

Postmetamorphic changes in parvalbumin expression in the hindlimb skeletal muscle of the bullfrog, *Rana catesbeiana*

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Abstract

Anuran amphibians, animals that spend a terrestrial life after metamorphosis, exhibit a marked development of hindlimbs during and after metamorphosis. In order to see whether changes occur in the muscle protein components in the course of postmetamorphic development, we subjected gastrocnemius muscle extracts from growing froglets to two-dimensional electrophoresis (2DE). As a result, we found two proteins to undergo a change in level. One spot, indicating a molecular mass of approximately 12 kDa and an isoelectric point (pI) of 5.0 first became detectable at 45 days after metamorphosis. Another spot, corresponding to a protein of 11 kDa and pI 4.8, was prominent until the former spot appeared. N-terminal amino acid sequence analysis and comparison of the spots with those of parvalbumin (PA) revealed that these two proteins were PA α and PA β . Northern blot analysis using PA α and PA β cDNAs as probes revealed that the PA β mRNA level declined whereas that of PA α mRNA rose as the frogs grew.

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1. Introduction

It has been well documented that in amphibians various organs exhibit morphological and functional changes as they progress toward the terrestrial life. Although the development of hindlimbs begins at a premetamorphic period, these limbs grow slowly, not attaining functional size until close to metamorphic climax. They still continue to develop during climax and even after metamorphosis.

Metamorphosis is initiated by thyroid hormone (TH) and some other hormones, namely, prolactin, growth hormone, and adrenocortical hormones, which antagonize or synergize with the action of TH [1]. The development of hindlimbs, including changes in gene expression in the skeletal muscle, has been reported to be controlled by those hormones in pre- and pro-metamorphic and climactic tadpoles [1–6].

Although the development of hindlimbs in postmetamorphic animals may involve a transformation of the skeletal muscle components, virtually no information has been published regarding this point.

For the investigation of transitions in protein expression, proteome analysis by two-dimensional electrophoresis (2DE), which was developed by O'Farrell [7] and improved by several other investigators [8–10], is considered to be one of the ideal techniques.

In the present experiment, conducted to see whether some changes occur in the muscle components in postmetamorphic froglets, 2DE analysis of extract of the gastrocnemius muscle of the juvenile bullfrog (*Rana catesbeiana*) revealed that the amount of two protein components changed as the animals grew. These were identified as two forms, α and β , of the calcium-binding protein parvalbumin (PA). Furthermore, cDNAs encoding both proteins were cloned. Using these cDNAs as probes, we investigated developmental changes in the expression levels of PA α and PA β mRNAs.

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2. Materials and methods

2.1. Animals

Bullfrog tadpoles at metamorphic climax and adults were purchased from a commercial source and were maintained at 23 °C under laboratory conditions. Froglets were obtained from the larval stocks that had completed metamorphosis. They were fed porcine liver until they reached the appropriate ages after metamorphosis.

2.2. Sample preparation and 2DE

Gastrocnemius muscles of the hindlimbs from climactic tadpoles, froglets at 1, 15, 30, 45, and 60 days after metamorphosis, and from fully grown adults were immersed in a solubilization solution consisting of 8 M urea, 2% (v/v) Nonidet P-40, 2% (v/v) Ampholine pH 3–10 (Amersham Pharmacia, Buckinghamshire, UK), and 5% (v/v) 2-mercaptoethanol, at a concentration of 0.1 g tissue/500 μ l solution and homogenized in a Phycotron (Microtec, Chiba, Japan) for 2 min on ice. Following centrifugation at 15,000 $\times$ g for 30 min at 20 °C, the resultant supernatant was collected and stored as sample at 4 °C for later analysis [10].

Thirty microliters of the sample and 95 μ l of the solubilization solution were mixed and loaded onto a 7-cm, pH 3–10, nonlinear immobilized pH gradient (IPG) gel strip (Amersham Pharmacia) and left overnight for rehydration of the gel. Isoelectric focusing (IEF) was conducted with a Cool Phorestar CF-200 (Anatech, Tokyo, Japan) at a temper-

ature of 20 °C, at 200 V for 20 min, at 1000 V for 1000 V h, at 2000 V for 2000 V h, and finally at 3500 V for 12 000 V h. Equilibration of IPG gels with sodium dodecyl sulfate (SDS) was performed by immersing the gels in equilibration buffer 1 [6 M urea, 2% (w/v) SDS, 10% (v/v) glycerol, 0.5% (w/v) dithiothreitol: DTT, 50 mM Tris-HCl, pH 6.8] for 10 min, and subsequently in equilibration buffer 2 [6 M urea, 2% (w/v) SDS, 10% (v/v) glycerol, 4.5% (w/v) iodoacetamide, 50 mM Tris-HCl, pH 6.8] for 10 min [8]. Next the gel was subjected to 15% SDS-PAGE, and finally, protein spots were detected by Coomassie brilliant blue (CBB) staining.

2.3. N-terminal amino acid sequencing

The spots on the unstained gel were transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting, the location of which had been identified by CBB staining. Each spot on the membrane to be identified was cut out, and the N-terminal amino acid sequence of the spot was determined by Edman degradation of the protein, followed by sequence analysis with a 477 A protein sequencer/120 A analyzer (Perkin Elmer, MA, USA).

2.4. Amplification of partial cDNAs encoding bullfrog PAs

Total RNAs were extracted from the gastrocnemius muscle of adult bullfrogs (for PA α) and prometamorphic tadpoles (for PA β) with ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan). Reverse transcription reactions were performed by using Superscript II reverse tran-

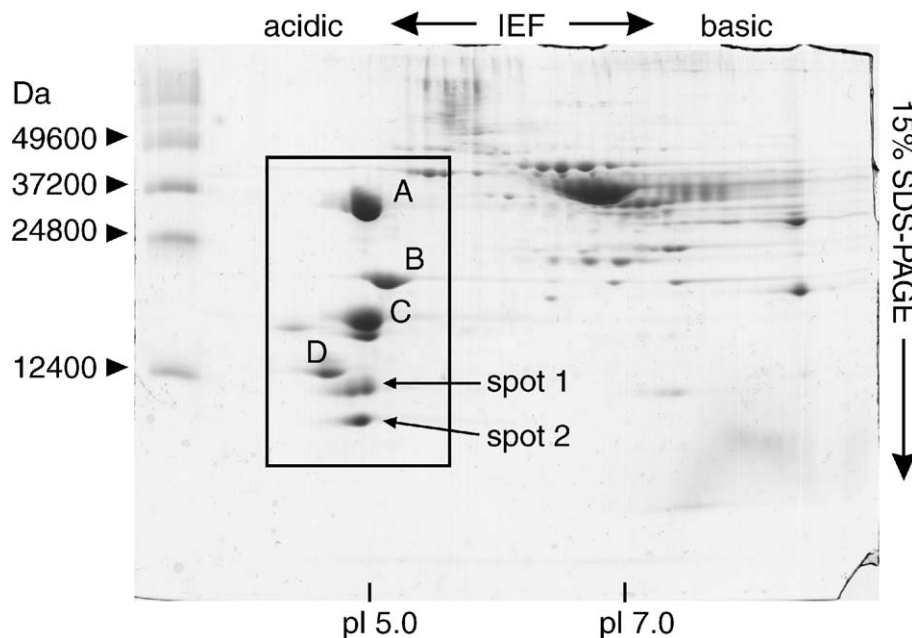


Fig. 1. Two-dimensional electrophoresis (2DE) of hindlimb skeletal muscle proteins from a fully grown adult frog. Proteins were extracted from the gastrocnemius muscle of an adult bullfrog, and were separated by 2DE (first dimension: pH 3–10 nonlinear gradient, second dimension: 15% SDS-PAGE) to create a protein map stained with CBB. Spot 1, which migrated to a position corresponding to a protein of approximately 12 kDa and pI 5.0, and spot 2, to one of about 11 kDa and pI 4.8, were subjected to further analyses; and hence spots in the rectangle were highlighted in Fig. 2. Spot A: tropomyosin; spots B to D: myosin light chains.

scriptase (Life Technologies, NY, USA). Amplification of the partial cDNAs encoding bullfrog PAs by the polymerase chain reaction was performed in *Ex-Taq* buffer containing 0.2 mM of each dNTP, 50 pmol of each synthetic degenerated primers designed based on PA sequences conserved in other species (sense primer: 5'-TTYATTGAGGARGAKGARCT-3'; antisense primer: 5'-CMACKCCRATTTTGCCATCAC-3') and 0.5 units *Ex-Taq* polymerase (TaKaRa Shuzo, Kusatsu, Japan) per 20 μ l of the reaction solution. The amplified cDNAs were subcloned into the pT7-blue T-vector (Novagen, WI, USA). The plasmid containing the cDNA encoding PA was used to transform JM109 competent cells (TaKaRa Shuzo) and was subjected to sequencing analysis.

2.5. Construction of cDNA libraries and screening of bullfrog PA cDNAs

Adult and larval gastrocnemius muscle cDNA libraries were constructed with a Timesaver synthesis kit (Amersham

Pharmacia), ligated with λ zapII (Stratagene, CA, USA), and packaged by using GigapackIII extracts (Stratagene). The phages of the cDNA libraries were plated on large LB agar plates (diameter, 140 mm) with LB containing 0.7% (w/v) agarose at a concentration of 2×10^4 pfu per plate. After having been transferred to membrane filters, the cDNAs were denatured, and then fixed to the membranes. Filters were prehybridized at 60 °C for 2 h in a hybridization solution consisting of sixfold standard saline citrate (SSC), 0.2% (w/v) bovine serum albumin, 0.4% (w/v) Ficoll 400, 0.4% (w/v) polyvinyl-pyrrolidone, and 1% (w/v) SDS. Hybridizations were performed with partial cDNAs encoding PA (PCR products) radiolabeled with a Bcabeat Labeling Kit (TaKaRa Shuzo) in the same solution at 60 °C for 16 h. The filters were washed twice for 30 min at 60 °C in 0.1-fold SSC containing 1% (w/v) SDS and placed in contact with an imaging plate by means of a BAS-2000 II (Fujifilm, Tokyo, Japan) for 30 min. The cDNAs giving positive signals were obtained by *in vivo* excision and the sequences

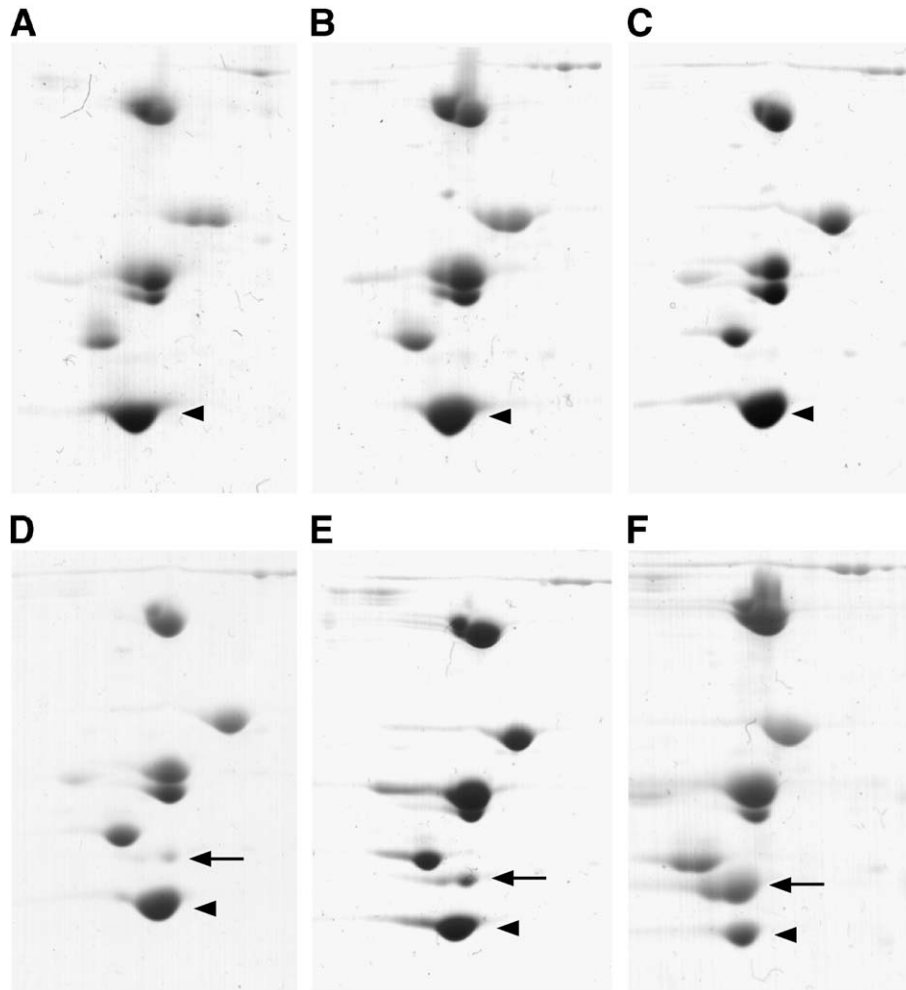


Fig. 2. Developmental changes in the electrophoretic profiles of skeletal muscle proteins from postmetamorphic froglets. Proteins in gastrocnemius muscle extracts of froglets at 1 (A), 15 (B), 30 (C), 45 (D), and 60 (E) days after metamorphosis, and from fully grown adults (F) were separated by 2DE and detected by CBB staining. Spot 1 (arrow) became first detectable at 45 days after metamorphosis, whereas spot 2 (arrowhead) was detectable throughout postmetamorphic life, but gradually decreased in size.

were analyzed by a cycle-sequence method using a fluorescence-labeled primer with a Thermo sequenase cycle sequencing kit (Amersham Pharmacia). The cDNAs were analyzed on a DNA sequencer model 4000 L (LI-COR, NE, USA).

2.6. Northern blot analysis

PA α and PA β mRNAs were assessed by Northern blot analysis using fragments from *Apa*LI–*Bam*HI double-digested PA α cDNA and *Bst*XI–*Eco*RI double-digested PA β cDNA as probes. The probes were radiolabeled by the random-priming method with a Bcabeast labeling kit (TaKaRa Shuzo) according to the manufacturer's instructions. Total RNA (3 μ g) extracted from froglets at 1, 15, 30, 45, and 60 days after metamorphosis, and from fully grown adults was electrophoresed in denaturing agarose gels (2.2 M formaldehyde) and transferred to a nylon membrane. The RNAs were fixed on the membrane by UV cross-linking. The membranes were prehybridized for 2 h at 55 °C for PA α or at 60 °C for PA β in hybridization solution (Express Hybridization Buffer; Clontech, CA, USA), and subsequently each radiolabeled probe was added, and the hybridizations were performed for 16 h. The membranes were washed with 10-fold diluted SSC containing 0.1% SDS twice for 30 min at temperatures described above and placed in contact for 30 min at room temperature with the imaging plate of a BAS-2000 II. Densitometry was performed on the Northern blot autoradiographs with the BASstation (Fujifilm). For normalization, the densitometry values for PAs were divided by those for the β -actin mRNA.

3. Results

A protein map of gastrocnemius muscle from an adult frog is shown in Fig. 1. Some of the spots were identified as tropomyosin and myosin light chains on the basis of their migratory patterns and immunostaining of the spots electroblotted on a PVDF membrane with specific antibodies (data not shown). Among the spots, we focused on two spots, one (spot 1) that migrated to the position corresponding to approximately 12 kDa and isoelectric point (pI) 5.0, and the other (spot 2) that migrated as a protein of about 11 kDa and pI 4.8, since they changed in size and density as the froglets grew. Spot 1 first became detectable at 45 days after metamorphosis (Fig. 2A–D). Thereafter, its signal became more intense as the frogs grew (Fig. 2E and F). The 2DE profiles of the muscle extract of climactic larvae were almost the same as those of froglets at 1 day after metamorphosis (data not shown). The densitometry values of the spots 1 and 2 were divided by those for the total spots on a gel for normalization, and were represented as protein contents. As shown in Fig. 3, the content of the protein spot 1 rose, whereas that of spot 2 slightly decreased with time after metamorphosis.

Sequencing of the protein in spot 1 revealed that the first 20 N-terminal amino acids had the sequence of MHMTDVLPA α DISKAVEAFA, which perfectly coincided with that of PA α reported by Sasaki et al. [11]. On the other hand, direct amino acid sequencing was impossible to elucidate the N-terminal amino acid of the protein in spot 2, suggesting that its N terminus was blocked. When this protein and authentic bullfrog PA β [12,13], the N terminus of which is known to be blocked (acetylated), were subjected to 2DE either separately or in a mixture, they migrated to the same position (data not shown). In order to confirm that spots 1 and 2 were indeed PA α and PA β , respectively, we cloned the cDNAs of both PAs. Using PCR products encoding partial PA α and PA β as probes, we isolated cDNAs encoding full-length PA α and PA β from adult and larval gastrocnemius muscle cDNA libraries, respectively. The deduced amino acid sequences from the cloned cDNAs encoding PA α (Fig. 4A) and PA β (Fig. 4B) corresponded to the complete amino

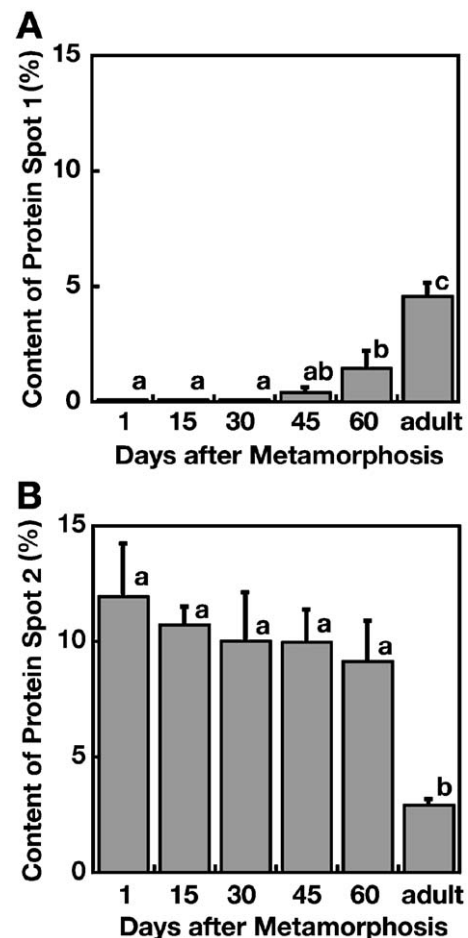


Fig. 3. Changes in the contents of spots 1 and 2 in the growing froglets. Protein contents of spots 1 (A) and 2 (B) were evaluated by densitometry of the electrophoretic profiles. The densitometry values for spots 1 and 2 were divided by the total of those for whole spots on the gel and expressed as percent. Each column and vertical bar represent the mean value for four samples and S.E., respectively. Values with the same superscript do not differ significantly from each other at the 5% level (by Duncan's multiple range test).

A		CGAGGTCGACGGTATCGATAAGCTTGATATCGAA	-61
-60	TTCGCGGCCGCTCTCAGCCCTTCCCACGGATCTGATCCATCCCCAAATCCAGAGCCGCA	-1	
1	ATGCATATGACTGATGTGCTCCCTGCTGGTGACATCTCAAAGGCTGTGGAGGCCTTTGCA	60	
	M H M T D V L P A G D I S K A V E A F A		
61	GCTCCTGACTCATTTAACCACAAGAAATTTTGAATGTGTGGCCTTAAGAGCAAAGGC	120	
	A P D S F N H K K F F E M C G L K S K G		
121	CCAGATGTAATGAAACAGGTTTTCGGCATCTTGGACCAGGACCGGAGTGGTTTCATTGAG	180	
	P D V M K Q V F G I L D Q D R S G F I E		
181	GAGGATGAGCTTTCCTTATGTTGAAAGGCTTCACCCAAATGCCAGAAGTCTGTCTGTC	240	
	E D E L C L M L K G F T P N A R S L S V		
241	AAGGAAACCACAGCCCTCCTCGCCGCTGGAGACAAGGATGGAGATGGCAAGATCGGCATG	300	
	K E T T A L L A A G D K D G D G K I G M		
301	GATGAGTTTCGTAACCCCTGGTTTCTGAATCCTAAGCCGTTCAAGCGCCATTCTTATTTGTG	360	
	D E F V T L V S E S *		
361	CACAGCTTTCCTTTCATTGGACACCACTTTTTTTTTTTTGCTCCCGTTTTTATACTCCACC	420	
421	TCTGGTCCCTGCCTCTTGGATCTCCGTGCTGGAAGTAAAGCATAAAACAACGCAATAAAGG	480	
481	GCATAAACACCTCGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	540	
541	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	579	
B		TGCCTAAACCACCACAAGAACAATA	-1
1	ATGTCTATCACCGACATTGTCTCCAGAAGGACATTGAGGCGCACTTGAGAGTGTGAAG	60	
	M S I T D I V S Q K D I E A A L E S V K		
61	GCTGAAGGTTTCATTCAATTACAAAACCTTTTCCAGAAGGTTGGCTTGGCCGGCAAGAGT	120	
	A E G S F N Y K T F F Q K V G L A G K S		
121	GCCAATGATGCCAAGAAGGTATTTCGAAATCTTTCAGAGGACAGGAGTGGATTTCATTGAG	180	
	A N D A K K V F E I L D R D R S G F I E		
181	GAAGAAGAACTTTCCTTCCAGAACTTCAAGTCTAACGCCAGAGCTCTGAGCAGT	240	
	E E E L C L F L Q N F K S N A R A L S S		
241	GCTGAGACTGCAGCTTTCCTGAAGGCTGGTGACTCTGATGGAGATGGCAAGATTGGAGTA	300	
	A E T A A F L K A G D S D G D G K I G V		
301	GAAGAATTCAGGCTCTGGTGAAGGCATAAACAGCCCAAGACCAAAGACTTCAAGAACG	360	
	E E F Q A L V K A *		
361	GAACCCCTCACTTTTCAACCAACAGACACCTCATTCACGGCTGGCACTTATTGTGCATTT	420	
421	TTTACTGTCTCATGGCATTGGTAAATGCCTGTATGTTAACTCTTTTGGGACTACTGA	480	
481	AAATCTAACAAATGTACAGGTATTCTGCTCACTCGATGTTACGTCTTAAGTGTATTGGA	540	
541	TGGCGCATAGTGATCTTGACCAATCACAATGTTGAAGCTGTGGCTGGAAGTTGGAAAAAC	600	
601	AAAAATAAAAAATTATTTAAATAAAATAATGTCTTGACAAAAA	649	

Fig. 4. Nucleotide sequences of cDNAs and deduced amino acid sequences of PA α (A) and PA β (B). An asterisk indicates the termination codon. Accession numbers—PA α cDNA: AB088845; PA β cDNA: AB088846.

acid sequences of bullfrog PA α and PA β , respectively, purified and characterized by Sasaki et al. [11] and Taka et al. [13], respectively.

Both PA α and PA β mRNA expression levels in total RNA aliquots from the gastrocnemius muscle of froglets at 1, 15, 30, 45, and 60 days after metamorphosis, and of fully grown adults were assessed by Northern blot analysis. The PA β mRNA was detected in every specimen examined. On the other hand, the expression of PA α mRNA was hardly detectable until 45 days after metamorphosis. PA α mRNA levels rose thereafter, whereas PA β mRNA levels gradually declined (Fig. 5).

4. Discussion

PAs are low-molecular-weight, high-affinity, calcium-binding proteins present in the sarcoplasm of vertebrate

skeletal muscles and specific nerve cells [14,15]. The highest concentrations of PA are found in the fast-contracting and fast-relaxing skeletal muscles, whereas in the slow-twitch skeletal muscles, cardiac or smooth muscle, little or no PA is detected [16,17].

PA is considered to be involved in the relaxation process by acting as a Ca²⁺ buffer that shuttles calcium from the contractile machinery to the storage site in the sarcoplasmic reticulum [18,19]. A gene knockout study demonstrated that PA-deficient mice could not properly regulate their intracellular Ca²⁺ concentration; consequently, the mutant muscles required a longer time to attain peak-twitch tension than their normal counterpart [20]. Furthermore, rat soleus muscles overexpressing PA required a much shorter time to produce twitch-relaxation than non-transfected muscles [21]; and PA gene transfer to the rat heart in vivo caused an acceleration of heart relaxation performance [22]. In addition, in GABAergic

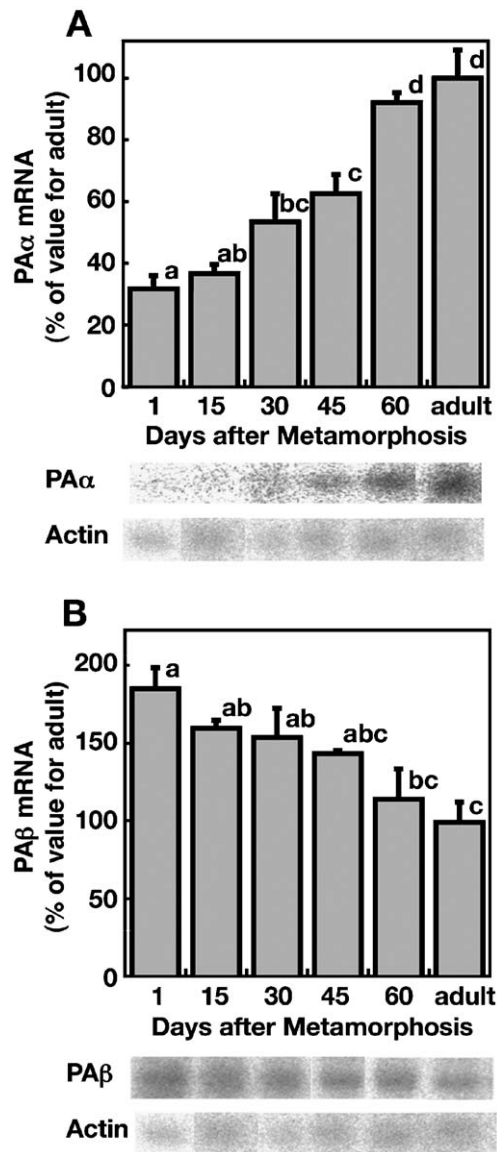


Fig. 5. Northern blot analysis of PA α and PA β . PA α (A) and PA β (B) mRNAs from gastrocnemius muscle of froglets at 1, 15, 30, 45, and 60 days after metamorphosis, and of fully grown adults were assessed by Northern blot analysis with specific probes for PA α and PA β . Densitometry was performed on the Northern blot autoradiographs. For normalization, the densitometry values for PAs were divided by those for the β -actin mRNA. Each column and vertical bar represent the mean value for four samples and S.E., respectively. PA mRNA was expressed as a percentage of the value for fully grown animals. Values with the same superscript do not differ significantly from each other at the 5% level (by Duncan's multiple range test).

neurons, PA possibly regulates short-term synaptic plasticity [23].

Although bullfrog PA α [11] and PA β [13] have been purified and their amino acid sequences have been determined, nothing is known at the cDNA level. The present report first demonstrates the nucleotide sequences of PA α and PA β . Comparison of the nucleotide sequence between *R. catesbeiana* PAs and known amphibian PAs revealed

that *R. catesbeiana* PA α showed homologies of 90.2%, 59.7%, and 64.1%, and *R. catesbeiana* PA β , 58.6%, 92.7%, and 66.6%, with *R. esculenta* PA α (accession number: AJ315959), PA β (accession number: AJ414730), and *Xenopus laevis* PA β [24], respectively.

In amphibians and fish, the presence of multiple, species-specific PA isoforms has been reported [11,13,25–28]. These isoforms are classified into two types, PA α ($pI > 5.0$) and PA β ($pI < 5.0$) [29]. In mammals, however, only a single isoform, PA α , is expressed in muscle. Interestingly, PA β was detected in tumor cells and placenta [30–32]. Up to now, isoforms of amphibian PA have been characterized in four anuran species, *R. catesbeiana* [11,13], *R. esculenta* [25,27], *X. laevis* [24], and *Leptodactylus insularis* [33], and in one urodele species, *Amphiuma means* [28]. Although Gerday et al. [33] speculated that the multiple PA isoforms may have kinetically different temperature-dependent Ca^{2+} - and Mg^{2+} -binding sites, reflecting molecular adaptation to changes in the temperature of their environment as the amphibians evolved, the role of particular isoforms is still not known.

The present experiments revealed that among the components in the gastrocnemius muscle of the bullfrog, PA α and PA β contents changed during the postmetamorphic period. According to the 2DE profiles and mRNA expression of PA α and PA β , we concluded there to be a gradual shift in terms of the content from PA β to PA α . To our knowledge, this is the first report of changes in PA components in amphibians. In three species of cat fish, changes in PA isoform composition in trunk muscles, extracts of which were examined by native and IEF polyacrylamide gel electrophoresis, occurred during development [26]. The larval isoforms predominated in the larval period and gradually disappeared as the fish grew or remained in the adult according to the type of isoform, whereas those of adults were not detectable until the juvenile stage. Focant et al. assume that the appearance of different types of PA isoforms at different times should reflect specific physiological needs (for mobility, feeding, etc.) at different developmental stages. Although PA expression was found to be significantly down-regulated in fast-twitch fibers of extensor digitorum longus muscles of rats during aging, it was possible to reverse this age-associated change through high-intensity training, suggesting that Ca^{2+} transport in aged fast-twitch muscles may be impaired but that its function could be maintained by exercising [34]. The changes in PA expression levels of α and β isoforms in the bullfrog may be related to the shift of the habitat from aqueous to terrestrial, where locomotion becomes intense, and body weight increases. Considering that there was no difference in the electrophoretic profiles between the male and female and that injections of gonadotropin and/or growth hormone to postmetamorphic froglets did not induce a precocious appearance of PA α in the hindlimb muscle (unpublished data), the PA α expression seemed to occur independently of sex steroids and growth hormone. Clar-

ification of the mechanisms involved in the changes in PA expression levels and of functional differences between PA α and PA β is awaited.

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