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Effects of sodium fluoride (NaF) on the cilia and microtubular system of *Tetrahymena*

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The effect of the nucleophilic reagent NaF on the microtubular system of *Tetrahymena* was studied by using scanning electron microscopy (SEM), confocal microscopy, and flow cytometry. Treatments with 40 mM NaF significantly reduced the amount of α-tubulin while 80 mM treatment did not alter its quantity. One possible explanation for this α-tubulin overexpression is that the higher amount of α-tubulin enables this organism to carry out the appropriate function of the cytoskeleton under this undesirable influence of higher amounts of 80 nM NaF. However, the amount of acetylated tubulin increased in a dose-dependent manner. The cilia became fragile under the effect of 80 mM NaF. Confocal microscopy revealed that after 40 mM NaF treatment transversal microtubule bands (TMs) and longitudinal microtubule bands (LMs) as well as basal bodies (BBs) were extremely strong decorated with anti-acetylated tubulin antibody and TM-localization abnormalities were visible. In the 80 mM NaF-treated cells, the deep fiber of oral apparatus was very strongly labeled, while the TMs and LMs were less decorated with anti-acetylated tubulin antibody, and LM deformities were visible. It is supposed that post-translational tubulin modifications (e.g., acetylation) defend the microtubules against the NaF-induced injury. NaF is able to influence the activity of several enzymes and G-proteins, therefore is capable to alter the structure, metabolism, and the dynamics of microtubular system. The possible connection of signaling and cytoskeletal system in *Tetrahymena* is discussed. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS — cytoskeleton; confocal microscopy; microtubuli; *Tetrahymena*; tubulin modification

INTRODUCTION

NaF as a nucleophilic reagent is able to inhibit or stimulate several enzymes. In *Tetrahymena*, it inhibits the activity of acid phosphatases,[1] and the activity of phospholipase C (PLC).[2] It prevents the autophosphorylation of the insulin receptor and also inhibits the activity of tyrosine kinase of this receptor.[3] NaF is inhibitory also to Ser/Thr-phosphatases.[4] The phospholipase D (PLD) activity of *Tetrahymena* is enhanced by sodium fluoride.[5] In addition, it has influence on the activity of G-proteins.[6] In platelets directly stimulates G-protein without receptor interaction.[7] G-proteins coupled to PLC were concentration-dependently activated by NaF.[8] G-protein activating fluorides (NaF, AlF_4, and BeF_3) influence the synthesis, metabolism, and breakdown of phospholipids (PIs) in *Tetrahymena*. After NaF treatments, the inositol PI content (PI, PIP, and PIP_2) of *Tetrahymena* decreased significantly.[9]

Microtubule assembly is known to be regulated by the phosphorylation of microtubule-associated proteins (MAPs), and it is sensitive to phosphatase inhibitors. Sodium fluoride stimulates tubulin and microtubule protein assembly by a nonspecific effect, probably involving water structure formation. Wyman analysis suggests an absence of direct or specific binding to tubulin ($d \ln K/d \ln [NaF] = 0.214$).[10] Tubulin forms the microtubule and regulates certain G- protein-mediated signaling pathways. Both functions rely on the GTP-binding properties of tubulin. Signal transduction through $G_{\alpha q}$-regulated PLC is activated by tubulin through a direct transfer of GTP

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from tubulin to $G_{\alpha q}$.[11] Tubulin forms complexes with specific G-proteins and these complexes might provide a locus for the interaction of cytoskeletal components and signal transduction cascades.[12] While NaF has effects on the above mentioned phenomena, these treatments offer a good possibility for the examination of these processes.

Tetrahymena is a superb tool for studies of microtubular system because it has a complex microtubule architecture comparable to that of the diverse cells of a multicellular organism. In addition, it also has signal reception and transduction systems similar to the higher ranked animals.[13–16] Because of this, in our laboratory several evidences have been provided for the connection of microtubular and signaling system of *Tetrahymena*.[17,18] Indomethacin, for example, causes significantly altered PI synthesis and it is able to influence the inositol PI signaling system in this unicellular organism.[19] At the same time, in the indomethacin-treated cells the cortical microtubular system became irregular, and also the divisional morphogenesis was disturbed.[20] On the basis of these, and the above mentioned facts it was supposed that NaF has some effects also on the microtubular system of *Tetrahymena*—mainly due to the effect on the PI-system, or on the phosphorylation of MAPs. The aim of the present study was to obtain additional data on the effect of NaF and on the connection of signaling- and cytoskeletal-systems in *Tetrahymena*.

MATERIALS AND METHODS

Materials

Mouse monoclonal anti-α-tubulin, anti-acetylated-tubulin antibody, FITC-labeled anti-mouse goat IgG, 2,2-dimethoxypropane (DMP), and tryptone were obtained from Sigma (St. Louis, MO, USA). Yeast extract was purchased from Oxoid (Unipath, Basingstoke, Hampshire, UK). All other chemicals used were of analytical grade available from commercial sources.

Tetrahymena cultures

In the experiments, *T. pyriformis* GL strain was tested in the logarithmic phase of growth. The cells were cultivated at 28°C in 0.1% yeast extract containing 1% tryptone medium. Before the experiments, the cells were washed with fresh culture medium and were resuspended at a concentration of 5×10^4 cells ml^{-1}.

Preparation of cells for SEM

Tetrahymena populations were treated with 40 or 80 mM NaF for 60 min. Untreated cells served as controls. After the treatments, the cells were washed with fresh culture medium, and were fixed in 0.5% OsO_4 containing Karnowsky fixative (4% paraformaldehyde in 2.5% glutaraldehyde dissolved with 0.2 M phosphate buffer, pH 7.0) for 60 min at 4°C. After fixation, the cells were washed with phosphate buffer, rinsed in 30% ethanol, dissolved in distilled water to remove the remnants of the buffer salts, and suspended in the acidified DMP: absolute ethanol = 1:3 (to 100 ml of this solution one drop of ccHCl was added) for 30 min, then pure DMP for 15 min.[21] After the DMP treatment (chemical dehydration), the cells were dropped onto aluminium SEM stubs and air dried. Specimens were coated *in vacuo* with evaporated gold before examination in a Hitachi S 2460 N scanning electron microscope (SEM).

Confocal scanning laser microscopic (CSLM) analysis of Tetrahymena cells labeled with monoclonal anti-acetylated-tubulin antibody

Tetrahymena cultures (5 ml) were treated with 40 or 80 mM NaF for 60 min. Untreated cells served as controls. To localize tubulin-containing structures, the cells were fixed in 4% paraformaldehyde dissolved in PBS, pH 7.2. After washing with wash buffer (WB; 0.1% BSA in 20 mM Tris-HCl; 0.9% NaCl; 0.05% Tween 20, pH 8.2), the cells were incubated with monoclonal anti-acetylated-tubulin antibody diluted 1:500 with antibody [AB] buffer (1% BSA in 20 mM Tris-HCl; 0.9% NaCl; 0.05% Tween 20, pH 8.2) for 45 min at room temperature. After three washings with WB, the cells were incubated with FITC-labeled anti-mouse goat IgG (diluted to 1:200 with AB buffer) for 45 min at room temperature. After incubation, the cells were washed four times with WB, and were mounted onto microscopic slides. The mounted cells were analyzed in a Bio-Rad MRC 1024 CSLM equipped with a krypton/argon mixed gas laser as a light source. Excitation was provided by the 480 nm line from the laser.

Flow cytometric (FACS) analysis of the tubulin and acteylated-tubulin content of Tetrahymena

Tetrahymena populations were treated with 40 or 80 mM NaF for 60 min. Untreated cells served as controls. After the treatments, the cells were fixed in

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4% paraformaldehyde dissolved in PBS, pH 7.2. After washing with WB, the cells were incubated with anti-α-tubulin or anti-acetylated-tubulin antibody diluted 1:300 with AB for 45 min at room temperature. After three washings with WB, the cells were incubated with FITC-labeled goat anti-mouse IgG for 45 min. After these incubations, the cells were washed four times with WB. To determine the nonspecific binding of secondary antibodies the primary antibodies were omitted as absolute controls. The measurement of fluorescence intensity was done in a FACS Calibur flow cytometer (Beckton Dickinson, San Jose, USA), using 10^4 cells for each measurement. For the measurement and analysis a CellQuest 3.1 program was used. During the evaluation, cell populations had been separated on the basis of size defined by "gating." In the identical cell populations, the FITC-labeled second antibody content inside the cells were measured. The evaluation of the results was done by the comparison of percentage changes of geometric mean channel values to the control groups. Student's *t*-test was used for the evaluation of all data, with $p < 0.05$ accepted as the level of statistical significance.

RESULTS

In the chemically dehydrated *Tetrahymena* cells in SEM, the cilia, undulating membrane and three membranelles of oral apparatus, transverse microtubule bands (TMs), and the pores of extruded mucocysts are clearly visible (Figure 1). After 40 mM NaF treatment the number of pores of extruded mucocysts was significantly elevated, but the structure of undulating membranes and the cilia were seemingly unaltered (Figures 2a and b). In case of 80 mM NaF-treatments the somatic cilia and the undulating membrane and three membranelles of oral apparatus were broken off, only the remnants of these structures (one file of basal bodies (BBs) in the undulating membrane and three files in the membranelles) were visible (Figures 2c and d).

In the control (untreated) cells the BBs, TMs, and longitudinal microtubule bands (LMs), pores of contractile vacuoles, undulating membrane and three membranelles of oral apparatus and the deep fiber are decorated with anti-acetylated-tubulin antibodies (Figure 3). This characteristic picture is altered only moderately after 40 and 80 mM NaF treatments. In case of 40 mM NaF treatment, the TMs, LMs, and BBs are extremely strong decorated with anti-acetylated-tubulin antibody, and frequently TM-aberration was visible (Figure 4) Similar situation

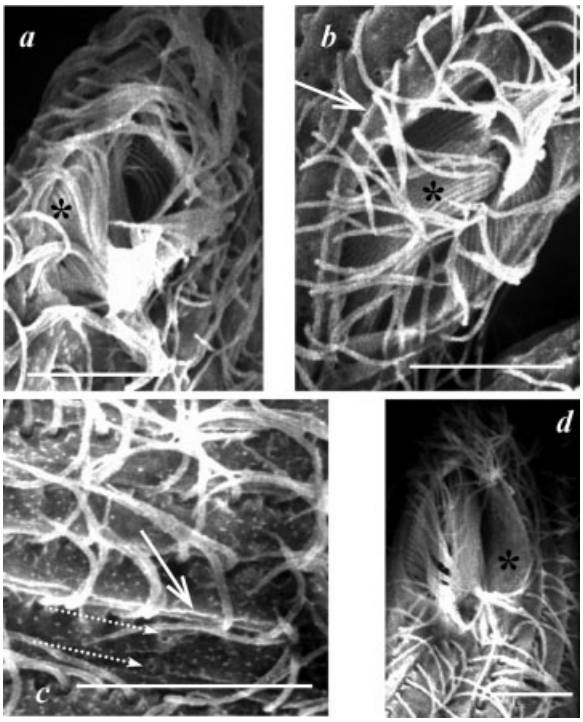

Figure 1. SEM pictures of control (untreated) *Tetrahymena*. "*" denotes oral apparatus (undulating membrane and three membranelles); arrow, longitudinal microtubule bundles; dotted arrows, pores of extruded mucocysts. Bars = 5 μm

was observed after 80 mM NaF treatments: in these cells the deep fibers were extreme strongly labeled but the LMs and TMs were weaker decorated with anti-acetylated-tubulin antibody. In these cells—beside the TM-irregularities—often also LM-deformities were visible. In some cells, the labeling of membranelles of oral apparatus was absent (Figure 5).

The amount of α-tubulin was significantly less in the 40 mM NaF-treated cells than in the controls, however treatment with 80 mM NaF caused no alteration. The amount of acetylated-tubulin increased in a concentration-dependent manner: the cells after 80 mM NaF treatment contained significantly higher amount related to the controls (Figure 6).

DISCUSSION

NaF as a nucleophilic reagent is able to influence the activity of several enzymes. In addition, it has influence on the activity of G-proteins: G-protein activating fluorides (NaF, AlF_4, and BeF_3) influence the synthesis, metabolism, and breakdown of PIs in

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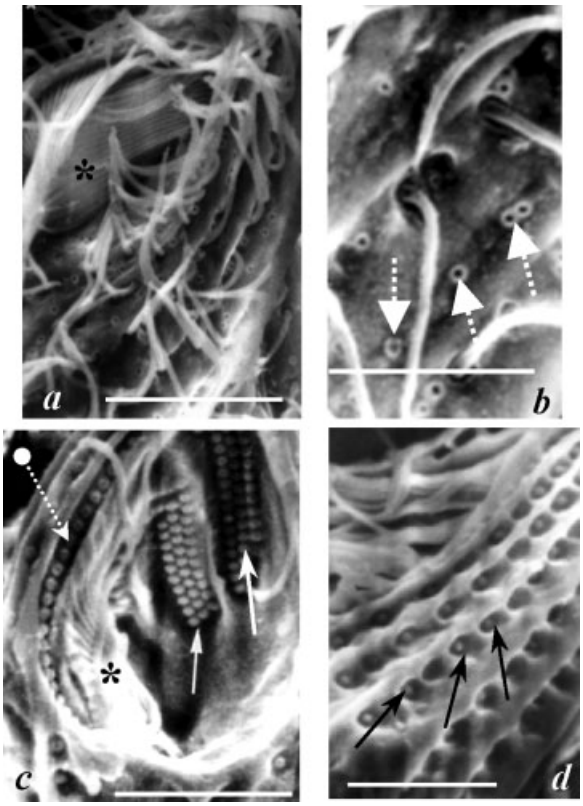

Figure 2. SEM pictures of 40 mM NaF- (a and b), and 80 mM NaF-treated (c and d) *Tetrahymena* cells. "*" denotes oral apparatus; white arrow, remnants of broken undulating membranelles (one file of basal bodies); dotted arrow with circle, remnants of membranelles (three files of basal bodies); black arrows, broken cilia; dotted arrows, pores of mucocysts. Bars: a and c = 5 μm; b and d = 3 μm

Tetrahymena.[9] Similarly to the NaF, indomethacin also influence the metabolism of PIs in *Tetrahymena*.[19] Both molecules decrease the amount of inositol PIs (PI, PIP, PIP_2) similarly and drastically decreased the amount of PE in *Tetrahymena* (which PI play important role in the conversion of PIs). In our earlier work,[20] we found that indomethacin treatments—among others—disturbed the cytoskeleton (mostly the TMs) of *Tetrahymena*. In the recent work, we found similar alterations: after 40 and 80 mM NaF treatments the cells showed frequently different kind of anomalies in the localization of TMs and LMs. These results may refer to a connection between the disturbance of PI (inositol PI) metabolism and the abnormalities of cytoskeletal structures.

The post-translational phosphorylation of both tubulin and MAPs is believed to be important in the regulation of microtubule assembly. The phosphorylation/dephosphorylation cycle of MAPs is especially important in the conversion of interphase cells and stable microtubules to mitotic cells and dynamic microtubules Because NaF can act as a protein phosphatase inhibitor, it could be suspected that after NaF treatment the stability of microtubules is transformed. This could explain the results of SEM analysis when after 80 mM NaF treatment the majority of somatic cilia and the oral apparatus (made up of four compound ciliary elements, the undulating membrane and three membranelles) were broken. Similar results were won by using anti-polyglutamylated-tubulin antibody (this tubulin post-translational modification occurs on the ciliary axonemes): according to the confocal microscopic pictures, the number of cilia on the cells was less than that of the controls, the majority of somatic cilia

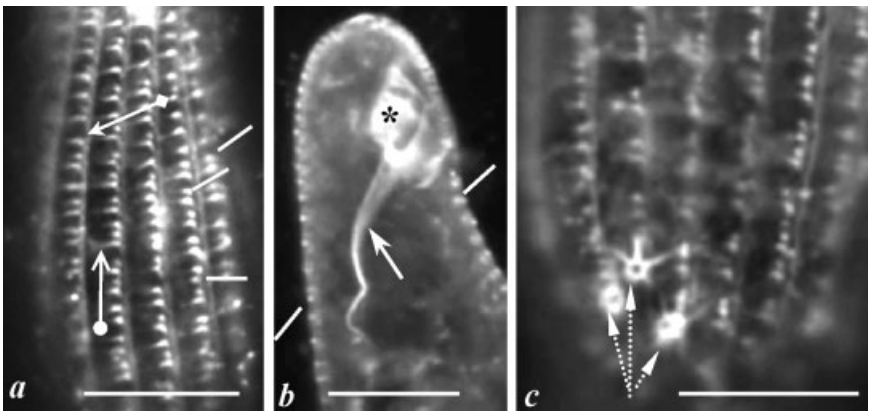

Figure 3. CSLM pictures of control (untreated) *Tetrahymena* cells decorated with anti-acetylated-tubulin antibody. "*" denotes oral apparatus; arrow, deep fiber; arrow with circle, transversal microtubule bands; arrow with square, longitudinal microtubule band; rods, basal bodies; dotted arrows, pores of contractile vacuoles. Bars = 5 μm

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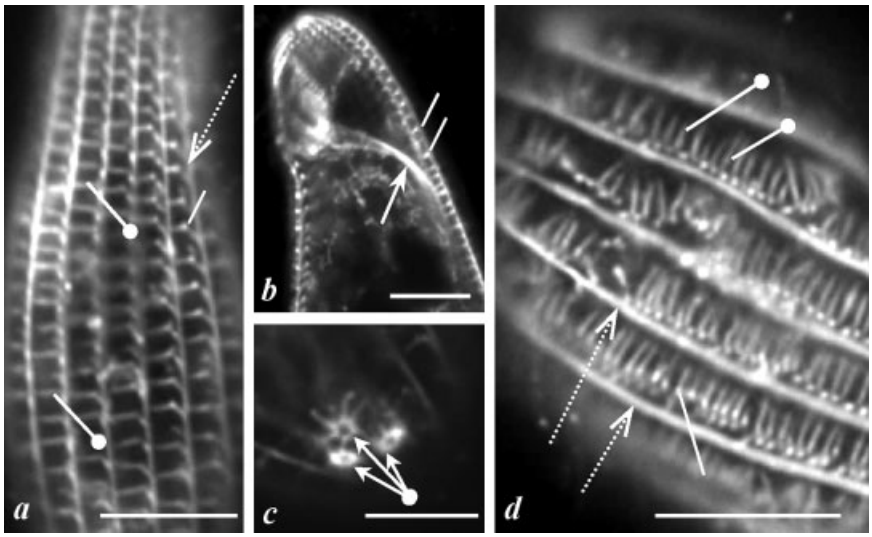

Figure 4. CSLM pictures of 40 mM NaF-treated *Tetrahymena* cells decorated with anti-acetylated-tubulin antibody. Arrow, deep fiber; arrows with circle, pores of contractile vacuoles; rods, basal bodies; dotted arrows, longitudinal microtubule bands; rods with circle, transversal microtubule bands. Anomaly of localization of TMs is clearly visible. Bars = 5 μm

were missing. Tubulin is modified by polyglutamyl side chains of variable length attached via an isopeptide bond to the γ-carboxyl group of selected glutamate residues in the C-terminal domain. The acidic C-terminal domains of tubulins are involved in the binding of MAPs. Axonemal tubulin in *Tetrahymena* is extensively polyglutamylated; polyglutamylation stabilizes the B-subfiber of outer doublets in

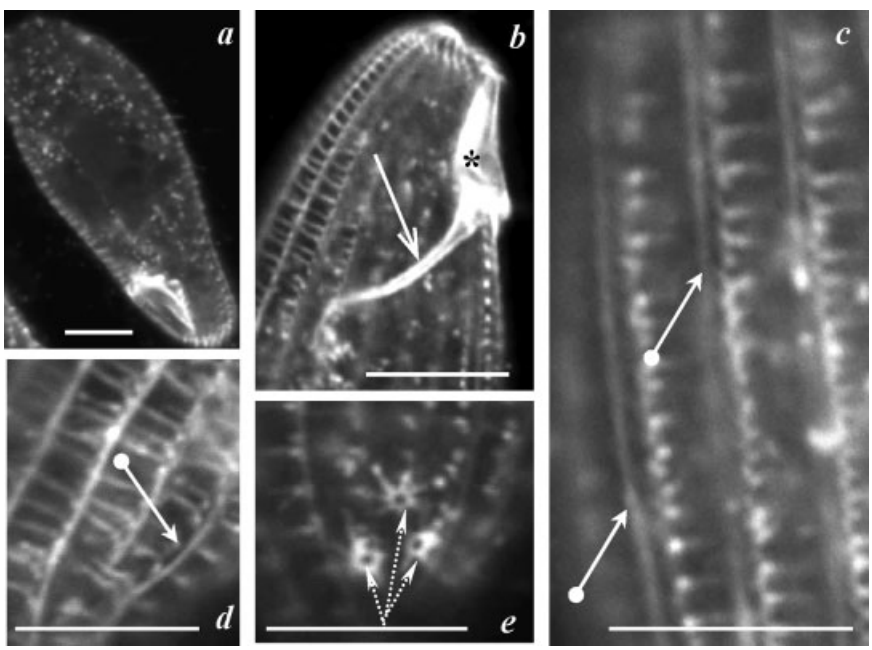

Figure 5. Pictures of 80 mM NaF-treated *Tetrahymena* cells decorated with anti-acetylated-tubulin antibody (a–e). Arrow, deep fiber; dotted arrows, pores of contractile vacuoles; arrows with circle, longitudinal microtubule bands. In (a) many strongly labeled dots are in the cytoplasm; the labeling of membranelles of oral apparatus is absent. Anomaly of localization of LMs in the picture (d) is clearly visible. Bars = 5 μm

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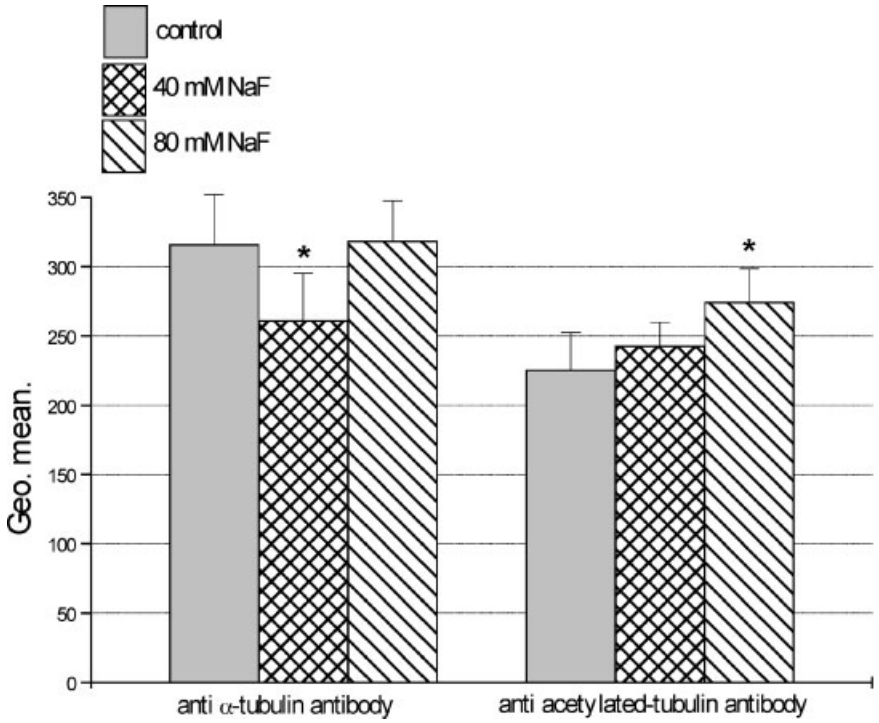

Figure 6. Flow cytometric analysis of control, 40 and 80 mM NaF-treated *Tetrahymena* populations decorated with anti-tubulin and anti-acetylated-tubulin antibodies. The data represent the mean (±SD) derived from three separated experiments. $*p < 0.05$ to the control

the cilia and affect the ciliary matrix.[22] Considering these facts can be surmized that NaF-treatments—mostly via the effect on MAPs—disturb the normal conditions in the ciliary axonemes, and thus these structures become fragile. This fragility is manifested in the ciliary breaking off during the use of scanning electron microscopical technics (chemical dehydration) after 80 nM NaF treatment (Figure 2) as well as in the loss of cilia.

After treatment with NaF the amount of α-tubulin decreased but with the higher concentration of NaF (80 nM) the α-tubulin content of the cells reached the control level. It seems difficult to explain the stimulatory effect of this higher NaF concentration on the amount of tubulin in the light of the inhibitory effects of 40 nM NaF. One possible explanation is that this treatment induced a reversible acetylation on the α-tubulin (or enzyme) overproduction; the higher amount of tubulin enables this organism to carry out the appropriate functions of the cytoskeleton under this undesirable influence. Similar phenomena were found in *Tetrahymena* also in the case of other treatments.[25]

NaF treatments in a concentration-dependent manner increase the acetylation of tubulin. Reversible acetylation on the ε-amino group of Lys40 stabilizes microtubule structures and may contribute in the regulation of microtubule dynamics.[23] It seems likely that the stronger acetylation of microtubular structures (see Figures 4 and 5) helps to avoid the undesirable effect of the NaF treatments. Post-translational tubulin modifications (e.g., polyglutamylation) affect the binding of MAPs to the microtubule wall [24] and presumably this saves the microtubules and elevates the amount of tubulin to the control level.

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