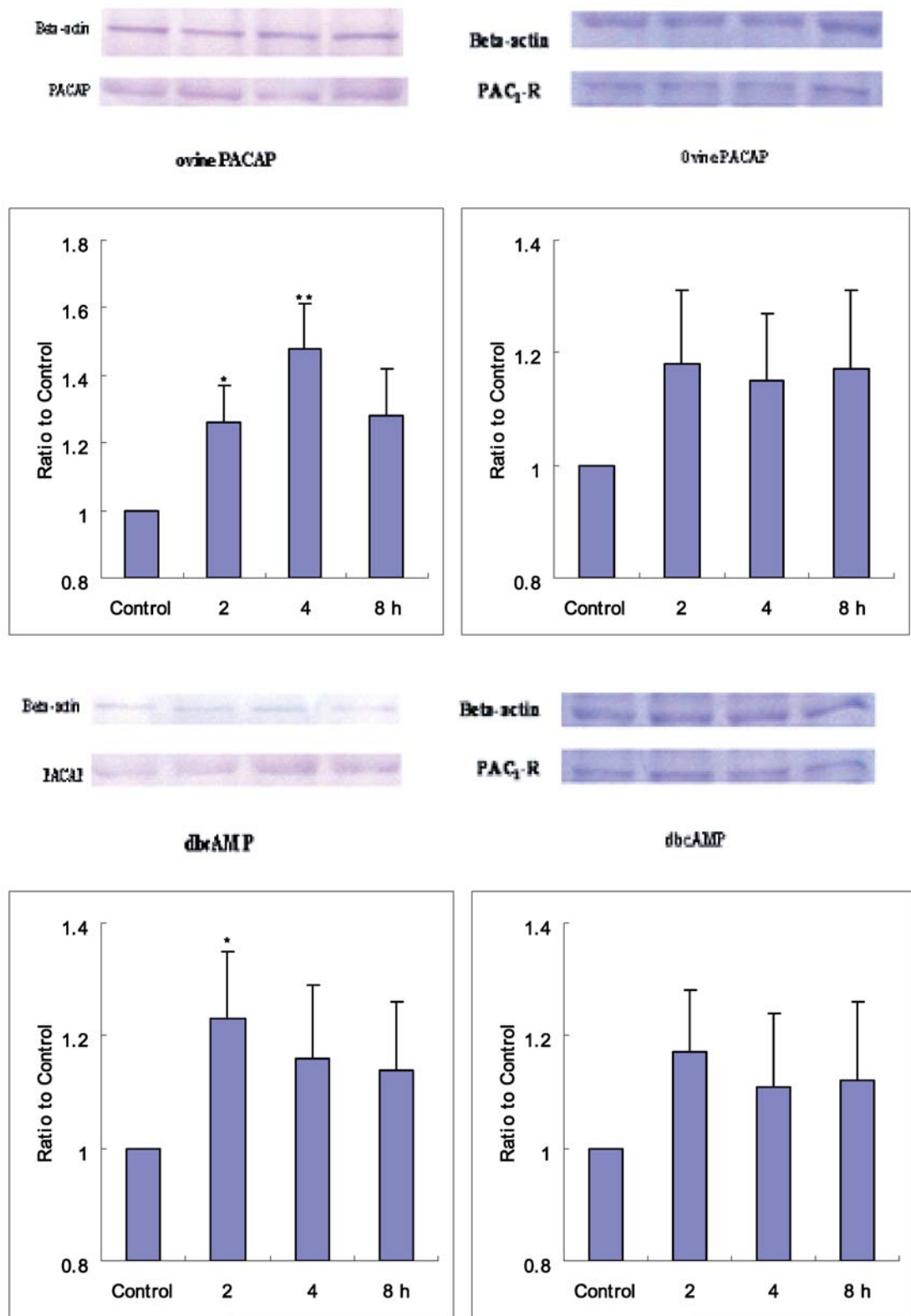
(C) Western blot
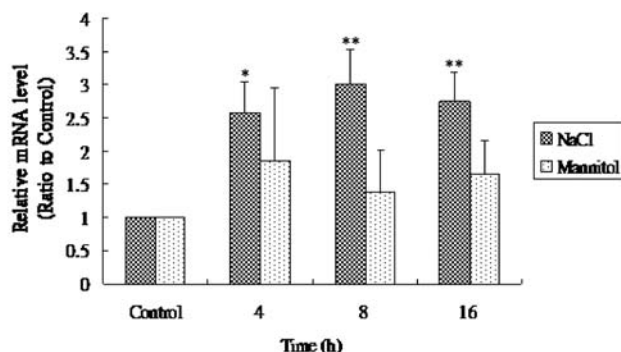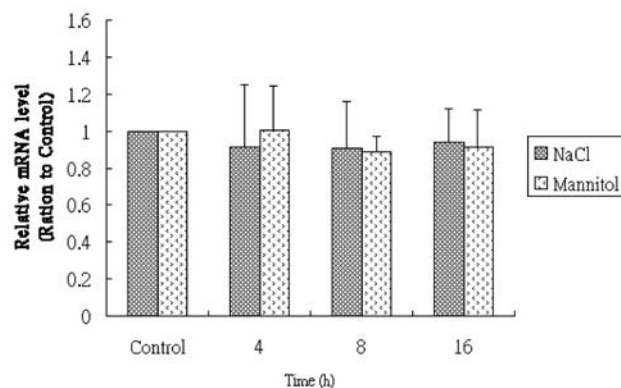

Figure 5. Continued.

(A) PACAP expression

(B) PAC₁-R expression

Figure 6. Effects of NaCl and mannitol (hyperosmolarity) on the expression of (A) $tPACAP_{38}$ and (B) PAC_1-R in cultured astrocytes of tilapia brain. The cells were treated with hyperosmolarity medium by adding NaCl and mannitol for various periods (0, 4, 8, or 16 hrs). The data obtained after β-actin normalization were presented as ratios with the data for the control. Three independent experiments were performed in triplicate. Asterisks (* and **) represent significant differences ($P < 0.05$ and $P < 0.01$) among treatments.

GFAP (Fig. 5A). They were not immunoreactive to microglial markers (ED1 or GSA-IB4) or oligodendrocyte markers (MBP and GalC). Particularly, they were virtually devoid of neurons (not immunoreactive to MAP-2 or neurofilament L; data not shown), which is consistent with the results of Franke et al. (48). The anti-$PACAP_{38}$ and anti-PAC_1-R antibodies were used to test the protein levels of $tPACAP_{38}$ and PAC_1-R in tilapia astrocytes (Fig. 5B and C). Based on our earlier findings, astrocytes also were separately treated with exogenous ovine $PACAP_{38}$ and db-cAMP for 4 hrs and 2 hrs, respectively, before immunocytochemistry. Astrocytes stained with anti-$PACAP_{38}$ antibody had a denser signal in the cytoplasm than the control group, indicating an increase in the expression of $tPACAP_{38}$ peptide. These results were confirmed by Western blot analysis (Fig. 5C). However, the control did not differ from the treated astrocytes following anti–PAC_1-R staining, revealing that treatment with exogenous ovine $PACAP_{38}$ did not affect the expression of PAC_1-R, which is consistent with the findings of RNA expression experiments.

Expression of $tPACAP_{38}$ Under Hyperosmotic Stress. The cultured astrocytes were treated with NaCl- and mannitol-hyperosmolarity medium for various periods. The data indicated that the PACAP expression of NaCl-treated astrocytes increased significantly for 4 hrs to a level sustained for up to 16 hrs (Fig. 6A). Mannitol treatment did not significantly change the expression of PACAP in tilapia astrocytes. Both NaCl and mannitol treatments did not alter the expression of PAC_1-R in tilapia astrocytes, even after 16 hrs (Fig. 6B). The anti-$PACAP_{38}$ and anti–PAC_1-R antibodies were used to determine the protein levels of $tPACAP_{38}$ and PAC_1-R in tilapia astrocytes, respectively (Fig. 7A and B). The astrocytes also were separately treated with NaCl and mannitol for 4 hrs before the immunocytochemical experiment was performed. A comparison with control group revealed that astrocytes stained with anti-$PACAP_{38}$ antibody had a denser signal in the cytoplasm, suggesting an increase in the expression of $tPACAP_{38}$ peptide following NaCl treatment but not mannitol treatment. This result was confirmed by Western blot analysis. However, the absence of any difference between the control and treated astrocytes after anti–PAC_1-R antibody staining demonstrated that the treatments of NaCl and mannitol did not influence the expression of PAC_1-R, which is consistent with the results of the mRNA expression experiments.

Discussion

VIP and PACAP have been shown as neuroprotective agents *in vivo* and exert several actions on neurons, glia, and inflammatory cells *in vitro* (49). Numerous studies have shown that PAC_1-R is present in rat astrocytes (17, 25, 50). Previous immunohistochemical works have identified the presence of $PACAP_{38}$ peptide in various areas of teleostian brain (28, 51, 52). However, the coexistence of PACAP and PAC_1-R in the brain and in specific neural or nonneural cells is uncertain. In our previous study, isolated full-length cDNAs encoded GHRH-PACAP from the brain of tilapia. The results of this present investigation verify the expression of GHRH-PACAP gene in the brain and pituitary of tilapia. Moreover, this is the first study to demonstrate the presence of GHRH-PACAP cDNA ($tGHRH$-$tPACAP_{38}$) in tilapia astrocytes. This work also shows that PAC_1-R is expressed concurrently in tilapia astrocytes. Three distinct subtypes of receptors possess differential affinities for PACAP and VIP: PAC1 receptors (PAC_1-R) show high affinity for PACAP and a much lower affinity for VIP, whereas VPAC1 and VPAC2 receptors ($VPAC_1$-R and $VPAC_2$-R) recognize PACAP and VIP with high affinity (20–22). Recently, both $VPAC_1$-R and $VPAC_2$-R were demonstrated to be present in various tissues, including brain of goldfish, zebrafish, fugu, and trout (53–55). We cannot rule out the possibility that effects of PACAP on astrocytes in the present study are mediated partially through VPAC-R. It also is possible that exogenous PACAP induced cytokine production in astrocytes and subsequently

acted back to the astroglial cytokine receptor to regulate PACAP expression. In mammals, most studies have focused on PACAP and VIP mediation of cytokine (such as interleukin-6) production and release (54). Relatively few experiments have addressed the regulation of PACAP by cytokines. Several studies indicate that VIP and certain other neuropeptides are regulated by leukemia inhibitory factor and other cytokines (56). The effects of exogenous or endogenous PACAP, exerting in an autocrine/paracrine manner, on the VPAC-R of tilapia astrocytes or the interaction of PACAP and cytokine in tilapia astrocytes deserves to be further explored.

The tGHRH-tPACAP$_{38}$ cDNA regions are those in other nonmammals as determined and described elsewhere (15). They are 5′ UTR, signal peptide, cryptic peptide, GHRH, PACAP, and 3′ UTR. RT-PCR analysis revealed two products of different sizes in tilapia brain, pituitary, and astrocyte. Sequence analysis of the PCR products showed that the large encodes both tGHRH and tPACAP$_{38}$, whereas the small product encodes only complete tPACAP$_{38}$ and some C-terminal amino acids of tGHRH, from which most of the tGHRH molecule is deleted. The shorter PCR product of tGHRH-tPACAP$_{38}$ is formed by exon skipping, a particular form of alternative splicing in which an exon is deleted during the formation of mRNA. This process has been shown to occur in lower vertebrates, including various species of fish (4, 6, 29, 57, 58), frog (10), and bird (11, 12). Mammals control the production of GHRH and PACAP by encoding these two peptides in separate genes. In the tilapia of interest here, exon 4 contains the tGHRH$_{(1–32\ aa)}$ that is deleted, allowing tPACAP$_{38}$ to be expressed independently of tGHRH. The deleted region corresponds to exon 4 of the rainbow trout GRF-PACAP gene (58). Exon skipping in the GHRH-PACAP mRNA probably represents the ancestral regulation of GHRH and PACAP production, increasing the ratio of PACAP mRNA to GHRH mRNA. However, the factors that regulate exon skipping of the GHRH-PACAP transcripts are not well understood. Several studies have reported that this mechanism operates as early as Embryonic Day 4 in fish (58) and developing turkeys (12), so the ratios of PACAP to GHRH-PACAP transcripts vary. These results also may support the findings that PACAP in fish more strongly promotes the secretion of GH and gonadotropins than does GHRH (28, 29). Alternative processing is an effective means of regulating the ratio of peptides from multipeptide coding genes in the PACAP/glucagon superfamily. In fish and birds, the proglucagon gene is alternatively spliced in a tissue-specific manner to generate either glucagon or glucagonlike peptides 1 and 2 (59). In birds, the vasoactive intestinal polypeptide can be formed without peptide histidine isoleucine (PHI) when the exon encoding of the latter is skipped (60). However, no evidence supports exon skipping in the mammalian vasoactive intestinal polypeptide transcript (61). These examples reveal that tissue-specific factors may activate exon skipping.

The expression of tilapia tPACAP$_{38}$ in astrocytes suggests a local role for peptides in regulating astrocytic functions. Locally generated PACAP acts as an autocrine and paracrine hormone such as a neurotransmitter or a neuromodulator in various organs (14). In follicle cells in zebrafish, PACAP acts as autocrine/paracrine in follicular maturation (38). As more evidence shows that PACAP is a potential local factor, understanding how it is regulated in astrocytes becomes increasingly important. In this study we verified that PACAP coexisted with PAC$_1$-R in tilapia astrocytes, suggesting that PACAP acts on tilapia astrocytes in an autocrine/paracrine manner. Evidence obtained here demonstrates that exogenous ovine PACAP$_{38}$ strongly upregulates tPACAP$_{38}$ in the tilapia astrocytes, which is consistent with the assumption that PACAP mediates neuromodulators. Like exogenous ovine PACAP$_{38}$, db-cAMP significantly promoted the expression of tPACAP$_{38}$ in a dose-dependent manner in tilapia astrocytes. Unlike tPACAP$_{38}$, PAC$_1$-R in tilapia astrocytes did not respond to exogenous ovine PACAP$_{38}$ at the mRNA level, as in a previous report that found that a reproductive hormone (gonadotropin) in rats does not affect PAC$_1$-R expression in granulosa cells (62). However, the expression of PAC$_1$-R in this work verified that PAC$_1$-R is present in astrocytes (63). Similar time course response (Fig. 5), as short as 2 hrs, the upregulations of PACAP mRNA in tilapia astrocytes were observed by dbcAMP and PACAP treatment. The upregulation of tPACAP$_{38}$ in response to db-cAMP treatment suggests that the activation of the cAMP-PKA signaling pathway dominantly mediates the upregulation of tPACAP$_{38}$ expression by exogenous ovine PACAP$_{38}$. These data are consistent with the results of other studies that found the presence of two cAMP response elements in the promoter of rat and mouse PACAP genes (64, 65) as well as with works that found that PACAP-induced differentiation is mediated by the production of cAMP in such cells as neuroblastoma (66), pheochromocytoma (67), cerebella granule (68), and embryonic stem (69) cells. This investigation verified that PACAP also may generate cAMP in tilapia astrocytes (data not shown) and in rat astrocytes (70). The strong upregulation of tPACAP$_{38}$ by added exogenous ovine PACAP$_{38}$ and db-cAMP reveals that PACAP in astrocytes acts as a paracrine communicator between an astrocyte (PACAP) and another astrocyte (PAC$_1$-R) or in an autocrine manner in astrocyte itself, (PACAP and PAC$_1$-R colocalization) to mediate the function of vertebrate astrocytes.

In rodents, PACAP has been found to have multiple functions, including the regulation of pituitary hormone release, intestinal motility, neuronal action, and insulin release, as well as cell division and differentiation (14). In addition, PACAP affects many defined behaviors, such as feeding behavior, exploratory activity, locomotor activity, face washing, body grooming, drinking and rearing behaviors, and rapid eye movement sleep when injected into the hypothalamic paraventricular nucleus or third ventricle, and there are more studies of feeding behavior
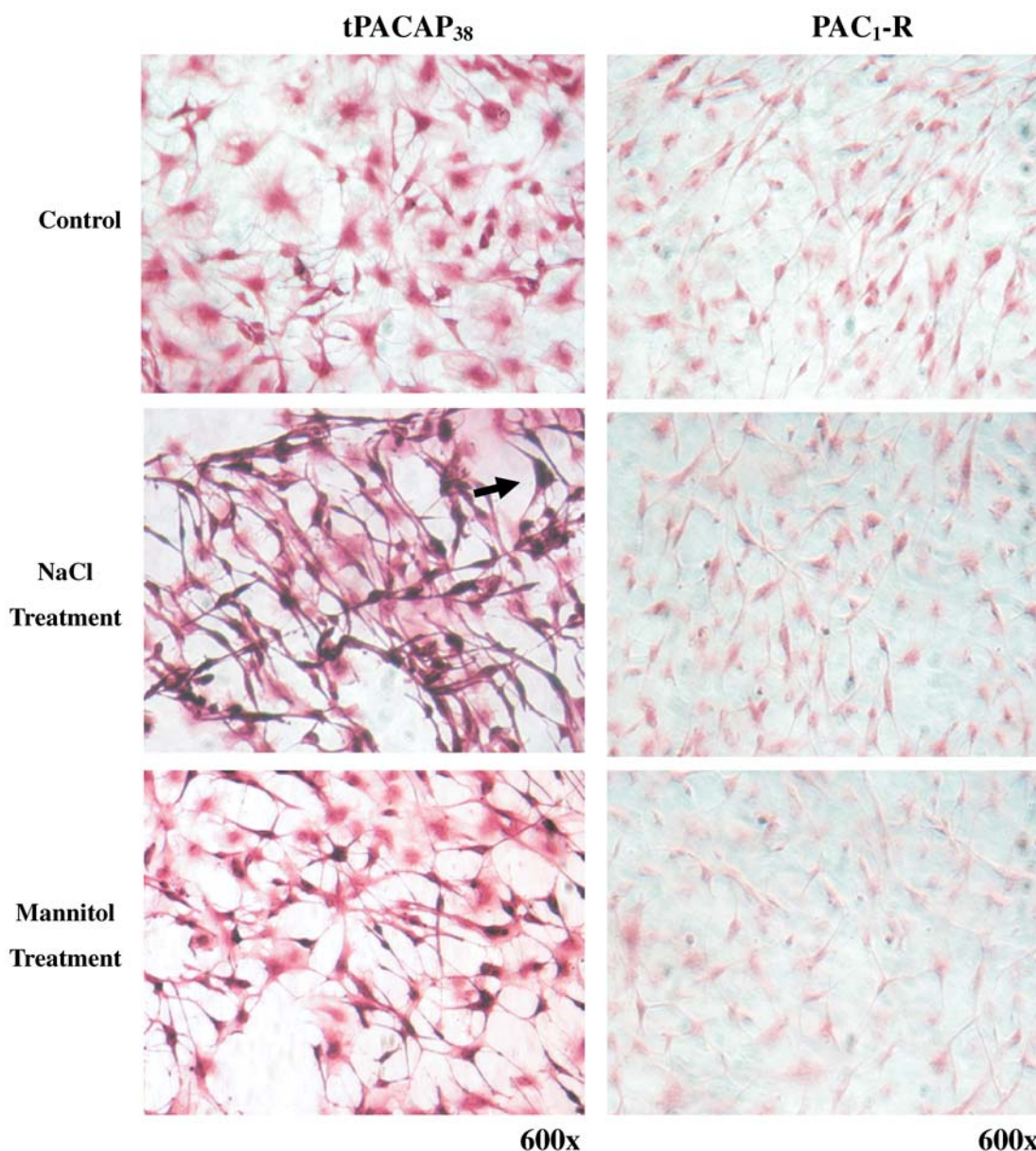
(A) tPACAP and PAC₁-R immunocytochemistry

Figure 7. (A) Immunocytochemistry of PACAP and PAC₁-R in cultured astrocytes treated with NaCl- and mannitol-hyperosmolarity medium (4 hrs). Magnification: ×600. The arrows indicate signals with different densities. (B) Western blot analysis of PACAP (3.8 kDa) and PAC₁-R (50 kDa) in cultured astrocytes treated with NaCl and mannitol for various periods (0, 4, 8, or 16 hrs). Three independent experiments were performed in triplicate. *Significant difference ($P < 0.05$) among treatments. Color version of this figure is available in the on-line journal.

in fish (55). Our preliminary data showed that the expression level of tPACAP₃₈ mRNA in tilapia brain was significantly increased at 8 hrs after transfer from FW to SW. We have postulated that PACAP may act in a paracrine and/or autocrine manner to mediate the *in vivo* effects of hyperosmolarity on brain PACAP expression. However, the role of PACAP in the osmoregulation of tilapia, particularly in brain and astrocytes, is becoming more interesting. Earlier investigations have reported that cells respond to

changes in osmotic stress with compensatory molecular adaptations, enabling them to reestablish the homeostasis of osmotically disturbed aspects of cell structure and function (71). Several studies have demonstrated that cultured astrocytes under various osmotic conditions exhibit augmentation of water channel mRNA and protein expression (43); expression of osmolytes, synthetic enzymes, and transporter (44); and activation of p38 kinase (45) in response to hyperosmotic stress accompanied by a change in
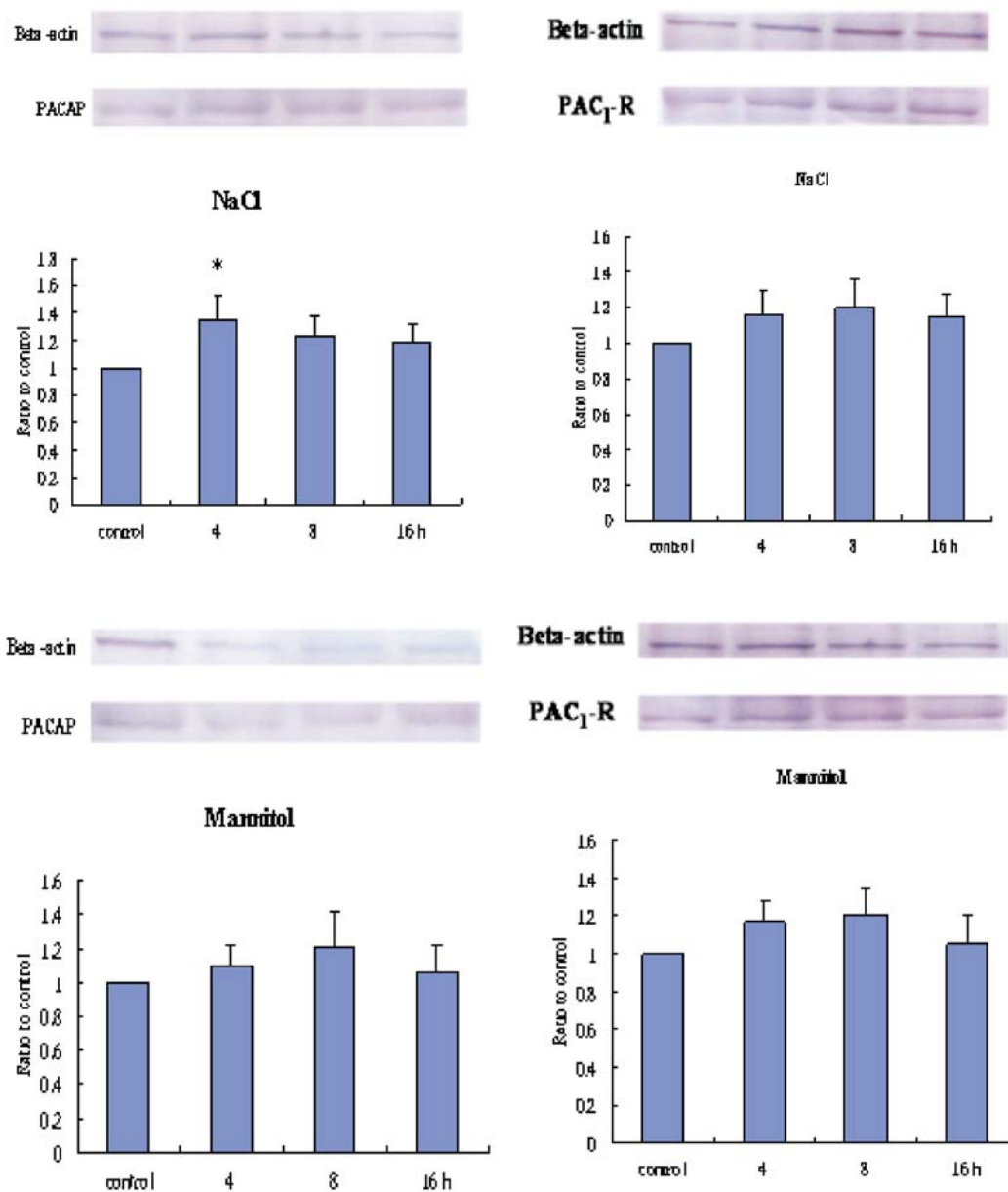
(B) Western blot

Figure 7. Continued.

the regulated volume through the chloride channel (46). Whereas the triggering of a calcium oscillation (47) in response to a hypoosmotic challenge has been reported. The effects of NaCl and mannitol on tilapia astrocytes were further examined to elucidate the involvement of osmolarity and ions in the osmoregulation of tilapia during SW transfer. As shown in Figures 6 and 7, the expressed level of PACAP mRNA in astrocytes was increased significantly up to 16 hrs after exposure to NaCl. A significant increase in protein content of PACAP was seen at 4 hrs. At 8 hrs and 16 hrs after NaCl exposure, the PACAP protein content in astrocytes remained higher, although it was not significantly

different from the control. This discrepancy could be due to an alteration at translational level. Previous reports from experiments with Xenopus oocytes have demonstrated that RNA splicing can influence subsequent events in the RNA processing pathway and result in change of translational yields (72). When mammalian cells (COS7 cells) are exposed to a variety of environmental stresses, translationally inactive messenger ribonucleoprotein particle (mRNP), 40S ribosomal subunits, and the mRNA binding proteins TIA-1 and TIAR in cytoplasm is formed and results in translational arrest of transcripts (73, 74). However, there no such reports have demonstrated stress-related changes of

cytoplasmic processing bodies in teleostian (tilapia). The underlying mechanism of our observation remains to be determined. This study is the first to show that NaCl treatment but not mannitol treatment significantly promoted the expression of PACAP in tilapia astrocytes, demonstrating that ions (Na$^+$ and Cl$^-$) under hyperosmolarity conditions induce the expression of tPACAP$_{38}$.

In conclusion, PACAP expresses in tilapia astrocytes and is upregulated by hyperosmolarity, and it subsequently acts on its own receptor by an autocrine and/or paracrine method. This fact suggests that PACAP may be involved in regulating the adaptation of tilapia to SW.

1. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler, MD, Coy DH. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 164:567–574, 1989.

2. Miyata A, Jiang L, Dahl RD, Kitada C, Kubo K, Fujino M, Minamino N, Arimura A. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). Biochem Biophys Res Commun 170:643–648, 1990.

3. McRory JE, Sherwood NM. Two protochordate genes encode pituitary adenylate cyclase-activating polypeptide and related family members. Endocrinology 138:2380–2390, 1997.

4. McRory JE, Parker DB, Ngamvongchon S, Sherwood NM. Sequence and expression of cDNA for pituitary adenylate cyclase activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH)-like peptide in catfish. Mol Cell Endocrinol 108:169–177, 1995.

5. Fradinger EA, Sherwood NM. Characterization of the gene encoding both growth hormone-releasing hormone (GRF) and pituitary adenylate cyclase-activating polypeptide (PACAP) in the zebrafish. Mol Cell Endocrinol 165: 211–219, 2000.

6. Parker DB, Coe IR, Dixon GH, Sherwood NM. Two salmon neuropeptides encoded by one brain cDNA are structurally related to members of the glucagon superfamily. Eur J Biochem 215:439–448, 1993.

7. Small BC, Nonneman D. Sequence and expression of a cDNA encoding both pituitary adenylate cyclase activating polypeptide and growth hormone-releasing hormone-like peptide in channel catfish (Ictalurus punctatus). Gen Comp Endocrinol 122:354–363, 2001.

8. Wong AO, Li WS, Lee EK, Leung MY, Tse LY, Chow BK. Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. Biochem Cell Biol 78:229–243, 2000.

9. Hu Z, Lelievre V, Tam J, Cheng JW, Fuenzalida G, Zhou X. Molecular cloning of growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide in the frog Xenopus laevis: brain distribution and regulation after castration. Endocrinology 141:3366–3376, 2000.

10. Alexandre D, Vaudry H, Jegou S, Anouar Y. Structure and distribution of the mRNAs encoding pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in the frog, Rana ridibunda. J Comp Neurol 421:234–246, 2000.

11. McRory JE, Parker DB, Sherwood NM. Expression and alternative processing of a chicken gene encoding both growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide. DNA Cell Biol 16:95–102, 1997.

12. Yoo SJ, You S, Kim H, Kim SC, Choi YJ, El Halawani M. Molecular cloning and characterization of alternatively spliced transcripts of the turkey pituitary adenylate cyclase-activating polypeptide. Gen Comp Endocrinol 120:326–335, 2000.

13. Matsuda K, Nagano Y, Uchiyama M, Onoue S, Takahashi A, Kawauchi H, Shioda S. Pituitary adenylate cyclase-activating polypeptide (PACAP)-like immunoreactivity in the brain of a teleost, Uranoscopus japonicus: immunohistochemical relationship between PACAP and adenohypophysial hormones. Regul Pept 126:129–136, 2005.

14. Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol Rev 52:269–324, 2000.

15. Sherwood NM, Krueckl SL, McRory JE. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagons superfamily. Endocr Rev 21:619–670, 2000.

16. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, Waschek JA. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol Rev 50:265–270, 1998.

17. Hashimoto H, Ishihara T, Shigemoto R, Mori K, Nagata S. Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. Neuron 11:333–342, 1993.

18. Ishihara T, Shigemoto R, Mori K, Takahashi K, Nagata S. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. Neuron 8:811–819, 1992.

19. Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G, Harmar AJ. The VIP2 receptor: molecular characterization of a cDNA encoding a novel receptor for vasoactive intestinal peptide. FEBS Lett 334:3–8, 1993.

20. Delporte C, Poloczek P, de Neef P, Vertongen P, Ciccarelli E, Svoboda M, Herchuelz A, Winand J, Robberecht P. Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide stimulate two signaling pathways in CHO cells stably transfected with the selective type I PACAP receptor. Mol Cell Endocrinol 107:71–76, 1995.

21. Pisegna JR, Wank SA. Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase-activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase C. J Biol Chem 271:17267–17274, 1996.

22. Schomerus E, Poch A, Bunting R, Mason WT, McArdle CA. Effects of pituitary adenylate cyclase-activating polypeptide in the pituitary: activation of two signal transduction pathways in the gonadotrope-derived alpha T3–1 cell line. Endocrinology 134:315–323, 1994.

23. Tatsuno I, Morio H, Tanaka T, Uchida D, Hirai A, Tamura Y, Saito Y. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a regulator of astrocytes: PACAP stimulates proliferation and production of interleukin 6 IL-6, but not nerve growth factor (NGF), in cultured rat astrocyte. Ann N Y Acad Sci 805:482–488, 1996.

24. Tatsuno I, Gottschall PE, Koves K, Arimura A. Demonstration of specific binding sites for pituitary adenylate cyclase activating polypeptide (PACAP) in rat astrocytes. Biochem Biophys Res Commun 168:1027–1033, 1990.

25. Tatsuno I, Gottschall PE, Arimura A. Specific binding sites for pituitary adenylate cyclase-activating polypeptide (PACAP) in rat cultured astrocytes: molecular identification and interaction with vasoactive intestinal peptide (VIP). Peptides 12:617–621, 1991.

26. Tatsuno I, Arimura A. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates intracellular free calcium concentration on rat cultured astrocytes in type-2, but not in type-1. Brain Res 662:1–10, 1994.

27. Moroo I, Tatsuno I, Uchida D, Tanaka T, Saito J, Saito Y, Hirai A. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates mitogen-activated protein kinase (MAPK) in cultured rat astrocytes. Brain Res 795:191–196, 1998.

28. Wong AO, Leung MY, Shea WL, Tse LY, Chang JP, Chow BK.

Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. Endocrinology 139:3465–3479, 1998.

29. Parker DB, Power ME, Swanson P, Rivier J, Sherwood NM. Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. Endocrinology 138:414–423, 1997.

30. Montero M, Yon L, Rousseau K, Arimura A, Fournier A, Dufour S. Localization of pituitary adenylate cyclase-activating polypeptide in the central nervous system of the European eel *Anguilla anguilla*: stimulatory effect of PACAP on GH secretion. Ann N Y Acad Sci 865:475–477, 1998.

31. Rousseau K, Le Belle N, Pichavant K, Marchelidon J, Chow BK, Boeuf G. Pituitary growth hormone secretion in the turbot, a phylogenetically recent teleost, is regulated by a species-specific pattern of neuropeptides. Neuroendocrinology 74:375–385, 2001.

32. Wirachowsky NR, Kwong P, Yunker WK, Johnson JD, Chang JD. Mechanisms of action of pituitary adenylate cyclase-activating polypeptide (PACAP) on growth hormone release from dispersed goldfish pituitary cells. Fish Physiol Biochem 23:201–214, 2000.

33. Chang JP, Wirachowsky NR, Kwong P, Johnson JD. PACAP stimulation of gonadotropin-II secretion in goldfish pituitary cells: mechanisms of action and interaction with gonadotropin releasing hormone signaling. J Neuroendocrinol 13:540–550, 2001.

34. Matsuda K, Kashimoto K, Higuchi T, Yoshida T, Uchiyama M, Shioda S. Presence of pituitary adenylate cyclase-activating polypeptide (PACAP) and its relaxant activity in the rectum of a teleost, the stargazer, *Uranoscopus japonicus*. Peptides 21:821–827, 2000.

35. Olsson C, Holmgren S. PACAP and nitric oxide inhibit contractions in the proximal intestine of the Atlantic cod, *Gadus morhua*. J Exp Biol 203:575–583, 2000.

36. Montpetit CJ, Perry SF. Vasoactive intestinal polypeptide- and pituitary adenylate cyclase-activating polypeptide-mediated control of catecholamine release from chromaffin tissue in the rainbow trout, *Oncorhynchus mykiss*. J Endocrinol 166:705–714, 2000.

37. Mathieu M, Ciarlo M, Trucco N, Griffero F, Damonte G, Salis A, Vallarino M. Pituitary adenylate cyclase-activating polypeptide in the brain, spinal cord and sensory organs of the zebrafish, *Danio rerio*, during development. Brain Res Dev Brain Res 151:169–185, 2004.

38. Wang Y, Wong AO, Ge W. Cloning, regulation of messenger ribonucleic acid expression, and function of a new isoform of pituitary adenylate cyclase-activating polypeptide in the zebrafish ovary. Endocrinology 144:4799–4810, 2003.

39. Gong HY, Wu JL, Huang WT, Lin CJ, Weng CF. Response to acute changes in salinity of two different muscle type creatine kinase isoforms, from euryhaline teleost (*Oreochromis mossambicus*) gills. Biochim Biophys Acta 1675:184–191, 2004.

40. Weng CF, Chiang CC, Gong HY, Chen MH, Lin CJ, Huang WT, Cheng CY, Hwang PP, Wu JL. Acute changes in gill Na$^+$-K$^+$-ATPase and creatine kinase in response to salinity changes in the euryhaline teleost, tilapia (*Oreochromis mossambicus*). Physiol Biochem Zool 75:29–36, 2002.

41. Lin LY, Chiang CC, Gong HY, Cheng CY, Hwang PP, Weng CF. Cellular distributions of creatine kinase in branchia of euryhaline tilapia (*Oreochromis mossambicus*). Am J Physiol Cell Physiol 284:C233–C241, 2003.

42. Weng CF, Chiang CC, Gong HY, Chen MH, Huang WT, Cheng CY, Wu JL. Bioenergetics of adaptation to a salinity transition in euryhaline teleost (*Oreochromis mossambicus*) brain. Exp Biol Med (Maywood) 227:45–50, 2002.

43. Arima H, Yamamoto N, Sobue K, Umenishi F, Tada T, Katsuya H, Asai K. Hyperosmolar mannitol simulates expression of aquaporins 4 and 9 through a p38 mitogen-activated protein kinase-dependent pathway in rat astrocytes. J Biol Chem 278:44525–44534, 2003.

44. Bitoun M, Tappaz M. Gene expression of the transporters and biosynthetic enzymes of the osmolytes in astrocyte primary cultures exposed to hyperosmotic conditions. Glia 32:165–176, 2000.

45. Xu D, Wang L, Olson JE, Lu L. Asymmetrical response of p38 kinase activation to volume changes in primary rat astrocytes. Exp Biol Med (Maywood) 226:927–933, 2001.

46. Parkerson KA, Sontheimer H. Contribution of chloride channels to volume regulation of cortical astrocytes. Am J Physiol Cell Physiol 284:C1460–C1467, 2003.

47. Fischer R, Schliess F, Haussinger D. Characterization of the hypo-osmolarity-induced Ca2+ response in cultured rat astrocytes. Glia 20:51–58, 1997.

48. Franke B, Figiel M, Engele J. CNS glia are targets for GDNF and neurturin. Histochem Cell Biol 110:595–601, 1998.

49. Dejda A, Sokolowska P, Nowak JZ. Neuroprotective potential of three neuropeptides PACAP, VIP and PHI. Pharmacol Rep 57:307–320, 2005.

50. Tatsuno I, Morio H, Tanaka T, Uchida D, Hirai A, Tamura Y, Saito Y. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a regulator of astrocytes: PACAP stimulates proliferation and production of interleukin 6 IL-6, but not nerve growth factor (NGF), in cultured rat astrocyte. Ann N Y Acad Sci 805:482–488, 1996.

51. Matsuda K, Nagano Y, Uchiyama M, Takahashi A, Kawauchi H. Immunohistochemical observation of pituitary adenylate cyclase-activating polypeptide (PACAP) and adenohypophysial hormones in the pituitary of a teleost, *Uranoscopus japonicus*. Zoolog Sci 22:71–76, 2005.

52. Montero M, Yon L, Rousseau K, Arimura A, Fournier A, Dufour S. Distribution, characterization, and growth hormone-releasing activity of pituitary adenylate cyclase-activating polypeptide in the European eel, *Anguilla anguilla*. Endocrinology 139:4300–4310, 1998.

53. Montpetit CJ, Shahsavarani A, Perry SF. Localization of VIP-binding sites exhibiting properties of VPAC receptors in chromaffin cells of rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 206:1917–1927, 2003.

54. Fradinger EA, Tello JA, Rivier JE, Sherwood NM. Characterization of four receptor cDNAs: PAC1, VPAC1, a novel PAC1 and a partial GHRH in zebrafish. Mol Cell Endocrinol. 231:49–63, 2005.

55. Matsuda K, Maruyama K, Miura T, Uchiyama M, Shioda S. Anorexigenic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: feeding-induced changes in the expression of mRNAs for PACAP and its receptors in the brain, and locomotor response to central injection. Neurosci Lett 386:9–13, 2005.

56. Armstrong BD, Hu Z, Abad C, Yamamoto M, Rodriguez WI, Cheng J, Tam J, Gomariz RP, Patterson PH, Waschek JA. Lymphocyte regulation of neuropeptide gene expression after neuronal injury. J Neurosci Res 74:240–247, 2003.

57. Adams BA, Lescheid DW, Vickers ED, Crim LW, Sherwood NM. Pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in sturgeon, whitefish, grayling, flounder and halibut: cDNA sequence, exon skipping and evolution. Regul Pept 109:27–37, 2002.

58. Krueckl SL, Sherwood NM. Developmental expression, alternative splicing and gene copy number for the pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GRF) gene in rainbow trout. Mol Cell Endocrinol 182:99–108, 2001.

59. Irwin DM, Wong J. Trout and chicken proglucagon: alternative splicing generates mRNA transcripts encoding glucagon-like peptide 2. Mol Endocrinol 9:267–277, 1995.

60. Talbot RT, Dunn IC, Wilson PW, Sang HM, Sharp PJ. Evidence for alternative splicing of the chicken vasoactive intestinal polypeptide gene transcript. J Mol Endocrinol 15:81–91, 1995.

61. Linder S, Barkhem T, Norberg A, Persson H, Schalling M, Hokfelt T. Structure and expression of the gene encoding the vasoactive intestinal polypeptide precursor. Proc Natl Acad Sci U S A 84:605–609, 1987.

62. Ko C, Park-Sarge OK. Progesterone receptor activation mediates LH-induced type-I pituitary adenylate cyclase-activating polypeptide receptor (PAC1) gene expression in rat granulosa cells. Biochem Biophys Res Commun 277:270–279, 2000.

63. Suzuki R, Arata S, Nakajo S, Ikenaka K, Kikuyama S, Shioda S. Expression of the receptor for pituitary adenylate cyclase-activating polypeptide (PAC1-R) in reactive astrocytes. Brain Res Mol Brain Res 115:10–20, 2003.

64. Yamamoto K, Hashimoto H, Hagihara N, Nishino A, Fujita T, Matsuda T, Baba A. Cloning and characterization of the mouse pituitary adenylate cyclase-activating polypeptide (PACAP) gene. Gene 211:63–69, 1998.

65. Park JI, Kim WJ, Wang L, Park HJ, Lee J, Park JH, Kwon HB, Tsafriri A, Chun SY. Involvement of progesterone in gonadotrophin-induced pituitary adenylate cyclase-activating polypeptide gene expression in pre-ovulatory follicles of rat ovary. Mol Hum Reprod 6:238–245, 2000.

66. Heraud C, Hilairet S, Muller JM, Leterrier JF, Chadeneau C. Neuritogenesis induced by vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, and peptide histidine methionine in SH-SY5y cells is associated with regulated expression of cytoskeleton mRNAs and proteins. J Neurosci Res 75:320–329, 2004.

67. Vaudry D, Chen Y, Ravni A, Hamelink C, Elkahloun AG, Eiden LE. Analysis of the PC12 cell transcriptome after differentiation with pituitary adenylate cyclase-activating polypeptide (PACAP). J Neurochem 83:1272–1284, 2002.

68. Gonzalez BJ, Basille M, Vaudry D, Fournier A, Vaudry H. Pituitary adenylate cyclase-activating polypeptide promotes cell survival and neurite outgrowth in rat cerebellar neuroblasts. Neuroscience 78:419–430, 1997.

69. Cazillis M, Gonzalez BJ, Billardon C, Lombet A, Fraichard A, Samarut J, Gressens P, Vaudry H, Rostene W. VIP and PACAP induce selective neuronal differentiation of mouse embryonic stem cells. Eur J Neurosci 19:798–808, 2004.

70. Hashimoto H, Kunugi A, Arakawa N, Shintani N, Fujita T, Kasai A, Kawaguchi C, Morita Y, Hirose M, Sakai Y, Baba A. Possible involvement of a cyclic AMP-dependent mechanism in PACAP-induced proliferation and ERK activation in astrocytes. Biochem Biophys Res Commun 311:337–343, 2003.

71. Kultz D, Burg M. Evolution of osmotic stress signaling via MAP kinase cascades. J Exp Biol 201:3015–3021, 1998.

72. Matsumoto K, Wassarman KM, Wolffe AP. Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. EMBO J 17:2107–2121, 1998.

73. Gilks N, Kedersha N, Ayodele M, Shen L, Stoecklin G, Dember LM, Anderson P. Stress granule assembly is mediated by prion-like aggregation of TIA-1. Mol Biol Cell 15:5383–5398, 2004.

74. Moore MJ. From birth to death: the complex lives of eukaryotic mRNAs. Sci 309:1514–1518, 2005.

75. Lo M-J, Weng C-F. Developmental regulation of gastric pepsin and pancreatic serine protease in larvae of the euryhaline teleost, *Oreochromis mossambicus*. Aquaculture 261:1403–1412, 2006.