Protein Phosphatase 2B (PP2B, Calcineurin) in *Paramecium*: Partial Characterization Reveals That Two Members of the Unusually Large Catalytic Subunit Family Have Distinct Roles in Calcium-Dependent Processes

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We characterized the calcineurin (CaN) gene family, including the subunits CaNA and CaNB, based upon sequence information obtained from the *Paramecium* genome project. *Paramecium tetraurelia* has seven subfamilies of the catalytic CaNA subunit and one subfamily of the regulatory CaNB subunit, with each subfamily having two members of considerable identity on the amino acid level (>55% between subfamilies, >94% within CaNA subfamilies, and full identity in the CaNB subfamily). Within CaNA subfamily members, the catalytic domain and the CaNB binding region are highly conserved and molecular modeling revealed a three-dimensional structure almost identical to a human ortholog. At 14 members, the size of the CaNA family is unprecedented, and we hypothesized that the different CaNA subfamily members were not strictly redundant and that at least some fulfill different roles in the cell. This was tested by selecting two phylogenetically distinct members of this large family for posttranscriptional silencing by RNA interference. The two targets resulted in differing effects in exocytosis, calcium dynamics, and backward swimming behavior that supported our hypothesis that the large, highly conserved CaNA family members are not strictly redundant and that at least two members have evolved diverse but overlapping functions. In sum, the occurrence of CaN in *Paramecium* spp., although disputed in the past, has been established on a molecular level. Its role in exocytosis and ciliary beat regulation in a protozoan, as well as in more complex organisms, suggests that these roles for CaN were acquired early in the evolution of this protein family.

Protein phosphatase 2B (PP2B), or calcineurin (CaN), is a Ser/Thr phosphatase (EC 3.1.3.16) consisting of two subunits, CaNA and CaNB, of ~60 and ~20 kDa, respectively (80). These represent the catalytic calmodulin (CaM) binding and the regulatory Ca2+ binding subunits, respectively. PP2B has broad substrate specificity, although only a restricted number of substrates are dephosphorylated with high activity (47). The catalytic subunit per se also possesses some, though restricted, phosphatase activity (30). In addition, it possesses a domain structure different from any other phosphatase (26). This includes a catalytic domain in the N-terminal half, a regulatory portion with a CaNB binding domain and a CaM-binding domain, followed by an autoinhibitory domain (48, 80). The CaNB subunit contains four EF-hand Ca2+ binding motifs (25) and binds tightly to activate subunit A.

CaN participates in a variety of cellular signaling events and activation processes (3, 98). For example, it has been implicated in the regulation of specific steps of the cell cycle, mitosis and meiosis (60, 61), development and cell differentiation (13), metabolism (54), and apoptosis. Its well-studied involvement in the immune response in mammals encompasses the regulation of transcriptional processes in connection with Ca2+ signaling (12). Its role in membrane processes has also been investigated. For example, it has been shown to regulate cation-influx channel activity (29, 98), and in fungi it has a role in regulating osmotic tolerance under stress conditions (51). Of significance to this report, CaN seems to be involved in some ill-defined way in exocytosis regulation, not only in a lower eukaryotic cell, *Paramecium* (63), but also in a variety of higher eukaryotic cells (22, 28, 88). Thus, CaN is involved in many calcium-dependent cytoplasmic, nuclear, and membrane processes in the cell, and how it accomplishes this wide range of tasks in a pathway-specific fashion is an area of active research.

CaN may act to regulate Ca2+-signaling processes indirectly through the action of other signaling proteins, such as protein phosphatases and protein kinases, or through adaptor or CaN binding proteins which might act to localize PP2B activity and facilitate the specificity of CaN action (3, 10, 16, 48). Its action through other signal proteins is demonstrated through its regulation of protein phosphatase cascades involving protein phosphatase type 1 (66). In addition, CaN helps mediate the regulation of intracellular Ca2+ release channels (10, 87) located in different subcellular Ca2+ stores. CaN may also regulate the action of membrane processes such as the dephosphorylation of dynamin (52), a large GTPase regulating organelle, and...
membrane fission, respectively, e.g., during endocytosis, internal vesicle budding as well as organelle division (76).

Genes encoding CaN are widely distributed among eukaryotes (26), unicellular organisms, including Dictyostelium (13) and Plasmodium (15), to mammals (80). Genes encoding the catalytic subunit, CaNA, have not been found in any of the plant or algae genomes sequenced to date (40). However, there do appear to be genes similar to the regulatory subunit, CaNB (called CaNB-like genes) that appear to have a role in stress response in plants (24). Although the CaNA gene has been identified in protozoans, there has been no characterization of CaN based on molecular biology in any ciliated protozoan. In fact, the occurrence of CaN in Paramecium spp. was originally disputed (50, 82), but several subsequent studies described a protein phosphatase with features characteristic of CaN (46, 63, 72), that localized by immunoelectron microscopy (EM) to established subcellular target structures of CaN activity (63). The publication of the Paramecium genome sequence confirmed the existence of CaN and the analysis here of the two families in Paramecium. We further demonstrate by using RNA interference (RNAi) technology combined with EM analysis, behavioral analysis, exocytosis stimulation, and Ca2+ fluorochrome imaging, that two members of the unusually large CaNA protein family have divergent and distinct roles in calcium-regulated processes in Paramecium.

MATERIALS AND METHODS

Cell cultures. The wild-type strains of Paramecium tetraurelia used were stock strains 7s and d4-2, derived from stock strain S15 (91). Cells were cultivated in a defined medium containing Klebsiella aerogenes (45).

PCR with genomic DNA. For PCR, total wild-type DNA was prepared from log-phase d4-2 cell cultures as described previously (27). For CaNAa, the following primer pair (MWG-Biotech, Ebersberg, Germany) was used: 5′-oligonucleotide 1 (5′-ATGGAATTCCTTTAAATGATCGGCAAGAATT-3′) and 3′-oligonucleotide 2 (5′-TCTACAGTTTGTGGTATGATG-3′). For CaNAb, we used 5′-oligonucleotide 3 (5′-ATGGAATTCCTTTAAATGATCGGCAAGAATT-3′) and 3′-oligonucleotide 4 (5′-TCTACAGTTTGTGGTATGATG-3′), and for CaNA2a, we used 5′-oligonucleotide 5 (5′-ATGGAATTCCTTTAAATGATCGGCAAGAATT-3′) and 3′-oligonucleotide 6 (5′-TCTACAGTTTGTGGTATGATG-3′). For CaNB, a 510-bp PCR amplification product was created using the primers 5′-oligonucleotide 7 (5′-ATGGAATTCCTTTAAATGATCGGCAAGAATT-3′) and 3′-oligonucleotide 8 (5′-TCTACAGTTTGTGGTATGATG-3′). Each PCR mixture (50 μl) contained 200 ng of DNA, a 200 nM concentration of each primer, 1 μl of Advantage 2 DNA polymerase (Clontech Laboratories, Inc., Heidelberg, Germany). Reactions were carried out for 1 cycle of denaturation (1 min, 95°C) and 35 cycles of denaturation (30 s, 95°C), annealing (1.5 s, 54°C), and extension (60 s, 68°C), with a final extension step (5 min, 68°C).

The CaNA-specific PCR products were cloned into the plasmid pCR2.1 by using a TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. After transformation into Escherichia coli (TOP10F− cells), positive clones were sequenced as described below.

PCR with cDNA. The open reading frames (ORFs) of CaNAa, CaNAb, CaNA2a, and CaNB were amplified by reverse transcriptase PCR (RT-PCR), using total RNA prepared according to the protocol by Haynes et al. (27). RT-PCR was performed in a programmable thermocycler T3 (Biometra, Göttingen, Germany) using oligonucleotide 8 and PowerScript reverse transcriptase (Clontech Labs) for first-strand cDNA synthesis (5′-oligonucleotide 9, 5′-AACCTGGAAGAATTCCTTTAAATGATCGGCAAGAATT-3′). The subsequent PCR (50 μl) was performed with Advantage cDNA polymerase mix (Clontech Labs) using the CaN-specific primers 1, 3, 5, and 7 combined either with the CaN-specific primers 2, 4, 6, and 8, respectively, or with 3′-oligonucleotide 10 (5′-CGCGGCGCCGGAATTATTATTTTTTTTTT-3′). Amplifications were performed with 1 cycle of denaturation (95°C, 1 min) and 39 cycles of denaturation (95°C, 45 s), and extension (68°C, 3 min), followed by a final extension step at 68°C for 5 min. CaN-specific PCR products were cloned into the plasmid pcR2.1 as described above.

Cloning. Sequencing was done by the MWG Biotech (Ebersberg, Germany) custom sequencing service or the Molecular Cellular Imaging Center (Ohio State University-OARDC, Wooster, OH). DNA and protein sequences were aligned by CLUSTAL W, integrated in the DNAStar Lasergene software package (Madison, WI) or MUSCLE, integrated in the Phylogeny.fr website (http://www.phylogeny.fr).

Computer analysis and modeling. In order to identify further paralogs of CaNA, the Paramecium database (http://paramecium.cgm.cnrs-gif.fr/pbatbl/) was screened using the nucleotide and amino acid sequences of CaNA1a and CaNA2a. Positive hits were further analyzed by performing BLAST searches at the NCBI database (2). Conserved motif searching was performed with either PROSITE (5, 37) or with BLAST2RQ, using tblastn entries of the corresponding CDD database (55).

The highly conserved catalytic domain and subunit B binding domains of CaNA were modeled according to Swiss model results as described previously (83). The domain structures of Paramecium calcineurin A isoforms were characterized according to the method of Ruskam and Mertz (80). Protein alignments were performed with CLUSTAL W (92). For the subunit B binding domain, the 21-residue sequence according to Klee et al. (48) was used as a reference (residues 348 to 368, H. sapiens α-CaNA, isoform CRA B, GenBank accession no. EAX06124). The 14-residue sequence of the CaN2−-dependent CaM binding motif “1-8-14 motif type A” (48, 78) from Homo sapiens α-CaNA (residues 396 to 409) was used as a reference for the search for a putative CaM binding motif.

Phylogenetic analysis was done as recommended by the Phylogeny.fr website using default settings (13). Alignments for Phylogeny.fr were done using MUSCLE with Gblocks on. Phylogenetic analysis included neighbor-joining, maximum-likelihood, and Bayesian analyses as implemented by Phylogeny.fr default settings. Gene conversion analysis was done using the RDP3 program (59, http://darwin.uvi.edu/rdp/rdp.html). RDP3 contains a variety of recombination detection programs including, RDP (56), GENECONV (69), BOOTSCAN (58), MAXCHI (90), SISCAN (7, 21), and 3seq (7). These six programs were run with default settings for detecting recombination events between subfamily members. P values were corrected for multiple comparisons, and only events detected by three or more programs were reported (to control for false positives). For analysis of within-subfamily (i.e., between oohnologs) gene conversion events, GENECONV was used exclusively since it allowed pairwise comparisons. GENECONV detects pairwise gene conversion events by identifying unusually long stretches of similarity given the overall similarity between the two sequences. Pairwise P values were determined by using 10,000 permutations and a cutoff P value of <0.05. Putative conversion events were all supported by visual inspection of the affected regions. For example, the intron locations seen in CaNA4 and CaNA5 are identical to the closely related isoform CaNA6, except in the suspected exchange region, where the intron locations (or absence) were identical with what is seen in CaNA2. In the case of the exchange between CaNA4 and CaNA8, the suspected region contains 67 mismatches per 100 bp, in contrast to an average of 6.3 mismatches per 100 bp in the aligned nucleotide coding sequences flanking this region. The identified exchange between CaNA7 and CaNA3 was less compelling since it was only 21 nucleotides long. However, these 21 nucleotides were identical between the four members, whereas the next longest stretch of identical residues between all four sequences was only eight residues.

Verification of introns. Introns were validated by RT-PCR using primers designed to straddle two introns predicted to be in the gene. The cDNA was generated from RNA isolated from log-phase Paramecium cells grown in wheatgrass media by using a PTC200 thermocycler (Bio-Rad, Hercules, CA) (denature DNA at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min). PCR fragments were electrophoresed on a 2% (wt/vol) agarose gel in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and sized by using the Gel Logic 100 imaging system (Kodak, Rochester, NY). Controls using introns in CaNA8 previously validated by sequencing cDNA were used to calibrate the size predictions (81).

Cloning, heterologous expression and purification of full-length CaNA1a and CaNB1a gene products. In order to express full-length CaNA1a and CaNB1a proteins in Escherichia coli, the corresponding sequences (accession numbers AF104922 and A354047) were first mutated to the universal code as described below and then cloned into the expression vectors pACYC184/pET28a (+) and pRV11a, respectively. For pACYC184/pET28a-CaNA1a, the mutated sequence of CaNA1a was first inserted into the expression cassette of pET28a (Novagen, Madison, WI) via the Ndel-Xhol restriction sites, before it was amplified and inserted into the expression vector pACYC184 (NEB, Frankfurt, Germany) by

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using the Clal restriction site. In the case of pRV11a-CaNB, the mutated sequence of CaNB was inserted into pRV11a (96) by using the restriction sites NdeI and Xhol. The His6-tagged fusion proteins were coexpressed with *E. coli* chaperone GroEL as described by Yasukawa et al. (99) and then purified in a two-step procedure. After 3 h of induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C, bacteria [BL21(DE3)] were centrifuged; the pellet was resuspended in double-distilled H2O containing 20 μg of lysozyme/ml and then stored overnight at −20°C. After thawing, Triton X-100 was added to a final concentration of 0.5%. Lyzed bacteria were sonicated (1 min; 80 W) and centrifuged at 30,000 × g for 20 min (4°C). The supernatant was further purified by using immobilized metal-ion-affinity-chromatography under native conditions according to the manufacturer’s protocol (Novagen).

**Western blots.** *E. coli* cells transformed with the plasmids described above containing the gene of CaNA1a or CaNA1b were induced by IPTG and, after up to 4 h of induction, were harvested, lyzed, and prepared for gel electrophoresis, followed by silver or Coomassie blue staining and Western blot analysis as previously described (46). Antibodies used were prepared in rabbits against bovine brain CaN (subunits A and B) as also described by Kissmehl et al. (46). For their detection, we used alkaline phosphatase- and peroxidase-coupled second antibodies from Sigma Immuno Chemicals (St. Louis, MO) and ICS Bio-medicals, Inc. (Aurora, CO), respectively.

**RNAi treatment.** *Paramecium* cells were fed bacteria containing a double-stranded RNA expressing vector as described previously (20, 79), with minor modifications as described below. Briefly, the target *Paramecium* CaNA1a and CaNA3a gene fragments were amplified by PCR and cloned into pL4440 using the Xbal and Xhol restriction enzyme sites. One positive clone for each for verificiation by DNA sequence analysis was used to transform *E. coli* HT115(DE3) cells and plated at 30°C on LB agar plates containing 50 μg/ml ampicillin. Individual colonies were selected from the plates and used to grow overnight cultures at 37°C in LB tetraacycline (5 μg/ml) plus ampicillin (50 μg/ml) medium. Cultures were grown to an optical density at 600 nm of 0.4 and induced with IPTG (125 μg/ml) as described previously (93). After a 4-h induction at 37°C, the bacteria were centrifuged, and the pellet was resuspended in an equal volume of un inoculated wheatgrass media to produce a 10× feeding stock. The 10× feeding stock was used to prepare a 1× feeding stock with un inoculated wheatgrass containing ampicillin (50 μg/ml) and IPTG (125 μg/ml). Approximately 80 starved *Paramecium* cells were placed into 5 ml of 1× feeding stock in a sterile 70-mm petri dish and incubated in a humidity chamber at 28°C overnight. The next day, 5 ml of a fresh 1× feeding stock was administered to the cultures. The cultures were incubated again for 24 h. The *Paramecium* cells were tested on the following 2 days (48- and 72-h treatments). Control cells were fed HT115(DE3) transformed with the empty vector, pL4440. Control cells fed untransformed HT115(DE3) gave results similar to those containing pL4440 and were not included in later experiments.

**Real-time RT-PCR.** RNA was isolated from RNAi treated cells by using the Ambion Ribopure kit (Ambion, Austin, TX) as described by the manufacturer. Cells were isolated from RNAi treatments of 48 and 72 h. The cultures were filtered through cheesecloth and centrifuged for 2 to 4 min at 800 × g. The supernatant was removed, and the cell pellets were homogenized in 10 to 20 volumes of TriReagent provided with the kit. The purified total RNA was quantified with a Nanodrop and cDNA was prepared as described by the RETROscript kit (Ambion) according to the two-step RT-PCR protocol. Primers for real-time RT-PCR were designed to straddle introns for both ohnologs of *Paramecium* RETROscript kit (Ambion) according to the two-step RT-PCR protocol. Primers for real-time RT-PCR were designed to straddle introns for both ohnologs of *Paramecium* genome database (http://aiaia.cgm.cnrs-gif.fr) contains seven “subfamilies” of nearly identical pairs (ohnologs) of CaN genes. As can be seen, both CaN subunits in *Paramecium* possesses a characteristic molecular size and domain structure, with little variation between family members (Fig. 1, Table 1). Interestingly, the number of CaN genes far outnumbers that reported in any other species (40, 80). The occurrence of genuine CaN genes is in contrast to the situation in higher land plants where only CaN-like genes are found (40) but is similar to what is observed in many other metazoans and some protozoans. The overall similarity on the amino acid level varies only slightly compared to other species (Table 2). This was additionally verified by expressing both CaN subunits heterologously in *E. coli* for Western blots analysis with anti-bovine CaN antibodies (see Fig. 5B in the supplemental material). The immuno reactive bands of an approx-imate size predicted for each of the two subunits provided additional proof of correct identification as calcineurin.

**Like metazoan CaNA subunits, Paramecium CaNA possesses a catalytic domain extending from near the N terminus to about two-thirds of the molecule. This is followed by a short CaNB binding domain, an equally short CaM-binding domain, and the autoimmune domain, characteristics of all CaNs (see Fig. 1). In the CaNA gene products, the first two domains are highly conserved, while the CaM-binding and the autoimmune domain are conserved to a lesser extent and more variable between the *Paramecium* paralogs (see Table SA in the sup-
For example, the CaM-binding domain varies between 50 and 93% identity between the Paramecium paralogs and between 14 and 57% identity compared to species from S. cerevisiae to H. sapiens (data not shown). In addition, when the CaNA1 amino acid sequence is threaded through the crystal structure of the human CaNA, it is clear the proteins can adopt similar structures (Fig. 2). The C-terminal end of the catalytic domain usually contains the motif, VYNN (including the shorter forms seen in the CaNA3 and CaNA7 gene products), as is typical for this domain in other species (80). Only in CaNA2a and CaNA2b ohnologs is the flanking region VYKN.

**Genetic structure of CaNA and CaNB.** The Paramecium CaNA and CaNB genes reveal a rather high degree of similarity to each other, especially between the ohnologs derived from the most recent whole-genome duplication (WGD) (Table 1). Intron predictions were made on the basis of stereotypic flanking regions in Paramecium (39, 81), and comparisons between genomic DNA and cDNA. Predicted introns were validated by RT-PCR and examination of the Paramecium Genome EST database. Interestingly, there are a variable number of introns, from two to ten, which often occur in similar positions but may vary somewhat, e.g., compare CaNA2 with CaNA1 (Fig. 3).
The RT-PCR validation of introns implies that at least one member of each of the CaNA gene pairs are expressed. Our RT-PCR analysis could not distinguish between the two gene pairs, and so it is possible that only one member is expressed for certain isoform pairs. However, an examination of the Paramecium EST database indicates that both members of the isoform pairs CaNA3, CaNA5, and CaNA6 are expressed and might suggest that all other isoform pairs are expressed as well. However, this is unproven at the moment.

An alignment of amino acid sequences indicates that there may have been several amino acid insertion and deletion events in this family as well (Fig. 3). Using parsimony as our guide, we hypothesize that CaNA2 had one deletion event, resulting in the loss of one amino acid, and that CaNA3 and CaNA7 each had two such deletions. In addition, our alignment also suggests that CaNA7 had an insertion event that led to the introduction of an additional amino acid (Fig. 3).

The Paramecium CaN subunit B is encoded by two slightly different genes resulting in identical amino acid sequences, including the typical four EF-hand domains (see Fig. SA in the supplemental material) (1). No introns were found in the two CaNB genes (Table 1). Similarity for these two genes with other organisms is between 50 and 54% identity with little variation from Plasmodium to humans (Table 2).

### TABLE 1. Characteristics of the Paramecium CaN genes

| Family | Gene<sup>a</sup> | Accession no. | Size (bp) | Size (aa) | Introns | % Identity within a group bp level | % Identity within a group aa level | % Identity between groups bp level | % Identity between groups aa level |
|--------|-----------------|---------------|----------|----------|---------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| CaNA   | CaNA1<sup>a</sup> | AF014922      | 1,779    | 509      | 10      | 22–29                             | 100                               | 100                               | 100                               |
|        | CaNA1<sup>b</sup> | CR932330      | 1,771    | 508      | 10      | 22–28                             | 87.7                              | 95.7                              | 95.7                              |
|        | CaNA2<sup>a</sup> | AJ567906      | 1,701    | 507      | 7       | 23–29                             | 86.9                              | 93.7                              | 72.2                              |
|        | CaNA2<sup>b</sup> | CR933485      | 1,698    | 507      | 7       | 21–29                             | 100                               | 100                               | 85.1                              |
|        | CaNA3<sup>a</sup> | CR932329      | 1,589    | 470      | 7       | 22–28                             | 96.5                              | 99.6                              | 58.4                              |
|        | CaNA3<sup>b</sup> | CR933228      | 1,591    | 470      | 7       | 23–28                             | 100                               | 100                               | 58.4                              |
|        | CaNA4<sup>a</sup> | CR933227      | 1,765    | 507      | 10      | 22–29                             | 89.8                              | 97.0                              | 83.5                              |
|        | CaNA4<sup>b</sup> | CR932326      | 1,766    | 507      | 10      | 22–28                             | 100                               | 100                               | 83.9                              |
|        | CaNA5<sup>a</sup> | CR932325      | 1,764    | 507      | 10      | 22–28                             | 96.1                              | 98.6                              | 84.9                              |
|        | CaNA5<sup>b</sup> | CR933484      | 1,775    | 509      | 10      | 22–28                             | 100                               | 100                               | 79.8                              |
|        | CaNA6<sup>a</sup> | CR933483      | 1,715    | 505      | 8       | 22–28                             | 100                               | 100                               | 55.1                              |
|        | CaNA6<sup>b</sup> | CR933224      | 1,715    | 504      | 8       | 22–27                             | 96.7                              | 99.0                              | 55.7                              |
|        | CaNA7<sup>a</sup> | CR933481      | 1,513    | 486      | 2       | 24–28                             | 100                               | 100                               | 43.5                              |
|        | CaNA7<sup>b</sup> | CR933482      | 1,511    | 486      | 2       | 24–26                             | 93.5                              | 100                               | 43.5                              |
| CaNB   | CaNB1<sup>a</sup> | AJ554047      | 510      | 169      | 0       | 100                               | 100                               | 100                               | 100                               |
|        | CaNB1<sup>b</sup> | AJ554048      | 510      | 169      | 0       | 93.5                              | 100                               | 100                               | 100                               |

<sup>a</sup> Sequences were aligned by using CLUSTAL W. Groups are defined as ohnolog pairs. aa, amino acids.

<sup>b</sup> *, verified at the cDNA level.

### TABLE 2. Comparison of Paramecium amino acid sequences with various species

| Gene      | Plasmodium yoelii yoelii | Saccharomyces cerevisiae | Dictyostelium discoideum | Drosophila melanogaster | Xenopus laevis | Rattus norwegicus | Homo sapiens |
|-----------|--------------------------|--------------------------|--------------------------|-------------------------|----------------|------------------|--------------|
| CaNA1<sup>a</sup> | 49                        | 42.7                     | 43.9                     | 43.9                    | 42.7           | 43.1             | 43.1         |
| CaNA1<sup>b</sup> | 50.7                     | 42.2                     | 44.8                     | 45                      | 44             | 44.8             | 44.2         |
| CaNA2<sup>a</sup> | 51.6                     | 40.2                     | 44.3                     | 42.9                    | 43.5           | 44.1             | 41.9         |
| CaNA2<sup>b</sup> | 52                       | 40.7                     | 44.9                     | 43.3                    | 44.7           | 45.7             | 42.9         |
| CaNA3<sup>a</sup> | 53.5                     | 42.9                     | 45.6                     | 44.6                    | 44.4           | 44.6             | 43.7         |
| CaNA3<sup>b</sup> | 53.5                     | 42.7                     | 45.6                     | 44.6                    | 44.2           | 44.8             | 43.7         |
| CaNA4<sup>a</sup> | 50.4                     | 40.7                     | 42.5                     | 44.1                    | 44.1           | 44.1             | 42.9         |
| CaNA4<sup>b</sup> | 50.4                     | 41.7                     | 42.1                     | 43.9                    | 42.9           | 43.3             | 42.3         |
| CaNA5<sup>a</sup> | 49.5                     | 40.8                     | 41.8                     | 43.6                    | 43.2           | 43.6             | 42.6         |
| CaNA5<sup>b</sup> | 49.6                     | 40.8                     | 41                       | 43.1                    | 42.2           | 42.5             | 42           |
| CaNA6<sup>a</sup> | 51.1                     | 42                       | 44                       | 41.8                    | 40.8           | 41.6             | 40.8         |
| CaNA6<sup>b</sup> | 50.9                     | 41.4                     | 43.6                     | 42.4                    | 42.6           | 43.6             | 42.2         |
| CaNA7<sup>a</sup> | 49.5                     | 44.6                     | 45.4                     | 42.7                    | 43.5           | 44.1             | 43.9         |
| CaNA7<sup>b</sup> | 49.7                     | 43.9                     | 46                       | 43.1                    | 42.7           | 43.3             | 43.1         |
| CaNB1<sup>a</sup> | 54.1                     | 50                       | 52.4                     | 51.2                    | 53.5           | 52.9             | 54.1         |
| CaNB1<sup>b</sup> | 54.1                     | 50                       | 52.4                     | 51.2                    | 53.5           | 52.9             | 54.1         |

<sup>a</sup> Based upon percent identity scores determined by using CLUSTAL W.
Paramecium CaNA protein family members cluster together with two identified Tetrahymena CaNA protein subunits and in a distinct clade within a larger protozoan clustering. The fungus and animal CaNA protein sequences cluster apart from the protozoan CaNA proteins, and the deep branching seen for the protozoan variants supports the hypothesis that the CaNA gene arose in protozoans prior to the development of multicellular life forms, as others have suggested (31).

A closer examination of the phylogenetic relationships revealed in the ciliate cluster indicates that the branching pattern does not readily fit a model in which the 14 isoforms are simply derived from three WGDs. The most recent WGDs are clearly evident, with the pairs of ohnologs produced by this event easily seen in Fig. 4. However, the earlier WGDs are more difficult to detect. For example, a simple model for the more ancient WGD would predict that the isoforms would branch in a succession of bifurcations after each duplication event, resulting in a more “balanced” tree with approximately equal numbers of isoforms on either side of a deep midpoint branching. Instead, we observe a tree that is not “balanced,” with successively more divergent individual isoforms branching out as one approaches the base of the tree. It is possible that there may have been significant selective pressure to diverge the amino acid sequences of individual isoforms for optimal function within different cellular contexts thus obscuring their derivation from earlier duplication events. Two other possibilities include extensive gene loss in some branches but not others as suggested by Aury et al. (4) and/or gene conversion between individual family members.

The CaNA gene family shows evidence of gene conversion. One mechanism that might obscure phylogenetic relationships is gene conversion. The members of the CaNA gene family have high levels of sequence identity that has been correlated with gene conversion events in other species (17, 68, 85, 97). CaNA family members were tested for possible conversion events between pairs of ohnologs and between nonohnologs (Fig. 3). For this analysis, we used the software package RDP3 (57). The analysis provided evidence for four recombination events between members of this family. Three were between nonohnologs (CaNA2 exchanging with CaNA4 and CaNA5; CaNA7 exchanging with CaNA3), and one was between a pair of ohnologs (CaNA3a exchanging with CaNA3b). The ex-
change between CaNA2 and both CaNA4 and CaNA5 is most parsimoniously explained as an exchange between CaNA2 and a common ancestor for CaNA4 and CaNA5 (based on Fig. 4). Thus, this was counted as one gene conversion event. The small exchange detected between CaNA7 and CaNA3 was considered an event, although its small size casts some doubt on its validity given the high degree of sequence conservation seen between subfamily members, especially in this region. However, these putative events were all supported by visual inspection of the affected regions as explained in the Materials and Methods. Despite the fact that we can identify gene conversion events in this family, they appear to have minimal effect upon the phylogenetic patterns observed, since removal of the regions suspected of undergoing exchange from our phylogenetic analysis resulted in no change in the branching patterns observed (data not shown).

Functional analysis of two CaNA family members using RNAi. The CaNA family of proteins is highly conserved and shows evidence of gene conversion events in its evolutionary history, suggesting some redundancy of function, as discussed by others (4). We decided to test whether the family members might in fact have diverse functions in the cell despite the high degree of conservation. For our purposes, we selected two relatively diverse members (CaNA1 and CaNA3) so as to maximize our chances of detecting differences in function, if they existed.

Considering the wide spectrum of CaN activities in a cell and the previous observation that, in Paramecium, microinjected antibodies can inhibit stimulated trichocyst exocytosis (63), we selected exocytosis and swimming behavior for a more detailed analysis by gene silencing through RNAi since these are two well-studied and characterized calcium-dependent processes in Paramecium. RNAi was accomplished by feeding an appropriate E. coli strain (see Materials and Methods) transformed with a vector containing the open reading frame of the gene to be silenced (20). This posttranscriptional homology-dependent RNAi-based method is known to efficiently silence paralogs with a nucleotide identity of >85% or greater (79). Thus, the sequences selected for gene silencing are likely to silence their corresponding ohnolog, i.e., the RNAi construct for CaNA1a is likely to only silence the pair of CaNA1 ohnologs. Similarly, the CaNA3a is likely to only silence the pair of CaNA3 ohnologs. Using real-time RT-PCR we verified that each RNAi treatment selectively reduced the mRNA levels for the

FIG. 4. Phylogenetic analysis of the Paramecium CaNA isoforms. A maximum-likelihood tree generated using the PhyML program as implemented by Phylogeny.fr (see Materials and Methods) is shown. Protein sequences were selected from a broad range of species. The lengths of the branches represent the distance between sequences, and a scale bar is shown on the left (substitutions per site). All bootstrap support values shown were ≥75%. Bootstrap values of <75% were collapsed. Neighbor-joining and Bayesian analyses were also conducted using Phylogeny.fr and gave equivalent branching patterns. Abbreviations and GenBank accession numbers were as follows: DrCaNA (Danio rerio CaNA: CaNA1, NP_001074063; CaNA2, NP_00101441; CaNA3, XP_686173; CaNA4, XP_001334569), HsCaNAx (Homo sapiens CaNAx: CaNAox, NP_000935; CaNAx, NP_001135826; TaCaNA (Trichoplax adhaerens CaNA, XP_1021111637), CcCaNA (Caenorhabditis elegans CaNA, CAB02719), DmCaNA (Drosophila melanogaster CaNA; CaNA1, NP_727985; CaNA2, NP_523373; CaNA3, NP_524600), ScCaNA (Saccharomyces cerevisiae CaNA; CaNA1, NP_013537; CaNA2, NP_013655), UmCaNA (Ustilago maydis CaNA, XP_757083), MgCaNA (Malassezia globosa CaNA, XP_001729627), AoCaNA (Aspergillus oryzae CaNA, XP_001824850), SpCaNA (Schizosaccharomyces pombe CaNA, NP_596178), PyyCaNA (Plasmodium yoelii yoelli CaNA; EAA21752), TgCaNA (Toxoplasma gondii), CpCaNA (Cryptosporidium parvum; XP_627753), TiCaNA (Tetrathyphema thermophila CaNA1; XP_001027523; CaNA1, XP_001012424), PtcCaNA(a or b) (Paramecium tetraurelia CaNA(a or b); 1a, AAB80918; 1b, CAI39156; 2a, CAD99184; 2b, CAI44580; 3a, CAI39155; 3b, CAI39154; 4a, CAI39153; 4b, CAI39152; 5a, CAI39151; 5b, CAI44589; 6a, CAI44588; 6b, CAI39150; 7a, CAI44587; 7b, CAI44586). All annotated Paramecium sequences have been deposited into the EMBL and NCBI databases.
target genes (ohnolog pairs) to a minimum of 5 to 8% of control levels after 48 h in the case of \( \text{CaNA1} \) and 48 to 72 h in the case of \( \text{CaNA3} \). \( \text{CaNA1} \) message levels had begun to recover by 72 h, reaching 45 to 55% of the control levels, whereas \( \text{CaNA3} \) had only recovered to ca. 10 to 15% of control levels in that time frame. Cells were phenotypically tested at 48 and 72 h. Message levels for \( \text{CaNA1} \) were not significantly affected when cells were subjected to \( \text{CaNA3} \) RNAi treatment and, similarly, \( \text{CaNA3} \) levels were not significantly affected by \( \text{CaNA1} \) treatment.

Silencing with \( \text{CaNA3} \) but not \( \text{CaNA1} \) in wild-type cells leads to increased backward swimming in 30 mM KCl testing solution. \( \text{Paramecium} \) swimming behavior is controlled by the coordinated action of a set of voltage- and calcium-dependent ion currents (33). The most significant of these is the voltage-dependent calcium current that initiates the action potential controlling backward swimming. This current can be indirectly evaluated by placing cells in a 30 mM KCl testing solution (34). When wild-type cells treated with \( \text{CaNA3} \) RNAi were tested in this solution, they displayed increased backward swimming times relative to controls, a finding indicative of an increase in the duration of the inward calcium current and/or an inability to clear calcium quickly from the cilia (Fig. 5A; for significance values, see the figure legend). In contrast, wild-type cells treated with \( \text{CaNA1} \) RNAi were not affected when tested in 30 mM KCl testing solution. This suggests that the \( \text{CaNA3} \) gene products have an important role in regulating calcium concentrations in response to stimuli. This action could be either through direct or indirect regulation of calcium ion channels.
that allow calcium in or through direct or indirect regulation of calcium downregulation.

During the course of these experiments it was observed that the phenotype displayed after treatment with CaNA3 RNAi was similar to the mutant Dancer phenotype (i.e., which reacts to depolarization with prolonged backward swimming due to delayed inactivation of the voltage dependent Ca\(^{2+}\) channel) (34). To investigate this further, we repeated the RNAi experiments using Dancer cells (Fig. 5B). Interestingly, the CaNA3-treated Dancer cells swam backward for the same duration as Dancer cells treated with control RNAi plasmid (pL4440), whereas the CaNA1-treated Dancer cells showed increased backward swimming times in the same test solution. These results were surprising and suggested that the Dancer mutation somehow overrode or masked the effects seen from silencing CaNA3 expression in wild-type cells. This could be due to the Dancer mutation affecting the CaNA3 gene products directly or indirectly through a common link between the Dancer and CaNA3 gene products. To address the first possibility, we sequenced the CaNA3a and CaNA3b genes and the CaNA1a and CaNA1b genes from Dancer 1, Dancer 7, and wild-type cells but did not find any mutations (data not shown). Thus, it appears that the Dancer gene product works cooperatively with CaNA3 gene product but is not the result of a mutation in either CaNA3 gene.

The revealed CaNA1 RNAi phenotype in the Dancer background was also surprising and suggested that the regulation of calcium levels in the cell is complex and perhaps controlled by more than one CaNA subunit either through regulation of different components or through some limited subunit redundancy and/or ability to compensate for each other.

Silencing of CaNA1 but not silencing of CaNA3 reduces exocytosis. In contrast to a clear role for the CaNA3 gene products in swimming behavior, CaNA3 did not have an identifiable role in exocytosis, whereas CaNA1 did. The results of our test for exocytosis capacity using picric acid (a fixative allowing easy quantification of protruding needlelike trichocysts) is shown in Fig. 6. In the wild-type cells that were treated with the control vector (empty pL4440), 76% of cells show full exocytosis (90% or greater). However, only 25% of CaNA1-RNAi-treated cells and 35% of ND7-RNAi-treated positive-control cells were capable of full exocytosis. Similarly, only 7% of wild-type cells had an exocytotic discharge of <50%, whereas 39% of CaNA1-RNAi-treated cells and 31% in ND7-RNAi-treated positive control cells displayed an exocytotic discharge of <50%. The differences seen in exocytosis efficiency were significant (P < 0.001) comparing CaNA3- and pL4440 RNAi-treated cells (no effect) with CaNA1- and pND7 RNAi-treated cells (reduced). In sum, the reduced exocytosis efficiency achieved by CaNA1 silencing is similar to that achieved with the positive control ND7-RNAi-treated cells.

Inhibition of exocytosis by CaNA1-RNAi treatment does not result from any observable structural defect. Since exocytosis was reduced, we wanted to determine on what level this was manifested. Using light and electron microscopic approaches, we examined whether a full set of normal trichocysts, with normal-looking docking sites, would be found in cells after CaNA1 silencing. Trichocyst docking appeared normal as seen in Fig. 6B, which provides a representative light microscopy result. Each cell seemed to have a normal endowment of trichocysts. The trichocysts have identical carrot shapes (when seen in longitudinal section), with a moderately electron-clear “trichocyst body” (round, when seen in cross-section) and a much shorter, barely visible (because of similar electron density as the surrounding cytoplasm) “trichocyst tip” for docking at the cell surface. Also note the very similar shapes and numbers of trichocysts in wild-type and silenced cells. When ultra-thin sections were analyzed by EM, no overt differences were found in the ultrastructure of trichocysts and their docking sites at the plasmalemma (data not shown). Thus, inhibition of exocytosis is not due to an observable structural defect.

CaNA1-RNAi treatment results in delayed [Ca\(^{2+}\)]\(_i\) decay. We next examined if Ca\(^{2+}\) dynamics after exocytosis stimulation was affected in CaNA1-RNAi-treated cells (Fig. 7). This was done at two different [Ca\(^{2+}\)]\(_o\) values, one at 100 \(\mu\)M and one at \(\sim\)30 nM. The latter is slightly below resting [Ca\(^{2+}\)]\(_o\) values, thus taking into account any effect of silencing on store mobilization (84). Higher [Ca\(^{2+}\)]\(_o\) values reflect the situation when Ca\(^{2+}\) store depletion is superimposed by influx, i.e., “store-operated Ca\(^{2+}\) influx” (74). In fact, when exocytosis was stimulated by AED, both wild-type and CaNA1-silenced cells show approximately the same response, except for the kinetics of the signal decay. Considering limitations due to the time required for a filter change in our double wavelength recordings (see Materials and Methods), the rise time may be identical. The signal height is lower in cells at low [Ca\(^{2+}\)]\(_o\). To make signal decay better visible, those parts of the decay curves were plotted separately where the decay curve shows a quasilinear fit within standard errors. At high [Ca\(^{2+}\)]\(_o\), the half time of [Ca\(^{2+}\)]\(_i\) decay (linear regression within standard errors) is 5.9 s (±0.3 [standard error of the mean]) and 12.3 s (±0.9) in normal and CaNA1-silenced cells, respectively. At low [Ca\(^{2+}\)]\(_o\), [Ca\(^{2+}\)]\(_i\) decays with half times of 6.3 ± 0.05 s and 8.3 ± 0.2 s in normal and silenced cells, respectively (Fig. 7).

In conclusion, only CaNA1-RNAi treatment of wild-type cells resulted in a reduction of exocytosis efficiency. This effect was not due to reduced trichocyst docking, gross changes in the docking architecture at the membrane, or decreased calcium influx coincident with stimulation. It was correlated with an increase in the half time for decay in the calcium signal after stimulation. However, it seems unlikely this causes reduced exocytosis efficiency, though it may indicate a reduced efficiency to regulate calcium levels in the cell (see below).

**DISCUSSION**

Identification of highly conserved CaNA and CaNB gene families in *Paramecium*. This report conclusively demonstrates the existence of both CaNA and CaNB gene families in *Paramecium*, as suggested by previous enzymatic (45, 46), and antibody binding studies (62, 63). Moreover, there is considerable evolutionary conservation of these families with both subunits recognized by antibodies generated to their mammalian counterparts. CaNA and CaNB genes have been identified more recently in other protozoans (31), but in the case of trypanosomes (65), the identified CaNA subunit lacks two of the regulatory domains typically seen in CaNA subunits (the calmodulin binding and autoinhibitory domains). The percent identity seen between the *Paramecium* CaNA subunits and the human CaNA (39 to 41%) is in the middle of the range seen when
comparing other protozoan CaNA subunits to human CaNA (34 to 48%). This includes a very strong conservation of the CaNB binding site in the CaNA protein (see Table SA in the supplemental material), a finding consistent with there only being two genes encoding identical versions of the CaNB subunit and thus presumably strong purifying selection acts on the binding site. Molecular modeling of the CaNA1 gene product (Fig. 2) further supports the conclusion that it closely resembles its human counterpart.

The CaNA gene family in Paramecium is exceptionally large and may have a complex evolutionary history. Several WGDs in the evolution of Paramecium (4) may account for the occurrence of seven CaNA subfamily pairs, with the final WGD duplication event resulting in the nearly duplicate pairs (ohnologs) seen in each subfamily (Fig. 4). Current estimates are that the Paramecium genome has been duplicated at least three times with subsequent gene loss (4). Thus, the CaNA family either started with a pair of CaNA genes prior to these three WGD events or underwent additional duplication events independent of the WGDs. Although it is clear that the most recent WGD is supported by the clustering of pairs of highly conserved sequences at the tips of each branch, it is difficult to reconcile the branching pattern seen with a simple model in which the gene family is successively duplicated after each

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**FIG. 6.** Inhibition of stimulated trichocyst exocytosis by silencing the CaNA1 gene. (A) Examples of picric acid-treated cells: pL4440 (A1)-, CaNA1 (A2)-, and ND7 (A3) RNAi-treated cells. Exocytosis was tested with saturated picric acid. The table in panel B compares the silencing effect using the different RNAi vectors indicated. Statistical analysis was as described in Materials and Methods, but only the relative distributions of the phenotypes are shown here for simplicity. The distribution of the CaNA1 and ND7 phenotypes was not statistically different from each other but were statistically different from pL4440- and CaNA3 RNAi-treated cells ($P < 0.001$). Silencing with an RNAi vector targeting CaNA3 yielded results similar to those obtained with cells fed the pL4440 control vector ($P > 0.5$). (B) Semithin sections of wild-type and CaNA1 silenced P. tetraurelia cells. The photographs were obtained from semithin sections of plastic embedded materials analyzed by light microscopy. Examples of wild-type (B1 and B2) and CaNA1 silenced (B3 and B4) cells do not reveal any overt difference in trichocyst (carrot-shaped bright organelles in B1 to B4) endowment. Images were at identical magnifications for survey (B1 and B3) and detail (B2 and B4). Bars, 10 μm.
WGD even when postulating a gene loss event to leave seven genes prior to the last WGD. One explanation might be that this particular family was expanded at some level through successive individual gene duplication events with subsequent divergence in sequence. Another possibility is that after the first two WGD events there was strong selective pressure to diverge individual members, leading to the pattern we observe today after the last, relatively recent WGD event. If true, such selective pressure might be expected to result in the selection for nonredundant functions for family members, as seen here. In addition, complicating factors such as the gene conversion events identified in Results may have obscured a clear phylogenetic signal or served to “homogenize” some gene sequences but not others.

Gene conversion in this family was examined to determine whether it was impacting our phylogenetic analysis, and three events were tentatively identified. The overall number of gene conversion events (three events between 14 genes [21%]) is higher than what has been reported for global gene conversion rates in a variety of species (2 to 7.4%) ranging from yeast to Drosophila to rice (17, 68, 85, 97). However, it is consistent with an analysis of large gene families in Arabidopsis (44%), which, like many plants, has a large number of duplicated genes (64). In general, higher frequencies of recombination are found between very similar sequences (such as recently duplicated genes), and thus relatively higher gene conversion rates would be expected in species with large numbers of duplicated genes such as seen in plants and in Paramecium. The size of the exchanges in CaNA genes (20 to 559 bases) is consistent with what has been reported in a range of species (7 to 2,958 bases) (17, 68, 85, 97). Nonetheless, the effect of the tentatively identified gene conversion events upon the relationships observed in Fig. 4 appears to be negligible. When the regions suspected of undergoing exchange were removed from our phylogenetic analysis, there was no significant change in the branching pattern (data not shown). This suggests that, while gene conversion may be a factor in explaining the phylogenetic relationships, it cannot explain the branching pattern we observe and thus other mechanisms must be invoked (such as preferential gene loss of certain lineages or extensive divergence in some lineages).

And finally, while the number of CaNB genes is similar to what is seen in other organisms, the seven CaNA gene subfamilies with a total of 14 members are without precedent. Our search of available databases and sequence alignments revealed that zebrafish have four, humans and Drosophila have Saccharomyces has two, and Caenorhabditis has one. Protozoans typically have only one copy of the CaNA gene, although Tetrahymena appears to have two. Interestingly, the entire family is thought to be absent in plant and algae genomes (40). Its tempting to speculate that the large number of

FIG. 7. Ca^{2+} signals occurring after exocytosis stimulation in wild-type and CaNA1 silenced cells at normal and reduced [Ca^{2+}]o. The time course of fluorochrome signals was determined in the cortex of Fura Red-loaded cells at the site of AED stimulation (at time zero). The abscissa shows the time scale (s); the ordinate shows the relative [Ca^{2+}]i signal. In the graphs on the left, no difference in the initial signal generation was found between control cells (fed with the empty vector, black) and CaNA1 silenced cells (red), both at normal and reduced [Ca^{2+}]o, i.e., 100 μM and ~30 nM, respectively. In contrast, the decay time was increased in CaNA1 silenced cells when experiments were performed at high and low [Ca^{2+}]o, respectively. For the graphs on the right, an evaluation of the quasilinear decay periods was performed (see the text). In this sequence (left to right, top to bottom), the R values are 0.9513, 0.9854, 0.9727, and 0.9775, respectively. Bars indicate the standard errors of the mean.
family members have differential localizations and functions in the rather complex Paramecium cells, as seen for other large gene families (44, 95), while recently formed subfamily members may act as a kind of gene amplification (4). This latter hypothesis may be particularly true for CaNB whose subfamily members are both expressed and are identical on the amino acid level. The requirement for precise binding by the CaNB subunit to its binding site on the CaNA subunits (see Table SA in the supplemental material) may have further constrained the two from any considerable variation. Although the CaNA subunit exerts some phosphatase activity on its own (30), it is increased when complexed with the B subunit (70), again stressing the requirement of a precise fit. A complex system of CaN binding or interacting proteins (3, 12, 86) may be as important as the interaction with the B subunit, an aspect requiring further analysis.

**Relation to previous functional data.** How do our findings fit with the type of phosphorylation and the kinases previously shown to be involved in trichocyst discharge and swimming behavior? In Paramecium, our previous work has concentrated on the exocytosis-sensitive CaN substrate, pp63 (45, 46) that is phosphorylated by a cGMP-activated kinase (43) and a casein kinase 2-type kinase (94). This fits the generally broad spectrum of CaN substrates that may be phosphorylated by widely different kinases (86) and the multifunctional activity of CaN (47, 80, 86). All of these molecules, including the kinases, together with CaN and pp63, colocalize to the narrow space between the cell membrane and cortical calcium stores, as well as around the docking sites of trichocysts (42, 62).

Few details are known about possible kinases or phosphatases involved in regulating swimming behavior in Paramecium. Several calcium-dependent kinases and calcineurin have been localized to the cilia (62), but no specific testing of their roles has been previously conducted (33). Antisense reagents targeting calmodulin (one of the activators of calcineurin) have revealed a role for calmodulin in swimming behavior but not with directly regulating the behavior tested in 30 mM KCl testing solution observed here (32). This may be because the levels of calmodulin reduction were not sufficient to disrupt its ability to activate CaN upon calcium stimulation. Nonetheless, the results presented here are consistent with what is seen in other species, in which CaN has been demonstrated to regulate various ion currents in excitable cells including voltage-dependent calcium currents (29, 98).

The RNAi results obtained with Dancer were unexpected. The Dancer mutation causes a much slower inactivation of the calcium channels involved in the behavioral response compared to wild-type calcium channels (39). Our results suggest distinct functions for the CaNA1 and CaNA3 proteins in this regulation. The fact that the silencing of CaNA3 in Dancer does not change the phenotype of the mutant cells despite affecting wild-type cells suggests that CaN may be involved in the phosphorylation state of the calcium channel or a regulator; the dephosphorylation of the channel by CaNA3 gene product being required for proper inactivation. If the phosphorylation of the calcium channel (or a regulator) is altered in the Dancer mutant, silencing of CaNA3 would have no further effect on the cellular phenotype, as seen here. Conversely, since silencing of the CaNA1 gene has no effect on the behavioral response of wild-type cells to 30 mM KCl but exaggerates the response of Dancer cells, a different cellular function is indicated. One explanation for this might be that CaNA1 gene product is involved in the activation of a calcium pump that rids the cilia of the increased calcium following the action potential. To support this speculation, there is evidence that CaN is involved in the modulation of plasma membrane Ca$^{2+}$-ATPases (36). Thus, silencing of CaNA1 expression would exaggerate the response of Dancer cells because not only is the calcium channel inactivation delayed by the Dancer mutation, but the excess calcium is removed slower than that seen in normal cells. However, without direct evidence of interaction, these remain hypotheses for the time being.

**What may be the physiological significance of the large number of CaNA isoforms?** The question about the large number of gene duplicates in Paramecium was first raised upon the release of the genome sequence (4). In general terms, it was suggested that there has been selection for increased expression in this species. Alternatively, there are numerous potential substrates and target structures of CaN/PP2B in Paramecium that could be differentially served by the distinct isoforms. Substrates may contain different docking sites for CaN interaction (53), and different isoforms of a protein may possess different affinities for CaN binding (36). As known from other protein phosphatases (38), strategic positioning allows the phosphatases to become rapidly active at specific sites where target molecules occur (16). The experiments described here suggest we can eliminate the simplest hypothesis that the highly identical family of CaNA protein subunits exists simply for gene expression purposes and act redundantly in the cell. By comparing RNAi results from two of the more distantly related isoforms, it is clear that these two isoforms have distinct but also partially overlapping functions in the cell. In the case of the CaNA1 gene products, a clear role in regulating exocytosis is seen, as well as perhaps a minor or redundant role in regulating swimming behavior. The CaNA3 gene products, on the other hand, do not have a clear role in regulating exocytosis, at least as measured here, but do have a significant role in the regulation of swimming behavior, most likely through regulation of calcium flux. The effects achieved after silencing of CaNA1 on [Ca$^{2+}$], signal decay recorded during AED stimulation and the prolongation of ciliary response upon depolarization in CaNA3 silenced cells may both be explained by retarded calcium downregulation, yet at different sites of the cell. The latter is supported by the occurrence of widely independent [Ca$^{2+}$], dynamics (74). CaNA1 silencing, however, must include still an additional disturbance, since exocytosis would not be inhibited by a slow signal decay.

Calcineurin isoforms in other species have also been assigned different roles in the cell based upon functional analyses similar to what is described here (9, 100). For example, studies in rabbit pulmonary artery smooth muscle cells revealed CaNA-α but not CaNA-β regulates Ca$^{2+}$-dependent currents. This difference in activity was correlated with an isoform-specific translocation of the CaNA-α isoform to the cell membrane after calcium stimulation. In addition, targeted gene knockouts in mice of CaNA/AED stimulation and the prolongation of ciliary response upon depolarization in CaNA3 silenced cells may both be explained by retarded calcium downregulation, yet at different sites of the cell. The latter is supported by the occurrence of widely independent [Ca$^{2+}$], dynamics (74). CaNA1 silencing, however, must include still an additional disturbance, since exocytosis would not be inhibited by a slow signal decay.
sensitivity to known inhibitors of calcineurin activity (71), providing a biochemical basis for isoform differences in function.

**CaN’s role in exocytosis in Paramecium.** In *Paramecium*, CaN has been shown to specifically dephosphorylate the 63-kDa phosphoprotein, pp63 (45, 46), a 63-kDa protein with multiple Ser/Thr phosphorylation sites on its surface (67), in strict correlation with synchronous (80 ms) exocytosis (35). In a recent survey, pp63 could be reasonably connected with several aspects of Ca\(^{2+}\) signaling, specifically in subplasmalemmal domains (73) where pp63 (42) colocalizes with CaN (62). This may include a role for CaN in Ca\(^{2+}\) store activation (10, 87), Ca\(^{2+}\) influx, superimposed to store activation in *Paramecium* (74), may also be affected, in analogy to other cells (29, 98). We demonstrate here that at least one isoform of CaNA is involved in a step subsequent to vesicle docking and prior to exocytosis. Our understanding of the role of calcineurin in the regulation of exocytosis is complex, with some systems showing that calcineurin acts to inhibit exocytosis (8) and others showing it facilitates exocytosis (77). Our results would suggest that at least one CaNA isoform in *Paramecium* acts to facilitate exocytosis since silencing it by RNAi results in less efficient exocytosis.

**Exocytosis versus Ca\(^{2+}\) signal generation after CaNAI silencing.** How to reconcile the inhibitory effect of CaNAI silencing on exocytosis and the simultaneous persistence of a strong Ca\(^{2+}\) signal upon exocytosis stimulation? Recall that after CaNAI silencing we found no changes in the occurrence of trichocyst docking at the cell surface (Fig. 6B) and in the (ultra)structural appearance of trichocyst docking sites (data not shown). Furthermore, the rise of the Ca\(^{2+}\) signal after AED stimulation was normal (Fig. 7). (One has to consider that the time required for filter change in our microscope does not allow us to clearly define the time of Ca\(^{2+}\) increase within the short time available.) Only the signal decay differs between controls and silenced cells.

From the discussion above, one might expect a reduced Ca\(^{2+}\) release from cortical stores, but instead a retardation of the signal decay is observed, regardless of whether AED stimulated exocytosis at high or low [Ca\(^{2+}\)]\(_{\text{cyt}}\). This could mean that release channels (possibly also influx channels [see above]) would remain open for longer times or that termination of the signal by sequestration and/or binding to immobile Ca\(^{2+}\) buffers, such as centrin (84), may be retarded. In fact, the degree of phosphorylation might instead affect centrin assembly in cortical filament bundles (41, 49), and this might affect down-regulation of the Ca\(^{2+}\) signal. This argument, however, was disproofed by exploiting the Ca\(^{2+}\)-dependent contraction of cortical centrin-filament bundles (6), since we found no difference in AED-mediated contraction between CaNAI-silenced and nonsilenced cells (data not shown).

Considering the inhibitory effects of CaN on exocytosis seen after CaNAI silencing, one also has to consider that CaN during exocytosis may regulate the degree of phosphorylation of a subset of phosphoproteins occurring in exocytotic systems (11). Thus, it is possible that CaNA1 regulates the readiness of additional proteins involved in the membrane fusion events leading up to and including exocytosis.

**Conclusion.** We document here an unusually large family CaNA genes present in the protozoan, *Paramecium tetraurelia*. This supports previous suggestions that CaNA evolved very early in the evolution of eukaryotes (31) and further suggests that CaN involvement in calcium channel regulation and exocytosis evolved early as well. The occurrence in *Paramecium* of many functions normally ascribed to CaN/PP2B, the multiplicity of isoforms in *Paramecium*, and our preliminary evidence suggest that perhaps the different CaNA isoforms (at least the ones tested here) have evolved distinct and yet partially overlapping functions. The conservation of CaNB amino acid sequence, with only small variations in the gene structure, together with the conservation of the CaNB binding site in the various isoforms of the CaNA gene products, suggest a highly coordinated coevolution. All this makes *Paramecium* a promising model system for understanding how duplicated genes evolve over time to serve the multiple needs of a cell.

The pleiotropic effects of CaN-mediated dephosphorylation processes, and its frequently antagonistic effects (18, 23), point to a complex role for CaN in many cells. Given the complexity of CaN regulatory pathways in general and the large family of CaNA subunits seen in *Paramecium* in particular, further work on the *Paramecium* CaN family may yield additional insight into the process by which duplicated genes are retained, modified, or eliminated in a species. Isoforms may exert site-specific functions, e.g., in the local regulation of spatially restricted Ca\(^{2+}\) signals. On the basis of the present work, much more diversified functional experiments now appear feasible in the future in order to determine to what extent the large gene families in *Paramecium* have diversified their functions.

In sum, the salient features of our work are as follows. (i) CaN definitely occurs in the protozoan, *Paramecium*, a point that has been disputed previously (50, 82). Its presence in ciliates and some other protozoans supports the hypothesis made by others that CaN evolved early in the evolution of eukaryotes (31). (ii) CaNA, in contrast to CaNB, occurs as an unusually large family composed of seven pairs of nearly identical proteins. (iii) The interaction between CaNB and its binding site on the CaNA subunits is strongly conserved. Apparently the CaNB subunit can fulfill its role in the cell without a similar expansion in family members as seen for the CaNA family. (iv) At least two of the CaNA subfamily members have distinct functional roles in exocytosis and ciliary beat regulation. (v) Identification of a role for CaN in exocytosis and the regulation of ciliary-based motility in protozoan provides evidence that CaN developed a role in these processes early in its evolution.

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