Review

Control Molecules in Protozoan Ciliary Motility

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Ciliated protozoa such as *Tetrahymena* or *Paramecium* have long been important organisms for the study of ciliary motility. The coordinated activity of many hundreds of cilia is responsible for the movement of these ciliates through water. Furthermore, changes in that activity are responsible for the behavior of the cells, in particular chemotactic and mechanotactic responses. Almost a century ago, Jennings (1906) described the avoiding reaction of *Paramecium*, whereby the cell backs away from an obstacle by changing the direction of ciliary beat, a so-called ciliary reversal. It was then shown that this response was produced when a voltage sensitive Ca^{2+} channel of the ciliary membrane opened, permitting Ca^{2+} to enter the cilium (Eckert, 1972; Eckert et al. 1976).

Ultrastructural studies beginning with Fawcett and Porter (1954) showed that virtually all motile cilia are comprised of a microtubule-based 9+2 axoneme surrounded by the ciliary membrane, an extension of the cell membrane. The ciliary membrane contains the receptors and channels for the transduction of environmental events into ciliary, and consequently, cell behavior. The signaling molecules, second messengers such as Ca^{2+} or cyclic AMP (cAMP), act on axonemal proteins to change ciliary beat characteristics. This can be demonstrated experimentally by removing the ciliary membrane with a detergent such as Triton X-100; the result is a naked axoneme directly exposed to the surrounding solution. In *Paramecium*, the cortical epiplasmic network maintains cell shape and ciliary position after detergent treatment (Lieberman et al 1988). When ATP is added, the cilia beat and the membraneless model swims just as the living cell (Fig. 1). When the appropriate second messenger, for example Ca^{2+}, is added the cilia change their beat characteristics, again mimicking the response of the living cell, with one exception. In the living cell, the cell adapts spontaneously. For example, when Ca^{2+} pumps in the ciliary membrane remove Ca^{2+} from around the axoneme, the cell resumes forward swimming. In the membraneless models, ciliary reversal continues until the ATP is depleted or the model falls apart. Naitoh and Kaneko (1972, 1973) and later Lieberman et al. (1988) carefully

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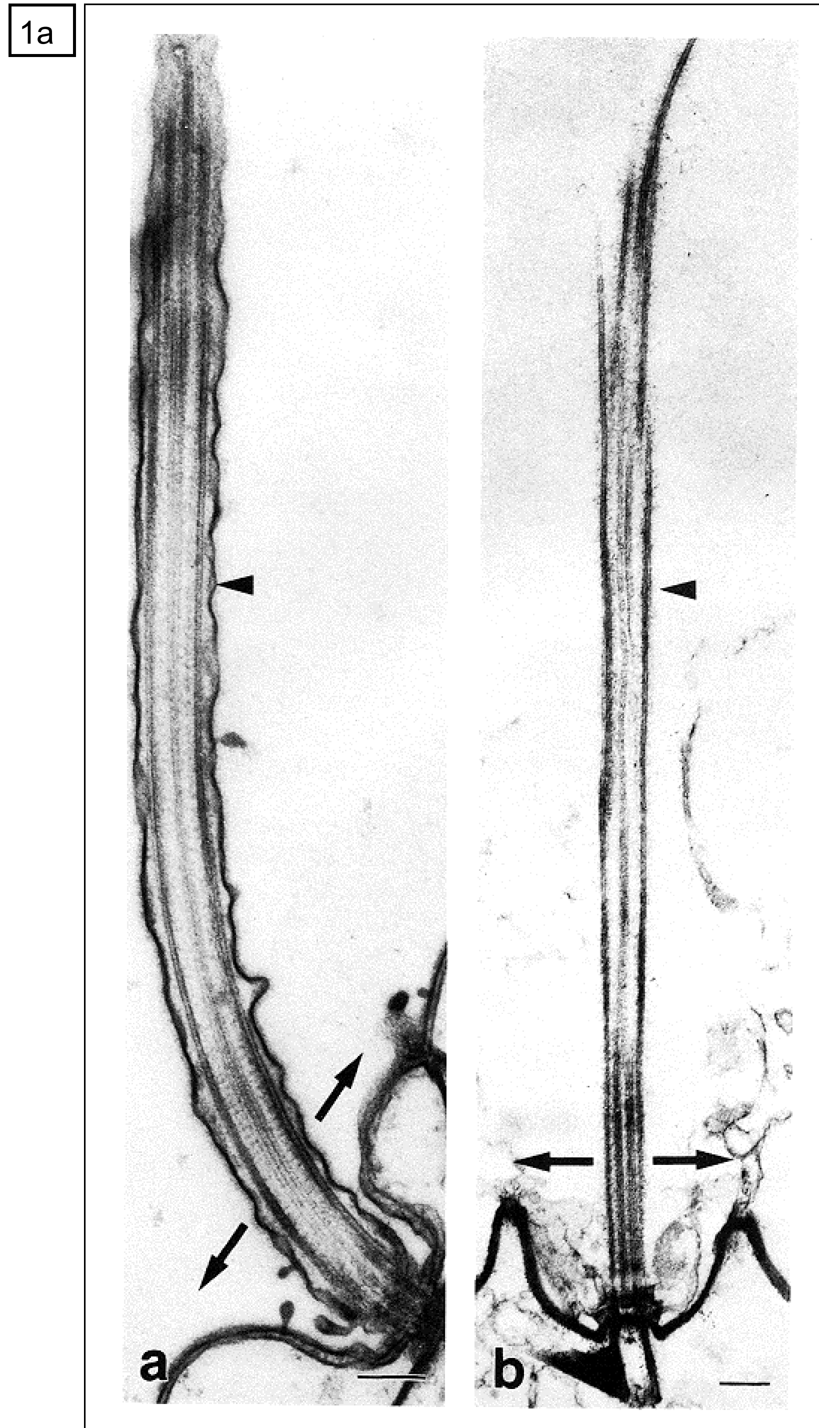


Fig. 1. Motility and control molecules are built into the axoneme. A: Permeabilization dissolves the ciliary and cell membranes of *Paramecium*. Ciliary membrane at arrowhead in (a), missing in (b). Arrows show width of cortical ridge; cell membrane present in (a), absent in (b). Bar 0.25 µm (from Lieberman et al., 1988 with permission). B: Fixed metachronal wave. (Above) Intact *Paramecium* swimming forward just before fixation. (Below) Membraneless *Paramecium* reactivated by ATP, swimming after permeabilization . Bar 10 µm (Satir et al., 1993 with permission).

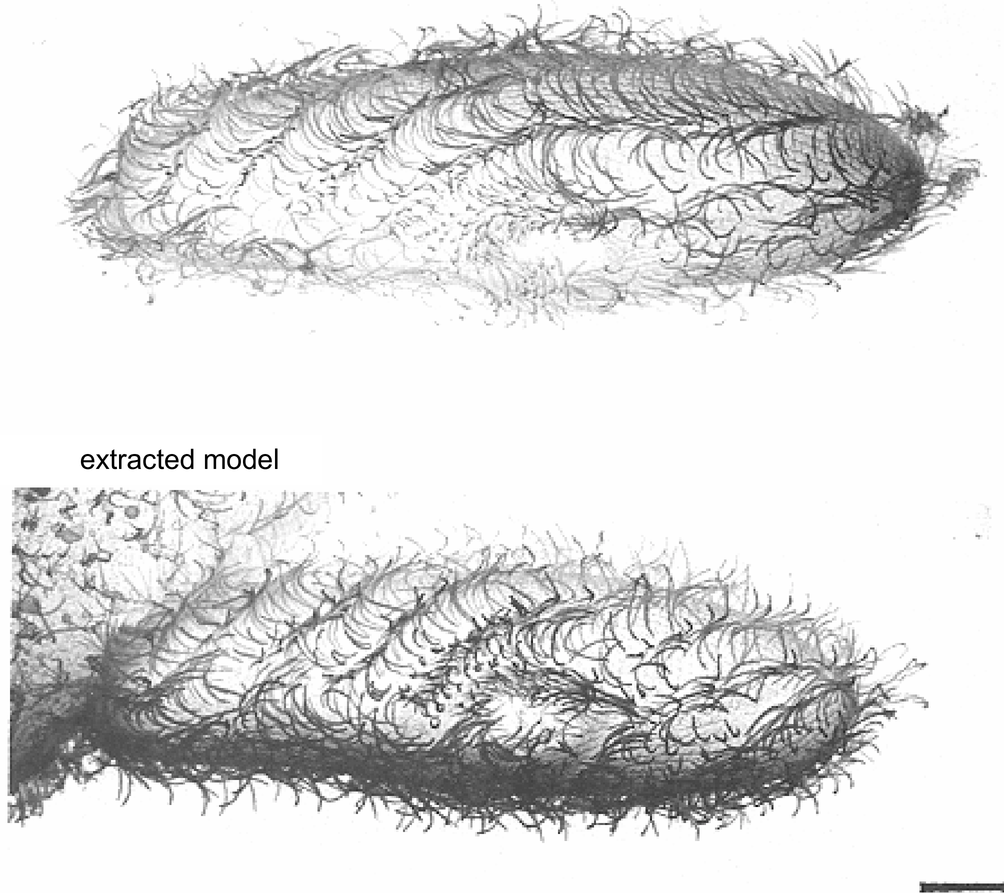


Fig. 1b. See p. 88 for explanation.

measured the Ca^{2+} responses of detergent treated *Paramecium*. When Ca^{2+} was 10^{-7}M (pCa7), the models swam forward; forward swimming speed decreased as Ca^{2+} rose toward 10^{-6}M (pCa6), until the model virtually stopped, and the individual ciliary axonemes beat in an uncoordinated helical fashion. Further increases in Ca^{2+} reactivated the swimming, but now the models swam backward with increasing speed as Ca^{2+} increased toward 10^{-5}M (pCa5).

The responding axoneme functions as a cellular nanomachine comprised of approximately 250 different protein components arranged into structural complexes including the axonemal microtubules, inner and outer dynein arms (IDAs and ODAs), spokes etc. Axoneme structure can be shown accurately in three-dimensional computer reconstruction (Taylor et al. 1999) (Fig. 2).

The structure and mechanism of motion of the axoneme is evolutionarily conserved from protozoa to man. All proteins necessary for

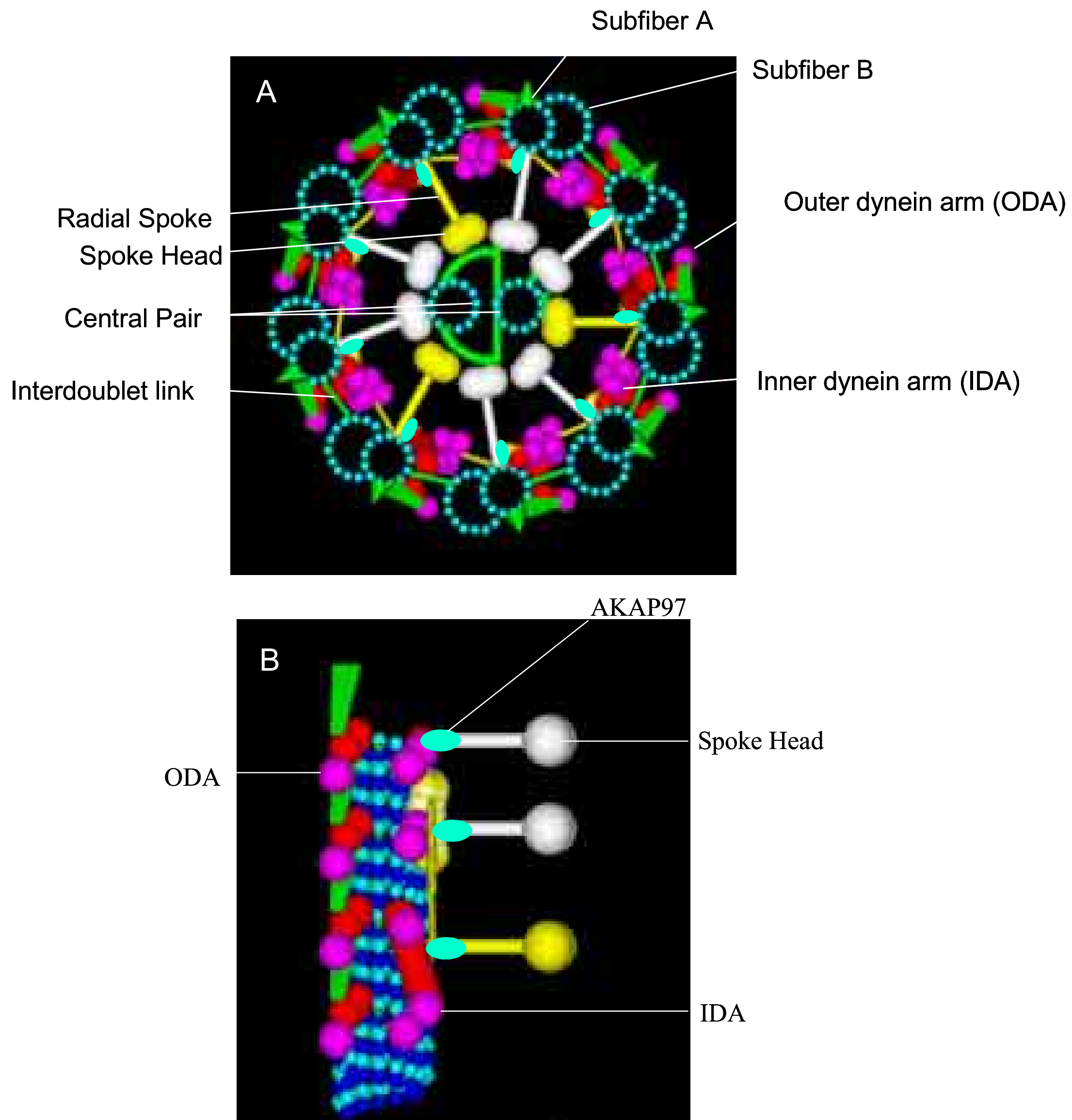


Fig. 2. A: Computer reconstruction of *Tetrahymena* axoneme with major structures labeled (modified from Taylor et al., 1999). B: Enface view of *Tetrahymena* doublet subfiber A with attachments showing arrangement of ODA and IDA subunits, radial spokes and AKAP97 (modified after Taylor et al., 1999)

motility and control of motility are built into the axoneme at specific locations. The mechanism of ciliary motion depends on a series of molecular motors built into the axoneme. These are the IDAs and ODAs, which act to produce a vectorial force that causes the doublet microtubules to slide relative to one another; the spoke and links transduce the force into a defined bend. The central pair divides the axoneme into opposite halves. The arms on doublets on one side of the axoneme are predominantly active during the effective stroke of the cilium; the arms on the other side are mainly active during the recovery stroke, and activity

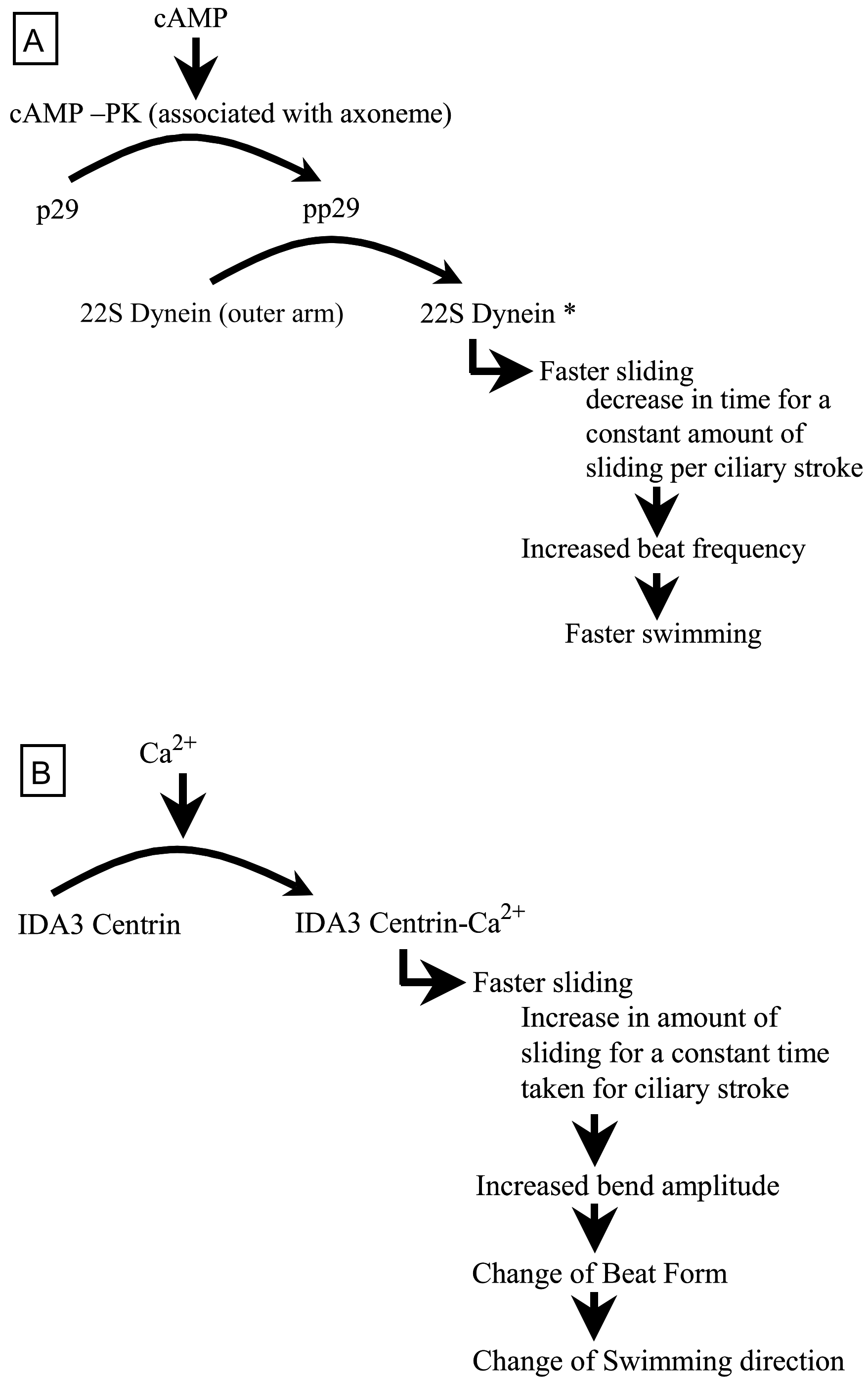


Fig. 3. Signal transduction cascades in the axoneme.

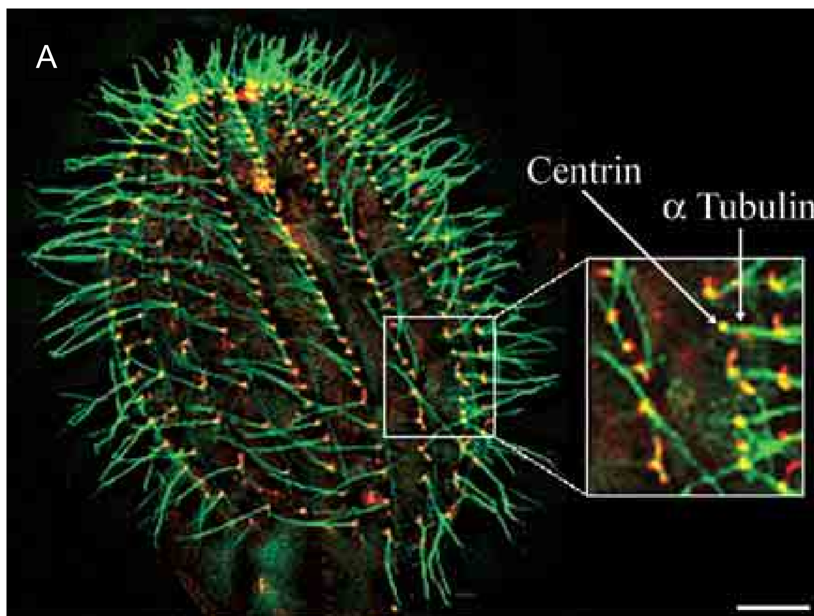
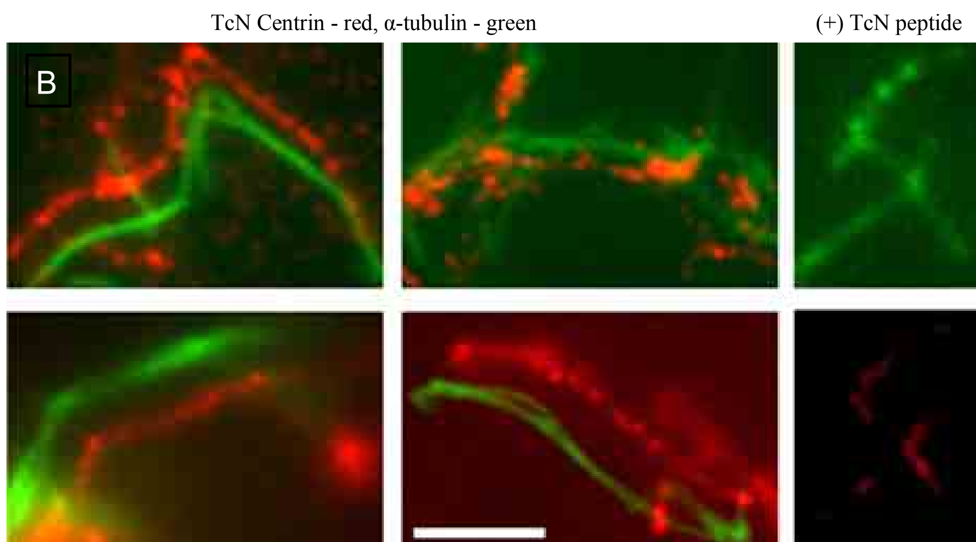
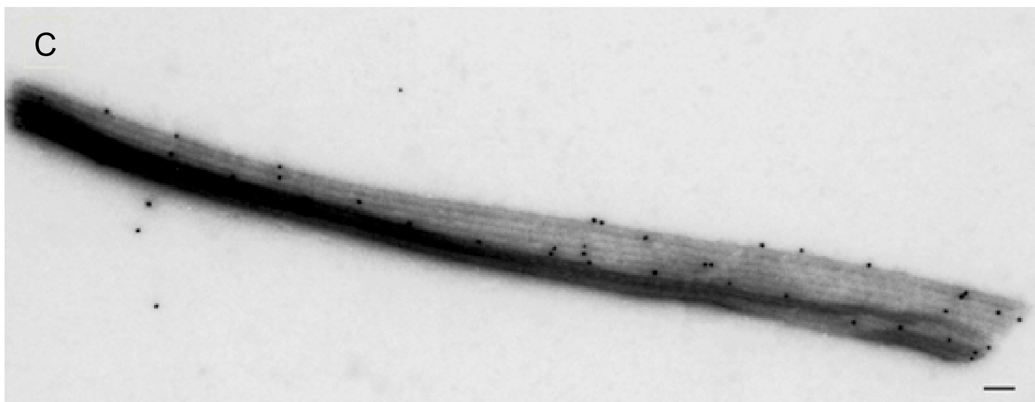


Fig. 4. Localization of centrin in *Tetrahymena* axonemes. A: In permeabilized cells, centrin localizes to basal bodies; not evident along axoneme. Bar 10 µm B: Isolation of axonemes reveals centrin. Co-localization with α-tubulin along axonemal length shown by displacement of images. Addition of competing peptide (TcN peptide) abolishes centrin but not a-tubulin localization. Bar 5 µm. C: Immunogold labeling of axoneme using centrin primary antibody. Bar 0.1 µm (From Guerra et al. 2003 with permission).





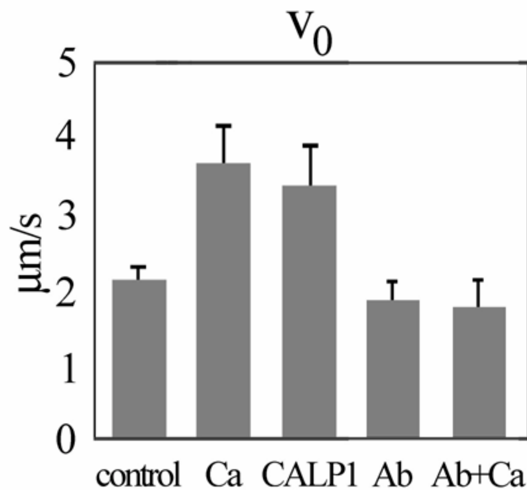


Fig. 5. Ca^{2+} binding to centrin speeds up IDA activity. Measurements of IDA translocation velocity (v_0) in the absence (control) and presence of Ca^{2+} (10 mM $CaCl_2$), the Ca^{2+} mimicking peptide CALP1 and/or an antibody to *Tetrahymena* centrin (from Guerra et al., 2003 with permission).

switches back and forth. The current state of understanding of this switch point mechanism is summarized in Satir (1997). Studies with *Chlamydomonas* swimming mutants show that ODA mutants have approximately normal beat form, while beat frequency falls to between 1/3 to 1/2 normal (Brokaw and Kamiya, 1987); IDA mutants, on the other hand, have almost normal beat frequency, but altered beat form. This implies that IDAs primarily control beat form, while the ODAs primarily control beat frequency. The end point of a response that alters cell swimming behavior by changing ciliary beat characteristics is a change in the sliding velocity of the axonemal microtubules caused by changes in dynein arm activity. In the absence of a change in beat form, beat frequency is directly proportional to ODA produced microtubule sliding velocity; likewise, in the absence of a change in beat frequency, beat form, as measured by bend amplitude in the effective and recovery stokes, is directly proportional to IDA produced microtubule sliding velocity.

cAMP is a signal transduction molecule that increases ciliary beat frequency. In *Paramecium*, cAMP is generated via a hyperpolarizing membrane K+ channel (Schultz et al., 1992). When a cell is stimulated appropriately, cAMP rises and beat frequency increases. In permeabilized cells, cAMP applied directly to the axoneme speeds up swimming by 60%. In the absence of cell shape or viscosity changes, swimming speed increases correlate with beat frequency increases. An ODA light chain, p29, is phosphorylated in the presence of cAMP (Hamasaki, et al., 1988). High Ca^{2+} abolishes the swimming speed increase by inhibiting p29 phosphorylation. 22S outer arm dynein can be extracted from cAMP or cAMP+Ca^{2+} treated axonemes and used for *in vitro* motility assays to measure microtubule sliding rate. The cAMP treated dynein with phosphorylated p29 produces a 40% increase in microtubule sliding rate (comparable to the increase in swimming speed) while cAMP+Ca^{2+} treated dynein without phosphorylated p29 shows no increase in microtubule sliding rate compared to controls. p29 can be extracted and readded to the 22S dynein (Barkalow et al., 1994; Hamasaki et al., 1995). When phosphorylated p29 is readded, microtubule sliding rate is increased in the same way as before, while addition of p29 which has not been phosphorylated produces no increase. It can concluded that cAMP works in a signal transduction cascade that produces phosphorylation of p29 of the 22S ODAs, which in turn causes faster microtubule sliding, increased beat frequency and faster swimming (Fig. 3A). An ortholog of p29, p34, present in *Tetrahymena thermophila*, is also a phosphorylatable ODA light chain that becomes phosphorylated in the presence of cAMP and causes an increase in microtubule sliding velocity (Christensen et al., 2001). A similar ortholog is present in mammalian cilia.

cAMP functions as a co-factor that activates a protein kinase (PKA) that is incorporated into the structure of the axoneme. The regulatory subunits of the protein kinases of *Paramecium* have been studied extensively by Nelson's group (Bonini et al., 1991). In the axoneme, the regulatory subunits of PKAs are docked by A-kinase anchoring proteins (AKAPs). In *Chlamydomonas*, AKAP97 is a radial spoke component (rsp3) located near the base of the spoke (Fig. 2B), while AKAP240 is a component of the central pair (Reviewed in Porter and Sale, 2000). Protein phosphatases can also be built into the axoneme, but the phosphatase that removes phosphate from phosphorylated p29 is extracted by detergent treatment, so that permeabilized *Paramecium* cannot adapt after cAMP treatment.

Although p29 is not the exclusive substrate for the axonemal PKAs and phosphatases, it is the substrate most closely linked to beat frequency and swimming speed changes where beat form is largely unchanged. Ca^{2+} affects ODA function in some measure by interaction with the PKA-phosphatase system that controls p29 phosphorylation. Between pCa7 and pCa6 in *Paramecium*, Ca^{2+} primarily affects beat frequency. However, between pCa6 and pCa5, Ca^{2+} causes changes in beat form and in microtubule activation patterns, resulting in ciliary reversal, independently of phosphorylation changes.

Ca^{2+} normally acts via Ca^{2+} binding proteins that activate specific enzymes. One class of such binding proteins are the 'EF hand' proteins, such as calmodulin. EF hand proteins could be part of the axonemal response mechanism responsible for changes in beat form in ciliate swimming. The obvious candidate enzyme for activation by a Ca^{2+} binding EF hand protein is inner arm dynein, since regulation of IDA sliding rate would affect bend amplitude, as discussed previously. Accordingly, we have recently examined the EF hand proteins of *Tetrahymena* with this possibility in mind (Guerra et al., 2003). There are four closely related EF hand proteins in *Tetrahymena*: TCBP23 and TCBP25 are found in the cell cortex; centrin and calmodulin are found in cilia.

Tetrahymena centrin is particularly interesting. LeDizet and Piperno (1995) found that centrin was a component of the *Chlamydomonas* inner dynein arm I3. Although centrin could be shown to be present in *Tetrahymena* and *Paramecium* basal bodies (Fig. 4) and elsewhere in cortical fibers (in *Paramecium* as a contractile cortical network), the protein was thought not to be present in the ciliary axonemes of these ciliates. Guerra et al. (2003) have cloned and sequenced *Tetrahymena* centrin and prepared an antibody to the unique N-terminal region of molecule. The antibody does not recognize calmodulin. Although centrin cannot be demonstrated by immunofluorescent or immunogold antibodies techniques in cilia of fixed cells (Fig. 4A), in isolated axonemes it is clearly localized all along the axoneme; immunofluorescent localization along the axoneme is abolished by preteatment with the N-terminal peptide to which the *Tetrahymena* centrin antibody has been made (Fig. 4B,C). Immunoblots against isolated dynein fractions show that centrin is localized to 14S IDA fractions, while calmodulin is not localized to dynein.

In *in vitro* motility assays using 14S IDA fractions, Ca^{2+} produces a highly significant increase in microtubule sliding velocity, measured as V_0 (Fig. 5) (Guerra et al., 2003). A similar increase is seen when CALP1, a synthetic peptide that mimics Ca^{2+} binding to EF hand proteins, is used in place of Ca^{2+}. The

increase is abolished by first treating the IDA fraction with the antibody to the N-terminal region of *Tetrahymena* centrin. These results indicate that centrin containing IDAs respond directly to the binding of Ca^{2+} by changing the velocity of microtubule sliding in the axoneme, such that the amount of sliding during a constant time of the ciliary stroke is increased (Fig. 3B). To provide ciliary reversal, Ca^{2+} must also work asymmetrically on the phases of the stoke; speculatively, this might be accomplished by Ca^{2+} binding to calmodulin associated with the central pair of microtubules, which could affect the switching mechanism.

We have come to a partial understanding of the signal transduction cascades governed by cAMP and Ca^{2+} that produce ciliary behavior, defining principal control molecules involved in regulating the dynein arms in each case: p29 or its ortholog, a regulatory light chain of the outer arms, whose phosphorylation state regulates ODA activity, and centrin, the Ca^{2+} binding EF hand protein of a group of inner arms, where Ca^{2+} binding directly regulates IDA activity, much as Ca^{2+} binding myosin light chains regulate myosin activity. IDA and ODA activity is, of course, regulated in a coordinated way that necessitates feedback between the two systems; how such co-ordination is achieved is unknown. Nevertheless, this approach has begun to indicate how specific molecules interact with the axonemal mechanism of motion to generate behavior and response in ciliated microorganisms.

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