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Regional Concentration and Chromatographic Characterization of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) in the Brain of the Bullfrog, *Rana catesbeiana*

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ABSTRACT—Pituitary adenylate cyclase-activating polypeptide (PACAP) is a regulatory neuropeptide which functions as a hypothalamic factor for pituitary hormone release, and as a neurotransmitter, neuromodulator and neurotrophic factor in both frogs and mammals. This study examined the quantitative distribution and chromatographic characterization of immunoreactive PACAP in the central nervous system (CNS) of the bullfrog, *Rana catesbeiana*, using an enzyme immunoassay (EIA), named avidin-biotin complex detectable EIA for PACAP, and high-performance liquid chromatographic (HPLC) analysis. The brain of adult bullfrogs contained relatively high levels of immunoreactive PACAP (344.63 pmol/g wet weight of tissue). The average concentrations of immunoreactive PACAP in the regions of the telencephalon, diencephalon, tectum, cerebellum, rhombencephalon, and spinal cord were 213.84, 767.14, 524.94, 192.71, 237.67, and 362.04 pmol/g wet weight of tissue, respectively. The concentrations of immunoreactive PACAP increased with the brain development during metamorphosis, and the concentration of immunoreactive PACAP in the brain of tadpoles at the end of metamorphosis was approximately 200 pmol/g wet weight of tissue. The predominant form of immunoreactive PACAP in the CNS of adult and tadpole was eluted closely with synthetic PACAP38, but another smaller immunoreactivity also appeared in a the fraction, which corresponded to the retention time of synthetic PACAP27, as analyzed by reverse-phase HPLC.

Key words: PACAP, brain, bullfrog, enzyme immunoassay, HPLC

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalami during an attempt to isolate a novel hypophysiotropic peptide possessing the ability to activate adenylate cyclase in cultured rat pituitary cells (Miyata *et al*., 1989). Two molecular forms of PACAP have been identified: an amidated 38-residue peptide (PACAP38) and an amidated 27-residue form (PACAP27) corresponding to the N-terminal 27 residues of PACAP38 (Miyata *et al*., 1990). The PACAP precursor in mammals contains PACAP-related peptide, PACAP38, and PACAP27, and prohormone convertases (PCs) such as PC1, PC2, and PC4, can cleave PACAP precursor to generate PACAP38 and PACAP27 (Li *et al*., 1998, 1999, 2000). Structurally, PACAP resembles vasoactive intestinal peptide (VIP), showing a 68% sequence similarity, and belongs to the secretin/glucagon superfamily of peptides, which includes gastric inhibitory peptide, growth hormone-releasing hormone, PACAP related peptide, and exendins (Campbell and Scanes, 1992; Hoyle, 1998; Pohl *et al*., 1998).

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PACAP exists in mammals in both the central nervous system (CNS) and peripheral organs, such as the gastrointestinal tract, lungs, testes and adrenal glands. PACAP is now considered to be a pleiotropic neuropeptide which functions as a regulatory factor for pituitary and peripheral hormone release, and as a neurotransmitter, neuromodulator, neurotrophic factor, vasodilator, and even as a regulator in the maturation of testicular germ cells and in the immune systems of mammals (Arimura, 1998).

The primary structure of PACAP has been markedly conserved during evolution among the phylum Chordata, including protochordates (Adams *et al.*, 2002; Chartrel *et al.*, 1991; Matsuda *et al.*, 1997, 1998; McRory and Sherwood, 1997; Montero *et al.*, 1998; Parker *et al.*, 1993, 1997; Yasuhara *et al.*, 1992). Reports describing the physiological functions of PACAP in nonmammalian animals have been increasing rapidly (Sherwood *et al.*, 2000). In such studies, it is important to establish a specific and sensitive method for determining PACAP levels in tissues. The endogenous PACAP levels in the CNS and adrenal glands of the European green frog, *Rana ridibunda* (Mathieu *et al.*, 2001; Yon *et al.*, 1993a, b), have been examined by a radioimmunoassay (RIA) using an antiserum against mammalian PACAP38 (Köves *et al.*, 1990) and ^{125}I-PACAP27. Research into PACAP in amphibians has advanced considerably with studies of *R. ridibunda* and *Xenopus laevis*, and has indicated that PACAP and its receptor are widely expressed in the CNS, and that PACAP functions as a hypothalamic factor for pituitary hormone release, and as a neurotransmitter, neuromodulator and neurotrophic factor (Alexandre *et al.*, 2000, 200l, 2002; Gracia-Navarro *et al.*, 1992; Hu *et al.*, 2002; Jeandel *et al.*, 1999; Mathieu *et al.*, 2001; Yon *et al.*, 1993b, 2001). Recently, we have developed a novel and specific EIA, named avidin-biotin complex detectable enzyme immunoassay (ABCDEIA), for measuring the tissue contents of PACAP in some vertebrates (Matsuda *et al.*, 2002), which has revealed that the concentrations of PACAP in the brains of the fish, such as the stargazer and stingray, are much higher than the levels in the brains of mammals, such as the macaque, rat and mouse. The aims of the present study were to determine PACAP-like immunoreactivities in the CNS of the bullfrog using an ABCDEIA system for PACAP and to examine the change of the levels of immunoreactive PACAP in the CNS during metamorphosis. This study was also extended to characterize immunoreactive PACAP in the CNS using high-performance liquid chromatographic (HPLC) analysis on a reverse-phase column.

MATERIALS AND METHODS

Animals

Young adult bullfrogs (*Rana catesbeiana*, 250–300 g body weight) of both sexes were purchased commercially. Bullfrog tadpoles at several developmental stages were collected from ponds in the suburbs of Toyama City, Japan. The developmental stages of tadpoles were determined according to Taylor and Kollros (1946). Animal experiments were conducted in accordance with Toyama University's guidelines for the care and use of laboratory animals.

Antiserum

The antiserum was raised in a rabbit against the keyhole limpet hemocyanin-conjugated synthetic stargazer PACAP27 deduced from the sequence of the purified stargazer PACAP38 (Matsuda *et al.*, 1997, 2000); the details of its production and characterization are available elsewhere (Matsuda *et al.*, 2002).

Synthetic peptides

Synthetic frog PACAP38, derived from *R. ridibunda* (Chartrel *et al.*, 1991), was purchased from American Peptide Company (Sunnyvale, CA, USA), and synthetic human PACAP27 and PACAP38, human glucagon, porcine secretin, and human VIP were purchased from Peptide Institute (Osaka, Japan). Peptide fragments with the N-terminal 10 and 15 residues of PACAP were synthesized as described previously (Matsuda et al., 2002). The sequence of frog PACAP38 is compared with those of human PACAP27 and PACAP38, stargazer PACAP27, peptide fragments of PACAP, and human VIP in Fig. 1. Biotin-labeled PACAP38 was also synthesized and selectively biotinylated on the α-amino group in the N-terminus of the peptides (Matsuda et al., 2002). They were dissolved in distilled water at concentrations of 0.1–1.0 mM and stored at –80°C until used.

EIA for PACAP

The EIA was performed using an ABCDEIA method (Matsuda *et al.*, 2002). The diluent used for the EIA consisted of 0.01 M phosphate buffered saline (PBS), 1% BSA, and 0.01% sodium azide at pH 7.5. The standards, samples, and biotin-labeled PACAP were diluted in this diluent. Each sample, frog or human PACAP standard was dissolved in 100 µl of the diluent and placed in a 12.5×77 mm

	1 5 10 15 20 25 30 35 40
Frog PACAP38[a]	H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R I K N K
Human PACAP38[b]	H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K
Human PACAP27[c]	H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L
Stargazer PACAP27[d]	H S D G I F T D S Y S R Y R K Q M A V Q K Y L A A V L
PACAP1-10	H S D G I F T D S Y
PACAP1-15	H S D G I F T D S Y S R Y R K
Human VIP[e]	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N

Fig. 1. Amino acid sequences of frog and human PACAPs, stargazer PACAP27, and peptide fragments of PACAP with N-terminal 10 and 15 residues aligned with the sequence of mammalian VIP. Amino acid residues are expressed as one-letter codes. White letters represent amino acid residues that are identical to those of frog PACAP. Superscripts a-e indicate citations to Chartrel *et al.* (1991), Miyata *et al.* (1989), Miyata *et al.* (1990), Matsuda *et al.* (1997), and Said and Mutt (1970), respectively.

polystyrene tube (Iwaki, Tokyo, Japan). We also added 200 µl of the diluent and 100 µl of appropriately diluted anti-stargazer PACAP27 serum in 0.05 M EDTA, 0.01 M PBS, 0.01% sodium azide, and 1% normal rabbit serum at pH 7.5. After incubation for 24 hr at room temperature, 100 ml of 25 nM biotin-labeled PACAP in the diluent was added, and the solution was incubated at room temperature for 24 hr. To precipitate the immune complexes, we added 200 µl of goat anti-rabbit IgG (a gift from Gunma University, Maebashi, Japan) diluted 1:50 in 0.05 M EDTA, 0.01 M PBS, 0.01% sodium azide, and 3.5% polyethylene glycol (M.W. 4000). After an 8-hr incubation at room temperature, the tubes were centrifuged at 1800 × *g* at room temperature for 15 min, and the supernatant was aspirated. The immunoprecipitate in each tube was suspended in 200 µl of the avidin solution (Vector Laboratories®, Burlingame, CA, USA) (diluted 1:500 in 0.01 M PBS) at room temperature for 30 min. This was then added to 200 µl of the biotin-conjugated peroxidase solution (Vector) (diluted 1:500 in 0.01 M PBS). After a 30-min incubation at room temperature, the tubes were centrifuged at 1800 × *g* at room temperature for 15 min, the supernatant was aspirated, and the pellets were washed two times with 0.01 M PBS for 15 min. After centrifugation and aspiration, the pellet from each tube was treated with 200 µl of 0.01% 3,3´, 5,5´-tetramethylbenzidine dihydrochloride (Sigma, St. Louis, MO, USA) and 0.006% H_2O_2 in 25 mM citric acid and 50 mM Na_2HPO_4 for 30 min at room temperature in the dark. The color development was then stopped and the solutionís color changed with 50 µl of 2 M H_2SO_4. Aliquots were transferred to microplate wells, and the absorbance of each well was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Extraction of tissues

Bullfrogs were anesthetized with MS-222 and decapitated, and the brains were dissected out and the following regions were collected: telencephalon including olfactory bulb, diencephalon, tectum, cerebellum, rhombencephalon, and spinal cord. Tadpoles at prometamorphic stage (stages XI–XIX), at the onset of climax (stage XX) and at climactic stage (Stages XXI–XXV), and adult frogs were also anesthetized and decapitated, and their whole brains were collected. Each tissue was weighed, placed immediately in liquid N_2, and stored at –80°C until used. Tissues were boiled for 15 min in 0.5 M acetic acid, homogenized in a sonicator (Tomy Seiko, Tokyo, Japan), and centrifuged at 1600 × *g* at room temperature for 15 min. The tissue extract was dissolved in 0.1% trifluoroacetic acid (TFA) and injected onto a Sep-Pak C18 cartridge (Waters Associates®, Milford, MA, USA) equilibrated with 0.1% TFA. The cartridge-bound substances were washed with 20% acetonitrile containing 0.1% TFA and eluted with 50% acetonitrile containing 0.1% TFA, and each eluate was lyophilized. An aliquot of each sample was then subjected to EIA for PACAP as described above.

Reverse-phase HPLC

Two adult bullfrog brains and ten brains of tadpoles at stages XI–XIX and XXI–XXV were collected and separately pooled, and their extracts were dissolved in 0.1% TFA and subjected to reverse-phase HPLC on a Puresil C18 column (4.6 mm i.d. × 150 mm; Waters) equilibrated with 0.1% TFA and 20% acetonitrile, at a flow rate of 1.0 ml/min. Linear-gradient elution was carried out for 40 min with 20–40% acetonitrile containing 0.1% TFA, and fractions (1 ml/tube) were collected and lyophilized. An aliquot of each fraction was dissolved and subjected to EIA for PACAP. Synthetic PACAP27 and PACAP38 were used as references for the retention time of immunoreactive PACAP in each fraction. The recovery of immunoreactive PACAP from the brain extract was about 60–63%.

Data analyses

Several separate extractions were carried out on each tissue preparation. Each tissue extract was assayed when more than three serially diluted points were performed at least in duplicate. The tissue content of immunoreactive PACAP was determined by comparing the absorbance (ABS) of each point with those of the serially diluted standards in a four-parameter logistic model by using the “KAIKI” computer program (http://homepage1.nifty.com/tombonak/kaiki.htm). Data are illustrated as the competitive binding ratio (B/Bo,%), and expressed as concentrations in picomoles/g wet tissue in the figures. Statistical analysis was performed by one-way ANOVA with Bonferroniís method. Statistical significances were determined at the 5% level.

RESULTS

EIA system for measuring immunoreactive PACAP

Antiserum diluted 1:1000 exhibited about 60% of the maximum reaction (ca 3.5 ABS) of the biotin-labeled PACAP38/avidin/biotin-conjugated peroxidase complex in the precipitate, compared with a background level of 0.15–0.20 ABS. The competitive binding was inhibited in a dose-dependent manner by unlabeled human PACAP38 in the range 0.098–12.5 pmol/tube (Fig. 2A). The minimum detectable level, defined as 2 SDs below the 100% bound point, averaged 0.48±0.10 pmol (mean±SEM) of unlabeled PACAP38 per 100 µl of assay buffer in ten assays. The

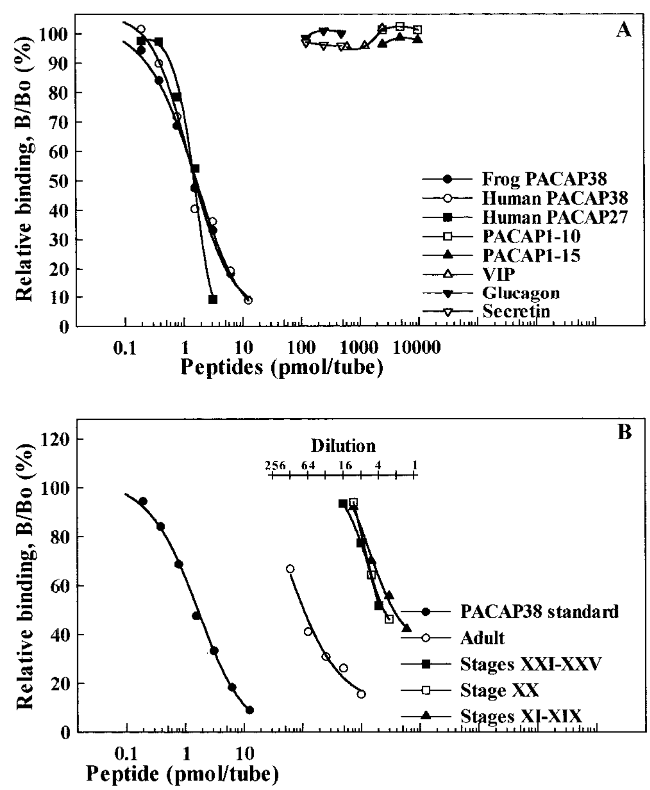

Fig. 2. Representative profiles of the ABCDEIA for PACAP38. An antiserum diluted 1:1000, frog PACAP38 as the standard, and biotinylated PACAP38 were used. (**A**) Displacement curves of biotin-labeled PACAP38 with frog PACAP38, human PACAP38, human PACAP27, peptide fragments of PACAP, glucagon, secretin and VIP at various concentrations. (**B**) The PACAP38 standard was compared with the extracts prepared from the adult and larval brains. Each point is the mean of two determinations.

intra-assay coefficient of variation of 7.9% was obtained by repeated determinations of 3.125 pmol PACAP38. The inter-assay coefficient of variation was 8.8% when the estimated dose at 50% inhibition was used in ten assays. This EIA recognized all of the PACAPs with 27 and 38 residues, but the binding was not altered by PACAP peptide fragments, glucagon, secretin or VIP. The binding of biotin-labeled PACAP38 was displaced by both adult brain and tadpole brain extracts, and the linear portion of their curves was parallel to that of PACAP38 (Fig. 2B).

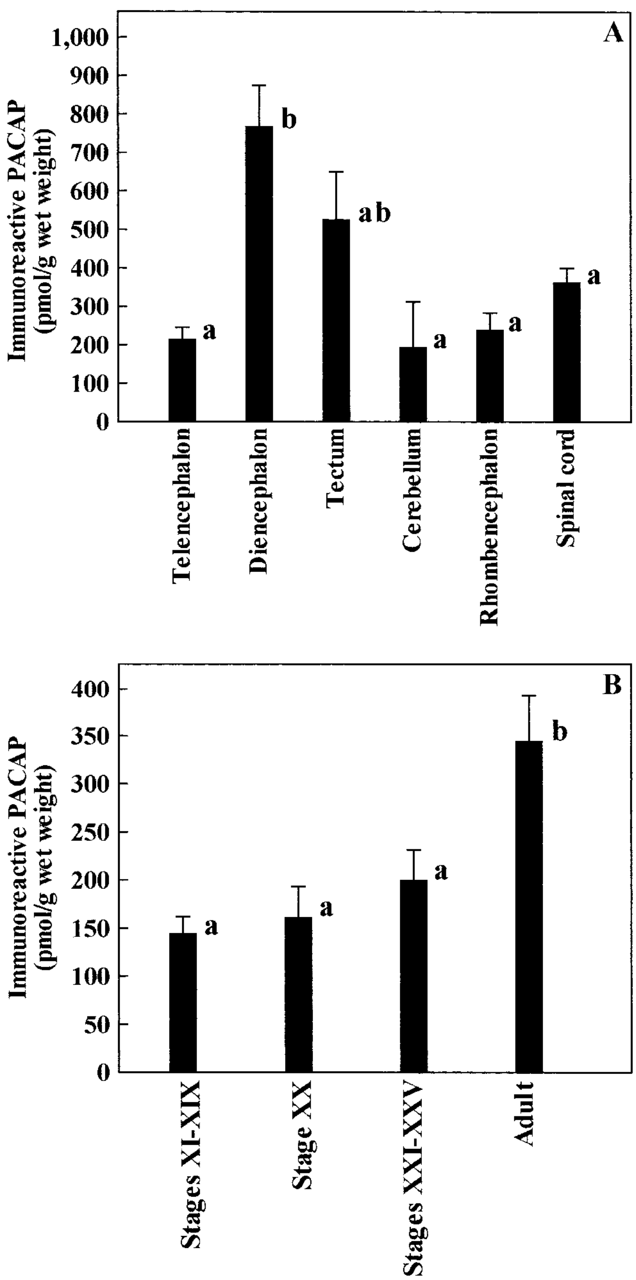

Fig. 3. Immunoreactive PACAP levels in the adult and larval brains of bullfrog. (**A**) The concentrations of immunoreactive PACAP in various regions of adult brain. Each column and bar are the mean and SEM derived from ten brains, respectively. Values with the same superscript are not statistically different at 5% level by one-way ANOVA with Bonferroni's method. (**B**) The concentrations of immunoreactive PACAP in the whole brains during metamorphosis. Each column and bar are the mean and SEM derived from 10–22 brains, respectively. Values with the same superscript are not statistically different at 5% level by one-way ANOVA with Bonferroni's method.

Determination of immunoreactive PACAP in the brain

Fig. 3A shows the concentration of immunoreactive PACAP in various regions of the adult bullfrog brain. Immunoreactive PACAP was measured by PACAP38 assay. The significant highest concentration of immunoreactive PACAP in the brain was in the diencephalon, although the other regions also contained high levels of immunoreactive PACAP. The concentrations of immunoreactive PACAP in the whole brains of adult and larvae are shown in Fig.3B. The concentrations of immunoreactive PACAP in the larval brains were approximately 140–200 pmol/g wet weight of tissue. Although the average value of PACAP concentrations showed a moderate increase as metamorphosis proceeds, no statistical difference was noted throughout metamorphosis. On the other hand, the concentration of immunoreactive PACAP in the adult whole brain was approximately 340 pmol/g wet weight of tissue, being significantly higher than in the larval brain.

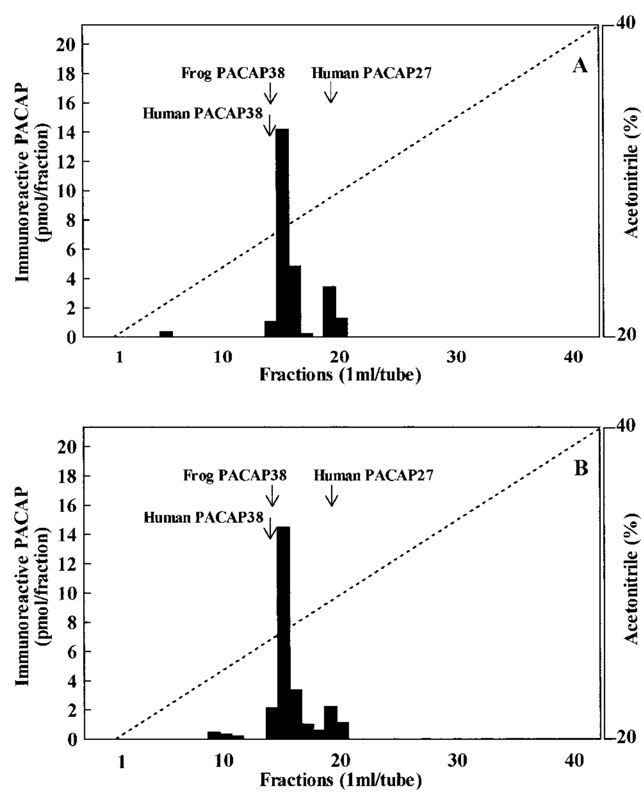

Fig. 4. Reverse-phase HPLC effluent profiles of immunoreactive PACAP in the brain extracts from adult (**A**) and tadpole (**B**) during the metamorphic climax. Black columns show PACAP-like immunoreactivities in the fractions obtained from a Puresil C18 column with a linear acetonitrile gradient (20–40%) containing 0.1% TFA. Arrows indicate the portions of eluted peaks of synthetic frog PACAP38, human PACAP38, and human PACAP27.

Characterization of immunoreactive PACAP in the brain

To characterize immunoreactive PACAP in adult and larval brains, we analyzed their brain extracts by HPLC, and assayed the obtained fractions by EIA for PACAP38. The effluent profiles of their immunoreactivities by reverse-phase HPLC are shown in Fig. 4. There was little difference in the profiles among the samples from prometamorphic tadpoles (data not shown), climactic tadpoles and adults. The major immunoreactive PACAP peaked in fraction 15, which showed only a slightly delayed retention time as compared with the retention times of synthetic frog PACAP38 (13.7 min) and human PACAP38 (13.6 min). Another smaller peak appeared in fraction 19, which corresponded to the retention time of synthetic human PACAP27 (19.1 min).

DISCUSSION

The endogenous PACAP levels in the CNS and peripheral tissues in mammals have been well investigated by RIA (Arimura *et al*., 1991), sandwich-EIA (Masuo *et al*., 1993), and time-resolved fluoroimmunoassay (Ito *et al*., 1997). These assay systems for mammalian PACAP were highly sensitive (pg/tube) for determining PACAP contents in mammalian tissues. In the present experiments, the antiserum used was raised against stargazer PACAP27-amide. The amino acid sequence of PACAP is very similar among vertebrates, and this antiserum has been shown to react with frog, mammal, and fish PACAPs (Matsuda *et al*., 2001). Using the antiserum, biotinylated PACAP, and a frog or human PACAP standard, we developed a specific EIA with a sensitivity level of ng/tube for measuring PACAP-like immunoreactivities in the bullfrog brain. The sensitivity of our EIA system was within the same range as that of the RIA for measuring frog PACAP (Mathieu *et al*., 2001; Yon *et al*., 1993a, b). We designated the system as the ABCDEIA for PACAP (Matsuda *et al*., 2002). The ABCDEIA methodology may also be suitable for measuring other immunoreactive substances, using a corresponding specific antiserum and biotin-labeled ligand as the antigen.

Recently, we have measured immunoreactive PACAP in the various tissues of fish, such as the stargazer and stingray, and in the mammalian brains, such as the macaque, rat, and mouse, using ABCDEIA for PACAP (Matsuda *et al*., 2002). The fish brains contained much higher levels of immunoreactive PACAP (72–433 pmol/g wet tissue) as compared with the levels in the mammalian brain (8–15 pmol/g). The concentration of immunoreactive PACAP in the adult and larval bullfrog brains as measured in the present experiment was also high (140–340 pmol/g), suggesting the importance of PACAP in the adult and larval brains. The amounts of immunoreactive PACAP in the CNS of *R. ridibunda* have been measured by RIA (Mathieu *et al*., 2001; Yon *et al*., 1993b). These reports indicated that the apparent concentrations of immunoreactive PACAP are high in the brain of tadpoles as well as of adults. In the present experiment, we measured PACAP concentrations in the whole brain of larvae and adults, and revealed that the amount of immunoreactive PACAP per wet weight in the adult brain is approximately twice as high as those in the larval brain. Moreover, we have presented the data on regional PACAP concentrations in the adult brain for the first time. It clearly indicated that PACAP concentrations are the highest in the diencephalon.

A previous immunohistochemical study indicated that the main populations of PACAP-like immunoreactive neuronal cell bodies were located in the diencephalon and telencephalon, that the hindbrain also contained substantial populations of PACAP-like immunoreactive cells, and that nerve fibers were present throughout the brain of *R. ridibunda* (Yon *et al*., 1992). The distribution of immunoreactive PACAP has also been investigated in the CNS of *R. ridibunda* during development (Mathieu *et al*., 2001). That report indicated that PACAP-like immunoreactive cells and fibers appeared very early in the CNS of tadpoles, and that PACAP-containing cells occurred in the thalamic region soon after hatching and in the hypothalamus, mesencephalon, cerebellum, rhombencephalon, and spinal cord both premetamorphosis and during metamorphosis. The cDNAs encoding two types of PACAP receptors have been cloned from some frogs, including *R. ridibunda*, *R. tigrina rugulosa* and *X. laevis*: one is PAC1-R, which possess four variants; and the others are VPAC1-R and VPAC2-R (Alexandre *et al*., 1999, 2000, 2001, 2002; Hoo *et al*., 2001; Hu *et al*., 2000). PAC1-R and VPAC-R mRNAs were found to be widely expressed in numerous regions of the frog brain (Yon *et al*., 2001). PACAP functions as a hypophysiotropic factor, and modulates the expression of various hypothalamic neuropeptides, and the proliferation and differentiation of nerve cells during development and acts as a neurotrophic factor in mammals (Arimura *et al*., 1998). The broad distribution of immunoreactive PACAP and PACAP receptors in the frog brain suggests that PACAP is involved in the regulation of various brain functions in amphibians as in the case of mammals (Gonzalez *et al*., 1998; Vaudry *et al*., 2000; Yon *et al*., 2001).

Characterization of immunoreactive PACAP by reverse-phase HPLC analysis combined with ABCDEIA measurement indicated that the predominant form present in the bullfrog brain corresponded to PACAP38, but showed only a slightly delayed retention time as compared with those of synthetic frog (*R. ridibunda*) PACAP38 and human PACAP38, and another smaller immunoreactive PACAP corresponded to synthetic human PACAP27. ABCDEIA for PACAP27 is PACAP27 specific (Matsuda *et al*., 2002), but we could not detected PACAP27 in the bullfrog brain with this assay system (data not shown). This smaller immunoreactive peak may also be the derivative of PACAP38, indicating that PACAP27 exists at relatively low concentrations. The effluent profile of immunoreactive PACAP in the larval brain resembled that of PACAP in the adult brain, indicating that the mechanism of proteolytic processing from the PACAP precursor in the tadpole brain may be identical with that of the adult. Although the primary structure of bullfrog PACAP is not yet clear, the effluent profiles suggest that the amino acid sequence of the bullfrog PACAP is slightly different from that of the frog (*R. ridibunda*) PACAP38. Previous reports have described PACAP38 as predominant molecule in the brains of mammals, turtle, frog and tadpole, and fish (Arimura *et al*., 1991; Mathieu *et al*., 2001; Montero *et al*., 1998, 2000; Reglödi *et al*., 2001; Yon *et al*., 1993b). We have also shown that PACAP38 or PACAP44 exists at very high levels in the brains of the stargazer and stingray, and that PACAP27 is almost absent in the tissues of both species (Matsuda *et al*., 2002).

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