Voltage-gated calcium channels of Paramecium cilia

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ABSTRACT

Paramecium cells swim by beating their cilia, and make turns by transiently reversing their power stroke. Reversal is caused by Ca\(^{2+}\) entering the cilium through voltage-gated Ca\(^{2+}\) (Ca\(_V\)) channels that are found exclusively in the cilia. As ciliary Ca\(^{2+}\) levels return to normal, the cell pivots and swims forward in a new direction. Thus, the activation of the Ca\(_V\) channels causes cells to make a turn in their swimming paths. For 45 years, the physiological characteristics of the Paramecium ciliary Ca\(_V\) channels have been known, but the proteins were not identified until recently, when the P. tetraurelia ciliary membrane proteome was determined. Three Ca\(_{V,1}\) subunits that were identified among the proteins were cloned and confirmed to be expressed in the cilia. We demonstrate using RNA interference that these channels function as the ciliary Ca\(_V\) channels that are responsible for the reversal of ciliary beating. Furthermore, we show that Pawn (pw) mutants of Paramecium that cannot swim backward for lack of Ca\(_V\) channel activity do not express any of the three Ca\(_{V,1}\) channels in their ciliary membrane, until they are rescued from the mutant phenotype by expression of the wild-type Pawn gene. These results reinforce the correlation of the three Ca\(_V\) channels with backward swimming through ciliary reversal. The PwB protein, found in endoplasmic reticulum fractions, co-immunoprecipitates with the Ca\(_{V,1}\)c channel and perhaps functions in trafficking. The PwA protein does not appear to have an interaction with the channel proteins but affects their appearance in the cilia.

KEY WORDS: Paramecium, Cilia, Voltage-gated calcium channel, Ca\(_{V,1}\), Pawn genes, Trafficking

INTRODUCTION

Cilia are slender organelles that protrude from eukaryotic cell surfaces. Depending on the cell type, there may be a solitary primary cilium or multiple cilia on a cell surface. These cilia have in common internal microtubule structures called axonomes, and those cilia with additional motor protein machinery in their axoneme can be motile. Primary cilia generally are immotile. The cilia of multi-ciliated cells usually are motile and contribute to the flow of spinal fluid or mucous in the airways. When cilia are dysfunctional, they contribute to a class of diseases called ciliopathies that are complex syndromes of developmental abnormalities. Regardless of superficial differences, all cilia are antennae for eukaryotic cells to sense many kinds of environmental stimuli (Berbari et al., 2009; Bloodgood, 2010; Brenker et al., 2012; Pazour and Witman, 2003; Singla and Reiter, 2006; Valentine et al., 2012).

As antennae, cilia detect stimuli such as odors or mechanical forces, and convert these stimuli to intra-ciliary signals (reviewed in Bloodgood, 2010; Kleene and Van Houten, 2014; Lishko and Kirichok, 2015). Although the identities of channels and the roles of ciliary versus cellular sources of Ca\(^{2+}\) are now under re-examination for mammalian primary and ependymal and nodal cilia (DeCaen et al., 2013; Delling et al., 2013, 2016; Blum and Vick, 2015; Lee et al., 2015; Doerner et al., 2015), for protists and sperm, the influx of Ca\(^{2+}\) into cilia is clearly through the ciliary voltage-gated Ca\(^{2+}\) (Ca\(_V\)) channels in response to chemical signals, mechanosensation or depolarization. Calcium levels in the cilia control signaling output, such as the beat form and force of motile cilia or cell proliferation (Bloodgood, 2010; Kleene and Van Houten, 2014).

Whether cilia are solitary or numerous, motile or non-motile, ion channels control the intra-ciliary calcium levels, which play important roles in sensory transduction (Bloodgood, 2010). For example, cyclic nucleotide-gated Ca\(^{2+}\) channels that are found only in the immotile cilia of olfactory neurons open as part of the signal transduction pathway initiated by odorant binding to its receptor (Pifferi et al., 2006; Kleene, 2008). Motile cilia similarly have ion channels that participate in sensory transduction. For example, the Ca\(_V\) channel of the Chlamydomonas flagellum is necessary for the change in waveform in response to light or mechanical stimulation (Fujii et al., 2011, 2009). The TRP family member polycystin-2 (PKD2) in Chlamydomonas flagella is crucial for the mating process that is dependent upon a Ca\(^{2+}\) influx (Huang et al., 2007). The CatSper Ca\(^{2+}\) channels of the sperm flagellum are responsible for the change in waveform in the vicinity of the egg (Brenker et al., 2012).

Because cilia are so highly conserved, it is possible to use a model system such as the ciliate Paramecium tetraurelia to provide insights into the ciliary calcium compartment and ciliary ion channel function. Paramecium tetraurelia, which is a cell covered in cilia, responds to sensory stimuli with changes in swimming behavior that result from changes in ciliary beating (Kung and Saimi, 1982; Machemer, 1988). Ciliary beat frequency and form are controlled by intra-ciliary Ca\(^{2+}\), which, in turn, is controlled by action potentials of Ca\(_V\) channels. The Ca\(_V\) channels, which are necessary for the action potential and increase in intra-ciliary calcium, are found exclusively in the cilia (Dunlap, 1977). Depolarizing stimuli, such as mechanical stimulation and high K\(^{+}\) concentrations, activate this channel and initiate the calcium action potential. The Ca\(_{V}^{2+}\) entering the cilium through the Ca\(_V\) channels affects the ciliary beating, reversing the power stroke and transiently sending the cell swimming backward. As ciliary Ca\(_{V}^{2+}\) levels return to normal, the cell pivots in place and then swims forward in a new direction. Thus, the activation of the voltage-gated calcium current \(I_{Ca(V)}\) of the Ca\(_V\) channels causes cells to swim backward transiently and make a turn in their swimming paths (Eckert, 1972; Machemer, 1988).

For almost 50 years, the physiological characteristics of the Paramecium ciliary Ca\(_V\) channels have been known (reviewed in Eckert, 1972; Machemer, 1988), but the proteins were not identified until recently, when the P. tetraurelia ciliary membrane proteome...
was determined. The three CaVα1 subunits that were identified among the proteins (Yano et al., 2013) were subsequently cloned and confirmed to be expressed in the cilia (Valentine et al., 2012; present study). Here we demonstrate using RNA interference (RNAi) that these channels function as the ciliary CaV channels that are responsible for the reversal of ciliary beating. Furthermore, we have turned to the important mutants of Paramecium called Pawns (pw), which are named for the chess piece because they cannot swim backward for lack of ciliary CaV channel activity (Satow and Kung, 1974). Here we show that pw mutants do not express the CaV1a, b or c channels in their ciliary membrane. However, when the pw mutants are rescued from the mutant phenotype (i.e. swim backward and turn) by expression of the wild-type PW gene, the CaV1a, b and c channels can be found in the ciliary membrane as in the wild type, reinforcing the correlation of these three CaV channels with the calcium action potential that causes backward swimming through ciliary reversal.

### MATERIALS AND METHODS

#### Stock and cultures

*Paramecium tetraurelia* wild-type (51s sensitive to killer), nd6 (non-trichocyst discharge mutant, courtesy of Dr Jean Cohen, Centre de Génétique Moléculaire, Gif-sur-Yvette, France), pwA (d4-94), pwA-nd6 double mutant (d4-94-nd6) and pwB (d4-95) cells were used. The cells were cultured in wheat grass infusion medium inoculated with *Paramecium tetraurelia* Stock and cultures were analyzed using Mann–Whitney U-tests. We also used 8 mmol l⁻¹ BaCl₂ in base buffer, pH 7.0, as the depolarization solution to induce backward swimming in pw mutants that were injected with wild-type sequences of the PWA or PWB genes.

#### Expressing tagged proteins from plasmids

The wild-type PWA (AF050753) and PWB (AF179276) genes were amplified by PCR using macronuclear genomic DNA. The primers used for amplification and restriction enzymes used for cloning into the expression plasmids are shown in Table S4. The PCR products were ligated to the vector L4440 (AddGene, Cambridge, MA, USA), using restriction enzymes (XhoI and XbaI, New England Biolabs, Ipswich, MA, USA) and a ligation kit (Ligate IT, Affymetrix, Santa Clara, CA, USA). The plasmid (empty or with insert) was transformed into strain HT115 of *Escherichia coli* following the manufacturer’s instructions. The feeding RNAi experiments were performed following a published protocol (Valentine et al., 2012).

### Backward swimming assay

All solutions for testing swimming behavior contain a base buffer of 1 mmol l⁻¹ Tris and 1 mmol l⁻¹ calcium citrate. 100 mmol l⁻¹ Tris was used to adjust the pH of solutions to pH 7.0. Cells from the control and test RNAi cultures were transferred to depression slides with a resting solution, 4 mmol l⁻¹ KCl in base buffer, pH 7.0, for 20 min. Cells were then transferred one by one to depressions with 1 calcium citrate. 100 mmol l⁻¹ BaCl₂ in base buffer, pH 7.0. The control cells first whirled or jerked and then swam backward. At the end, they whirled again and started swimming forward. The durations of backward swimming, measured using a stopwatch, were analyzed using Mann–Whitney U-tests.
Formulated as a selective filter through which divalent cations can pass (Mikami et al., 1989; Starr et al., 1991; McRory et al., 2001). In the mammalian CaV1 and 2 subfamilies, the consensus sequence found in each of the four pore loops that forms the filter site for divalent cation selection is TxExW. The four glutamic acid residues (E) are thought to be the site for the binding and selection of the divalent cation. In P. tetraurelia CaV1c, all four pore loops have an E (red oval). Two other conserved sites, FxxExxxK located in s2 and NxxD located in s3, are shown as purple and orange boxes, respectively.

When we analyzed the CaV1c protein for a putative calmodulin binding site, we located one in the C-terminal cytoplasmic tail (blue oval). Among the cytoplasmic loops, the loop between domains I and II is the longest, estimated to be 684 amino acids. The CaV1c paralogs CaV1a and 1b have the same predicted structure.

Genomic DNA from the cell lines was used as template in PCR to determine which lines had the highest concentration of exogenous gene copies for a second injection of a different plasmid or further experiments. To do so, the endogenous and exogenous (plasmid) DNAs were separately amplified by PCR, using a forward primer from the coding region, and a reverse primer from its 3′ untranscribed region (for endogenous sequences) or calmodulin 3′ untranscribed region of pPXV plasmid (for exogenous sequences). The intensity of PCR bands from endogenous and exogenous mRNAs were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to compare indirectly the relative amounts of plasmid copies in cell lines. Cells injected with plasmids containing the epitope tag sequence only were used as a control to match the experimental cells in each experiment.

Whole-cell lysates

Two to three milliliters of packed cells were collected from 3 to 6 liters of control and test cultured cells. The cells were washed twice in Dryl’s solution (1 mmol 1−1 Na2HPO4, 1 mmol 1−1 NaH2PO4, 2 mmol 1−1 trisodium citrate, 1.5 mmol 1−1 CaCl2) and once in cold LAP200 (50 mmol 1−1 HEPES, pH 7.4, 200 mmol 1−1 KCl, 1 mmol 1−1, EGTA, 1 mmol 1−1 MgCl2) with protease inhibitors [1 mmol 1−1 phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St Louis, MO, USA), 0.1 µg ml−1 Pepstatin (Research Products International, Mt Prospect, IL, USA), 0.1 µg ml−1 Leupeptin (Research Products International) and protease inhibitor cocktail (Sigma-Aldrich) at a final concentration of 0.1%]. The washed cells were resuspended in 5 ml of LAP200 buffer with the protease inhibitors and maintained at 4°C while being homogenized until 95% of the cells were ruptured.

Pellicles and subcellular P2 and P10 pellets

For work flow, see Fig. 2. The pellicles (cell surface membranes with cytoskeleton) were prepared as described previously (Wright and Van Houten, 1990). In this preparation, cells were washed three times in cold HM buffer (20 mmol 1−1 maleic acid, 20 mmol 1−1 Tris, 1 mmol 1−1 Na2EDTA, pH 7.8) instead of Dryl’s solution. The cells were homogenized in HM buffer with the protease inhibitors at 4°C. The pellet was washed, collected as the ‘pellicle’ and further treated with detergent and centrifugation to produce a supernatant for immunoprecipitation (IP). The first supernatant from the pellicle preparation was vortexed vigorously for 5 min and centrifuged at 2000 g for 10 min at 4°C. The supernatant was then centrifuged at 19,800 g for 30 min at 4°C to produce the pellet P2. The resulting supernatant was centrifuged at 100,000 g for 1 h at 4°C to produce the pellet P10. Intracellular membranes with an enzyme signature (glucose-6-phosphatase) of the endoplasmic reticulum (ER) are reported to be enriched in the P2 and P10 pellets (Haga et al., 1984; Wright and Van Houten, 1990; S. Lodh, Characterization of PWA and PWB proteins in Paramecium, PhD dissertation, University of Vermont, 2012). The pellet, P2 or P10, were resuspended in membrane buffer (10 mmol 1−1 Tris buffer, pH 7.4, 50 mmol 1−1 KCl, 5 mmol 1−1 MgCl2, 1 mmol 1−1 EGTA) or LAP200 buffer with the protease inhibitors (1 mmol 1−1 PMSF, 0.1 µg ml−1 Pepstatin, 0.1 µg ml−1 Leupeptin and 0.1% protease inhibitor cocktail) at 4°C (Haga et al., 1982, 1984; Wright and Van Houten, 1990).

Cytoplasmic factors that cure the three pw mutants and restore action potentials and backward swimming are thought to be in the subcellular membrane fraction, the fraction that we refer to as P10 is the equivalent of the ‘P2’ fraction that Haga and others used to cure P. tetraurelia pw mutants by injection (Haga et al., 1982, 1984).

Cilia isolation

The cilia from the control and test cells were isolated from 2–3 ml of packed cells collected from 3–6 liters of cell culture following the protocol of Yano and co-workers (Yano et al., 2013). The isolated cilia were suspended in 1 ml of membrane buffer or LAP200 with protease inhibitor at the same concentration previously described at 4°C.

Protein assay

The protein concentration of each sample was measured using the Pierce Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). The cilia samples from the control and test were adjusted to the same volume and protein concentration for further experiments.

Immunoprecipitation (IP)

Triton X-114 for solubilizing CaV1s, calcium ATPases (PMCA) or Pw proteins, or Triton X-100 for solubilizing Pw proteins (as...
indicated in each specific figure) was added to the whole-cell lysate, pellet, P2, P10 or cilia suspension to achieve a final concentration of 1%. Each sample was agitated by rocking at 4°C for 1 h followed by centrifugation at 48,400 g (pellicle and cilia) or 100,000 g (whole-cell lysate and P10) at 4°C for 30 min. The supernatant was incubated with 20 µl Protein A beads (Amersham Pharmacia/GE HealthCare, Pittsburgh, PA, USA) at 4°C for 1 h to clarify the supernatant. Prior to use, the Protein A beads had been washed in membrane buffer or LAP200 buffer with 1% Triton and 1% (w/v) bovine serum albumin (BSA). After removing the beads by centrifugation at 48,400 g at 4°C for 30 min, the clarified supernatant was incubated with 20 µl of anti-FLAG M2 affinity agarose (Sigma-Aldrich), or anti-c-Myc (polyclonal antibody) affinity agarose (Sigma-Aldrich) at 4°C for 1 h, which had been pre-washed in the membrane buffer or LAP200 with 1% Triton and 1% BSA.

Next, the antibody-conjugated beads were washed three times by centrifugation at 8000 g in the membrane buffer or LAP200 with 1% Triton and 1% BSA and three times in the buffer without Triton and BSA. The beads were suspended in 50 µl of 2× sodium dodecyl sulfate (SDS) buffer (6.25 mmol l\(^{-1}\) Tris, 1.5% SDS, 1% glycerol, 0.001% Bromophenol Blue, pH 6.8) with or without 3% β-mercaptoethanol, and boiled for 10 min. After centrifuging at 14,000 g at 4°C, the supernatant was loaded onto a 4–18% or 7–18% gradient SDS-polyacrylamide gel (SDS-PAG) and run at the constant current of 20 mA. To confirm that approximately the same amounts of protein went into the control and test IP, we removed 20 µl of the test and control samples, clarified the Triton-treated supernatants and analyzed the proteins on western blots using α-tubulin as a protein that should not vary between control and test samples.

For the IP of the CaV channels from the ciliary membrane, plasma membrane calcium ATPases (PMCA2) (anti-CBD2 antibody) (Van Houten, 1998) at 4°C for 1 h followed by the incubation with Protein A beads for IP.

**Western blot analysis**

We examined the subcellular localization of expressed epitope-tagged PWA and PWB proteins by western blot analysis. A total of 100 µg of protein from the pellet, P2, P10, or cilia suspension that was prepared from both the test and control cells were run on 12% SDS-PAG. The proteins separated in the SDS-PAGE (SDS-PAG electrophoresis) were transferred to BioTrace nitrocellulose blotting membrane (PALL Life Sciences, Pensacola, FL, USA). The western blots were treated with blocking buffer, incubated with primary antibody, followed by development with alkaline phosphatase or enhanced chemiluminescence, as previously described (Yano et al., 2003). The blots were probed for the protein of interest with the proper primary antibody: rabbit or mouse anti-FLAG antibodies (Sigma-Aldrich, F3165-5MG, F7425-0.2MG), 1:2500 dilution; rabbit or mouse anti-Myc antibodies (GenScript, Piscataway, NJ, USA, A00704), 1:2000 dilution; rabbit anti-CBD2 (for the plasma membrane calcium ATPases), 1:5000 dilution; or mouse anti-α-tubulin (loading control) (Sigma-Aldrich, T6199), 1:10,000 dilution. Secondary antibodies were goat anti-mouse or rabbit conjugated to alkaline phosphatase or horse radish peroxidase used in a 1:10,000 dilution.

When the rabbit antibody was used for IP, the precipitated proteins were detected on western blot with the appropriate mouse primary antibody. When the mouse antibody was used for IP, a rabbit antibody was used for western blot analysis. For re-probing, the blots were incubated in the stripping buffer (50 mmol l\(^{-1}\) dithiothreitol, 50 mmol l\(^{-1}\) Tris HCl, 70 mmol l\(^{-1}\) SDS, pH 7) at 70°C for 30 min before washing in TBS-T (16 mmol l\(^{-1}\) Tris HCl, 4 mmol l\(^{-1}\) Tris, 137 mmol l\(^{-1}\) NaCl, 0.1% Tween 20, pH 7.5) and re-blocking and re-probing the blot as just described.

**Mass spectrometry**

We used mass spectrometry (MS) to confirm that the bands precipitated with anti-FLAG M2 affinity agarose from whole-cell...
lysates of PWA-FLAG- and PWB-FLAG-expressing cells were FLAG-tagged PwA and PwB proteins, respectively. The precipitated proteins were separated on 12% SDS-PAGE. The gels were silver stained using the FAST Silver Kit (G-Biosciences, St Louis, MO, USA). The region corresponding to PwA- or PwB-FLAG was cut from the gel for MS analysis (see immediately below).

We examined whether the CaV1c channel could be immunoprecipitated with PwA from the P10 pellet in Fig. 2. Cultures of cells expressing both FLAG-CaV1c and PWA-Myc could not be cultured in sufficient quantities to analyze the results of IPs by western blots. Therefore, we used cells that grew better, i.e. expressing FLAG-CaV1c that had been transformed with untagged PWA expression plasmid. The P10 fraction was prepared from the cells expressing PWA and FLAG-CaV1c or PWA and the control FLAG epitope. The P10 fractions were normalized for protein concentration and solubilized with 1% Triton X-114 before centrifugation at 100,000 g.

After centrifugation, we performed the IP from the resulting supernatant using anti-FLAG M2 affinity agarose using the method described above. The precipitated proteins were separated on 4–18% gradient SDS-PAGE. The resulting gel was silver stained and the regions corresponding to CaV1c (200–270 kDa) and PWA (20–30 kDa) were cut out. Each band was diced, destained in 30 mmol L⁻¹ K₂Fe(CN)₆ and 100 mmol L⁻¹ Na₂S₂O₃, then rinsed in distilled water, and subjected to in-gel digestion with trypsin or trypsin/sodium acetate buffer (5% CH₃CN, 25 mmol L⁻¹) overnight at 37°C. The resulting peptides were analyzed by LC-MS/MS in an LTQ-XP linear ion trap mass spectrometer (Thermo Fisher Scientific) following the protocol of Yano and co-workers (Yano et al., 2013). The resulting protein data were searched simultaneously against the Paramecium tetraurelia database (Thermo Fisher Scientific) and against the combined database of murine and rat (Yano et al., 2013) using Scaffold 4 (Proteome Software, Portland, OR, USA) with a precursor tolerance of 2 Da and a fragment ion tolerance of 0.5 Da. In our experience, the majority of cysteine residues following reducing conditions and SDS-PAGE are identified with an acrylamide adduction. For increased throughput and simplicity, we conducted searches with a static increase in 71.0 Da for acrylamide addition. Differential modification of 16.0 Da on methionine residues was permitted. The search results were filtered using a delta correlation (dCn) score of 0.1 and cross-correlation (Xcorr) values of 1.9, 2.6, 3.2 and 3.4 for singly, doubly and quadruply charged ions, respectively. Peptides identified in these filtered lists that had two or more peptides were retained, and the false-positive ratio was zero in this list.

**RESULTS**

**Consensus domain analysis of three CaV channels in Paramecium**

In our proteomic analysis of the P. tetraurelia ciliary membrane (Yano et al., 2013), peptides from the putative CaV1α-subunit were identified. These CaV1α peptide sequences correspond to CaV1a, 1b and 1c. CaV1a and CaV1b are 87% identical at the nucleotide level and are likely to be derived from a recent whole genome duplication (WGD) (Aury et al., 2006). Their amino acid sequences are so close that the peptides we identified did not distinguish between CaV1a and 1b, but the peptides allowed us to establish that one or both of them are in the ciliary membrane. While CaV1c is 75% identical at the nucleotide level to CaV1a and 1b, CaV1c can be distinguished from these other CaV proteins through peptides that we identified in our mass spectrometry analysis. The gene for CaV1c is probably separated from the more ancient paralogs at the intermediate WGD and survived after the more recent WGD that created CaV1a and 1b (Aury et al., 2006). A FLAG-tagged sequence for CaV1c was previously used to confirm the presence of the protein in cilia (Valentine et al., 2012; Yano et al., 2013).

Blast searches showed that CaV1a, 1b and 1c are most closely related to the CaV1α subunits from the mammalian subfamily called CaV1. When the Paramecium database was searched using the mouse and rat sequences for CaV1.1 (NP_055008 for mouse and NP_446325 for rat) that are α subunits of typical high voltage-activated (L-type) channels, the Paramecium CaV1a, 1b and 1c have Expect (E) values of 6E⁻⁹⁵, 8E⁻¹⁰⁶ and 4E⁻⁹⁸ to mammalian CaV1.1, respectively (see Table S1).
The *P. tetraurelia* ciliary CaV α-subunits share conserved domains with the C termini of vertebrate CaV subfamilies CaV1–3. Our analyses of predicted structure show that *P. tetraurelia* CaV1a, 1b and 1c all have the expected four copies of the ion transporting domain comprising six transmembrane domains (s1 to s6) and a pore loop in each unit (Tyson and Snutch, 2013) (Fig. 1). These four channel domains come together to form a highly charged selectivity filter that confers specificity for Ca\(^{2+}\) on the channel. The sequences found in the *P. tetraurelia* CaV1a sequences have glutamic acids (E) in a critical position in each pore loop, giving these CaV α subunits the identity of the ‘EEEE’ motif in common with vertebrate CaV1 and 2, which are associated with high voltage-activated calcium channels. All CaV subgroups have transmembrane segments S3 and S4 that generally have an NxxD voltage sensor motif, respectively. The *P. tetraurelia* CaV1a-c is very long (around 680 amino acids) as compared with that of the mammalian CaV1 and 2.

RNAi demonstrates that CaV1a-c contribute to ciliary reversal and backward swimming in depolarizing solutions

Backward swimming is known to depend upon an \(I_{\text{CaV}}\) in the cilia. The Ca\(^{2+}\) conductance is proportional to the duration of backward swimming induced by depolarization with high potassium (Haga et al., 1984; Hiwatashi et al., 1980). We used RNAi to examine whether reduction of CaV1a, 1b, 1c, or all three affected backward swimming, and, indirectly, whether their channel activities participate in the backward swimming behavior.

Segments of the CaV1a, 1b and 1c sequences (see Materials and methods) were amplified by PCR and sub-cloned into the RNAi vector L4440 for feeding RNAi. *Paramaecium tetraurelia* cells were fed bacteria with the RNAi vector with the CaVc insert or the empty RNAi vector (L4440) as a control. Cells were first tested in 30 mmol l\(^{-1}\) KCl in buffer to induce backward swimming after 24, 48 and 72 h of feeding on the RNAi bacteria, but changes in the backward swimming duration were most dramatic at 72 h of feeding. Therefore, we present here the data collected at 72 h of feeding RNAi. The RNAi with each CaV1a, 1b or 1c sequence individually caused significantly shorter backward swimming in high KCl compared with the control fed the empty vector (Mann–Whitney U-test; Fig. 4). Moreover, the RNAi for the mixture of CaV1a, 1b and 1c showed the shortest backward swimming as compared with the control or with RNAi for each individual calcium channel (Fig. 4).

Off-target effects by the RNAi sequences developed to downregulate CaV1a-c showed that the potential 23-mer nucleotide products from RNAi processing of the double-stranded RNA for CaV1a-c could bind many sequences within the mRNA for CaV1b and vice versa, marking these sequences for degradation (Table S3). However, there are very few potential off-target effects of CaV1a or 1b on CaV1c and of CaV1c on either CaV1a or 1b.

As a negative control for our RNAi of CaV1a-c, we carried out RNAi for gene sequence GSPATG0005636001, which has been identified previously (Ben-Johny et al., 2014; Taiakina et al., 2013) as a putative CaV channel \(\alpha1\)-subunit based on sequence homology to mammalian CaV1.1. This putative CaV \(\alpha\)-subunit has not been found in the proteomic analysis of the ciliary membrane. RNAi for this sequence does not reduce backward swimming (Fig. 5) as seen for the RNAi of CaV1a, 1b or 1c shown in Fig. 4.
Wild-type PW gene sequences rescue the wild-type phenotype when injected into pw cells, but overexpression of CaV1a, 1b or 1c does not

Haynes and others have shown that injection of the wild-type sequence for PWA or PWB into the mutant cell nucleus will rescue the wild-type phenotype (Haynes et al., 2000, 1998). In order to carry out the present study, we reproduced the outcome that the wild-type PW sequences could rescue the wild-type phenotype, i.e. restore the ability of pw cells to swim backward in depolarizing solutions. Table S5 shows that injection of the wild-type PWA or PWB sequence restores the ability of pwA or pwB mutants, respectively, to swim backward in 8 mmol l\(^{-1}\) BaCl\(_2\), although the duration is not as long as the backward swimming of wild-type controls. The PWA or PWB sequences with epoipeptide tags similarly restore the ability of pwA or pwB mutant cells to swim backward in 8 mmol l\(^{-1}\) BaCl\(_2\) solutions (Table S5).

In contrast, expression of FLAG-CaV1a, 1b or 1c in pwA or pwB cells does not result in the restoration of backward swimming tested in 30 mmol l\(^{-1}\) KCl solutions (Table S5). Even with these additional exogenous sequences for CaV1a, 1b or 1c injected into the pw cells, the wild-type phenotype is not rescued in pw mutants.

CaV1a, 1b and 1c proteins are found in cilia, but pw mutants do not show these CaV1s in cilia unless they are rescued by expression of the wild-type PW sequence

The FLAG-tagged CaV1c can be immunoprecipitated from the ciliary membrane of wild-type cells (Fig. 6A) and its expression in wild-type cells increases their backward swimming (Table S5). Similarly, FLAG-CaV1a or 1b were immunoprecipitated from the ciliary membrane of wild-type cells expressing FLAG-CaV1a or 1b, respectively (Fig. S1). However, the cells expressing tagged CaV1a or 1b showed the same backward swimming duration as the control cells (Table S5). The expression of the tagged CaV1s’ proteins in wild-type cilia made it possible for us to investigate the possibility that the pw mutants do not activate the CaV1s to initiate backward swimming because the CaV1s are not in their cilia.

FLAG-CaV1c was expressed in mutant pw cells or in pw cells whose phenotypes had been rescued with wild-type PW sequences (Fig. 6A). In each case, the cells were tested for restoration of backward swimming before the cilia were isolated from transformed cells. The isolated cilia were treated with 1% Triton X-114 and the proteins were precipitated using anti-FLAG M2 affinity agarose, then analyzed by western blot. Note that in lane C of both pwA and pwB cells shown in Fig. 6A, the FLAG-CaV1c is not immunoprecipitated from the cilia of pwA or pwB cells co-expressing only the pPXV plasmid. However, once the pw mutants are rescued with the wild-type sequence for PWA or PWB, the FLAG-CaV1c protein can be immunoprecipitated from the cilia (lane T of pwA and pwB cells in Fig. 6A).

As a concentration control for Fig. 6A, the plasma membrane calcium ATPases 2, 3 and 4 (PMCA) (CR932147, CR932150 and CR933346) were precipitated from the cilia with anti-CBD2 antibody (Van Houten, 1998). The western blot showed that the intensity of bands corresponding to the PMCAs were almost the same, indicating that injection of pPXV plasmid with either FLAG-CaV1c or FLAG-CaV1a did not affect the expression of the PMCAs in either wild-type or mutant cells (Fig. 6B).

**Fig. 6.** Wild-type PWA and PWB genes are required for the presence of CaV1c in cilia of P. tetraurelia. (A) Cilia isolated from wild-type (51s), pwA and pwB mutant cells expressing FLAG-CaV1c (lane T) or only FLAG (lane C) were solubilized with 1% Triton X-114. The FLAG-CaV1c that was precipitated with anti-FLAG M2 was detected with anti-FLAG polyclonal antibody on western blots. The pwA cells expressing both FLAG-CaV1c and the PWA gene show backward swimming, i.e. the wild-type phenotype is rescued (Table S5). However, the pwA cells expressing only FLAG-CaV1c do not show backward swimming, i.e. the pwA mutants are not rescued (Table S5). The band corresponding to FLAG-CaV1c (arrow) was detected in the ciliary membrane of pwA cells expressing both FLAG-CaV1c and the PWA protein (lane T), but not in the ciliary membrane of pwA cells expressing only FLAG-CaV1c (lane C). Similarly, pwB cells expressing FLAG-CaV1c show no backward swimming in depolarizing solutions (Table S5), while the pwB mutant cells expressing both FLAG-CaV1c and PWB are capable of swimming backward (Table S5). The band corresponding to FLAG-CaV1c was detected in the ciliary membrane of pwB mutant cells that express both the FLAG-CaV1c and PwB protein (lane T), but not in the ciliary membrane from pwB cells that express only the FLAG-CaV1c protein (lane C). IP: immunoprecipitation; ID: immunodevelopment. (B) Loading control. After IP with anti-FLAG, plasma membrane calcium ATPases (PMCAs) were precipitated with anti-CBD2, which was produced against the calmodulin binding domain of the Paramecium plasma membrane calcium ATPase 2 (Van Houten, 1998). The PMCAs were detected with anti-CBD2 in lanes C (control) and T (test). Approximately the same amount of PMCA seems to be solubilized in the ciliary sample of control and test cells with 1% Triton X-114.
same between the cilia of cells expressing the FLAG-CaV1c and control vector pPXV, and the cilia co-expressing FLAG-CaV1c and the PWA or PWB wild-type gene (Fig. 6B).

Similar results were obtained when the tagged channel genes for CaV1a and 1b were co-expressed with the wild-type PWA in pwA cells or PWB gene in pwB cells. Under these conditions, FLAG-CaV1a and 1b proteins could be immunoprecipitated from the cilia of pwA or pwB cells (see Fig. S1).

Subcellular localization of PwA and PwB proteins, and potential interaction with CaV1c
The injection of cytoplasm, especially fraction P10 (see Fig. 2 and Materials and methods), from the wild-type cells into the pwA or pwB cells caused the mutants to regain the voltage-activated calcium conductance without new protein synthesis (Haga et al., 1984). Therefore, we included in our determination of the subcellular localization of Pw proteins the P10 fraction used by Haga and co-workers to cure pw phenotypes (Haga et al., 1984) and also the P2 fraction, because both fractions were reported to be enriched in ER (Haga et al., 1984; Wright and Van Houten, 1990).

We used western blots to locate Pw proteins in cell fractions. Fig. 7A shows western blots of the P2 fraction and the pellicle, and western blots of IPs from the cilia of cells expressing PwA-FLAG (test, T lane) or FLAG (control, C lane) (see Materials and methods). We considered bands to be from PwA-FLAG only if they were found in T lanes and not C lanes, as only the T lanes should have the expressed tagged protein. The bands of the P2 fraction show three bands (30, 27 and 25 kDa) in the T lane and one (30 kDa, blue arrow) in the C lane. We discounted the 30 kDa band from further analysis because it was in both the T and C lanes. The P2 band at 27 kDa (upper black arrow, lane T only) matches the expected size of the PwA-FLAG protein. The P2 band of 25 kDa (lower black arrow, T lane only) matches the PwA-FLAG protein without the signal sequence.

Blots of the pellicles of cells expressing PwA-FLAG and FLAG show three bands of 25, 27 and 28 kDa in the T lane only. The pellicle band of 28 kDa (arrowhead) matches a glycosylated form of the PwA-FLAG protein, which has two putative glycosylation sites. The lower band in the pellicle blot at 25 kDa matches the predicted size of PwA-FLAG without the signal sequence. In the blot from the IP of PwA-FLAG from cilia, two bands of 25 and 28 kDa were detected in the T lane only, similar to the pellicle western blot.

Proteins were immunoprecipitated with anti-FLAG M2 from the Triton-X114 extracts of P2, P10, pellicle and cilia from pwB cells expressing PWB-FLAG (T lane) or FLAG (C lane; Fig. 7B). Western blots show bands at 35 kDa in the T lane in the P2 and P10 fractions (Fig. 7B, black arrow), which are consistent with PwB-FLAG protein. The PwB-FLAG protein was not found in the pellicle or cilia IPs.
To examine whether the PwB protein interacts (directly or indirectly) with CaV1c in the P10 fraction, we carried out reciprocal IPs of FLAG-tagged CaV1c and PwB-Myc proteins expressed in wild-type cells. Fig. 8A,B shows the western blot results of reciprocal IPs of FLAG-CaV1c and PwB-Myc proteins from the P10 fraction. After the IP with the anti-Myc beads, a FLAG-CaV1c band of 250 kDa was detected in lane T (Fig. 8A). As expected, the PwB-Myc protein of 35 kDa was also detected. From the IP with anti-FLAG M2 affinity agarose, the PwB-Myc protein was detected at 35 kDa (Fig. 8B). The loading control is shown in Fig. 8C,D.

To examine whether the PwA protein interacts with FLAG-CaV1c in the P10 fraction, FLAG-CaV1c and PwA-Myc (test) and empty vectors containing FLAG and Myc (control) were co-expressed in wild-type cells. We could not grow the PWA-Myc transformed cells to sufficient density for the IPs, which led us to look for the presence of the PwA protein by overexpressing the untagged version and using MS to examine the gel of the IP products. IP with anti-FLAG was carried out, and the immunoprecipitated proteins were analyzed by separating the precipitate into a sample for a blot to confirm the successful IP of CaV1c and a sample for a silver-stained gel that would be analyzed by MS/MS. Two regions, 200–270 kDa and 20–30 kDa, corresponding to CaV1c and PwA proteins, respectively, were cut out from the silver-stained gel (Fig. 9) and analyzed by MS. The same experiments were repeated three times. Although the peptides of CaV1c were detected from the regions corresponding to FLAG-CaV1c, no peptides from the PwA protein were detected.

**DISCUSSION**

The power stroke of the cilia of *P. tetraurelia* is controlled by the activity of the CaV channels in the ciliary membrane. Through proteomic analysis of the ciliary membrane, we found three proteins and their corresponding genes that could potentially be responsible for the action potential that controls the ciliary beat form (Yano et al., 2013). The cloning and epitope tagging of these large genes was challenging, but allowed us to demonstrate that the proteins from the expression vectors were located in the ciliary membrane. The first channel, CaV1c, was the first of three that we cloned and expressed (Valentine et al., 2012). The expressed CaV1a and 1b channels are shown in the present study. The epitope tags allowed us to surmount the challenge of immunoprecipitating these large proteins to concentrate them for definitive identification in wild-type and rescued *pwA* and *pwB* mutants.

Using RNAi, we were able to downregulate the expression of the CaV1a, 1b and 1c genes singly and together to demonstrate that...
These CaV α-subunits contribute to the action potential that causes the cells to turn. RNAi generally does not cause a complete removal of a protein as a null mutation would. The mRNA for these channels is not eliminated by RNAi, as shown by RT-PCR (Fig. 3). However, the extreme reduction of backward swimming by RNAi treatment, especially that seen in the cells depleted of all three CaV1a-c channels, gives us confidence that these channels are the major, if not the only, contributors to the calcium action potential.

Another related sequence not found in the cilia by proteomics was the large channels allowed us to address the very old question of the cause of the failure of Pawn mutants to reverse their ciliary beat because they lacked the CaV channels in their cilia or whether the pw mutants had CaV channels in their ciliary membranes but these channels could not be activated with depolarization. In the present study, we could not find the CaV1a-c expressed channels in the ciliary membranes of Pawn mutants pwA or pwB, suggesting that these mutants cannot swim backward for lack of the CaV current from the channels. Expressing the genes for these channels in the mutants did not restore the ability to reverse ciliary beating and these expressed channels could not be found in the ciliary membrane. However, restoring the wild-type versions of the PWA or PWB mutant genes not only restores the ability to reverse swimming, but also restores the CaV1a-c channels in the ciliary membrane. These results reinforce our contention that the presence of one of the channel types CaV1a, b or c is necessary and perhaps sufficient for backward swimming.

The Pawn proteins do not resemble vertebrate CaV channel α1 or other subunits that are thought to assist in trafficking of the vertebrate CaV α-subunits to the cell surface (Dolphin, 2012). However, they appear to be involved in trafficking of the P. tetraurelia CaV1a-c proteins to the ciliary membrane. Because the PwB protein appears to be limited to the ER, its role in trafficking may be to assist the Ca2+ current from the channels. Expressing the genes for these proteins to traffic into the cilia (Valentine et al., 2012). In that same study, we found that CaV1c did not require the BBS8 protein of the BBSome complex to successfully reach the ciliary membrane.

Previously, we showed that other channels of the P. tetraurelia ciliary membrane, a calcium-activated K+ channel (SK1a) and polycystin-2 (PKD2), require Bardet–Biedel syndrome (BBS) proteins to traffic into the cilia (Valentine et al., 2012). In that study, we found that CaV1c did not require the BBS8 protein of the BBSome complex to successfully reach the ciliary membrane.

### Fig. 9. Western blot and silver-stained gel from IP with anti-FLAG M2 affinity agarose in P. tetraurelia.

We examined whether the PwA protein was co-precipitated with the IP with anti-FLAG M2 affinity agarose for the FLAG-CaV1c channel using mass spectrometry (MS) analysis. The P10 fraction from cells expressing FLAG-CaV1a and PWA (lane C) or only FLAG and empty vector (lane M) was solubilized with 1% Triton X-114, and the Triton X-114 extract was used for IP. (A) Ten percent of the precipitated protein was analyzed by silver stain. The areas corresponding to CaV1a (200–270 kDa) and PwA (20–30 kDa) were cut out from the gel. (B) The remaining protein was analyzed by western blot. The bands of FLAG-CaV1a were detected with anti-FLAG antibody (arrows). (C) The same amount of Triton extract was used as a loading control. α-Tubulin was detected by anti-α-tubulin antibody and serves as a control for the protein solubilized in both the test and control samples.


