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2-Arachidonoylglycerol as an Endogenous Cue Negatively Regulates Attachment of the Mussel *Perna viridis*

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Endocannabinoids play important roles in the functioning of various physiological systems in humans and non-mammalian animals, including invertebrates. However, information concerning their roles in physiological functions in members of the phylum Mollusca is scarce. Here the hypothesis that the endocannabinoids are involved in mediating settlement of marine invertebrates was tested. Two endocannabinoids [N-arachidonoyl ethanolamide (AEA) and 2-arachidonoyl glycerol (2-AG)], and two endocannabinoid-like lipids [N-Oleoylethanolamide (OEA) and N-Palmitoylethanolamide (PEA)] were detected in the green mussel *Perna viridis*. In particular, 2-AG was present at significantly higher levels in unattached *P. viridis* compared with attached mussels. The *in vivo* level of 2-AG was inversely correlated with the attachment activity of *P. viridis*. Furthermore, exposure to synthetic 2-AG inhibited attachment of *P. viridis* in a reversible manner. Transcriptomic analysis suggested that up-regulation of 2-AG synthase (Phospholipase C-β, PLC-β) and down-regulation of its degrading enzyme (Monoacylglycerol lipase, MAGL) resulted in higher levels of 2-AG in unattached mussels. A putative mechanism for the negative regulation of mussel attachment by 2-AG is proposed that involves a Ca^{2+}- Nitric oxide (NO)- cyclic guanosine monophosphate (cGMP) pathway. This study broadens our understanding of the evolution and roles of the endocannabinoid system in animals, and reveals an endogenous regulatory cue for mussel attachment.

Keywords: endocannabinoid, 2-AG, mussel, *Perna viridis*, attachment

Abbreviations: 2-AG, 2-Arachidonoyl glycerol; AEA, N-arachidonoyl ethanolamide; AMPAR, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; cGMP, Cyclic guanosine monophosphate; CNRIP1, Cannabinoid receptor-interacting protein 1; DAG, Diacylglycerol; DEG, Differentially expressed genes; DMSO, Dimethyl sulfoxide; FAAH, Fatty acid amide hydrolase; FP1V, Foot protein 1 variant; FPKM, Fragments Per kb per Million reads; FSW, Filtered seawater; $GABA_A$, γ-Aminobutyric acid type A; GPCR, G Protein-Coupled Receptor; GTP, Guanosine-5′-triphosphate; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, Liquid chromatography–mass spectrometry; MAGL, Monoacylglycerol lipase; MAPK, Mitogen-activated protein kinase; mGluR, Metabotropic glutamate receptor; NAAA, N-Acylethanolamine Acid Amidase; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; NO, Nitric oxide; PEA, N-Palmitoylethanolamide; PKA, Protein kinase A; PKC, Protein kinase C; PLC-β, Phospholipase C β; PPARα, Peroxisome proliferator-activated receptor alpha; S1P, Sphingosine 1-phosphate; THC, Δ^9-tetrahydrocannabinol; TRPV1, Transient receptor potential cation channel, subfamily V, member 1; VGCC, Voltage-gated calcium channels.

INTRODUCTION

The plant *Cannabis sativa* has been used by humans for centuries, and is currently the most widely used illicit drug in many countries (Hall et al., 1999; Salhab, 2017). Its use is controversial due to its psychological effects on humans (Bloomfield et al., 2011; Bridgeman and Abazia, 2017). The major psychoactive component of cannabis is Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1964; Wachtel et al., 2002). Studies on the mechanism of action of THC led to the discovery of cannabinoid receptors (Matsuda et al., 1990; Munro et al., 1993; Howlett et al., 2002), which in turn led to the discovery of natural endogenous cannabinoids (Devane et al., 1992; Mechoulam et al., 1995; Mechoulam and Hanuš, 2000). The two most well-studied endocannabinoids are the lipids, N-arachidonoyl ethanolamine (AEA or anandamide) and 2-arachidonoylglycerol (2-AG). The endocannabinoid system has been found to play important roles in the functioning of various physiological systems in humans including the nervous system, the immune system and the cardiovascular system (Montecucco and Marzo, 2012; Cabral et al., 2015; Zou and Kumar, 2018). Interestingly, the endocannabinoid system is also present in non-mammalian animals, including invertebrates (Salzet and Stefano, 2002; McPartland et al., 2006; Silver, 2019; Clarke et al., 2021). This system also occurs in Hydra (De Petrocellis et al., 1999), the earliest animal described to date to have evolved a neural network. Many other invertebrates such as the sea urchin *Paracentrotus lividus*, the mollusc *Mytilus galloprovincialis*, and the annelid *Hirudo medicinalis* were also found to contain endocannabinoids (Thalheim, 1997; Sepe et al., 1998; Matias et al., 2001).

Endocannabinoids are thought to play several roles in invertebrates, including control of reproduction, control of feeding behavior, and neuroimmune modulation (Salzet and Stefano, 2002; Silver, 2019; Clarke et al., 2021). For benthic invertebrates like hydra and mussels, attachment is a critical stage in their natural lifestyle that is important for successful reproduction and dispersal to new habitats. However, the molecular mechanisms that regulate attachment in benthic invertebrates are poorly understood. Investigations into the physiological role of endocannabinoids in invertebrates may provide insights into the functions of small endogenous molecules and lead to a deeper understanding of the evolutionary origins of endocannabinoid systems in higher vertebrates and mammals (Salzet and Stefano, 2002).

Mussels are an important group of bivalves from both ecological and economic perspectives. Mussels such as the blue mussel, *Mytilus edulis*, are an important food resource. Mussel beds, formed by dense aggregations of mussels can be very large and are found in intertidal zones, at deep-sea hydrothermal vents, and at cold seeps (Commito et al., 2008, 2018; Turnipseed et al., 2010). Mussel beds also provide an important habitat for other organisms and have been recognized as ecosystem engineers (Commito et al., 2008, 2018). In contrast, some invasive mussel species such as the zebra mussel *Dreissena polymorpha* have negative impacts on aquatic ecosystems (Karatayev et al., 2015). Mussels are one of the most problematic macrofoulers. They can settle on submerged artificial surfaces such as ship hulls by producing proteinaceous byssal threads. Mussel fouling is particularly troublesome when it is responsible for clogging water pipelines in power plants and water treatment plants (Hosler, 2011; Prescott et al., 2013). To address this serious issue, current antifouling technology commonly uses toxicants that can have adverse effects on the marine environment.

In the search for antifoulants with reduced environmental damage, Angarano et al. (2009) found that exposure to synthetic AEA inhibited zebra mussel byssal attachment in a non-toxic manner. AEA and endocannabinoid-like compounds such as PEA (an endogenous lipid known to mimic several effects of endocannabinoids) have previously been detected in lipid extracts of the mussel *M. galloprovincialis* (Sepe et al., 1998). It has been speculated that exogenous AEA and 2-AG may stimulate nitric oxide (NO) release from immunocytes of *M. edulis* tissues (Stefano et al., 1996, 2000). However, whether the endocannabinoids naturally present in the mussels are involved in the mediation of byssal attachment is unknown. Based on the previous findings of Sepe et al. (1998) and Angarano et al. (2009), it is reasonable to hypothesize that the endocannabinoids may play a role in regulation of mussel attachment.

The green mussel *Perna viridis* is widely distributed as a native species in the Asia-Pacific and Indo-Pacific waters and is an invasive species in Australia, the Caribbean, North America and South America (Benson, 2001; Baker et al., 2007; Stafford et al., 2007; Donaghy and Volety, 2011). This species is a valuable source of seafood in Asia. It is also notorious for fouling power plant pipes, ship hulls, and other submerged artificial structures in large clusters and is used as a model species in antifouling research (Benson, 2001; Yaqin et al., 2014; Basu et al., 2020).

In the natural environment, mussel attachment (which is achieved by byssus threads) can be broken by storms and other physical disturbances (Lee et al., 1990). After detaching from their substratum, the juvenile and adult mussels tend to crawl across surfaces using their foot and reattach to new locations by producing new byssus threads (Kavouras and Maki, 2003; Rajagopal et al., 2005; South et al., 2021). During preliminary studies, we noticed that when attached green mussels of *P. viridis* were cut from their byssus and subsequently cultured in seawater for 24 h, most mussels would reattach by producing new byssus, but there were some individuals that did not secrete any byssus threads and remained unattached. This led to the hypothesis that there may be naturally occurring substance(s) in mussels that mediate the reattachment process and that these mediator(s) may be present in different amounts in the reattached mussels compared to the unattached mussels. We further hypothesized that endocannabinoids (or endocannabinoid-like lipids) could function as such mediator(s) in *P. viridis*. The aims of this study were to test our hypotheses, to improve our understanding of the molecular mechanism underlying mussel attachment, and to provide underpinning knowledge which may be useful in developing environmentally friendly methods for the control of mussel fouling. We measured the levels of endocannabinoids and endocannabinoid-like compounds in the reattached and unattached *P. viridis*, examined the change in the content of 2-AG in *P. viridis* during its attachment, and investigated the

effect of exogenous 2-AG on mussel attachment. Furthermore, comparative transcriptomic analysis of attached and unattached mussels was performed to provide possible clues to the regulation mechanism of 2-AG on mussel attachment.

MATERIALS AND METHODS

Mussels

Specimens of the green mussel *P. viridis* (shell length, 2.1 ± 0.3 cm) were collected from submerged ropes on a fish farm near Dadeng Island, Fujian Province, P.R. China (24°34′N, 118°22′E), during March 2017. In the laboratory, the mussels were held without food in an aquarium with aerated natural seawater (collected from the cost of Xiamen, Fujian Province, P.R. China) for 1–2 days prior to use for assays.

Analysis of Endocannabinoids and Endocannabinoid-Like Lipids in Attached and Unattached *P. viridis*

Green mussels attached with byssus were chosen and their byssus was gently cut off with sharp scissors. Eight of these mussels were randomly chosen and immediately dissected (referred to as Group I). Their shell free tissues were weighed, frozen in liquid nitrogen and stored at −80°C until use for extraction of endogenous cannabinoids. Meanwhile, the other mussels with removed byssus were placed into 12-well plates, with one mussel in each well containing 5 mL 0.22 μm filtered seawater (FSW). After 24 h, all mussels were still alive. Most mussels attached to the surface of wells by secreting byssal threads. A few mussels did not secrete the byssus and remained unattached. Eight mussels were randomly selected from the attached mussels (referred to as Group II), and another eight mussels were randomly selected from the unattached mussels (referred to as Group III). For each group, there were eight replicates, with one mussel considered as a replicate. The mussels were dissected and their shell-free tissues were weighed, frozen in liquid nitrogen and stored at −80°C until use for extraction. A schematic illustration of the sampling for extraction is shown in **Figure 1A**.

The endocannabinoids and endocannabinoid-like lipids were extracted from mussel tissue and analyzed with liquid chromatography–mass spectrometry (LC-MS/MS) by the procedure of Yang et al. (2015) with minor modifications. Briefly, each batch of shell-free mussel tissue from the groups I, II, and III was homogenized, and added to a glass tube. Then, 1 mL solution with 1 nmol heptadecanoyl ethanolamide (C17:1 FAE, as internal standard) in methanol and 1 mL double distilled H_2O were added into each tube, which was subsequently vortexed for 10 s. Then 4 mL chloroform was added into each tube and the tubes were vortexed again for 2 min. After centrifugation at 1,000 × g for 10 min at 4°C, the lipid layer was concentrated by nitrogen flow and subjected to column chromatography using silica gel eluted with the mixed solvent chloroform/methanol (9: 1, V/V). The eluate was dried by nitrogen flow and reconstituted into 0.1 mL chloroform/methanol mixture (1: 3, V/V), and 15 μL of the solution was used for LC-MS/MS.

The analyses were conducted using an Agilent 1100 G1316A HPLC system (Agilent, Shanghai, China) coupled with Mass Spectrometry 3200 Q TRAP (Applied Biosystems, Foster City, CA, United States). An Agilent Eclipse C18 (250 mm × 4.6 mm, 5 μm) column was used and the column temperature was kept at 30°C. The mobile phase for HPLC analysis consisted of methanol and ultra-pure water. The gradient elution, at a flow rate of 1 mL/min, was as follows: 85% methanol for the first 3 min, followed by a linear gradient from 85 to 100% methanol for 2 min and then 100% methanol for 10 min. The elution condition was finally returned to 85% methanol before the next run. The mass spectra were acquired by ion detection at APCI + -MRM mode. Here two endocannabinoids AEA and 2-AG, and two endocannabinoid-like lipids PEA and OEA were analyzed. The molecular ions were monitored at *m/z* 300.20/62.00 for PEA, *m/z* 326.10/62.00 for OEA, *m/z* 348.00/62.00 for AEA, *m/z* 379.10/287.10 for 2-AG, and *m/z* 313.10/62.00 for C17:1 FAE.

Change in the Content of 2-AG in *P. viridis* During Its Attachment

Since the amount of 2-AG was significantly different between the attached and unattached mussels 24 h after having the byssus removed, to further understand how 2-AG is involved in the attachment of *P. viridis*, the change in 2-AG content in mussels over a 24 h period of incubation was measured. Six hundred green mussels were detached by cutting the byssus threads and immediately put into 12-well plates, with one mussel in each well containing 5 mL FSW. These were separated into two groups (A and B, each group with 300 mussels), with group A for observing the reattachment of mussels, and the group B for measuring the change in 2-AG content in mussels during reattachment. During the 24 h incubation period, for group A, the number of the mussels that had reattached was counted at 0.167, 0.5, 1, 1.5, 2, 4, 6, 12, and 24 h after being put into the wells. Three replicates were set up, with each replicate including 100 mussels. The new attachment rate of the mussels at each time point was calculated by formula (1). At each time point, for group B, three mussels (with one mussel as a replicate) were randomly selected, frozen in liquid nitrogen and stored at −80°C until used for extraction of 2-AG. The extraction and LC-MS/MS analysis of 2-AG were performed as described above.

$$R_t = (A_t - A_{t-1})/100 \times 100\%, \quad (1)$$

where R_t: the new attachment rate of mussels at certain time point; A_t: the number of attached mussels at a certain time point; and t: time points ($t_0 = 0$, $t_1 = 0.167$, $t_2 = 0.5$, $t_3 = 1$, $t_4 = 1.5$, $t_5 = 2$, $t_6 = 4$, $t_7 = 6$, $t_8 = 12$, and $t_9 = 24$ h).

Effect of Exogenous 2-AG on Attachment of *P. viridis*

To further investigate the effect of 2-AG on attachment of *P. viridis*, mussels were exposed to synthetic 2-AG and their response observed. This attachment assay was performed following the method described in Angarano et al. (2009) with minor modification. The tested mussels were detached by removing their byssus. The 2-AG (Sigma-Aldrich, United States)

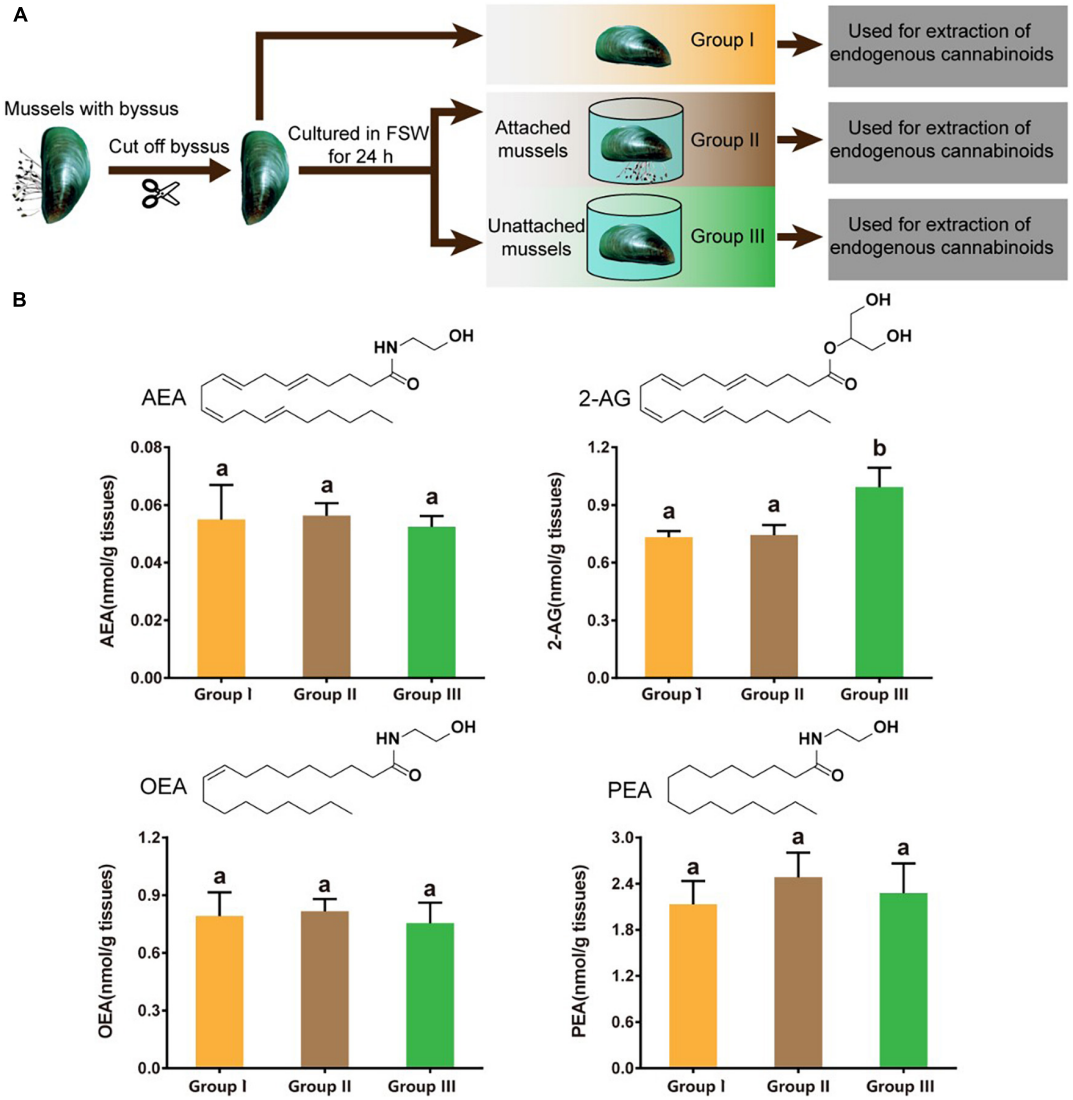

FIGURE 1 | 2-AG is present at significantly higher levels in unattached *P. viridis*. **(A)** Schematic illustration of the sampling for extraction of endogenous cannabinoids. **(B)** Comparison of amounts of AEA, 2-AG, OEA, and PEA in three groups of *P. viridis*. Group I: Mussels with the byssus removed. Group II: Mussels that had the byssus removed and that were then incubated in FSW for 24 h and reattached. Group III: Mussels with byssus removed, then incubated in FSW for 24 h and which remained unattached. Data shown are means + SE of replicates (n = 8). Different letters above columns indicate significant differences among groups ($P < 0.05$).

was dissolved in dimethyl-sulfoxide (DMSO). A total of 25 μL 2-AG solution and 4.975 mL FSW were added into each well (12-well plates), after which one mussel was put into each well. The final test concentrations of 2-AG were 10, 20, 50, and 100 μM. The FSW control and the solvent control (0.5% DMSO in FSW, V/V) were used in this experiment. There were three replicates for each treatment. Each replicate included 12 mussels, with one mussel in one well of a 12-well plate. After a 24 h exposure period, the numbers of mussels attached, or alive but unattached were counted. To test whether 2-AG had a residual effect on *P. viridis*, after the above 24 h exposure period, the attached mussels were gently detached by cutting off the byssus threads with sharp scissors, and washed with FSW. The living but unattached mussels were also washed with FSW. Then, these mussels were put into new 12-well plates, with each well containing 5 mL FSW and one mussel. After 24 h of the post-exposure incubation, the numbers of mussels attached or alive but unattached were counted. The number of byssus threads produced by each mussel was also counted (Winkle, 1970; Rajagopal et al., 2003). No dead mussels were observed in this bioassay. The viability of mussels was tested following Matthews and McMahon (1999).

RNA Extraction and Transcriptome Sequencing

To investigate the events that occur upon detachment, we sequenced transcripts of *P. viridis* under two states: individuals of *P. viridis* that attached at 24 h after being detached by

removing their byssus threads (AT group), and mussels that remained unattached at 24 h after being detached by removing their byssus threads (UAT group). Each group had three replicates, each replicate containing RNA from three mussels. Total RNA was extracted from shell-free tissues of mussels from the AT and UAT groups using TRIzol® Reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. The purity of the RNA was checked using a NanoDrop 2,000 spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, United States). The concentration of RNA was then measured using Qubit® RNA Assay Kit by a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). The integrity of RNA was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2,100 system (Agilent Technologies, Santa Clara, CA, United States). The cDNA library construction and transcriptome sequencing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, United States) following the manufacturer's instructions. Sequencing was carried out using an Illumina Hiseq 4000 platform.

Transcriptome Assembly and Annotation

Raw reads generated using the Illumina Hiseq 4000 platform were filtered to remove adaptors, reads with unknown bases (N) > 10%, and low-quality reads (those consisting of more than 50% bases whose quality scores were $\leq$ 20) to achieve clean reads. The clean reads were used for *de novo* assembly of the transcriptome using Trinity (Grabherr et al., 2011). All of the unigenes were annotated against several databases: Nr (NCBI non-redundant protein sequences) and Swiss-Prot using diamond v0.8.22 with *e*-value = 1e-5 (Buchfink et al., 2015); KOG (Eukaryotic Orthologous Groups) with *e*-value = 1e-3; Nt (NCBI non-redundant nucleotide sequences) using NCBI blast v2.2.28 + with *e*-value = 1e-5 (Altschul et al., 1997); KO (KEGG Ortholog database) using KAAS r140224 with *e*-value = 1e-10 (Moriya et al., 2007); and Gene Ontology (GO) using Blast2GO with *e*-value = 1e-6 (Stefan et al., 2008).

Analysis of Differentially Expressed Genes (DEGs)

The clean reads were mapped to unigenes using RSEM (Li and Dewey, 2011), and the expression levels of unigenes were calculated using the Fragments Per Kilobase per Million reads (FPKM) method (Trapnell et al., 2010). DEGs between the AT and UAT groups were identified using DESeq v1.10.1 (Anders and Huber, 2010). Genes with *p*-value $\leq$ 0.05 and the absolute value of $\log_2$ (fold change) $\geq$ 1 were considered differentially expressed. GO enrichment analysis of DEGs was performed using GOseq v1.10.0 (Young et al., 2010). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs was performed using KOBAS v2.0.12 (Mao et al., 2005). GO terms and KEGG pathways with *p*-value $\leq$ 0.05 were considered to be significantly enriched.

Validation of Differentially Expressed Genes by Quantitative Real-Time PCR (qRT-PCR)

To confirm the transcriptome results, qRT-PCR analysis was performed for six unigenes (**Supplementary Table 1**). FP1V-1, FP1V-2, and FP4V-1 are foot proteins involved in mussel attachment. PLC-β and MAGL are important enzymes in metabolism of 2-AG. PKA is involved in the retrograde endocannabinoid signaling pathway. To generate single-stranded cDNA, 1 μg of total RNA sample was reverse-transcribed using the PrimeScript™ RT reagent Kit (TaKaRa, Shanghai, China). qRT-PCR was performed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) with a FastStart Universal SYBR® Green Master Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The qRT-PCR program was as follows: one cycle of 95°C for 5 min; and 40 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 30 s; followed by a melting curve analysis. The qRT-PCR reactions were performed in triplicate for each gene in each sample and there were three biological replicates. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method and normalized to the expression of 18S rRNA. The gene-specific primers used in the qRT-PCR were designed using Primer premier v5.0 (**Supplementary Table 1**).

Statistical Analysis

The results were analyzed with SPSS 18.0 software. In the assay measuring the levels of endocannabinoids and endocannabinoid-like lipids in mussels and the assay testing exogenous 2-AG on mussel attachment, one-way analysis of variance (ANOVA) was performed with Tukey's *post hoc* test for analysis of significant differences between groups or treatments. In the qRT-PCR assay, Student's *t*-test was used to evaluate the statistical significance between two data sets. The concentration of 2-AG that inhibited attachment by 50% relative to the control (EC_{50}) was calculated using GraphPad Prism software, Version 6.01.

RESULTS

AEA, 2-AG, OEA, and PEA in Attached and Unattached *P. viridis*

AEA, 2-AG, OEA, and PEA were all detected in *P. viridis*, with PEA being most abundant (**Figure 1B**). In the mussels of group I that had just had their byssus threads cut off, AEA, 2-AG, OEA, and PEA were present at the following concentrations (means ± SE): 0.055 ± 0.012, 0.732 ± 0.032, 0.773 ± 0.122, and 2.131 ± 0.304 nmol/g, respectively, indicating the background levels of these lipids in normal *P. viridis*. At 24 h after removing the byssus, most mussels reattached by secreting new byssus threads and these mussels (group Π) showed similar amounts of these four compounds as in the mussels of group I. However, in the mussels that remained unattached 24 h after their byssus was removed (group III), the amount of 2-AG was significantly higher than in the groups I ($P = 0.032$) and Π ($P = 0.042$), although for AEA, OEA, and PEA, there was no significant difference between

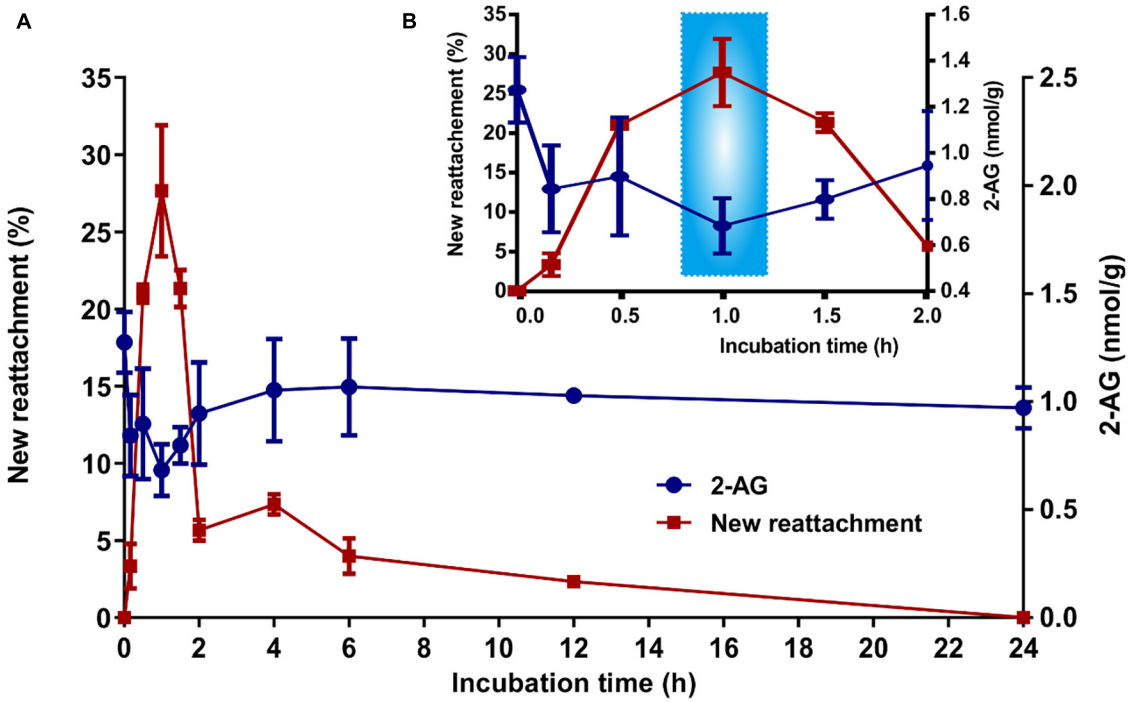

FIGURE 2 | Changes in the content of 2-AG in *P. viridis* during attachment. The level of 2-AG in *P. viridis* is inversely correlated with reattachment activity. **(A)** Changes in *P. viridis* reattachment and content of 2-AG during the first 24 h of incubation after removal of the byssus. **(B)** The enlarged view during the first 2 h. Data shown are means ± SE of replicates (n = 3).

these three groups (**Figure 1B**). This suggests that 2-AG may play a role in mediating mussel attachment.

2-AG Levels in *P. viridis* Changed During Attachment

As shown in **Figure 2**, during 1 h after the byssus was removed, there was an increase in reattachment activity, and the amount of 2-AG decreased. The highest new attachment rate was found at 1 h. It is noteworthy that the lowest level of 2-AG was also found at 1 h. For the 1–2 h period, the reattachment activity decreased, but the amount of 2-AG increased. At 2 h, 79.0% of the mussels had reattached, and following this the reattachment activity remained low. Correspondingly, 2-AG levels remained stable level after 2 h. The finding that the amount of 2-AG decreased when *P. viridis* was active at attachment was consistent with the finding that attached *P. viridis* contained a lower level of 2-AG compared to unattached organisms (**Figure 1B**).

Effect of Exogenous 2-AG on Attachment of *P. viridis*

Attachment of *P. viridis* was significantly inhibited in the presence of exogenous 2-AG at a concentration of 100 μM when compared to the solvent control (P = 0.003, **Figure 3A**). The EC_{50} value of 2-AG for mussel attachment was 80.67 μM. Although there was no significant difference in attachment percentage between the treatment of 50 μM 2-AG and the solvent control (P > 0.5), the byssus thread production of *P. viridis* decreased significantly in the treatment of 50 μM 2-AG when compared with the solvent control (P = 0.004, **Figure 3B**), indicating an inhibitory effect on byssus thread production at this concentration. The EC_{50} value of 2-AG for byssus thread production was 46.29 μM. Exposure to 2-AG had no obvious residual effect on mussel attachment, because when 2-AG was removed, almost all mussels in each treatment group attached during the 24 h post-exposure period, with the percentage of attached mussels not significantly different from the control (**Figure 3C**). A similar effect was observed for byssus thread production (**Figure 3D**). This suggests that 2-AG inhibits attachment and byssus thread production in *P. viridis* in a non-toxic manner (which is consistent with the observation that there was no mussel mortality over both the exposure and post-exposure periods in all treatments), and that this inhibitory effect is reversible.

Transcriptomic Analysis of Attached and Unattached *P. viridis*

The results of transcriptome sequencing and assembly are shown in **Supplementary Tables 2, 3**. All of the RNA-seq data have been deposited in the NCBI Short Read Archive (SRA) under accession number PRJNA601624. For functional annotation, all unigenes were annotated against databases including Nr, Swiss-Prot, Nt, KOG, KO and GO, with 68,446 unigenes annotated (**Supplementary Figure 1**). Specifically, the genes involved in endocannabinoid signaling were found to be

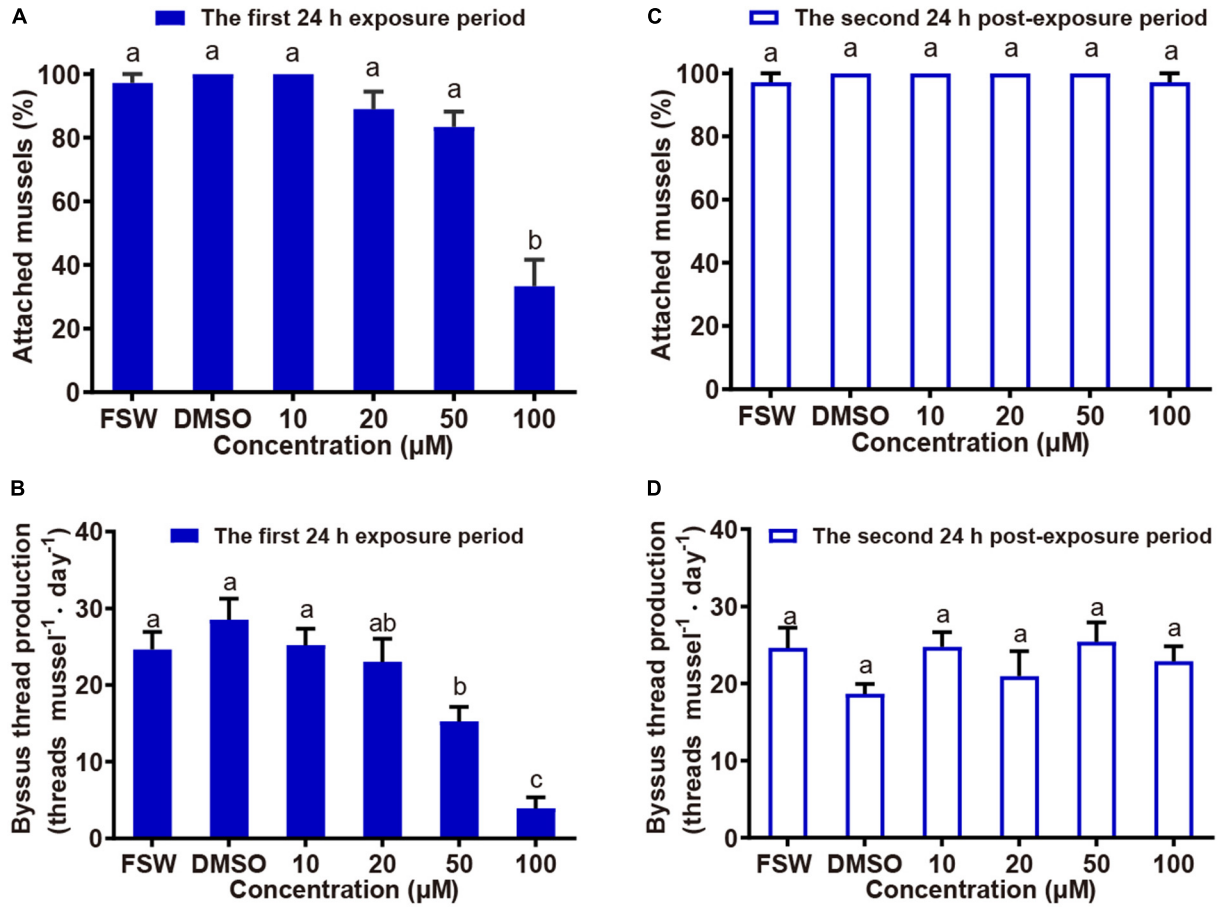

FIGURE 3 | Effect of exogenous 2-AG on attachment of *P. viridis*. Attachment and byssus thread production of mussels following exposure to 2-AG for a 24 h period (exposure effect) and in the absence of 2-AG in the subsequent 24 h post-exposure period (residual effect). FSW: The FSW control. DMSO: The solvent only control (0.5% DMSO in FSW). Different letters above columns indicate significant differences ($P < 0.05$). Data shown are means + SE of replicates ($n = 3$ for attachment percentage in **(A,C)**; $n = 36$ for the number of byssus threads in **(B,D)**.

present in *P. viridis* (**Table 1**). The genes encoding enzymes for 2-AG synthesis (PLC-β and diacylglycerol lipase, DAGL) and degradation (MAGL), enzymes for AEA, OEA, and PEA synthesis (N-acyl phosphatidylethanolamine phospholipase D, NAPE-PLD) and their degradation (Fatty acid amide hydrolase, FAAH and N-Acylethanolamine acid amidase, NAAA) were annotated here. The genes for proteins involved in the retrograde endocannabinoid signaling pathway, including metabotropic glutamate receptors (mGluRs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptor, AMPAR), Voltage-gated calcium channels (VGCC), γ-Aminobutyric acid type A ($GABA_A$), Mitogen-activated protein kinase (MAPK), Protein kinase A (PKA), and Protein kinase C (PKC) were also annotated. Although the expression of two well-known vertebrate cannabinoid receptors 1 and 2 (CB1 and CB2, two G-protein-coupled receptors) was not found in the present transcriptomic data of *P. viridis*, the genes for TRPV1 and PPARα, which can also interact with endocannabinoid or endocannabinoid-like compounds, and the gene for CNRIP1 (or CRIP1), which is a regulator of CB1 signaling, were found among the annotated genes (**Table 1**). These findings, combined with the above discovery of the presence of endocannabinoids in *P. viridis*, indicate the presence of an endocannabinoid system in *P. viridis*.

In total, 3,380 differentially expressed genes (DEGs) were identified between attached and unattached *P. viridis*, including 1,637 up-regulated DEGs and 1,743 down-regulated DEGs in the unattached group compared with the attached group (**Figures 4A,B**). The results of GO and KEGG enrichment analyses of DEGs are shown in **Supplementary Figure 2**. Particular attention was paid to DEGs between attached and unattached *P. viridis* that were related to the metabolism and action of 2-AG (**Table 1**). As shown in **Table 1**, the expression level of PLC-β which is involved in synthesis of 2-AG, was significantly up-regulated ($P = 0.001178$), and the transcription level of MAGL which is involved in degradation of 2-AG, was down-regulated ($P = 0.077479$). This indicates a possible mechanism for why unattached mussels had higher levels of 2-AG than attached mussels. Furthermore, it was noteworthy that there were three DEGs (mGluRs, AMPAR, and VGCC) enriched in the pathway of retrograde endocannabinoid signaling in the unattached *P. viridis* (**Table 1**).

TABLE 1 | List of annotated genes involved in endocannabinoid signaling.

Transcript ID	log2 ratio	*P*-value	Gene description	Gene name
Cluster-9497.109380	**Inf**	**0.001178**	**Phospholipase C beta**	**PLC-β**
Cluster-9497.18690	−1.9829	0.11894	Sn1-specific diacylglycerol lipase beta-like isoform X1	DAGL
Cluster-9497.102202	−1.3094	0.077479	Monoacylglycerol lipase abhd6-B-like	MAGL/ ABHD6
Cluster-1785.2	0.18486	0.76466	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D-like isoform X1	NAPE-PLD
Cluster-40913.1	1.4829	0.15791	Fatty-acid amide hydrolase	FAAH
Cluster-9497.62394	−0.63014	0.28658	N-acylethanolamine-hydrolyzing acid amidase	NAAA
Cluster-9497.63247	**Inf**	**0.002208**	**Ionotropic glutamate receptor activity/metalloendopeptidase activity**	**AMPAR**
Cluster-9497.107242	**−3.6923**	**0.001129**	**Metabotropic glutamate receptor 3-like**	**mGluRs**
Cluster-9497.71136	**−1.7318**	**0.008598**	**Voltage-dependent calcium channel**	**VGCC**
Cluster-9497.65478	−0.41028	0.74213	GABAA receptor -associated protein	GABAa
Cluster-9497.83566	0.027224	0.96309	p38 MAPK protein mRNA, complete cds	MAPK
Cluster-9497.97822	1.539	0.10376	cAMP-dependent protein kinase catalytic subunit	PKA
Cluster-42035.0	1.5283	0.17465	PKC- activated protein phosphatase-1 inhibitor	PKC
Cluster-9497.6560	**−5.2092**	**0.027036**	**Transient receptor potential cation channel subfamily V member 1**	**TRPV1**
Cluster-9497.88713	**2.495**	**0.021773**	**Peroxisome Proliferator Activated Receptor Alpha**	**PPARα**
Cluster-9497.46776	−0.099552	0.88672	CB1 cannabinoid receptor-interacting protein 1-like	CNRIP1
Cluster-9497.108668	**Inf**	**0.008698**	**Nitric Oxide Synthase, brain-like isoform X2**	**NOS**

The differentially expressed genes (DEGs) are shown in bold.

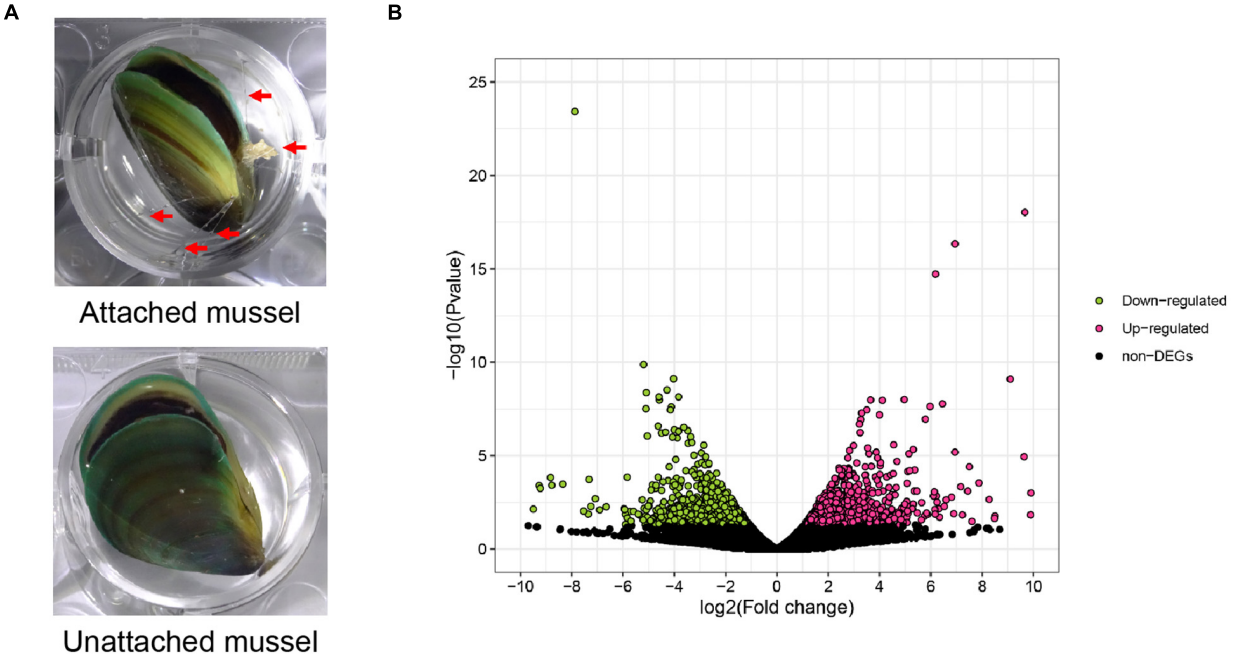

FIGURE 4 | Transcriptomic analysis of attached and unattached *P. viridis*. **(A)** The mussels attached 24 h after byssus thread removal (AT group for transcriptome analysis) and the mussels unattached 24 h after byssus thread removal (UAT group for transcriptome). Red arrows indicate byssus attachment sites. **(B)** Volcano plots of differentially expressed genes between AT and UAT mussels. Red and green spots indicate the up-regulated expressed genes and the down-regulated expressed genes between the AT and UAT groups, respectively. Black spots indicate genes that were not differentially expressed.

Validation of the transcriptome data using qRT-PCR was performed with six unigenes [FP1V-1 (Foot protein 1 variant), FP1V-2, FP4V-1, MAGL, PLC-β, and PKA]. As shown in **Supplementary Figure 3**, the transcription profiles of all six selected unigenes as determined by qRT-PCR were consistent with those determined by RNA-seq, confirming the reliability and reproducibility of the transcriptome data.

DISCUSSION

Mussel attachment is critical for the formation of mussel beds, thus underpinning the propagation and persistence of mussel population in the marine ecosystem (Commito et al., 2014). Many chemical cues, including exogenous factors (or environmental chemical cues, such as metabolites from conspecifics and

bacteria) and endogenous factors (such as catecholamines, serotonin, and acetylcholine) have been suggested to promote mussel attachment (Li et al., 2014; Morello and Yund, 2016; Joyce and Vogeler, 2018). Most of these studies on endogenous cues used exposure assays with synthetic compounds to confirm the effect of chemical cues on mussel attachment. However, little information is available concerning the relationship between the level of endogenous cues in the mussels and their attachment activity. Here, the strong inverse relationship found between the levels of 2-AG in *P. viridis* and its attachment behavior suggests that 2-AG may function as a negative regulator of mussel attachment. Interestingly, an inverse correlation between the *in vivo* level of 2-AG and animal behavior has also been reported in mice (Chevalier et al., 2020), wherein the effect is manifested as a depressive-like behavior.

The understanding of negative cues of mussel attachment is far less advanced than the study of inducing cues. The exposure assay carried out here found that by adding exogenous 2-AG, attachment of *P. viridis* could be inhibited, demonstrating the negative effect of 2-AG on mussel attachment. Consistent with these findings are the observations that structural analogs of 2-AG, including AEA, linoleyl ethanolamide, and noladin ether, inhibit byssal attachment in a different subclass of mussel (in *Dreissena polymorpha*) (Angarano et al., 2009), indicating that endocannabinoids might also inhibit attachment in other mussels.

Transcriptomic sequence data indicated the existence of several endocannabinoid synthesizing and degrading enzymes in *P. viridis*. There are very few studies of endocannabinoid metabolic enzymes in molluscs, only the FAAH-like enzymatic activity has been reported in *Mytilus galloprovincialis* (Sepe et al., 1998). This study confirmed the presence of the gene for FAAH in molluscs. Furthermore, the present transcriptomic analysis on metabolic enzymes suggests a possible underlying mechanism for the higher level of 2-AG in unattached mussels. Expression of PLC-β was significantly up-regulated in unattached mussels. PLC-β plays a pivotal role in catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to yield inositol trisphosphate (IP3) and diacylglycerol (DAG) (Kadamur and Ross, 2013). DAG can be further hydrolyzed by DAGL to produce 2-AG (Kano et al., 2009). In other words, PLC-β is important for the synthesis of 2-AG. In contrast, expression of MAGL was down-regulated in unattached mussels (with a *P*-value of 0.077479, near to 0.05). MAGL is an important enzyme in degradation of 2-AG to arachidonic acid (AA) and glycerol (Lambert et al., 2010; Savinainen et al., 2012). We therefore suggest that the up-regulation of the synthase and down-regulation of the degrading enzyme for 2-AG results in increased concentrations of 2-AG in unattached mussels.

The discovery of the endocannabinoid system in mammals also raises interest in its evolutionary origins (Elphick and Egertova, 2001). The endocannabinoid system is generally suggested to consist of endocannabinoids, enzymes responsible for their synthesis and degradation, and cannabinoid receptors (Buznikov et al., 2010). The endocannabinoids (such as AEA and 2-AG) and their metabolic enzymes have been found in various species of non-mammalian vertebrates and invertebrates, indicating they are phylogenetically widespread (Soderstrom, 2009; Elphick, 2012). For cannabinoid receptors, genes encoding the mammalian CB1/CB2 receptors have been identified in non-mammalian vertebrates (Elphick and Egertová, 2009; Elphick, 2012), the urochordate *Ciona intestinalis* (Elphick et al., 2003), and the cephalochordate *Branchiostoma floridae* (Elphick, 2007) (the two invertebrate species are closely related to the vertebrates). However, CB1/CB2 receptor-like genes have not been found in non-chordate invertebrates. Consistent with this, the present transcriptome sequencing of the mussel *P. viridis* did not reveal any CB1/CB2 genes. Nevertheless, investigations using radiolabeled cannabinoids revealed the presence of specific binding sites for cannabinoids in non-chordate invertebrate species such as the sea urchin *Strongylocentrotus purpuratus* (Chang et al., 1993), the leech *Hirudo medicinalis* (Stefano et al., 1997), the mussel *M. edulis* (Stefano et al., 1996), and the cnidarian *Hydra vulgaris* (De Petrocellis et al., 1999), indicating the existence of non-CB1/CB2-type cannabinoid receptors. As Elphick and Egertova (2001) suggested, not all cannabinoid receptors are necessarily orthologs of vertebrate CB1/CB2 genes.

Phylogenetic analysis of the relationship of vertebrate CB receptors with other GPCRs suggested that CB receptors, lysophospholipid receptors, melanocortin receptors and adenosine receptors have evolved from a common ancestral gene (Elphick and Egertova, 2001). Yin et al. (2009) found that AEA and 2-AG showed weak agonist activity at the lysophospholipid S1P1 receptor in *Mus musculus*. Here the finding of the genes for the S1P receptor and adenosine receptor in *P. viridis* implied the possibility of these receptors as primitive cannabinoid receptors in mussels, although this would need to be verified experimentally. In addition, recent studies have found that besides CB1 and CB2, other proteins including PPARα and TRPV1 can be activated by endocannabinoid or endocannabinoid-like compounds (Muller et al., 2019). PPARα was found to bind with OEA and then activate the downstream transcriptional activity in *Mus musculus* (Sun et al., 2007). Yuan and Burrell (2013) discovered that by activating TRPV-type receptors, 2-AG mediates retrograde synaptic signaling in the nervous system of the leech *H. medicinalis*. The present finding of the gene for TRPV1 in *P. viridis* suggests the possibility of 2-AG binding with TRPV1 to mediate mussel attachment.

It has been reported that endocannabinoids serve as retrograde messengers in vertebrates (Kreitzer and Regehr, 2002; Ohno-Shosaku et al., 2012). Released endocannabinoids can activate the receptors at presynaptic terminals and suppress the release of neurotransmitters by inhibiting Ca^{2+} channels (Twitchell et al., 1997; Di Marzo, 1998; Diana and Marty, 2004). The CB1 receptor has been reported to be able to suppress Ca^{2+} influx via VGCC (Zou and Kumar, 2018). Ca^{2+} has been previously suggested to play an important role in settlement of marine invertebrates, including the barnacle *Balanus amphitrite* (Clare, 1996), the sea urchin *Strongylocentrotus purpuratus* (Amador-Cano et al., 2006), and the oyster *Ostrea edulis* (Smyth et al., 2018). The suppression of Ca^{2+} influx by receptor activation and the significant down-regulation of VGCC in unattached *P. viridis* could lower intracellular Ca^{2+} in unattached *P. viridis*. Ca^{2+} regulates NO synthase (Schmidt et al., 1993).

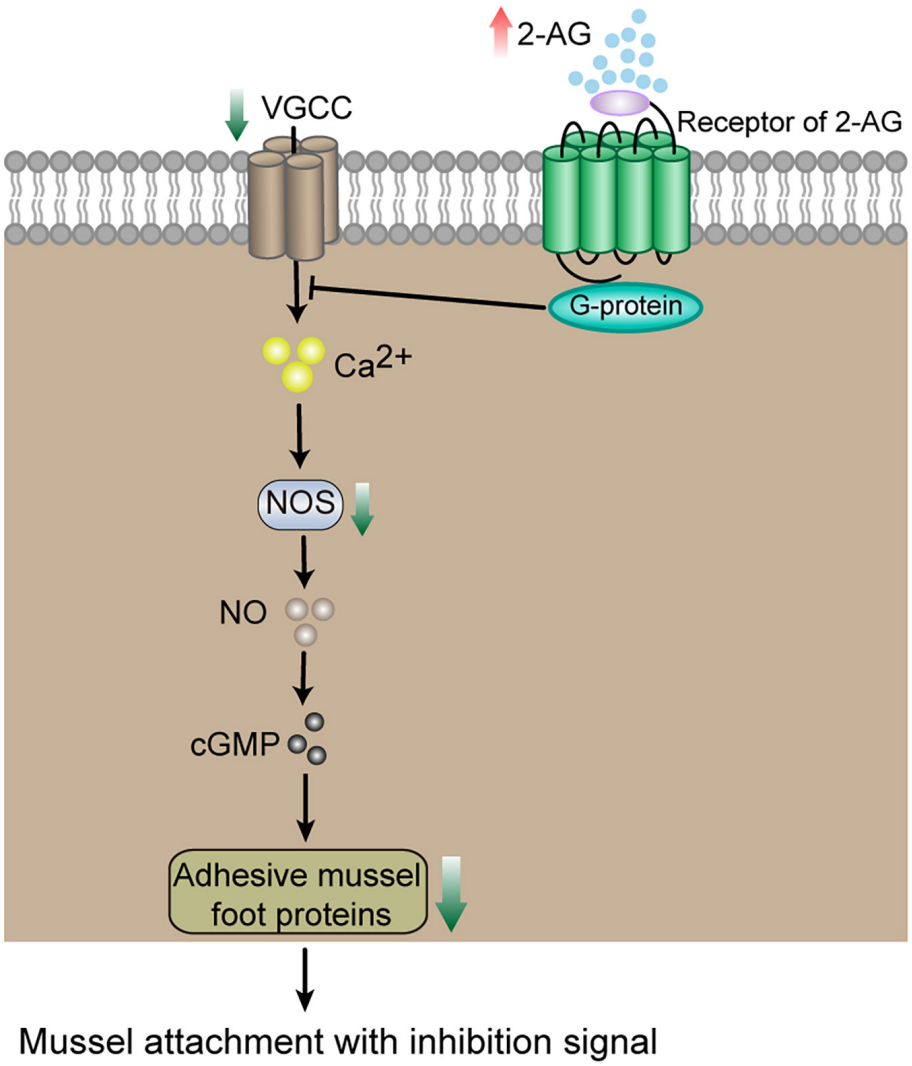

FIGURE 5 | A suggested pathway for the inhibitory effect of 2-AG on mussel attachment based on transcriptomic data. Red arrows indicate higher levels. Green arrows indicate lower levels.

Furthermore, significant differences in expression of the NO synthase gene were found between attached and unattached *P. viridis* (down-regulation in unattached mussels, **Table 1**). NO synthase can convert L-arginine to NO, a diffusible gaseous signaling molecule in many organisms (Tejero and Stuehr, 2013). It is noteworthy that the NO signaling system has been previously reported to regulate settlement of many marine invertebrates (Bishop and Brandhorst, 2003; Say and Degnan, 2020; Zhu et al., 2020). In the barnacle *B. amphitrite* and the sponge *Amphimedon queenslandica*, it was suggested that NO regulates their settlement through cGMP signaling, in which NO activates Guanylate cyclase (GC); GC is converted to Guanosine-5′-triphosphate (GTP) and then to the important second messenger cGMP, which mediates downstream effectors (Lucas et al., 2000; Zhang et al., 2012; Sahoo and Khandeparker, 2018; Say and Degnan, 2020). The NO-cGMP pathway may also play a regulatory role in settlement of *P. viridis*. Based on the above analysis, one possible explanation for the observed inhibition of attachment is suggested (**Figure 5**).

The occurrence of the endocannabinoid system has been suggested in different phyla of animals from *Hydra* to humans, indicating its fundamental importance to animals (Elphick and Egertova, 2001; Soderstrom, 2009). Investigation of this system in invertebrates may therefore shed light on important questions such as how the endocannabinoid system evolved, the roles of this system are in controlling behavior in non-mammalian animals, and what clues it can provide for the neurophysiology of humans (Salzet and Stefano, 2002). As the review by Elphick (2012) pointed out, there has been relatively little investigation of the endocannabinoid system in molluscs. The present work suggests the existence of a functional endocannabinoid system in *P. viridis*, which provides a basis for deeper investigation. For example, the question of whether endocannabinods are also involved in regulating settlement in other marine benthic invertebrates.

CONCLUSION

The present results suggest that the endocannabinoid system might be present in *P. viridis* and in particular, 2-AG is an endogenous cue for mediating attachment of *P. viridis*. The up-regulation of the synthase (PLC-β) and down-regulation of the degrading enzyme (MAGL) for 2-AG resulted in a higher level of 2-AG in unattached mussels. A potential mechanism for the negative regulation of 2-AG on mussel attachment via the Ca^{2+}-NO-cGMP pathway is proposed. Our study reveals an endogenous cue for mediating mussel attachment, thereby enhancing our understanding of the attachment mechanism of mussels. The findings herein may also be of relevance for controlled enhancement of settlement, for example in aquaculture of mussels, and for the prevention of attachment, for example in the development of new antifouling strategies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

QD, Y-QS, and D-QF designed the research. QD, Z-XW, Y-QS, and PS conducted the experiments. Z-WW and YQ performed the data analysis. QD, Z-XW, and D-QF wrote the manuscript. C-HK and D-QF supervised the project and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.719781/full#supplementary-material

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