Targeting cyclic nucleotide phosphodiesterases as potential nematicides using chemical and molecular agents

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Introduction

Phytoparasitic nematodes are responsible for ~$100B in annual crop losses. Current chemical controls are only partially effective and pose significant adverse consequences for human health and the environment. Cyclic nucleotide signaling pathways are critical for regulating nematode behavior and developmental progression, but the phosphodiesterases (PDEs) and cyclases responsible for regulating many of these physiological processes are poorly characterized. Work from our laboratory (Schuster et al., 2019) has determined that six PDE genes are present not only in *C. elegans* but throughout the nematode phylum that are orthologous to vertebrate PDEs (Table 1). Structural and pharmacological comparisons of *C. elegans* and human PDE4 catalytic domains demonstrated that a non-specific PDE inhibitor bound equally well to both enzymes, whereas compounds selective for human PDE4 bound with lower affinity to nematode PDE4 due to differences in the amino acid residues lining the inhibitor binding pocket (Schuster et al., 2019). This work supports the feasibility of designing compounds specifically targeting parasitic nematode PDEs without adverse effects on vertebrate animals or agricultural crops (Cote et al., 2023).

To further evaluate nematode PDEs as nematicidal targets using chemical or genetic controls, we utilized *C. elegans* to generate transgenic strains containing an ablated PDE gene as well as employing interfering RNA (RNAi) to knock down expression of *C. elegans pde-1* through *pde-6*. We conducted phenotypic screens of each knockout strain in the absence or presence of RNAi to evaluate the physiological and behavioral consequences of disrupting one or multiple nematode PDEs.

C. elegans PDE gene	Canonical sequence	Vertebrate ortholog	Name	Protein domains	Substrate specificity
pde-1	T04D3.3a.1	PDE1	Ca^{2+}-calmodulin-dependent PDE	PDEase; calmodulin binding?	cAMP, cGMP
pde-2	R08D7.6a	PDE2	cGMP-stimulated PDE	PDEase; GAFb; GAFa?	cAMP, cGMP
pde-3	E01F3.1e.1	PDE3	cGMP-inhibited PDE	PDEase	cAMP > cGMP
pde-4	R153.1d.1	PDE4	cAMP-specific PDE	PDEase; upstream conserved region	cAMP
pde-5	C32E12.2.1	PDE10	cAMP-inhibited PDE	PDEase; GAFa, GAFb	cAMP > cGMP
pde-6	Y95B8A.10	PDE8	high affinity cAMP-specific PDE	PDEase; PAS-like?	cAMP

Table 1. Cyclic nucleotide phosphodiesterase (PDE) genes in *C. elegans* and vertebrate orthologs.

Methods

Creation of transgenic *C. elegans* strains with ablated PDE genes. Ablation of each of the six PDE genes in *C. elegans* was performed by In Vivo Biosystems by targeted genome editing using CRISPR/Cas9. For each PDE knockout (KO), guide RNAs were designed to (at minimum) remove the catalytic domain (Fig. 1). The PDE 1/4 double KO was generated by genetic crosses of the two individual knockout strains. The PDE5 KO strain was inadvertently lost during the COVID pandemic and is being regenerated. Whole genome sequencing by the Hubbard Center for Genome Studies (HCGS) at UNH confirmed the targeted deletion of each of the PDE KOs, with no off-target effects of genome editing observed (when compared to the *C. elegans* N2 strain).

Use of RNA interference (RNAi) to knock down expression of *C. elegans* PDE genes. *E. coli* HT115 transformed with the L4440 plasmid containing inserts coding for double-stranded RNA targeting *C. elegans pde-1, pde-2, pde-4, and pde-5* were kindly provided by the Walhout lab (Univ. Massachusetts). Plasmids subcloned with inserts for *pde-3* and *pde-8* were prepared in our lab, and all six plasmids were sequenced verified by the HCGS prior to use. Starved L1-stage *C. elegans* [either PDE KO strains on an N2 background or RNAi hypersensitive strain *rrf-3(pk1426)II* (Simmer et al., 2002)] were transferred to agar plates with *E. coli* HT115 lawns harboring one or two PDE RNAi constructs (or empty L4440 plasmid as a negative control) and nematode growth and behavior monitored at 20°C (Timmons and Fire, 1998).

Growth and locomotion assays. Synchronized L1 nematodes were cultured at 20°C for various times on the indicated bacterial lawn, then washed and transferred to NGM agar plates and allowed to disperse for 10 min prior to recording videos with the WormLab Imaging System (MBF Bioscience). Nematode growth (length, µm) and locomotion (velocity, µm per sec) were quantified (~150 worms per treatment) with WormLab software (v 3.0.0) at a resolution of 1280 x 960 pixels at 15 frames per sec (Roussel et al., 2014).

Fecundity assay. The reproductive capacity of nematodes exposed to RNAi were assayed following an established protocol (Brooks and Johnson, 1991). L1 stage nematodes were transferred to plates containing a *E. coli* HT115 lawn with or without PDE RNAi-containing plasmids and allowed to grow at 20°C to the L3 stage. Individual L3-stage nematodes were transferred daily to 6-well plates containing the same bacterial lawn to quantify the total number of progeny produced over the subsequent 3-day period.

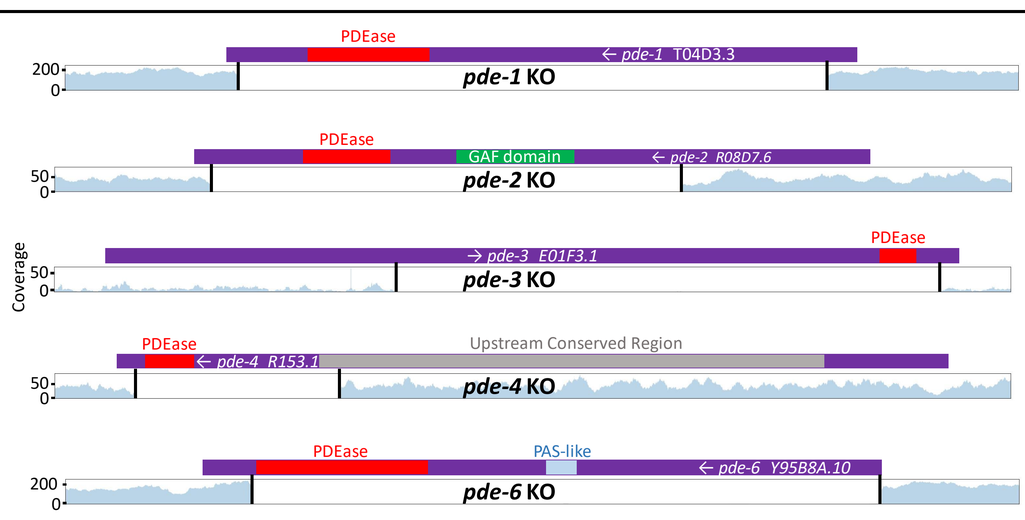

Fig. 1. Whole genome sequencing analysis of transgenic *C. elegans* strains with PDE gene ablation. DNA was extracted from each PDE KO strain and genomic sequencing and bioinformatic analyses performed by the Hubbard Center for Genome Studies. Light blue shows the depth of sequencing coverage flanking the site of deletion of each PDE gene. The catalytic domain (red) and putative regulatory domains (green, gray, and light blue) are shown within the genomic position of each PDE gene (purple).

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Results

Characterization of *C. elegans* PDE knockout (KO) strains

- Ablation of individual PDE genes in *C. elegans* (or the PDE 1/4 double KO) does not result in observable changes in locomotion, development, or fecundity.
- The PDE1 KO strain lacks a chemotactic response to 2-butanone, as does the N2 strain exposed to a human PDE1-selective inhibitor or a non-selective PDE inhibitor (Fig. 2).
- Exposure of *C. elegans* to the human PDE10-selective inhibitor MP-10 (PF-2545920) reduced viability and fecundity, whereas ablating *C. elegans pde-5* (orthologous to vertebrate PDE10) was without effect. Other PDE10-selective inhibitors had no effect on physiology or behavior, indicating that MP-10 exerted an off-target effect.

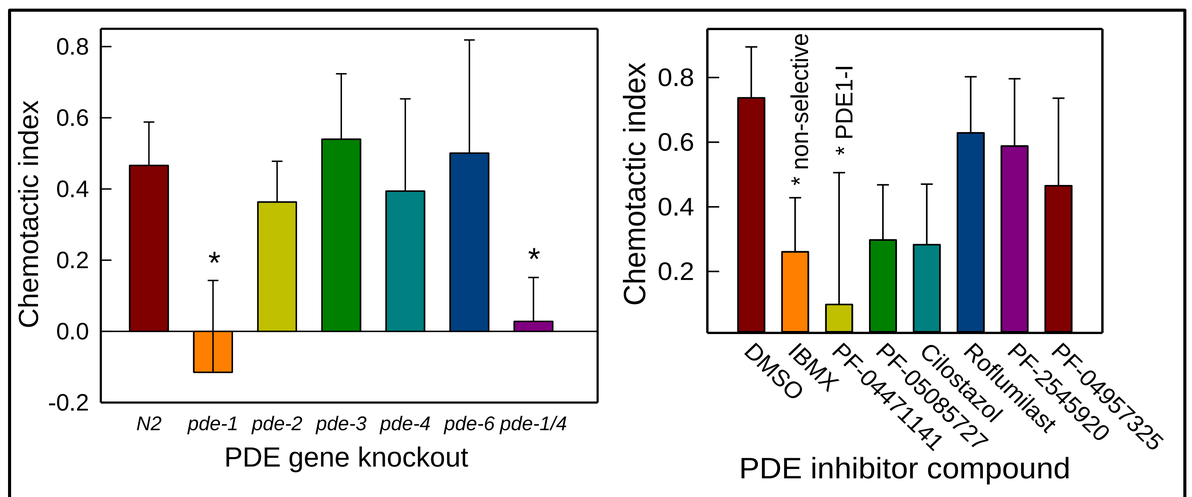

Fig. 2. Chemoattraction to 2-butanone is abolished in PDE1 KO strains and upon exposure to a human PDE1-selective inhibitor. *Left:* L3 stage nematodes of each PDE KO strain were assayed for their chemotactic response to the attractant 2-butanone (2 µl of 10% solution). *Right:* L3 stage *C. elegans* (N2) were pre-incubated for 1 h with a pan-specific inhibitor (IBMX) or human PDE family-selective compounds at concentrations near their individual aqueous solubility limit (ranging from 500 µM for IBMX to 25 µM for PF-05085727, with most inhibitors at ~125 µM; final [DMSO] = 0.5%) before initiating the chemotaxis assay. In both panels, * denotes $p < 0.05$ (Holm-Siak pairwise test; n = 3).

Disruption of multiple PDEs are required to cause defects in locomotion and development

RNAi #1	RNAi #2	Phenotype at 4 days
RNAi1,2,3,4,5 or 6	none	no phenotype
RNAi1	RNAi2,3,4, or 6	L3-L4 plaques absent
RNAi3	RNAi4 or RNAi6	L3-L4 plaques absent
RNAi4	RNAi6	L3-L4 plaques absent

Table 2. Effects of single or dual RNAi exposure on the hypersensitive *rrf-3* strain. *C. elegans rrf-3* strain were fed *E. coli* containing PDE RNAi to knock down the activity of the corresponding PDE (see Methods). Nematodes were screened for developmental progression at 4 days post transfer to the bacterial lawn. All dual RNAi combinations exhibited a lethargic phenotype, but only those listed above were developmentally delayed.

KO strain	RNAi	Phenotype
N2	one or two RNAi	none
1	one or two RNAi	none
2	4+5 or 4+6	V↓; V↓
3	4+5 or 4+6	V↓; V↓
4	3	V↓
6	2	V↓
1,4	3	V↓ L↓, F↓

Table 3. Phenotypes of PDE KO strains exposed to PDE RNAi. L1 nematodes were transferred to lawns containing RNAi-containing bacteria (see Methods). Phenotypes evaluated: V↓, reduced velocity (Fig. 3, *left*), L↓, reduced length, and F↓, reduced fecundity (Fig. 3, *right*).

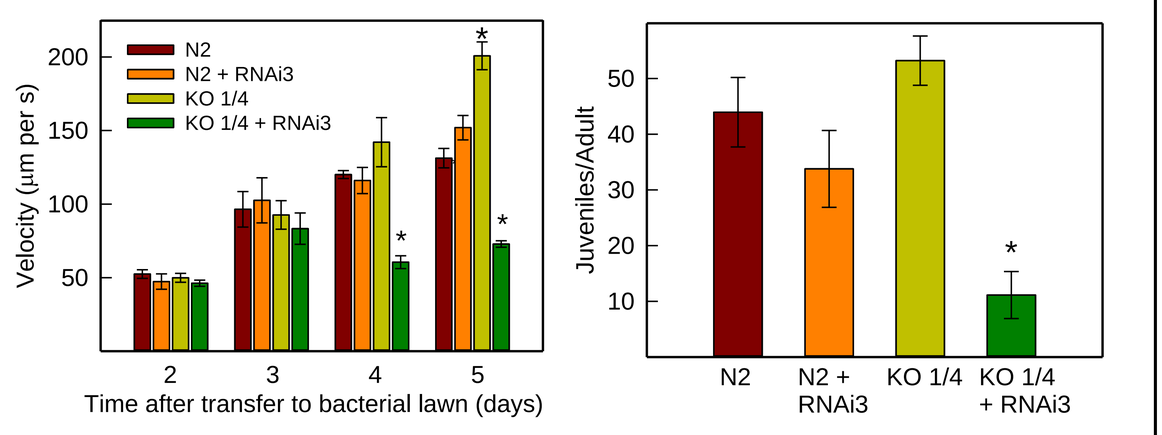

Fig. 3. Disruption of *pde-1, pde-3,* and *pde-4* markedly reduce growth rate and fecundity. L1 stage N2 or PDE 1/4 KO strains were grown in the presence of bacteria containing RNAi3 or the empty L4440 vector. Upon reaching L2 stage, one portion of each treatment was transferred to agar plates for locomotion assays (*left panel*). The following day, individual L3 animals were replica plated daily onto fresh bacterial lawns containing the same vector and total progeny produced over three days was determined (*right panel*). * Indicates statistically significant difference ($p < 0.001$; n = 3) using Dunnett's t-test.

Conclusions and Future Directions

- Disruption of an individual PDE by gene deletion or RNAi knockdown does not result in a significant locomotion or developmental phenotype.
- Deletion of *pde-1* mimics exposure to a PDE1 inhibitor in abolishing chemoattraction.
- Disruption of *pde-1, pde-3,* and *pde-4* through a combination of knockouts and knockdown resulted in developmental delays and lethargic movement.
- cAMP and cGMP metabolism appears to be coordinated by multiple PDEs in most cells; disruption of one PDE gene may be compensated for by upregulation of other PDEs.
- Future directions for this work (suggestions welcome!):
  - Additional phenotypic assays?
  - Screen additional triple PDE "knockout/knockdown" combinations for phenotypes?
- In summary, using both chemical and genetic approaches will accelerate identification of nematicidal agents as well as advance our understanding of the complex regulation of cyclic nucleotide signalling pathways by nematode PDEs.
- ***Interested in using our PDE KO strains in your research; inquire!***

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