

# Specific Expression of *Aplysia* Phosphodiesterase 4 in Bag Cells Revealed by *in situ* Hybridization Analysis

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Phosphodiesterases (PDEs) play a key role in the regulation of cyclic adenosine monophosphate (cAMP), which in turn mediates various cellular functions including learning and memory. We previously cloned and characterized three PDE4 isoforms (ApPDE4) from *Aplysia kurodai*. Using reverse transcription polymerase chain reaction (RT-PCR), we found that ApPDE4 isoforms are primarily expressed in the central nervous system. However, the detailed distribution of ApPDE4 mRNA in *Aplysia* individual ganglions was not evident. In this study, to determine the distribution of ApPDE4 mRNAs in *Aplysia* ganglions, we performed *in situ* hybridization (ISH) using a probe targeting ApPDE4, including the PDE catalytic domain. Interestingly, we found the strongest ISH-positive signals in the symmetrical bag cell clusters of the abdominal ganglion. The R2, R14, L7, L2 and L11 neurons in the abdominal ganglion, LP1 neuron in pleural ganglion, and metacerebral (MCC) neurons were ISH-positive. Mechanosensory neurons of the sensory cluster were also stained on the ventral aspect of the right and left pleural ganglia. Taken together, we found the detailed distribution of ApPDE4 mRNA in *Aplysia* ganglion and support their roles in serotonin (5-HT)-induced synaptic facilitation of *Aplysia* mechanosensory neurons.

**Key words:** *Aplysia*, phosphodiesterase 4, bag cell, *in situ* hybridization

## INTRODUCTION

Phosphodiesterases (PDEs) are key regulators of cyclic adenosine monophosphate (cAMP), an important signaling molecule implicated in various physiological functions including addiction and memory [1-3]. We previously cloned long-, short- and supershort-isoform of *Aplysia* PDE4 (ApPDE4), which

are mammalian PDE4D homologues [4,5]. Overexpression of the supershort ApPDE4 isoform impaired serotonin (5-HT)-induced synaptic facilitation in a depressed synapse [6], and knockdown of the ApPDE4 isoforms impairs 5-HT-induced synaptic facilitation [4,5]. Short- and long-form of ApPDE4 were localized to the plasma membrane and to both the plasma membrane and presynaptic terminals, respectively [4]. We also showed that the 20 N-terminal amino acids of the long-form are sufficient for membrane expression in HEK293T cells and targeting to the presynaptic terminals of *Aplysia* sensory neurons [4,7]. The targeting mediated by the 20 N-terminal amino acids may be due to direct association with the membrane as evidenced by the ability of the first 16 N-terminal amino acids of ApPDE4

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long-form to associate with the membrane through hydrophobic interactions [7]. Additionally, the short-form was found to target the plasma membrane via nonspecific electrostatic interactions, including the membrane affinity of the hydrophobic N-terminal, basic domain, and the interaction between the upstream conserved region 1 (UCR1) and UCR2 domains, located between the extreme N-terminal and the catalytic region [7]. Moreover, oligomerization of the short- or the long-form, mediated through an interaction between the UCR1 and UCR2 domains, further enhanced targeting to the plasma membrane [7]. Thus, the different ApPDE4 isoforms localize to distinct cellular regions and regulate cAMP signaling differently.

Previously, using reverse transcription polymerase chain reaction (RT-PCR) with isoform-specific primer sets, mRNAs of ApPDE4 isoforms were found to be primarily expressed in various tissues including the central nervous system [4]. However, the detailed distribution of ApPDE4s mRNAs in *Aplysia* individual ganglion was unclear. In this study, we performed *in situ* hybridization (ISH) using a probe containing the PDE catalytic domain of ApPDE4 to clearly determine the distribution of all ApPDE4 mRNAs in *Aplysia* ganglia. Interestingly, the strongest ISH-positive signals in the symmetrical bag cell clusters of the abdominal ganglion. Mechanosensory neurons from the sensory cluster also were stained on the ventral surfaces of the right and left pleural ganglia. Our data support an evidence that ApPDE4 is expressed in mechanosensory neurons in *Aplysia* and play key roles in 5-HT-induced synaptic facilitation in *Aplysia* mechanosensory neurons.

## MATERIALS AND METHODS

### Whole-mount *in situ* hybridization

*Aplysia kurodai* was purchased from a local supplier in Yong-Deok and Po-Hang, South Korea, and prior to use, it was maintained in recirculating seawater tanks at 14°C for at least a week. *Aplysia* ganglia were dissected and digested with 1% protease (type IX, Sigma) for 1 h at 34°C. The treated ganglia were trimmed and fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffered saline (PBS). The ganglia were washed several times with artificial seawater (ASW) and de-sheathed. Dehydration of the ganglia was performed using a methanol series followed by rehydration by washing with 0.1% Tween-20 in PBS and 0.3% Triton X-100 in PBS. To increase ganglion permeability, they were treated with proteinase K (10 µg/ml) for 1 h at room temperature. After this reaction, the proteinase was neutralized with glycine (2 mg/ml) diluted in PBS and incubated with 4% tetraethylammonium (TEA)-HCl. After these treatments, the ganglia were washed several times with PBS and

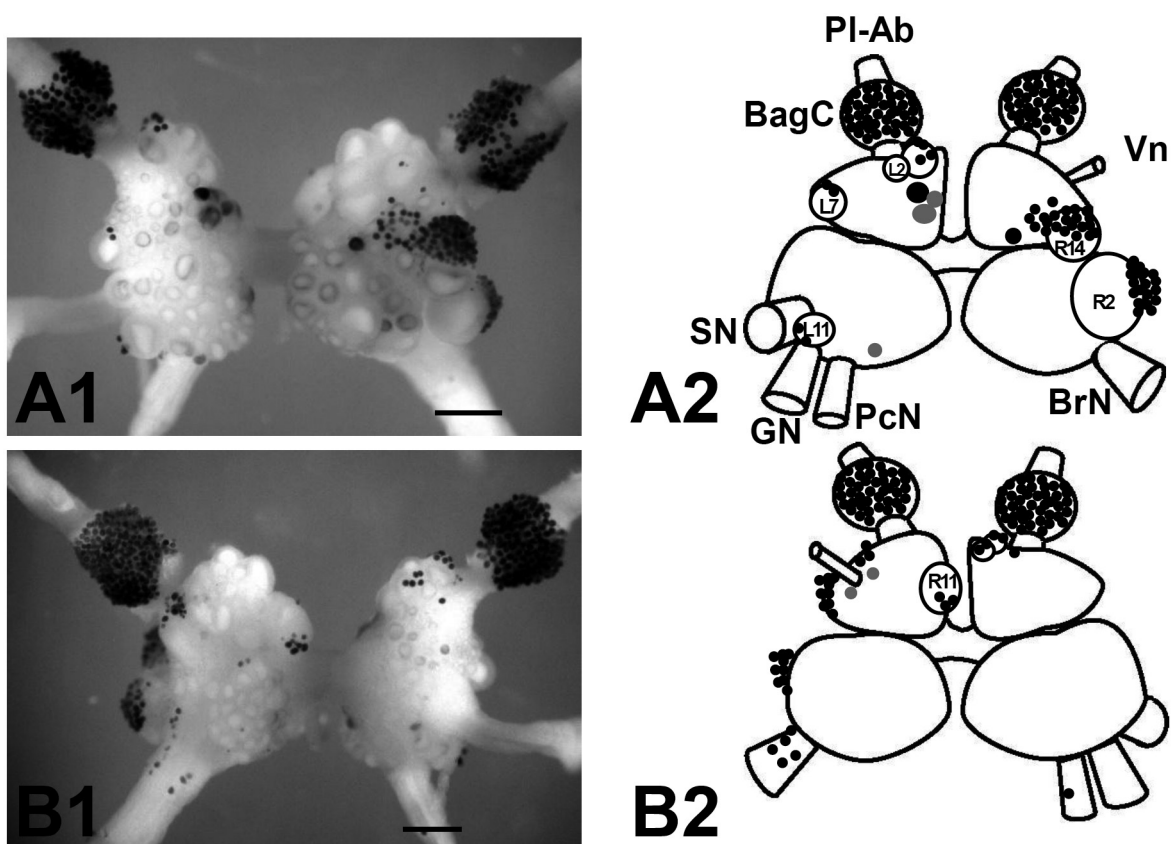
prehybridized in hybridization buffer (50% formamide, 5 mM ethylenediaminetetraacetic acid (EDTA), 5× SSC, 1× Denhardt's solution, 0.1% Tween-20, 0.5 mg/ml yeast tRNA for 8 h at 50°C.

A digoxigenin-labeled ApPDE4 RNA anti-sense or sense probe from the PDE catalytic domain corresponding to 510~842 amino acids of ApPDE4 long-form was synthesized using T7 or T3 RNA polymerase with the ApPDE4 cDNA clone as the template, respectively. The probe was dissolved in hybridization buffer (1 µg/ml), and the ganglia were hybridized overnight at 50°C and then washed at 60°C in washing solution A (50% formamide, 5× SSC, 1% SDS), washing solution B (50% formamide, 2× SSC, 1% SDS), and washing solution C (0.2× SSC).

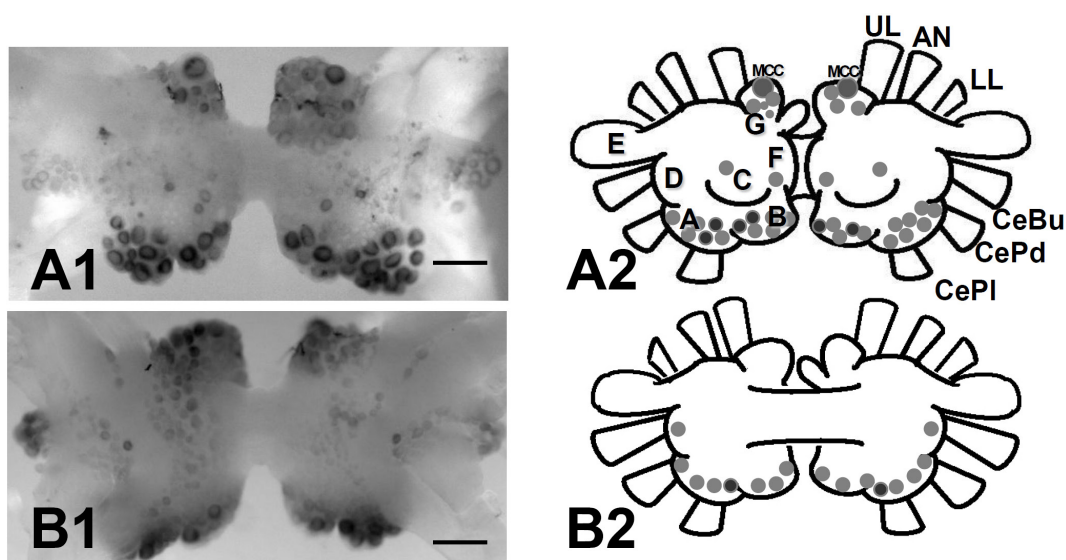
The ganglia were washed with PBS and then blocked for 2 h with 10% normal goat serum in PBS at RT. After blocking, the ganglia were incubated overnight with alkaline-phosphatase-conjugated anti-digoxigenin antibody diluted 1:1500 in PBS containing 1% goat serum at 4°C. These ganglia were rinsed with detection buffer (100 mM NaCl, 50 mM MgCl<sub>2</sub>, 0.1% Tween-20, 1 mM lavamisol, 10 mM Tris-HCl, pH 9.5) and then developed with 4.5 µl of NBT and 3.5 µl of BCIP in 1 ml of detection buffer. The staining reaction was monitored and subsequently stopped with 4% formaldehyde in methanol when the signal became readily apparent. The ganglia were observed and photographed using a microscope (Nikon) attached with a digital camera (Nikon CoolPix 995). We used three animals for each experimental groups and picked the typical one for the presentation.

## RESULTS AND DISCUSSION

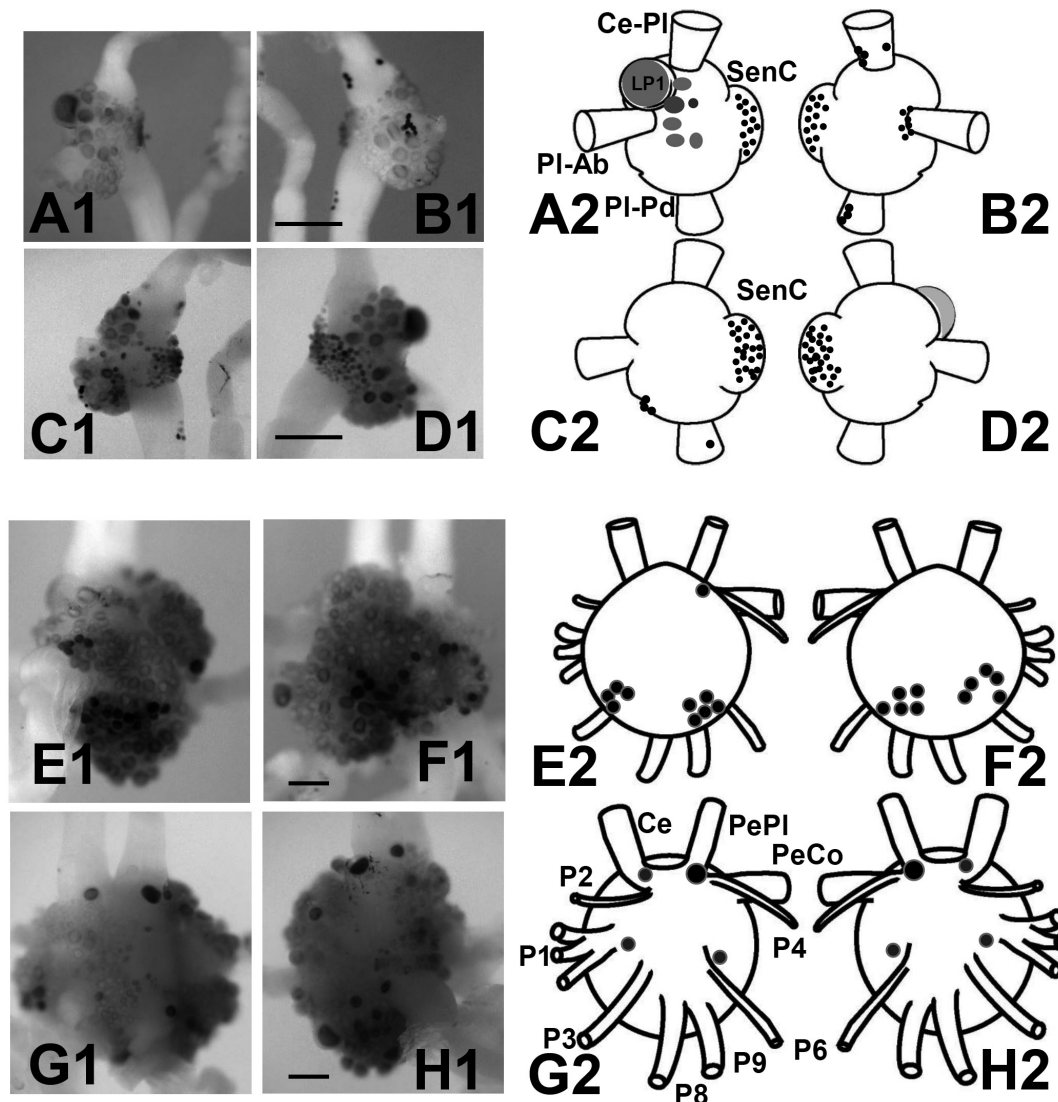
In this study, we examined distributions of all PDE4 mRNA isoforms in *Aplysia* ganglia. Previously, we identified three ApPDE4 isoforms sharing the identical PDE catalytic activity domain [4-7], but it is not clear how many different isoforms exist in *Aplysia*. We thus chose a probe corresponding to the PDE catalytic domain, which is commonly found in all ApPDE4 isoforms. Digoxigenin-labeled ApPDE4 anti-sense RNA was used to detect the ApPDE4 isoforms. Its sense probe was used as a negative control and no signal was detected (data not shown). On the other hands, ISH signals were found in ganglionic nerve cells by ApPDE4 anti-sense probe (Fig. 1-3): the strongest signals in the abdominal ganglion (Fig. 1) and relatively weak signals in other ganglia (Fig. 2 and 3). Notably, symmetrical bag cell clusters were stained most strongly in the abdominal ganglion (Fig. 1-A1 and A2). Other types of neurons including R2, R14, L7, L2, and L11 were stained weakly but significantly on the dorsal surface of the abdominal ganglion. Additional ISH-positive neurons were detected in the medial aspect of the right and left rostral quarter



**Fig. 1.** *In situ* hybridization of ApPDE4 isoforms in *Aplysia* abdominal ganglia. ApPDE4-positive neurons in *Aplysia* ganglia. A1, B1: Dorsal (A1) and ventral (B1) surface of abdominal ganglion. A2, B2: Diagrams summarizing the locations of ApPDE4-positive neurons on the dorsal (A2) and ventral (B2) abdominal ganglia. PI-Ab, pleural-abdominal connection; BagC, bag cell cluster; SN, siphon nerve; GN, gill nerve; PcN, pericardiac nerve; BrN, brachial nerve; Vn, vulvar nerve. C1, D1. Intensity of gray color of circle indicates the signal intensity of ISH-positive neurons. Scale bar 500  $\mu$ m.



**Fig. 2.** *In situ* hybridization of ApPDE4 isoforms in *Aplysia* cerebral ganglia. A1, B1: Dorsal (A1) and ventral (B1) surface of cerebral ganglia. A2, B2: Diagrams summarizing the localization of ApPDE4-positive neurons on the dorsal (A2) and ventral (B2) cerebral ganglia. MCC, metacerebral neurons; UL, upper lip nerve; AN, antenna nerve; LL, lower lip nerve; CeBu, cerebro-buccal connection; CePd, cerebro-pedal connection; CePl, cerebro-pleural connection. Intensity of gray color of circle indicates the signal intensity of ISH-positive neurons. Scale bar 500  $\mu$ m.



**Fig. 3.** *In situ* hybridization of ApPDE4 isoforms in *Aplysia* pleural-pedal ganglia. Dorsal surface of left (A1) and right (B1) pleural ganglia. A2, B2: Diagrams summarizing the localization of ApPDE4 positive neurons on the dorsal left (A2) and right (B2) pleural ganglia. C1, D1: Ventral surface of right (C1) and left (D1) pleural ganglia. C2, D2: Diagrams summarizing the localization of ApPDE4 positive neurons on the ventral right (C2) and left (D2) pleural ganglia. Ce-Pl, cerebral-pleural connection; LP1, LP1 giant neuron; SenC, sensory cluster; PI-Ab, pleural-abdominal connection; PI-Pd, pleural-pedal connection. E1, F1: Dorsal surface of left (E1) and right (F1) pedal ganglia. E2, F2: Diagrams summarizing the localization of ApPDE4 positive neurons on the dorsal left (E2) and right (F2) pedal ganglia. K1, L1: Ventral surface of right (E1) and left (F1) pedal ganglia. E2, F2: Diagrams summarizing the localization of ApPDE4 positive neurons on the ventral right (E2) and left (F2) pedal ganglia. PePl, pedal-pleural connection; Ce, cerebral connection; PeCo, pedal commissure. Intensity of gray color of circle indicates the signal intensity of ISH-positive neurons. Scale bar 500  $\mu$ m.

ganglia. On the ventral surface of the abdominal ganglion, ISH-positive endocrine cells were dispersed around the brachial nerve, the vulvar nerve, and the R11 cell (Fig. 1-B1 and B2). Normally, bag cells have a high resting potential, and they do not show any spontaneous activity. If an electrical stimulus is applied to the pleuro-abdominal connectives (PI-Ab in Fig. 1-A2), the bag cells fire repeatedly for 30 min, performing what is called an “afterdischarge” [8]. During the afterdischarge, the level of cAMP inside bag cells increases [9], thereby leading to modulation of

potassium channels [10,11] and subsequent stimulation of ELH release. Therefore, our data suggest that each ApPDE4 isoform play a different role in modulating ELH release via cAMP regulation, as similar with different roles of ApPDE4 isoforms in *Aplysia* sensory neurons [4-6]. Therefore, our data suggest that each ApPDE4 isoform plays a different role in modulating ELH release via cAMP regulation, as similar with different roles of ApPDE4 isoforms in *Aplysia* sensory neurons [4-6]. Future studies examining the regulatory roles of each ApPDE4 isoform in the afterdischarge in



bag cells are warranted.

ISH-positive cells were also observed in cerebral, pleural and pedal ganglion. First, the dorsal and ventral surfaces of the cerebral ganglion contained clusters positive for ApPDE4s, but the signals were weaker than the bag cells' (Fig. 2). On the dorsal surface of the cerebral ganglion (Fig. 2A), A, B and G cluster cells were stained strongly and symmetrically. Interestingly, metacerebral (MCC) neurons were stained in the G cluster. C, D and F cluster cells were stained less than the other clusters, but the ISH-positive neurons were detected symmetrically on both sides. On the ventral surface of the cerebral ganglion, ISH-positive neurons were observed in the outer layers of the A, B, and G clusters. The serotonergic MCC neurons are involved in food-induced arousal by direct modulation of ingestion-related B21 neurons in the buccal ganglion [12]. The MCC neurons receive long-lasting excitatory synaptic input by nitric oxide (NO) and histamine which induce cAMP-mediated signaling pathways [13]. Therefore, ApPDE4 might play a role in food-induced arousal via indirect pathways such as cAMP-dependent signaling.

Second, we found ISH-positive neurons in each pleural ganglion (Fig. 3A-D). The giant cell, LP1 which was a homologous giant neuron of R2, was stained on the dorsal surface of the left pleural ganglion as similar intensity level with R2 (Fig. 3A and 2A). The LP1 and R2 are cholinergic neurons involved in mucus release from the body wall [14]. This suggests that ApPDE4 has a role in mucus release. Some ISH-positive cells were observed near the pleura-abdominal, cerebro-pleural and pleura-pedal connections of the right pleural ganglion. On the ventral surface of both the pleural ganglia, several mechanosensory neurons from the sensory cluster were stained (Fig. 3C and D). Last, ISH-positive cells were detected in the pedal ganglion and these also showed the symmetrical staining pattern (Fig. 3E-H). On the dorsal surface of both pedal ganglia, ISH-positive neurons were located near the base of P3 and the middle pedal nerve (P9) (Fig. 3E and F). Additional ISH-positive neurons were found on the ventral surface near the pedal-pleural connective, cerebral connective, and P1 nerve in a symmetrical pattern (Fig. 3G and H). Previous study using RT-PCR showed that the short- and long-form of ApPDE4 were strongly expressed in the pleural ganglion, but the supershort-form was weakly expressed [4]. In consistent with our previous results, we found the mRNA expressions of ApPDE isoforms in mechanosensory neurons of pleural ganglion (Fig. 3-C and D). These data suggest that short- and long-form of ApPDE4 in the pleural ganglion have main functions in 5-HT-induced synaptic plasticity in *Aplysia*.

## ACKNOWLEDGEMENTS

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