Detecting pituitary adenylate cyclase-activating polypeptide in cartilaginous fish brain

ABSTRACT

Here we evaluate gene expression of the hypophysiotropic factor pituitary adenylate cyclase-activating polypeptide in cartilaginous fish brain. Total RNA was isolated from cartilaginous fish brain using the SV Total RNA Isolation System (Cat.# Z3101). RNA was reverse transcribed using ImProm-II™ Reverse Transcriptase (Cat.# A3802), and the cDNA was amplified using Taq DNA Polymerase.

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Introduction

The molecular detection of substances in different taxa and comparison of the corresponding related sequences may provide useful data to evaluate evolutionary relationships between organisms. This study investigates the presence of a small hypophysiotropic peptide, pituitary adenylate cyclase-activating polypeptide (PACAP), in the brain of the cartilaginous fish Torpedo marmorata. The expression of PACAP has been widely investigated in vertebrates (1). Since it was first isolated from the hypothalamus of sheep (2), investigators have demonstrated that PACAP is well conserved within vertebrates; the primary sequence differs by only a few amino acid residues in all vertebrates studied so far, suggesting strong selective pressure in the molecular evolution of this neuropeptide (3). Despite a number of studies with other vertebrates (4)(5)(6)(7)(8), information about PACAP in cartilaginous fish is still poor (9). This work shows the expression of PACAP mRNA in an elasmobranch for the first time, providing new insights on vertebrate evolution.

Total RNA Isolation

Six Torpedo marmorata were captured in the Bay of Naples (southern Italy). Brains were removed, and total RNA was extracted using the SV Total RNA Isolation System (Promega). Brains were homogenized in RNA Lysis Buffer. To an aliquot of the lysate, RNA Dilution Buffer was added, and samples were heated at 70°C for 3 minutes. The lysate was centrifuged at 13,000 × g for 10 minutes and transferred to a new tube, and 200µl of 95% ethanol was added. Samples were transferred to a Spin Column Assembly and centrifuged at 13,000 × g for 1 minute. Total RNA was washed with RNA Wash Solution, and the tubes were centrifuged at 13,000 × g for 1 minute. After a DNase treatment at room temperature, another wash step and centrifugation was carried out. Finally, RNA was eluted in diethylpyrocarbonate (DEPC)-treated water (Sigma) to avoid RNase contamination.

RT-PCR

cDNA synthesis was carried out using 4µg of DNase-treated total RNA, ImProm-II™ Reverse Transcriptase and oligo (dT)15 primer at 37°C for 1.5 hours. Polymerase chain reaction (PCR) amplification was executed using 3µl of cDNA, 1.25 units of Taq DNA Polymerase, 0.4mM of each dNTP, 2.5mM MgCl2 and 0.4µM of each primer. PCR primers were designed within conserved regions, which were identified by comparing vertebrate PACAP coding sequences with Clustal X version 1.81. The
reaction was subjected to 35 cycles in an Applied Biosystems 2720 thermal cycler (Applied Biosystems). The PCR conditions were as follows: 94°C for 90 seconds, 48°C for 1 minute and 72°C for 1 minute. A negative control reaction without the reverse transcribed cDNA was performed. Ten microliters of amplification product was analysed on a 2% agarose gel and stained with ethidium bromide for visualization. The PCR product was purified using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281): Briefly the gel slice was dissolved in Membrane Binding Solution at 65°C for 10 minutes, the mixture was centrifuged at 16,000 × g for 1 minute and the spin column was washed with Membrane Binding Solution and centrifuged as described above twice. DEPC-treated water was added to elute DNA from the spin column by centrifugation. The purified DNA was sequenced with an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems).
Results and Conclusions

The aim of this study was to search for PACAP mRNA in cartilaginous fish brain. This goal was difficult to achieve since few molecular biology reagents are optimized for the use in lower vertebrates, and some technical problems were encountered. With reagents from other suppliers, we encountered poor RNA integrity and subsequently cDNA of low quality after reverse transcription. However, the RT-PCR system used in this study seemed to work well in our animal model. Our results showed that the PACAP gene transcript was readily detected in the brain of *Torpedo marmorata*. PCR using specific primers designed by comparing coding regions of vertebrate PACAP amplified a product of approximately 200bp from brain total RNA (Figure 1). The negative control reaction showed no amplification product (Figure 1). The nucleotide sequence of the cloned cDNA contained an open reading frame of 138bp coding for an inferred 45 amino acid polypeptide. The mature protein consists of 38 amino acids, which correspond to a molecular weight of 4.56kDa. Sequence analysis revealed high homology between the deduced amino acid sequence and vertebrate PACAP. These data strongly suggest that PACAP has a basic role in the physiology of cartilaginous fish brain. In conclusion, by detecting this neuropeptide in cartilaginous fish, we demonstrate that this procedure is suitable for routine use in other lower vertebrates to determine mRNA expression levels.

![Amplification of the PACAP gene sequence](image)

Figure 1. Amplification of the PACAP gene sequence. Four micrograms of total RNA from *Torpedo marmorata* brain was reverse transcribed using ImProm-II™ Reverse Transcriptase (Promega) and oligo (dT)15 primer. Three microliters of cDNA was amplified using PCR primers designed by comparing vertebrate PACAP coding sequences to identify conserved sequences. Ten microliters of amplification product was analysed on a 2% agarose gel and stained with ethidium bromide for visualization.

For additional information about the techniques used and results generated, see the complete published article in the *Annals of the New York Academy of Sciences* (10).

REFERENCES


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