

Molecular characterization of G-protein-coupled receptor (GPCR) and protein kinase A (PKA) cDNA in *Perinereis aibuhitensis* and expression during benzo(a)pyrene exposure

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Background. G-protein-coupled receptors (GPCRs) are one of the most important molecules that transfer signals across the plasma membrane, and play central roles in physiological systems. The molecular architecture of GPCRs allows them to bind to diverse chemicals, including environmental contaminants.

Methods. To investigate the effects of benzo(a)pyrene (B[a]p) on GPCR signaling, GPCR and the protein kinase A (PKA) catalytic subunit of *Perinereis aibuhitensis* were cloned. The expression patterns of these two genes during B(a)p exposure were determined with real-time fluorescence quantitative PCR.

Results. The full-length cDNAs of PaGPCR and the PaPKA catalytic subunit were 1,514 and 2,662 nucleotides, respectively, encoding 339 and 351 amino acids, respectively. Multiple sequence alignments indicated that the deduced amino acid sequence of PaGPCR shared a low level of similarity with the orphan GPCRs of polychaetes and echinoderms, whereas PaPKA shared a high level of identify with the PKA catalytic subunits of other invertebrates. B(a)p exposure time-dependently elevated the expression of *PaGPCR* and *PaPKA*. The expression of both *PaGPCR* and *PaPKA* was also dose-dependent, except at a dose of 10 µg/L B(a)p.

Discussion. These results suggested that GPCR signaling in *P. aibuhitensis* was involved in the polychaete's response to environmental contaminants.

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Abstract

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Methods. To investigate the effects of benzo(a)pyrene (B[a]p) on GPCR signaling, GPCR and the protein kinase A (PKA) catalytic subunit of *Perinereis aibuhitensis* were cloned. The expression patterns of these two genes during B(a)p exposure were determined with real-time fluorescence quantitative PCR.

Results. The full-length cDNAs of PaGPCR and the PaPKA catalytic subunit were 1,514 and 2,662 nucleotides, respectively, encoding 339 and 351 amino acids, respectively. Multiple sequence alignments indicated that the deduced amino acid sequence of PaGPCR shared a low level of similarity with the orphan GPCRs of polychaetes and echinoderms, whereas PaPKA shared a high level of identify with the PKA catalytic subunits of other invertebrates. B(a)p exposure time-dependently elevated the expression of *PaGPCR* and *PaPKA*. The expression of both *PaGPCR* and *PaPKA* was also dose-dependent, except at a dose of 10 µg/L B(a)p.

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Introduction

Benzo(a)pyrene (B(a)P), a kind of polycyclic aromatic hydrocarbon (PAH), can cause genetic damage, immune and endocrine dysfunction, and malformation in humans and other organisms. Its high lipophilicity allows it to absorb to organic matter and other particulate matter and thus accumulate in sediments. Recent increases in offshore oil production and transportation and the sewerage discharge of domestic and industrial wastewater have led to environmental pollution in coastal regions, and B(a)P has been widely detected in sediments around the world, even in China. The levels of B(a)P in the sediments of Dalian Bay vary from 10.5 to 3421.2 ng/g (Zhang,

2008), and in the sediments around the drilling platform in the Bohai Sea, the concentration of B(a)p is up to 27.69 ng/g (Yang et al, 2016). PAHs such as B(a)p can be absorbed by benthic organisms via ingestion and through their body surfaces, and B(a)p is reported to have serious effects on deposit feeders. Therefore, the toxicity and bioavailability of B(a)p are important factors in the assessment of sediment pollution.

G-protein-coupled receptors (GPCRs) are the largest superfamily of cell membrane proteins (Fredriksson et al, 2003). The molecular architecture of the GPCRs allows them to bind to diverse organic and inorganic molecules. GPCRs mediate cell proliferation and survival by transmitting signals from a range of extracellular ligands across the cell membrane to signaling pathways. In vertebrates, they are key regulators of the innate and adaptive immune responses and have been investigated as potential targets in drug discovery (Garland, 2013). However, examples of GPCRs in invertebrates are limited. Miller et al. (2015) reported that *Caenorhabditis elegans* with mutations in the GPCR follicle-stimulating hormone receptor 1 (FSHR-1) died significantly more quickly in the presence of cadmium than wild-type nematodes, which suggests that this GPCR pathway protects the nematode against cadmium-induced damage. They also found that FSHR-1 antagonizes the capacity of *C. elegans* to resist cold stress, and the mutants lacking *fshr-1* survived better than wild-type worms at low temperatures. Dong and Zhang (2012) identified a putative GPCR gene, *HP1R*, in the red swamp crayfish *Procambarus clarkia*, and the expression of *HP1R* was significantly increased in the presence of Gram-negative bacteria.

Because of the aromatic structures present a number of GPCR ligands, GPCRs are potential targets of aromatic pollutants such as B(a)p (Ferrec and Ovrevik, 2018). Mayati et al. (2012) reported the interaction between B(a)p and the β_2 -adrenergic receptor (β_2 ADR) in endothelial HMEC-1 cells and the consequent increase in intracellular Ca^{2+} , which influenced the expression of cytochrome P450 B1. This suggests that β_2 ADR, a kind of GPCR, is potentially involved in the deleterious effects of PAHs. Factor et al. (2011) also observed the reduced expression and function of β_2 ADR in airway epithelial cells and smooth muscle cells after their exposure to a mixture of PAHs. This implies that the β_2 ADR signal transduction pathway is affected by PAHs. These data indicate that PAHs, including B(a)p, modulate the concentrations of intracytosolic cyclic adenosine monophosphate (cAMP) or Ca^{2+} via G-protein-dependent mechanisms (Bainy, 2007; Nadal et al., 2000).

The marine polychaete *Perinereis aibuhitensis* is widely distributed in the mudflats and estuarine sediments that occur widely along the coasts of Southeast Asia. They spend most of their lives within the sediments, ensuring their continuous contact with any sediment-associated contaminants. Chen et al. (2012) identified a *CYP4* gene of *P. aibuhitensis* and showed that exposure to petroleum hydrocarbons significantly induce the expression of this gene. To clarify whether GPCRs were involved in modulating the toxicity of aromatic pollutants, the full-length GPCR and protein kinase A (PKA) cDNAs were cloned and the expression patterns of these two genes were determined in this study. Our results provide important information on the function of GPCRs in polychaetes.

Materials & Methods

B(a)p exposure

Perinereis aibuhitensis specimens (10–15 cm, 2.0 ± 0.5 g wet weight) were collected from an estuary of Jinzhou Bay in Dalian, China. The animals were transferred to the laboratory and acclimatized in filtered seawater (salinity 31–32, temperature $16 \pm 0.5^\circ\text{C}$) for a week before the experiment. During acclimatization, the *P. aibuhitensis* were fed a powdered mix containing kelp powder, gulfweed powder, fishmeal, yeast, and spirulina. The worms were deprived of food during their exposure to B(a)P.

Based on the standard seawater quality of the People's Republic of China (GB 3097-1997), four B(a)P concentration groups were established: 0.5, 5, 10, and 50 $\mu\text{g/L}$. A blank control (seawater only) group and an acetone solvent group (100 $\mu\text{L/L}$) were also established. Three repetitions of each concentration group were set up. Ten worms were randomly placed in 2L beakers containing different concentrations of B(a)P. During the experiment, the temperature of the seawater was $16 \pm 0.5^\circ\text{C}$, and the seawater was renewed every 24h. On days 4, 7, and 14 of the experiment, four individuals were randomly sampled from each concentration group, and the body wall was removed and preserved at -80°C .

Cloning the full-length GPCR and PKA cDNAs of *P. aibuhitensis*

Each sample was ground to powder and the total RNA was extracted with RNAiso™ Plus (TaKaRa, Dalian, China). The quality of the RNA was determined with 1% agarose gel electrophoresis. The RNA (500 ng) was reverse transcribed to cDNA for the rapid amplification of cDNA ends (RACE) using the SMARTer® RACE Kit (Clontech, Palo Alto, CA, USA). The 3' and 5' RACE primers were designed with the Primer 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA) according to the confirmed partial sequences of GPCR and PKA obtained from *P. aibuhitensis* transcriptome sequences. The primers used in this study are shown in Table 1.

The 3' RACE amplification of *P. aibuhitensis* GPCR (*PaGPCR*) was performed using the 3' RACE cDNA as the template. The PCR system (50 μL) for *PaGPCR* contained 15.5 μL of PCR-grade water, 25.0 μL of 2× SeqAmp Buffer, 1.0 μL of SeqAmp DNA Polymerase, 2.5 μL of 3' RACE cDNA, 5.0 μL 10 × UPM (Universal Primer Mixture), and 1.0 μL of primer GPCR-F1 (10 μM). The thermal cycling conditions were: 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30s, and extension at 72°C for 3 min. The 3' RACE amplification of *PaPKA* was performed with nested PCR. The outer PCR reaction system for *P. aibuhitensis* PKA (*PaPKA*) was the same as that for *PaGPCR*, except that a specific primer was used. The reaction conditions for the outer PCR were: 35 cycles of denaturation at 94°C for 30 s, annealing at 63.9°C for 30 s, and extension at 72°C for 3 min. The outer PCR product (5.0 μL) was diluted with 245 μL of TE buffer, and 5.0 μL of the diluted product was used as the template for the inner PCR. The reaction conditions and system for the inner PCR were the same as for the outer PCR of *PaPKA*.

The 5' RACE product of *PaGPCR* was amplified with nested PCR. The outer PCR reaction system (50 μL) for *PaGPCR* contained 15.5 μL of PCR-grade water, 25.0 μL of 2× SeqAmp

Buffer, 1.0 μ L of SeqAmp DNA Polymerase, 2.5 μ L of 5' RACE DNA, 5.0 μ L of 10 \times UPM, and 1.0 μ L of primer GPCR-R1 (10 μ M). The reaction conditions for the outer PCR were: 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min. The outer PCR product (5.0 μ L) of *PaGPCR* was diluted with 245 μ L of TE buffer and 5.0 μ L of the diluted product was used as the template for the inner PCR. The reaction system (50 μ L) for the inner PCR of *PaGPCR* contained 5.0 μ L of the diluted outer PCR product, 17.0 μ L of PCR-grade water, 25.0 μ L of 2 \times SeqAmp Buffer, 1.0 μ L of SeqAmp DNA Polymerase, 1.0 μ L of UPM Short, and 1.0 μ L of primer GPCR-R2 (10 μ M). The reaction conditions were: 20 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min. The 5' RACE product of *PaPKA* was amplified with ordinary PCR, and the reaction system and conditions were the same as those for *PaGPCR*.

The PCR products were detected with 1% agarose gel electrophoresis and purified with the Agarose Gel DNA Purification Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The PCR products were sequenced by TaKaRa Biotechnology Co. Ltd.

Bioinformatic analysis of *PaGPCR* and *PaPKA*

The amino acid sequences of *PaGPCR* and *PaPKA* were deduced with the Expert Protein Analysis System (<http://www.us.expasy.org/tools>). The conserved domain in each amino acid sequence was analyzed with the Motif Scan (<http://www.hits.isbsib.ch/cgi-bin/PESCAN>) and Expasy (<http://www.au.expasy.org/prosite/>). The protein localization sites in the cell were predicted with the Psort software (<http://psort.hgc.jp/form2.html>). The transmembrane (TM) helices in the protein were predicted with the TMHMM software (<http://www.cbs.dtu.dk/services/TMHMM/>). The tertiary structures of GPCR and PKA were predicted with the Swiss-Model software (<http://swissmodel.expasy.org/interactive>). Multiple sequences were aligned with the Clustal W software (<http://www.ebi.ac.uk/clustalW>). Phylogenetic analyses of GPCR and PKA were performed in MEGA 5.0. The tree topologies were evaluated with bootstrapping, using 1,000 replicates.

Expression of *PaGPCR* and *PaPKA* genes during B(a)p exposure

Real-time fluorescence quantitative PCR was used to investigate the expression of the two genes in *P. aibuhitensis* during B(a)p exposure. The β -actin gene was used as the reference gene, according to our previous study (*Li et al., 2018*). The primer information is shown in Table 1. Amplification was performed in 20 μ L reactions containing 10 μ L of SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Dalian, China), 0.8 μ L of each primer (10 μ M), 0.4 μ L of 50 \times ROX Reference Dye II, 2.0 μ L of cDNA, and 6.0 μ L of H₂O. The reaction conditions were: 95°C for 30 s, then 40 cycles of 95°C for 5 s and 60°C for 34 s. The melting curves were analyzed after the real-time quantitative analyses. The standard curves were tested with serial 10-fold sample dilutions. The slopes of the standard curves and the PCR efficiency were calculated to confirm the accuracy of the real-time PCR data.

Statistical analysis

The relative quantitative ($2^{-\Delta\Delta C_t}$) method was used to analyze the expression of the *GPCR* and *PKA* genes. The data are expressed as means \pm standard deviations (SD), and one-way analysis

of variance was used to analyze the significance of the differences among the different concentration groups at each sampling point, with the SPSS 19.0 software. *P* values ≤ 0.05 were considered statistically significant.

Results

Molecular characterization of *PaGPCR*

The full-length cDNA of *PaGPCR* was 1,514 bp and included a 5' untranslated region (UTR) of 213 bp, a 3' UTR of 284 bp, and an open reading frame (ORF) of 1,017 bp, encoding 338 amino acids with a predicted molecular weight of 38.799 kDa and a theoretical isoelectric point of 9.38 (Figure 1). This nucleotide sequence was deposited in the GenBank database under accession number KX792261. The seven-transmembrane (7TM)-helix bundle (304–1,146 bp) that defines the GPCR protein family was present in *PaGPCR*. The glutamic acid/aspartic acid-arginine-tyrosine (E/DRY) motif (amino acids 124–126) at the border between TM III and intracellular loop 2 and the NPXXY motif (amino acids 298–302) of TM VII near the inner cell membrane were detected in the deduced protein sequence, indicating that the protein sequence belonged to the rhodopsin family. In an amino acid comparison, *PaGPCR* shared 33% similarity with the orphan GPCR of *Platynereis dumerilii* and 30%–33% similarity with galanin receptor type 2 of echinoderms (Figure 2).

The predicted cellular localization of the *PaGPCR* protein showed it mostly located on the cell membrane (52.2%), and seven TM helices were predicted in the deduced protein sequence (Figure 3). The three-dimensional structural analysis of *PaGPCR* showed that it contained seven α -helices, similar to the GPCRs of other animals (Figure 4). Its three-dimensional structure and protein localization confirmed that this protein sequence was a GPCR.

Molecular characterization of *PaPKA*

The total length of *P. aibuhitensis* PKA cDNA was 2662 bp, containing a 3' UTR of 1,483 bp, a 5' UTR of 126 bp, and an ORF of 1,053 bp encoding 350 amino acids (Figure 5). The predicted molecular weight of *PaPKA* was 40.28 kDa and its theoretical isoelectric point was 8.35. The nucleotide sequence was deposited in GenBank under accession number KX839259. A glycine-rich loop GTGSFGRV (amino acids 50–57), Ser/Thr active site RDLKPEN (amino acids 165–171), PKA-regulatory-subunit-binding site LCGTPEY (amino acids 198–204), DFG triplet (Asp–Phe–Gly) for orienting the γ -phosphates of adenosine triphosphate (ATP) for transfer, APE motif (Ala–Pro–Glu) to stabilize the structure of the large lobe of PKA, and conserved phosphorylation site (Thr197) were detected in this deduced amino acid sequence. The presence of these conserved regions indicated that *PaPKA* was the catalytic subunit of PKA. An amino acid comparison indicated that *PaPKA* was highly similar to other PKA catalytic subunits (Figure 6).

The predicted location of *PaPKA* in the cell was predominantly in the cytoplasm (47.8%). The three-dimensional structural analysis of *PaPKA* showed that it folded into a two-lobed structure (Figure 7). The small lobe had a predominantly β -sheet structure, which was responsible for anchoring and orienting the nucleotide, and the large lobe had a predominantly α -helix structure,

and was primarily involved in binding the peptide substrate and initiating phosphotransfer (Hanks and Hunter, 1995). Ser53, Phe54, and Gly55 formed hydrogen bonds with ATP β -phosphate oxygens, and Leu49 and Val57 formed a hydrophobic pocket enclosing the adenine ring of ATP.

Phylogenetic analysis of PaGPCR and PaPKA

Phylogenetic trees were constructed from the amino acid sequences of GPCR and PKA, (Figure 8 and Figure 9, respectively). Figure 8 indicates that PaGPCR shared great identity with the orphan GPCRs of other polychaetes. Figure 9 shows that PaPKA shared identity with mollusk PKAs, which clustered together on a single branch.

Effects of B(a)P on PaGPCR and PaPKA expression in *P. aibuhitensis*

Figure 10A shows the expression of the *PaGPCR* gene of *P. aibuhitensis* during B(a)p exposure. There was no difference in its expression between the acetone control group and the blank control group, indicating that acetone as a solvent had no toxic effect on the nematodes. During exposure to B(a)p, the expression of the *PaGPCR* gene increased both time- and approximately dose-dependently. On day 4, *PaGPCR* expression was significantly upregulated ($P < 0.05$) in all but the 0.5 $\mu\text{g/L}$ B(a)P group. The expression of *PaGPCR* in the 5, 10, and 50 $\mu\text{g/L}$ B(a)P groups was 2.32-, 3.46-, and 3.15-fold higher than in the control group, respectively. On day 7, the expression of *PaGPCR* in the 0.5, 5, 10, and 50 $\mu\text{g/L}$ B(a)P groups was 3.10-, 2.91-, 3.59-, and 3.28-fold higher than in the control group, respectively ($P < 0.05$). The expression of *PaGPCR* in each concentration group reached its highest level on day 14, at 4.30-, 6.60-, 4.79-, and 6.36-fold higher than the control group, respectively ($P < 0.01$).

The expression pattern of the *PaPKA* gene during B(a)P exposure was the same as that of *PaGPCR* (Figure 10B). The expression of *PaPKA* increased as the time of exposure increased. On day 4, the expression of *PaPKA* was slightly higher in all but the 0.5 $\mu\text{g/L}$ B(a)p concentration group, at 1.79-, 1.21-, and 2.21-fold higher in the 5, 10, and 50 $\mu\text{g/L}$ B(a)P groups, respectively, than in the control group. The expression of *PaPKA* in each concentration group was higher on day 7 than on day 4, at 1.25-, 1.90-, 2.52-, and 2.89-fold higher in the 0.5, 5, 10, and 50 $\mu\text{g/L}$ B(a)P groups, respectively, than in the control group ($P < 0.05$). On day 14, the expression of *PaPKA* reached its highest level in each concentration group, at 3.19-, 5.03-, 3.10-, and 5.02-fold higher than the control group, respectively ($P < 0.05$).

Discussion

To investigate the reaction of GPCR to B(a)p exposure in *P. aibuhitensis*, the full-length cDNAs of the *PaGPCR* and *PaPKA* were isolated and characterized in *P. aibuhitensis* for the first time. The sequence of *PaGPCR* contained 1514 bp, encoding 338 amino acids. The deduced protein sequence of PaGPCR contained a 7TM helix bundle domain, flanked by the extracellular N-terminal region and the intracellular C-terminus. As part of the functional mechanism of GPCR, the E/DRY motif (amino acids 124–126), which plays an important role in regulating the conformational state of GPCR, occurred at the border between TM III and intracellular loop 2 in this sequence. The protein sequence of PaGPCR also contained the NPXXY motif (amino acids 298–302) in TM VII, which confirmed that it belonged to the rhodopsin family, the largest of the

five families involved in many signaling processes (Fredriksson *et al.*, 2003). A multiple protein sequence alignment showed that PaGPCR shared almost 33% homology with the galanin receptor of echinoderms. A phylogenetic analysis showed that it clustered most closely with the orphan GPCRs of other polychaetes, and the galanin receptor of echinoderms. It is well-known that proteins with similar sequences often display comparable functions if the sequence identity exceeds 30% (Kakarala and Jamil, 2014). However, a prediction based on sequence identity alone may not be reliable. Therefore, further study is needed to prove the function of PaGPCR. In contrast to the low sequence identity of GPCR, PKA in *P. aibuhitensis* shared high sequence identity with the PKA catalytic subunits of other species. *PaPKA* contained 2,662 bp, which encoded 350 amino acids. The deduced amino acid sequence of PaPKA contained all the conserved domains that were necessary for kinase activity, such as the conserved Thr in the activation loop, the ATP-binding site (GTGSFGRV), the serine/threonine kinase active site (RDLKPEN), and the PKA-regulatory-subunit-binding site (LCGTPEY). The highly conserved amino acids at the ATP-binding site played important roles in ATP binding and phosphotransfer. The high homology among the PKA catalytic subunits suggested that they have a conserved role in intracellular signaling in both vertebrates and invertebrates.

GPCRs comprise the largest and most important family of cell-surface proteins, transmitting signals from extracellular ligands. In vertebrates, they are key regulators of the innate and adaptive immune responses and have been used as potential targets in drug discovery (Garland, 2013). However, they have been inadequately investigated in invertebrates. Miller *et al.* (2015) reported that FSHR-1 mutants of *C. elegans* died significantly more quickly during cadmium exposure than wild-type nematodes, which suggests that the GPCR pathway protects *C. elegans* against pollutant damage. Dong and Zhang (2012) reported that Gram-negative bacterial infection induced the expression of the *HP1R* gene in *Procambarus clarkia*. In the present study, we found that B(a)p exposure induced the expression of *PaGPCR*, which increased with time in each concentration group. This result was similar to reports in other invertebrates, and implied that GPCR played an important role in reducing the deleterious effects of B(a)p in *P. aibuhitensis*. GPCRs interact with diverse chemical structures, which increases cAMP production, which then stimulates phospholipase C activity and the subsequent mobilization of Ca^{2+} . Mayati *et al.* (2012) observed an interaction between B(a)p and β_2 ADR in endothelial HMEC-1 cells, which altered the levels of intracellular Ca^{2+} and the expression of cytochrome P450 B1. Factor *et al.* (2011) observed the reduced expression and function of β_2 ADR in airway epithelial cells and smooth muscle cells after their exposure to a mixture of PAHs. In the present study, we also observed that the expression of *PaPKA* in *P. aibuhitensis*, was higher during B(a)p exposure than the control level in all but the 0.5 μ g/L B(a)p concentration group. The expression of *PaPKA* was significantly and exposure-time-dependently induced by 50 μ g/L B(a)p. The increased expression of *PaPKA* and *PaGPCR* suggested that the GPCR signal transduction pathway in *P. aibuhitensis* was affected by PAHs.

B(a)p can be metabolized by organisms through a series of enzymatic and nonenzymatic reactions. Typically, B(a)p is metabolized by the phase I enzyme cytochrome P450 (CYP) and

phase II enzymes such as glutathione S-transferase (GST). The induction of CYP gene expression has been detected in *P. aibuhitensis* (Chen et al., 2012; Zhao et al., 2014). In vertebrates, the induction of the CYP enzymes involved in the biotransformation of PAHs is mediated by the aryl hydrocarbon receptor (AhR) pathway. Both α - and β -type AhR proteins have been reported in bivalves (Fabbri and Capuzzo, 2010). However, no AhR homologues have been identified in other invertebrates, including marine polychaetes (Jorgensen et al., 2008). Ferrec and Ovrevik (2018) reported that a number of GPCR ligands contain aromatic structures, and that B(a)p modulates the concentration of intracytosolic cAMP through the GPCR pathway without the involvement of conventional nuclear receptors. In this study, we demonstrated the induction of *GPCR* and *PKA* expression during B(a)p exposure, so we hypothesized that the GPCR pathway is also involved in the biotransformation of PAHs in *P. aibuhitensis*. Further study of the relationship between GPCR and CYP expression is required to test our hypothesis.

Conclusions

GPCR represents a critical point of contact between cells and their surrounding environments. This is the first study in which *P. aibuhitensis* GPCR and PKA cDNAs have been cloned. We have also demonstrated that the expression of *GPCR* and *PKA* was induced in *P. aibuhitensis* by B(a)p exposure, and that their expression was affected, to some extent, by the B(a)p concentration and the exposure time. These results should be useful in investigating the biotransformation of PAHs by marine polychaetes.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (No. 41306138), the key laboratory of mariculture and stock enhancement in North China's Sea, Ministry of Agriculture and Rural Affairs, Dalian Ocean university, P. R. China(2018-KF-15) and Liaoning Scientific instrument sharing Platform(L201810).

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382

Figure 1

Nucleotide sequence and deduced amino acid sequence of GnRHR from *perinereis aibuhitensis*.

Initiation codon and termination codon are included in the boxes; Terminator is showed as *;
The approximate locations of predicted transmembrane domains 1 through 7 are noted.

1 TAAACAGTGGCATTACGCAGAGTACATGAGGGCAGTTTAAAGTGATTCAATTTATGGGAATGGGTTTCCCTGGTG
76 ATTAGTTAGCAAAAATATATATTTCTGGAATATTTGGACTTCAGAAATTGAAGGATTTTATACTTGTCTTCGGC
151 TTAATGGAAATTTTCATCTTGTGATTGAGGATTTAATCGGTGAATTTTATTACATGTTAACATTATGGATAACACA
1 M D N T
226 ACATTCAACAGAACGTTTGATGGGAGTTTGAACCCTAACTTCAACTACATTGGAGATTTTGTGGTGTACATAGTA
5 T F N R T F D G S L N P N F N Y I G D F V V Y I V
301 ATCGGATGTCTTGGGATTTTAGATAATGGATTGTTATTATAGTCATTCTCCATAGCAGAAAGATGAGGAATAAA
30 I G C L G I L D N G F V I I V I L H S R K M R N K
376 CTGTGCAATTTATTCATCCTTAATCAAAGTGTGGTAGACCTGGTGGCTTCTGTGTTTCTCCTGTGCAATTCTCCG
55 L C N L F I L N Q S V V D L V A S V F L L C N S P
451 TCTGTTCCGACCTTAGGGTCAGCGTCGAATATTAGTCTGGAGTTTTATTGCCGCATTTGGGATTCGAACTATCTC
80 S V P T L G S A S N I S L E F Y C R I W D S N Y L
526 TTCTGGGCTGCCGTCACATGGTCAACTTACAACCTAGTCGCCATCACAATCGAACGTTACTTAGAGGTCGTTTAC
105 F W A A V T W S T Y N L V A I T I E R Y L E V V H
601 CCACTTCGGTACAGATCATTCTTACGCGGAGACGTGCAAAGGTCATTGTCTGCTGTCTGCTGTTGGTTGGATTCT
130 P L R Y R S F F T R R R A K V I V A V V W L V G F
676 ACCATACCTATCGTGACGTCAGTTATCACCAGTCTGCGGGAGCAGACGGCACTTGTGAGAAGCACAGAGCGTGG
155 T I P I V T S V I T S P A G A D G T C Q K H R A W
751 TCCTCCCGACTCATGGCTGCCCTCGTAGGATTTTACGCCCTCTTCTTCGGATTTCTTTTACCTGTCTGCATATAATG
180 S S R L M A A L V G F Y A L F F G F L L P V V I M
826 ATCGTTTGCTACACTCAGATGATCATGACCTTCAACTTGAAGGTCCGACCCTCCGACCCAGCACAATGATCTCC
205 I V C Y T Q M I M T F N L K V R P S D P S T M I S
901 GAAAGTGAGAAACGTCGAAGCGAAAGGATGTTGAGGGTCCGTAAGGCCTCATCAAGACAATGTTGATGGTTTCT
230 E S E K R R S E R M L R V R K S L I K T M L M V S
976 ATCGTCTTTGTGATCTGTTGGATCGGCGACCAAGTTTATTTCTTCTTCAACATCAGAGTCATCAAAGACCTT
255 I V F V I C W I G D O V Y F F L F N I R V I K D L
.051 CAACAACTCTTACGACTATCGTTGTTTCGTTAGCTTTCTTCAATTGCTGCATTAATCCATTCAATTTACACCTGC
280 Q Q T L T T I V V S L A F L N C C I N F F I Y T C
.126 CAATATAACGACTTCCAAGAAGCTACAAGAAGACTCTTAAAAATCAAAAAGGAGAGTGAAAACAGTGAAAGGTCT
305 Q Y N D F Q E A T R R L L K I K K E S E N S E R S
.201 ACGTTGGATCTGTCTAACCAAAAAGTCTAATCCACATGAACTAATTAACATATTGATTGACCAATTTTAAAC
330 T L D L S N Q K V *
.276 TTTCTTACAGATTTACCAAAAATTAATTTTAAATTTTAGTTACCTCTATTTATTTAATATTTTCATGTACAGCGCC
.351 TCTGAAGATTTTACTTTCCGAGATCTCGCTACATAAATAAGTCATTATTTGTATTTATTTTAAAGAGGTAATGA
.426 CAAATTGTAAAGATGTTCTTAATTTGTGATGGTCTCCATTAAATTTGACATGGTCATTTACTAAAAAAAAAAAAA
.501 AAAAAAAAAAAAAA

Figure 2

Multiple alignment analysis of PaGPCR with other GPCR protein.

The identical amino acid are shaded. The GenBank accession number for these proteins are as following: (A) *Platynereis dumerilii*, AKQ63061.1. (B) *Strongylocentrotus purpuratus*, XP_003727596.1. (C) *Acanthaster planci*, XP022098630.1. (D) *Apostichopus japonicus*, PIK48567.1.

```

Perinereis_aibuhitensis -----ILLHVNIMDNITTFNRTFDGSLNPNFNYIGDFVYVI 37
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii MAATTNVTGDIITQNFNLQSEHYFETLPTQSLNVNQSAEEVDASASINTDYHLIIYYVI 60
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus -----MASLNGDNLTSPMIPSPATVLTITANPDQGGGGGAVLNELYLKHIIYALI 50
galanin_receptor_type_2-like_Acanthaster_planci -----MAENDLPITIFQSIV 15
putative_galanin_receptor_type_2-like_Apostichopus_japonicus -----MDPTGFVTTGSVYTTEGIPTSTS-----VNDVLLFVLYHII 36

Perinereis_aibuhitensis GCLGLDNGFVITVILHSRKMNRKLNLFILNQSVVDLVAVFLLCNSSPSVP-----TL 91
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii GACGIFGNILVCIYMFSSATLARKITNWFHINQSLVDFAVSFFLTQADVQ-----GE 113
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus GLLGIYVNGIVCLVFTIKRRQFSITNYLILNQSVIDLDSIFFILIHYPNGPISETA 110
galanin_receptor_type_2-like_Acanthaster_planci GITGICGNALVGVVIKIR-FMHTLTNAFENQALIDLFGSFMILLNNLVF-----IPD 68
putative_galanin_receptor_type_2-like_Apostichopus_japonicus GLGLIFGNGVVIIVFLANRKFVRSINLLILNQAIMDFVVAIIFLLDRFGP-----SLYK 91

Perinereis_aibuhitensis GSASNISLEFYCRHWDSNYLFWAAVTWSTYNLVAITERYLEVVDLRYRSFFTRRRKRV 151
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii PPHSGIAGELYCRHWATKFLWGLVSVSTYNLVALTHERYLAVVHPIWHTTSFSKTKATV 173
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus NIPSRGLAEFFCRHWDSYPLWALYIASTANLVLSLERYFATCRPVIIHRNFTVRRKRW 170
galanin_receptor_type_2-like_Acanthaster_planci PLPSNSNGVLLCRHWLSGYFNWALFVSVSTYNLVALTHERYLAIVFPFRYQVLGTRKNALI 128
putative_galanin_receptor_type_2-like_Apostichopus_japonicus LITNETLSEMLCRHWDSYELLWAFYIASTQNLVMSLERYFATCHEVVKHNYFTIRGAKI 151

Perinereis_aibuhitensis IVAVVWLVGFTIPIVTSVITSPAG-----ADGTCQKHRAWSSRLMAALVGFYALFFGELL 206
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii LIALVWLVGFPVWNASYMIVTSSNED-----GVCAIYAIWPSITVRRFPGLTVLLQLYLI 227
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus AMLCQVWVYGAVYQLYWLPVQTFS-----DWR-CHPNWPNRVVQRFGVLLFSLEYLI 222
galanin_receptor_type_2-like_Acanthaster_planci IIVIVWVSAFLFTSYGVFIIRYK-----GQC-----KQLVAHPEVLGAVFAVTELL 177
putative_galanin_receptor_type_2-like_Apostichopus_japonicus GIAGIWLFGLVYQSYWIFVFFFEF-----SSQSCFPLWNTRTLQACMGVVFVLMEXLI 204

Perinereis_aibuhitensis PVVIMIVCYTQMIMTNFLKVRPSDPS-----TMISESEKRRSERMLRVRSLSIKTML 258
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii PHTFIIFAYGSIALKLYRKLKSD-----GAAGKDETIQGRMNTVKTTLV 272
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus PLIIMTCSYVSIILMLNRNRIK-----SHGKVQVNAFQRAKRNVTITLC 265
galanin_receptor_type_2-like_Acanthaster_planci PVIVMLVVVAHITVVLKRGAGRIQPGPAA---AVPSTGTAPEGQGESLMRARNITFKTLL 234
putative_galanin_receptor_type_2-like_Apostichopus_japonicus PLIVMTFSYVNIILMLKKR-----GQKSGS-VFQRAKRNVTITLC 244

Perinereis_aibuhitensis PVVIMIVCYTQMIMTNFLKVRPSDPS-----TMISESEKRRSERMLRVRSLSIKTML 258
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii PHTFIIFAYGSIALKLYRKLKSD-----GAAGKDETIQGRMNTVKTTLV 272
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus PLIIMTCSYVSIILMLNRNRIK-----SHGKVQVNAFQRAKRNVTITLC 265
galanin_receptor_type_2-like_Acanthaster_planci PVIVMLVVVAHITVVLKRGAGRIQPGPAA---AVPSTGTAPEGQGESLMRARNITFKTLL 234
putative_galanin_receptor_type_2-like_Apostichopus_japonicus PLIVMTFSYVNIILMLKKR-----GQKSGS-VFQRAKRNVTITLC 244

Perinereis_aibuhitensis MVSIVSVICWIGDQVYFLFN--IRVIKDLQTLTTIVVSLAFLNCCINFFIYTCQYND 316
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii LVAICSVACWSWNQIYLLMMN--LGFREDYSSNFYHTVIAVFINSCVNMIMYALKRYDPF 330
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus IVFVSVVVCWTPTELSYLLAYN--LGHDYNFESAVHDVLKGLVACNLGVNFFIYAFKRYEHF 323
galanin_receptor_type_2-like_Acanthaster_planci LVFVASTICWTPNEVFLLEN--LGVDVNLITSTIFFVIVAMVSTNSCLNFFIYAIKKYQF 292
putative_galanin_receptor_type_2-like_Apostichopus_japonicus LVFVSVVICWTPTEFGIILYN--CGRPYDFEGTFHYVATVVLVLCNMCTAFIYTFKRYEQF 302

Perinereis_aibuhitensis QEATRRLLKIKKESENSERSTLDLSNQKV----- 345
orphan_G-protein_coupled_receptor_56_Platynereis_dumerilii KKAAYQLFCTKMLGIRPNAIEDQS----- 354
galanin_receptor_type_2-like_Strongylocentrotus_purpuratus QSELKKIFCSMCAAGNRIESTSRGTVLSPANNSNNTP----- 361
galanin_receptor_type_2-like_Acanthaster_planci RKALKTLFG---RQGGIEDESTLATVATAHD----- 320
putative_galanin_receptor_type_2-like_Apostichopus_japonicus QNYLKRMFGRCLGVNRIDVNTVSAVAEPDQTLNRRSDTGKQAATTA----- 350

```

Figure 3

Analysis of transmembrane region of PaGPCR

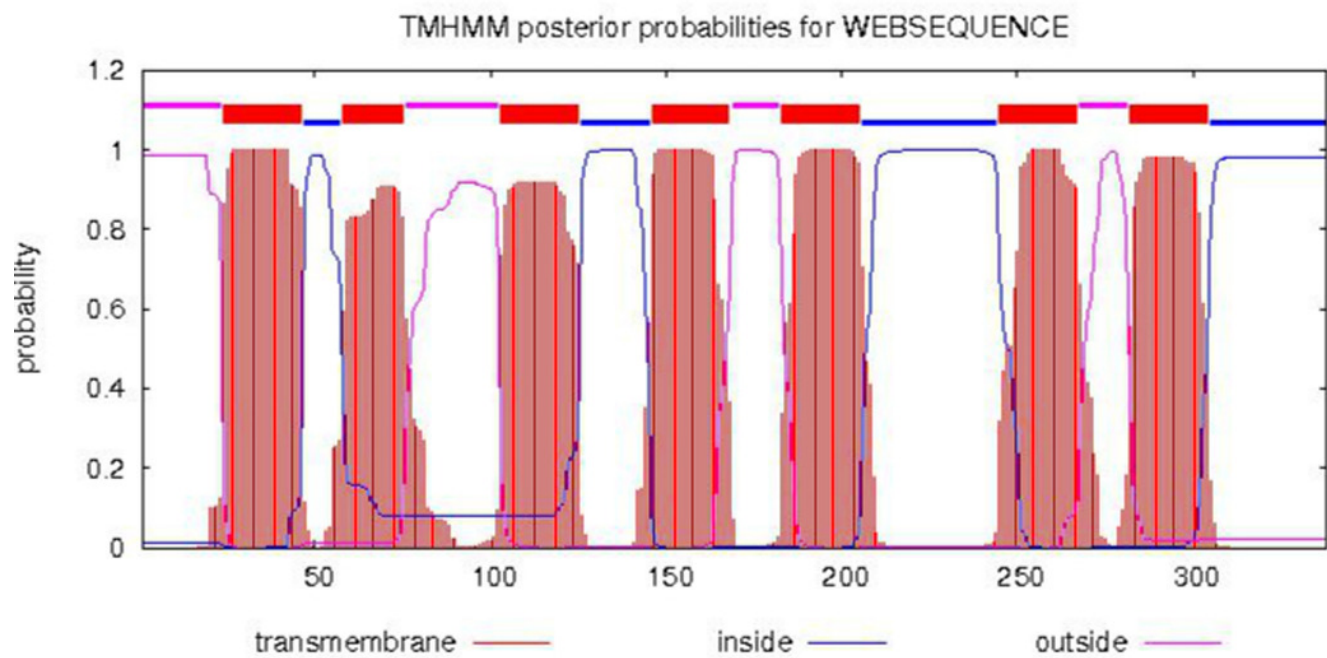


Figure 4

The three dimensional structure of PaGPCR

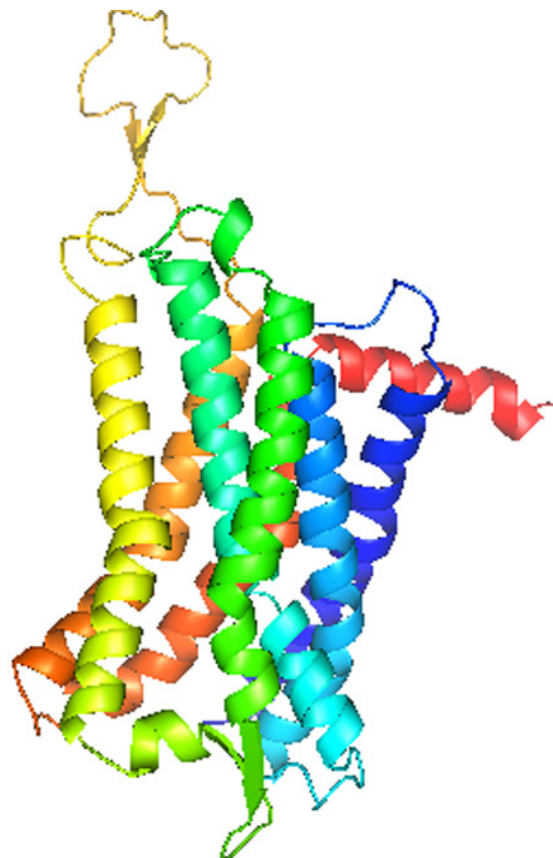


Figure 5

the full cDNA sequence and deduced amino acid sequence of PaPKA.

Red fonts show initiation codon and termination codon; Terminator is showed as*; The red bars under the sequences indicated the ATP active site, Ser /Thr active site and PKA regulatory subunit binding site was underlined with red line, conservative phosphorylation site is boxed in green.

1 GTCGAAGAGATCGAGGTGGAATATTACAGACATAATTTTGGAGAAGCTGGTTCTGAGAGTTCTCTGATTTCCTGGCCGGTAATTCTCTGGATCACCACGGACTAG
106 GTAGTTTACCACACGGTAGCCATCGGAAATGCTGCAACAGCAAAGAAAGGCGATCCAGAGAATGTCAAAGAGTTCTTAGCCAAAGCTAAGAGGACTTCAACAAG
1 M G N A A T A K K G D P E N V K E F L A K A K E D F N K
211 AAATGGGAAGAGCCATCATGTAACTGCATCACTAGATGACTTCGACAGAATTAACACCTGGGAACAGGGTCATTGGACGGGTCATGCTGGTTCAGCACAAA
29 K W E E P S C N T A S L D D F D R I K T L G T G S F G R V M L V Q H K
316 GCCACGAAGGAGTACTATGCCATGAAGATTTTGTAGATAAACAAAGGCTAGTGAACTGAAGCAAGTTGAACACACATGAATGAAAAAGAAATTTCTGTCGCCATA
64 A T K E Y Y A M K I L D K Q K V V K L K Q V E H T L N E K K I L S A I
421 TCATTTCATTCTTAGTGAGCCTAGAGTACAGTTTTAAGGATAAECTCAAATTTGTACATGGTATTGGAGTTCGTGACAGGAGGTGAAATGTTCTCACATCTGCGA
99 S F P F L V S L E Y S F K D N S N L Y M V L E F V T G G E M F S H L R
526 AGAATTGGCCGATTAGTGAAACTCACAGCGATTTTATGCTGCACAAGTATGCATGGTATTGAATATCTGCACAATCTAGACACACTGTACAGAGATTTGAAG
134 R I G R F S E T H S R F Y A A Q V C M V F E Y L H N L D T L Y R D L K
631 CCAGAAAAATATTCTGATTGATGACACTGGTCACTTGAGAGTAACAGACTTCGGTTTTGCCAAACGCGTAAAAGGCAGGACATGGACGTTGTGTGGCACACCAGAG
169 P E N I L I D D T T G H L R A V T D F G F A K R V K G R T W T L C G T T P E
736 TACCTGGCCCCAGAAATCATCTTTAGCAAGGGCTACAACAAGCCGTAGACTGGTGGCGCTTGGAGTCCTTGTTTGAATGAGCAGCTGGATACCCCACTTTTC
204 Y L A P E I I L S K G Y N K A V D W W A L G V L V Y E M A A G Y P P F
841 TTGTCTGACCAGCCAATCCAATCTATGAGAAGATTGTCTCAGGAAAGGTGCGCTTCCCATCTCACTTTAGTTCTGATTGAAGGATCTTTGAAGAATCTGCTA
239 F A D Q P I Q I Y E K I V S G K V R F P S H F S S D L K D L L K N L L
946 CAGGTAGACTTGACAAAACGTTATGGAACCTGAAGATGGGGTCAACGATATCAAGAATCACAGTGGTTCTCCACCACAGACTGGATTGCTATCTACAGAAA
274 Q V D L T K R Y G N L K N G V N D I K N H K W F S T T D W I A I Y Q K
1051 AAGTTGAAGCACCCCTTATCCCAAGTGCAAAGGCCAGGTGACTACAGCAACTTTGATGACTATGAGGAAGAACCAGTGAAGATTTCTGCAACGGAAAAATGT
309 K V E A P F I P K C K G P G D Y S N F D D Y E E E P L R I S S T E K C
1156 GCCAAGGAGTTTCAGACTTCGAGACCTTGAGACATTTGGCTTGAGTGGCTAGTGTGTGTTGGGGGAACTAAAGACTGTGAGCACTTCGTGTTCTATGGTCTACT
344 A K E F A D F *
1261 GGCAGCTGGCTGAAGATCTGCGGCCACATACCACACAATCTCTCAAAGAATACATCCTTTGTGCATTAACAAATGTATATATACTTAATCATATCACATTGCGT
1366 AATTGTAACAAGGTGAATTCAGTTTCATATGTGTCAATGAGGTAAGAGAGAGGGCTTCGATCCCAGGTGTGGCATGTCCCTGAGGCTGCGCTATTGGTCCGAGGT
1471 AAGCCAGGTGCGTTCACTGCTCGACCCAGGCTGCTCAAAGCTACCTTGCTGCTACTCGCCGAAC TAGT CATTTACCTTGCTGTGTGGCCTTCCAGATTTGT
1576 GTGGGGCAGACTGGTGGAGGATTAGGCTTTAATTGCTTTGCTGAAGTGCCCCACACGGGTCCAGAAGCCAAGGCTATCTTCAAATACACAGTTAATACCATATAT
1681 TAATCTATCATTAACAACCTTACTGACCACTTGTTCACATTTAGGTCATCTCTGCACCTTTATTGTATACAGGTGGTCCACTGAAACAATCTCTGAACATTTG
1786 AATTATATCCATGTGAAGGAAGTGCACAACTTCTCTGTGCTGTGCTCTTCTGCACCCGTGCTGCTCTTCTGCTGTGAACCTTTGAATCAGAGAGAAACAA
1891 CACTTTATTGTATGATGTCAAATATTGTACTTTTGGTAAGGCCACCTCCTAACTGGGTGGTCACTAAGACTGTATATAGTTTGTGAGCAGTTGGCTTTTGGGT
1996 AGAGTAGGAGTTTTCTGTTTCATCACTGGTGGGATGACACCATCTCCCGCATACCAGTAGTACTGTGTAGTAGCAATAGCCGGCCATAAGGCCTATGAGTCTCC
2101 ATTGTTTCAGCTAATGTAGCTAGGCTTCAAGTCATAAGTCATGATAACATATAATGGCATTATTGTAGTACTGATGATGAGAGAATATTATCTTTGTCAAGACA
2206 AACCTATTTTACTTTTATATAACTGACTTGTATGTACAGACGTCGAAGGCTGTATGAACTGAGAATTTGTGCTTGTATGTGTAGTGTATAAAATGTGTATATCT
2311 ATCTCTGCATATTTTGTGTTTGATTGTCAATCCACAAATGCTTCAAATTTATCTTGAAACCATGTACTTGACTAATCTAATTTGTGATTTGAAAAGGTAATTGGA
2416 GTTGTGCTGAATTAGATAGGTATGCTTCTGACATGTGCTGAACACTACTACAGTGAGTCCCATTTGTGATGGTCTTACTCATGAATGTTGTTATATTTGTATA
2521 TACTAAAAATAAATTTATGCTGGTTCATGAAGATGTGAAAAAATATTTTCAAATGGCTAAGGATGGTTTGTATGAAATGTTTACACTAAGGTCAGCAGTTAA
2626 CAAACCCCATGAAAAAAAAAAAAAAAAAAAAAAAA

Figure 6

Multiple alignment of PaPKA with other PKA.

The identical amino acid are shaded. The GenBank accession number for these proteins are as following: (A) *Aplysia californica*, NP_001191420.1. (B) *Xenopus tropicalis*, NP_001164667.1. (C) *Branchiostoma floridae*, XP_002600447.1. (D) *Danio rerio*, NP_001030148.1. (E) *Octopus bimaculoides*, XP_014777153.1. (F) *Lingula anatina*, XP_013409439.1. (G) *Crassostrea gigas*, XP_011439335.1. (H) *Biomphalaria glabrata*, XP_013072294.1. (I) *Salmo salar*, XP_014071121.1. (J) *Gallus gallus*, XP_015146370.1.

Perinereis_aibuhitensis	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSCNTASLDDFDRIKTLGTGSFGRVMLVQHH-ATKEYYA	70
Aplysia_californica	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSCNTASLDDFDRIKTLGTGSFGRVMLVQHHGSRNFYA	72
Xenopus_tropicalis	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSCNTASLDDFDRIKTLGTGSFGRVMLVQHHGAEQY	71
Branchiostoma_floridae	MAAKATPKGSGHGKATAIGKISKHGDGSGSYSDSVKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHH-ATQNFYA	89
Danio_rio	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHH-ASDQY	71
Octopus_bimaculoides	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHH-ANKEYYA	71
Lingula_anatina	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHH-QSKQY	71
Crassostrea_gigas	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHH-ANKDY	71
Biomphalaria_glabrata	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHHGENKSY	72
Salmo_salar	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHH-GTEQY	71
Gallus_gallus	MGNAATAK-----KGDPAEN---VKBFLLAKAKEDFNKKWBEPSQNTAALDDFDRIKTLGTGSFGRVMLVQHH-ATEQY	74
Perinereis_aibuhitensis	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVCMVFYELHNL	160
Aplysia_californica	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	162
Xenopus_tropicalis	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	161
Branchiostoma_floridae	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	179
Danio_rio	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	161
Octopus_bimaculoides	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	161
Lingula_anatina	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	161
Crassostrea_gigas	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	161
Biomphalaria_glabrata	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	162
Salmo_salar	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	161
Gallus_gallus	MKILDKQKVVVKLQVEHTLNEKKILSAISFPFLVSLLEYSKDNSNLYMVVEFVTGGEMFSLRRIGRFSEPHSRFYAAQVIVLVLEYLHNL	164
Perinereis_aibuhitensis	DTLYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	250
Aplysia_californica	DIMYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	252
Xenopus_tropicalis	DLIYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	251
Branchiostoma_floridae	DIIYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	269
Danio_rio	DLIYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	251
Octopus_bimaculoides	DIIYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	251
Lingula_anatina	DIMYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	251
Crassostrea_gigas	DIMYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	251
Biomphalaria_glabrata	DLIYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	252
Salmo_salar	DLIYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	251
Gallus_gallus	DLIYRDLKPENLLIDDTGHLRVTDGFAKRVKGRWTWLCGTPEYLAPEIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYEKI	254
Perinereis_aibuhitensis	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	340
Aplysia_californica	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	342
Xenopus_tropicalis	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	341
Branchiostoma_floridae	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	359
Danio_rio	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	341
Octopus_bimaculoides	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	341
Lingula_anatina	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	341
Crassostrea_gigas	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	341
Biomphalaria_glabrata	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	342
Salmo_salar	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	341
Gallus_gallus	VSGKVRFPFHFSSDLKDLRLNLLQVDLTFRYGNLKNVNDIKHKKWFATTDWIAIYORKVEAPFVFKCKGPGDTSNFDDYEEBEPURISST	344
Perinereis_aibuhitensis	EKCAKEFAADF	350
Aplysia_californica	EKCAKEFAADF	352
Xenopus_tropicalis	EKCAKEFAADF	351
Branchiostoma_floridae	EKCAKEFAADF	369
Danio_rio	EKCAKEFAADF	351
Octopus_bimaculoides	EKCAKEFAADF	351
Lingula_anatina	EKCAKEFAADF	351
Crassostrea_gigas	EKCAKEFAADF	351
Biomphalaria_glabrata	EKCAKEFAADF	352
Salmo_salar	EKCAKEFAADF	351
Gallus_gallus	EKCAKEFAADF	354

Figure 7

The three dimensional structure of PKA from *P. aibuhitensis*



Figure 8

Molecular phylogenetic analysis of PaGPCR related to GPCR of other invertebrates and vertebrates

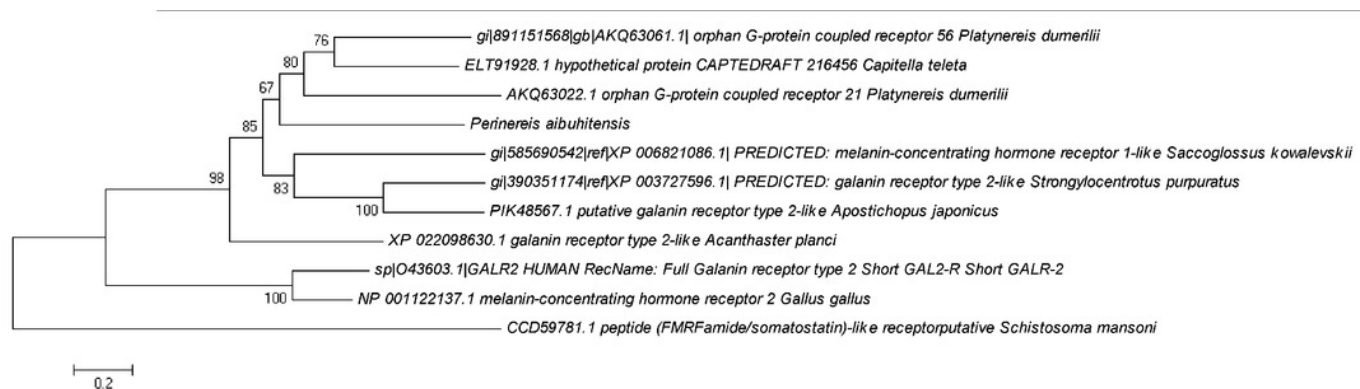


Figure 9

Molecular phylogenetic analysis of PaPKA related to PKA of other invertebrates and vertebrates

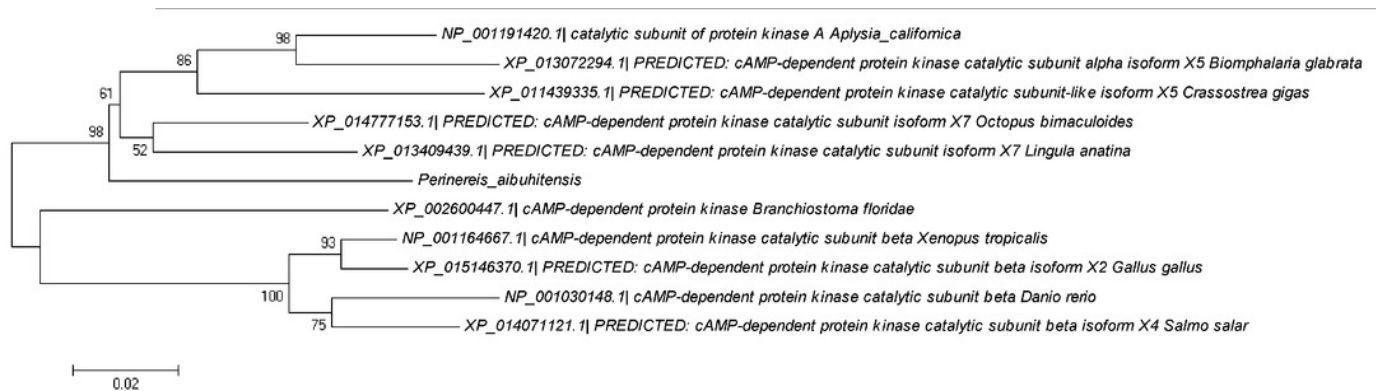


Figure 10

The relative expression level of PaGPCR and PaPKA cDNAs under various B(a)p concentration exposure. A PaGPCR, B PaPKA. Different lowercase letters indicate significant difference ($P < 0.05$). all data as mean+SD. N= four worms.

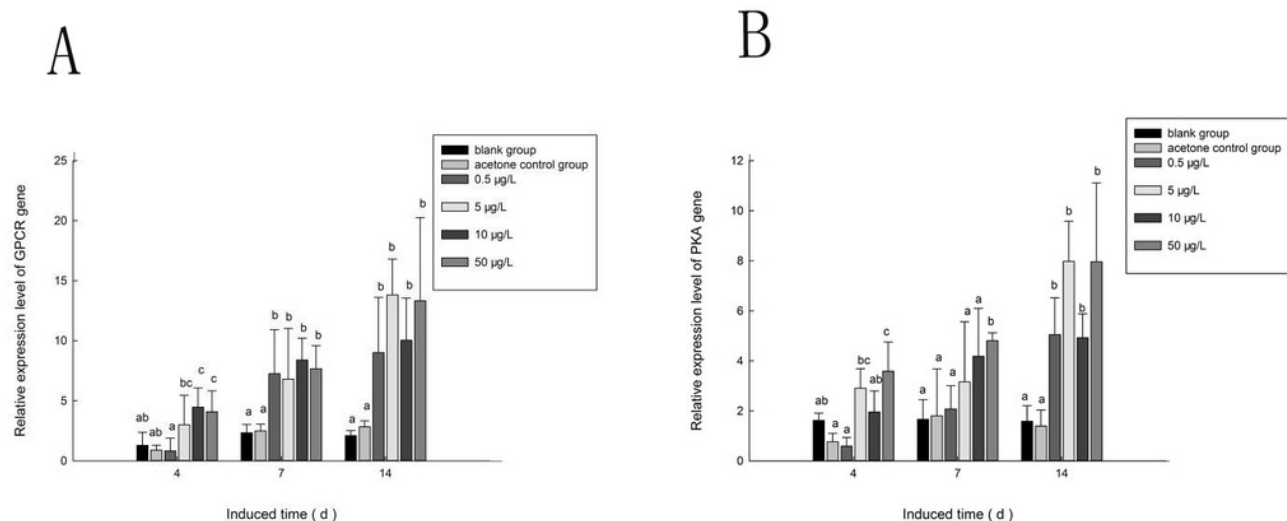


Table 1 (on next page)

The primers used in this study

Primer name		sequence (5'-3')
RACE	GPCR - F1	TGAGAAACGTCGAAGCGAAAGG
	GPCR - R1	ATATTCGACGCTGACCCTAAGG
	PKA-F1	GATACCCACCTTTCTTTGCTGACC
	PKA-F2	GGTGCGCTTCCCATCTCACTTT
	PKA-R1	CAATAGCGCAGCCTCAGGGACA
	UPM Long	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT
	UPM Short	CTAATACGACTCACTATAGGGC
Real time PCR	β -actin-R	CGAAGTCCAGAGCAACATAG
	β -actin-F	GGGCTACTCCTTCACCACCA
	GPCR-R3	CCGTAAAAGCCTCATCAAGACA
	GPCR-F3	TTGGCAGGTGTAAATGAATGG
	PKA-F3	GACCAGCCAATCCAAATCTATG
	PKA-R3	GACCCCATTCCTTCAGGTTTCC

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