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Dinoflagellate amphiesma at different stages of the life cycle

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Summary

Dinoflagellates possess the complex cell covering called amphiesma which undergoes rearrangements at different stages of the life cycle. In the present work, we summarize available data on such rearrangements obtained after the publication of the classical review by Morrill and Loeblich (1983). We provide a generalized model of the amphiesmal structural alterations through the dinoflagellate life cycle which can be useful in planning the physiological experiments on plasma membrane of these protists.

Key words: amphiesma, dinoflagellates, cell covering, ecdysis

Introduction

Dinoflagellates represent a group of eukaryotic microorganisms, which play the key role in aquatic ecosystems and display many unique morphological, molecular and physiological features, e.g. permanently condensed chromosomes which lack histones, unusual type of closed mitosis with an extranuclear spindle, particularly large nuclear genome, production of nasty bloom toxins, etc. (Steidinger and Cox, 1980; Raikov, 1995; Cembella, 2003; McEwan et al., 2008). Of special interest in these protists is a very complex cell covering with its not yet fully deciphered structure and genesis (Dodge and Crawford, 1970; Dodge, 1971; Loeblich, 1970; Morrill and Loeblich, 1983). In most cases, the complexity of the dinoflagellate cell covering termed amphiesma essentially hampers physiological studies on these microorganisms. For example, ion channels of dinoflagellates are still poorly studied *in situ* due to difficulties in application of the patch-clamp technique to these cells: amphiesma prevents formation of gigaohm contact with an electrode. In order to overcome existing obstacles for physiological studies on these flagellates, we need comprehensive information about amphiesmal structure and its changes through the life cycle of dinoflagellates.

Amphiesma is a type of alveolar cell covering common in the protistan supergroup Alveolata (Dinoflagellata, Ciliata, Apicomplexa). It consists of the continuous plasma membrane underlied by membrane vesicles, called amphiesmal vesicles (AVs), which may contain glucan thecal plates and/or the pellicle layer (Morrill and Loeblich, 1983; Hausmann and Hülsmann, 2010). One of the first generalizations of amphiesmal structure was made by Dodge and Crawford (1970). They suggested a range of amphiesmal arrangements based on the degree of complexity: from unarmored to heavely armored forms. Later, that range was supplemented with several substantial additions by Morrill and

Loeblich (1983), who also incorporated the pellicle as a common component of the amphiesma. Even now, the Morrill and Loeblich's review is still basic on this subject. However, after 1983, several very important publications appear that describe amphiesmal structure and development in more details, which will be summarized in the present review. Nevertheless, it is reasonable to start with a short summary of the classical Morrill and Loeblich's schemes.

Morrill and Loeblich's schemes

Morrill and Loeblich analized works on amphiesmal fine structure available at that time and suggested a conventional amphiesmal arrangement composed of three membranes: outermost membrane (OM), outer ampiesmal vesicle membrane (OAVM), and inner amphiesmal vesicle membrane (IAVM) (Fig.1). Moreover, they reinterpreted the data of some authors which reported four-membranes composition of the dinoflagellate cell covering (Mornin and Fransis, 1967; Messer and Ben-Shaul, 1969; Loeblich, 1970; Steidinger et al., 1978; Spector and Triemer, 1979; Spector et al., 1981). In contrast to those researchers, Morrill and Loeblich treated the fourth membrane as a pellicular layer or a membrane of an inner vacuole.

Unarmored or "naked" dinoflagellates that have no thecal plates (for instance, the genera *Oxyrrhis, Amphidinium* and *Noctiluca*) possess the simpliest amphiesmal arrangement. Their cell covering consists of OM flatterned by separated AVs (Fig. 1A). Other unarmored dinoflagellates (*Gymnodinium breve, G.fuscum*) have AVs containing discontinuous pellicle layers. Finally, some naked organisms have large fused AV containing a continuous pellicle (*G. micrum*). Similar types of amphiesmal arrangement were proposed for armored organisms with a difference that they additionally have thecal plates within AVs (genera *Heterocapsa, Ceratium, Peridinium, Prorocentrum, Zooxanthella*). Therefore, the most complex arrangement, e.g. in *Zooxanthella microadriatica*, includes OM, continuous OAVM, thecal plates, continuous pellicle and continuous IAVM (Fig. 1B).

In case the cell covering consists of many continous membranes, the question arises: which of the membranes is the plasma membrane? Morrill and Loeblich provided convincing evidences that OM is the plasma membrane. One of the facts supporting this point of view is continuity between OM and the flagellar membrane. Indeed, if the flagellar membrane is continuous with OM, so the latter must be plasmalemma. Such continuity

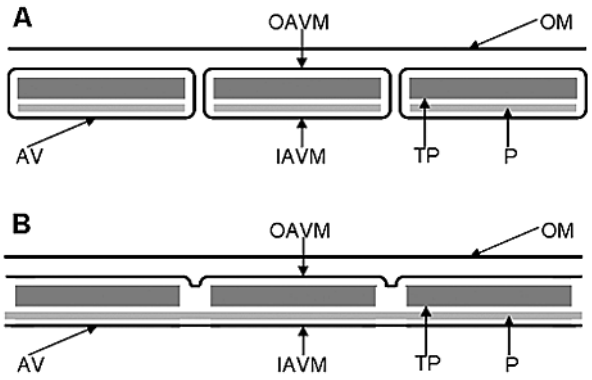

Fig. 1. Dinoflagellate amphiesmal arrangements according Morrill and Loeblich (1983). A – arrangement with separated amphiesmal vesicles; B – arrangement with fused amphiesmal vesicles. *Abbreviations*: AV – amphiesmal vesicle, IAVM – inner amphiesmal vesicle membrane, OAVM – outer amphiesmal vesicle membrane, OM – outermost membrane, P – pellicle (present in pelliculate organisms), TP – thecal plates (absent in unarmored dinoflagellates).

between OM and flagellar membrane was shown on micrographs by Soyer (1970) and then more clearly by Morrill (1984). Therefore, Morrill and Loeblich answered two questions in their review: (1) how many membranes are present in amphiesma, and (2) which membrane is the plasma membrane.

Changes in amphiesmal arrangement through the life cycle

Amphiesma is a dynamic structure. It undergoes chages through the life cycle of an organism (Morrill, 1984). Some dinoflagellates have intricate life cycles including both sexual and asexual phases (*Alexandrium* spp.), others have quite simple cycles comprising only asexual phase (*Prorocentrum* spp.). Remarkably, any dinoflagellate life cycle consists of motile and non-motile stages (Pfiester and Anderson, 1987). The organisms with simple asexual life cycles appear the "easiest" objects to investigate chages in amphiesmal arrangement, for example: *Heterocapsa niei* (Morrill, 1984; Höhfeld and Melkonian, 1992), *Amphidinium rhynchocephalum* (Höhfeld and Melkonian, 1992), *Glenodinium foleaceum* (Bricheux et al., 1992), *Symbiodinium* sp. (Wakefield et al., 2000), *Scrippsiella hexapraecingula* (Sekida et al., 2001), *Crypthecodinium cohnii* (Kwok and Wong, 2003).

There are several main processes leading to cell covering reorganization: two types of asexual reproduction, termed desmoschisis and eleuthero-

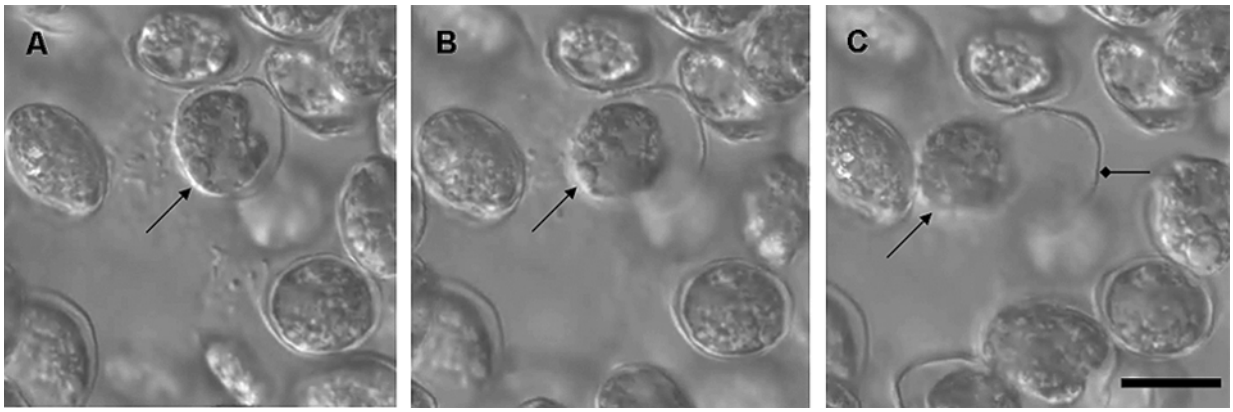

Fig. 2. Stress induced ecdysis in *Prorocentrum minimum* (our data). A – Start of ecdysis (ecdysing cell arrowed); B – ecdysis in progress; C – end of ecdysis: cell completely discarded its covering (square-headed arrow). Scale bar: 10 μm.

schisis, and the process of cell covering shedding, termed ecdysis (Fig. 2). In desmoschisis, each daughter cell inherits a half of the parent cell covering and completes the other half during or after cytokinesis (*Prorocentrum, Ceratium, Dinophysis*). In eleutheroschisis (Fig. 3), a non-motile parental cell undergoes fission within the old cell covering, which is then shed by motile daughter cells (*Glenodinium, Scrippsiella, Peridinium*) (Pfiester and Anderson, 1987). Under stress conditions organisms of some taxa shed their covering at the non-motile stage, discarding the old amphiesma and pellicle (if present) (Fig. 2). Such shedding is not associated with cell fission (Morrill, 1984). In contrast, dinoflagellates reproducing by eleutheroschisis udergo two consecutive sheddings that associated with cell fission (Fig. 3). The first shedding occurs during transformation of a motile cell into a non-motile cell. In this prosess, termed ecdysis *sensu stricto*, an old amphiesma is being substituted by a pellicle. The second shedding occurs after fission of a non-motile cell. As a result, new motile cells leave the parent covering (Bricheux et al., 1992).

Pellicle formation models

Morrill investigated the pellicle and new amphiesma formation in *Heterocapsa niei* exploiting stress conditions to induce ecdysis (Morrill, 1984). Cells were fixed at several time points after the stress treatment. In cells fixed immediately after stress amphiesma possessed a conventional three-membrane arrangement: OM, continuous OAVM, thecal plates, continuous pellicle and continuous IAVM. It means that *H. niei* cells have already had a continuous pellicular layer at the non-motile stage before they began to ecdyse. Therefore, the continuous pellicular layer formation occured at the stage preceding ecdysis, and ecdysis itself represented discarding of OM, OAVM, thecal plates and, finally, the pellicle (Fig. 4A).

Taylor reconsidered previous data on amphiesma formation obtained before 1987 and suggested that the pellicle had developed from an amorphous layer beneath AVs. In other words, the pellicle was situated beneath IAVM (Taylor, 1987). Based on discrepancy between Morrill's (1984) and Taylor's (1987) views, Höhfeld and Melkonian (1992) reinvestigated the amphiesmal structure of *H. niei* during ecdysis.

Höhfeld and Melkonian (1992) noticed that in non-centrifugated cells AVs did not fused. Each AV contained a thecal plate and a dark-staining layer beneath the plate corresponding to discontinuous pellicle in works by Morrill and Loeblich (1983) and Morrill (1984). Authors concluded that centrifugation could be a cause of AVs fusion. Since Morrill (1984) exploited centrifugation as a stress factor, they reinterpreted coalescence of the dark-staining layer in that work as an artefact. In Höhfeld and Melkonian's model, AVs fusion occurred during ecdysis but not before.

Höhfeld and Melkonian (1992) reported that the covering of ecdysed *H. niei* cells consisted of the outermost "honeycomb-patterned layer" derived from the dense layer of AVs, the "pellicular membrane", and the "pellicular layer" beneath the "pellicular membrane" (Fig. 4C). They interpreted this three-layered structure as pellicle *sensu lato*, and the "pellicular layer" as pellicle *sensu stricto*. According to the authors, the "pellicular membrane" derived from IAVMs and the "pelliclular layer" was formed in the cortical layer of cytoplasm. It is important that ecdysing cell sheds its old plasmalemma, and the question about the source of a new plasma mebrane arises again.

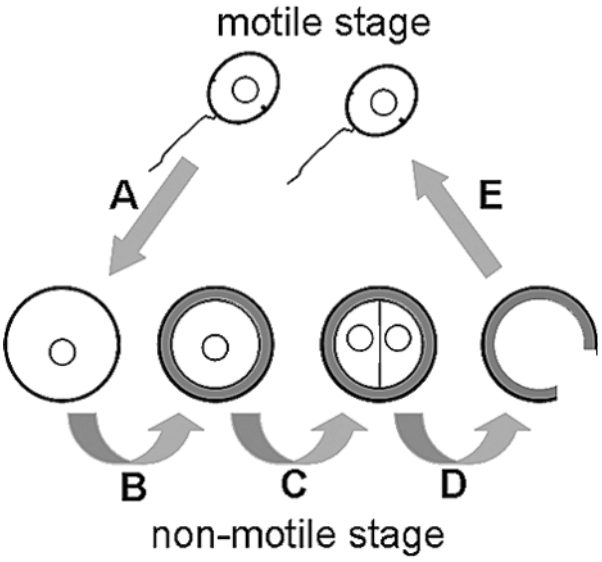

Fig. 3. Scheme of eleutheroschisis. A – Motile cell undergoes deflagellation and becomes non-motile; B – development of the pellicle; C – cell fission; D, E – new motile cells leave parental covering.

Fig. 4. Models of the pellicle arrangement. A – Morrill and Loeblich model; B – pellicle arrangment according to Brichuex et al. (1992); C – Höhfeld and Melkonian model. *Abbreviations*: DL – dense layer, FL – fibrous layer, HL – homogeneous layer, HPL - honeycomb-patterned layer, P – pellicle, PL – pellicular layer, PLM – pelliclular membrane, PM – plasma membrane.

Bricheux and colleagues (1992) studied the pellicle formation in the other member of order Peridinales, *Glenodinium foliaceum*, which reproduces by eleutheroschisis. In general, their data reconcile with Morrill's model of ecdysis, but it must be noted that they also used centrifugation during their work, which could affect the results. The pellicle of *G. foliaceum* was composed of three layers concordant with Höhfeld and Melkonian (1992). However, Bricheux and colleagues characterized the layers as follows: the fibrous layer, the dense layer, and the homogeneous layer (Fig. 4B). The dense layer was apparently derived from the pellicle of an ancestral motile cell and therefore corresponded to the pellicle in Morrill's model.

Thus, there are two alternative models of the mature pellicle of ecdysing dinoflagellate. In Morrill's model, pellicle occurs outside the new plasmalemma that derived from fused IAVMs. This model was supplemented with the three-layered structure of the pellicle introduced by Bricheux et al. (1992) (Fig. 4A, B). In the model of Höhfeld and Melkonian, the pellicle *sensu lato* was also composed of three layers: the "honeycomb-patterned layer" situated above the membrane, and the pellicle *sensu stricto* situated beneath the membrane structure (Fig. 4C).

Morrill's model seems to be more realistic for several reasons. First, it is not in conflict with the hypothesis of the IAVM origin of the new plasma membrane. This hypothesis is supported by the fact that fused IAVMs are the only continuous membrane in the pelliclulate stage of the life cycle. Second, the Höhfeld and Melkonian's data can be reconsidered. The "honeycomb-patterned layer" corresponds to the fibrous layer in Bricheux et al. (1992) (Fig. 5 in Höhfeld and Melkonian, 1992; Figs 5 and 6 in Bricheux et al., 1992). The "pellicular membrane" corresponds to the dense layer, and the "pellicular layer" - to the homogeneous layer. The plasmalemma does not seem to be resolved in their work. However, the data obtained on naked dinoflagellates *Nocticula miliaris* (Melkonian and Höhfeld, 1988) and *Amphidinium rhynchocephalum* (Höhfeld and Melkonian, 1992) cannot be explained by Morrill's model. The authors described the cortical layer of cytoplasm as a pellicular layer in these organisms. However, the layer which was termed pellicular in these articles had already been described as a dark band of cytoplasm in armoured *Ceratium tripos* (Whetherbee, 1975).

Sekida et al. (2001) investigated development of amphiesma in *Scrippsiella hexapraecingula* which belongs to the order Peridinales, same as *Heterocapsa* and *Glenodinium*. The *S. hexapraecingula* mode of reproduction is eleutheroschisis. Ecdysis occurs immediately after transformation of a motile cell into a non-motile cell. Authors harvested the cells with a micropipette instead of centrifugation. They revealed separated AVs in pre-ecdysing cells that conforms to Höhfeld and Melkonian (1992) research. However, the pellicle formarion reconciles with Morrill's model. During ecdysis a non-motile cell sheds covering mebranes, except continuous

IAVM – the new plasma membrane. Discontinuous pellicle fuses into the pellcular layer that increases in thickness during the pellicle maturation. The mature pellicle includes two layers thermed PI and PII. Outer PI layer consists of five laminae in the form ABABA, where A is an electron-dense layer and B is a less electron-dense layer. The PII is being formed just beneath PI layer and appears to be homogeneous. Similar data were obtained on symbiotic dinoflagellate Simbiodinium (Wakefield et al., 2000).

It should be noted, whereas pellicle formation preceeds ecdysis in *H. niei* (Morrill, 1984), it is the result of ecdysis in *S. hexapraecingula* (Sekida et al., 2001). This defference can be explained by features of the objects. *H. niei* is the pelliclulate dinoflagellate meaning that AVs contain the pellicle layer besides the thecal plates (Morrill and Loeblich, 1983). AVs of *S. hexapraecingula* contain only the thecal plates (Sekida et al., 2001).

Membranes in the flagelar area

In eukaryotes the plasma membrane must be continuous with the flagellar membrane. After flagella shedding, at least one membrane (plasmalemma) must enclose the entire cell. Morrill (1984) clearly indicated that OM, OAVM and IAVM are continuous membranes in the flagellar area after deflagellation. The author also showed that flagella of *H. niei* are formed under the pellicle during ecdysis, and their membranes are continuous with IAVM (the new plasma membrane). Sekida with colleagues (2001) proved it to be the case in *S. hexapraecingula*, where flagellar extention occurred after the pellicle thickening.

It should be noted that there is a stage where OM, i.e. the old plasmalemma, and IAVM, that is precursor of the new plasmalemma, are both continuous during ecdysis. It may be suggested that transformation of IAVM into the new plasma membrane occures at this stage, after or during pellicle formation. In the process of such transformation the vesicular transport should play a crucial role.

Development of amphiesmal vesicles and thecal plates

In general amphiesma is a plasmalemma underlied by vesicles (AVs), that may contain thecal plates and layers of immature pellicle (Morrill and Loeblich, 1983). As mentioned above, the plasmalemma appears to be derived from IAVM (Morrill and Loeblich, 1983; Morrill, 1984; Wakefield et al., 2000; Sekida et al, 2001). On the other hand, origin of AVs is still not determined.

Wetherbee (1975) showed the elongated vesicles in *C. tripos*, which seemed to originate from Golgi cisterns. Those vesicles contained an electron dense material that was suggested to be a thecal plate's precursor (TPP). Initially the vesicles were relatively small, but then became larger due to fusion with each other. Wetherbee reported about two ways of the TPP deposition. First way is a fusion of the elongated vesicles at the sutures, the places of contacts of two adjacent AVs. Second way of the TPP deposition is a fusion of TPP materials along the entire area at the base of AVs. Similar vesicles with electron dense contents were observed by Morrill (1984) and Morril and Loeblich (1984) in *H. niei*. Some of those vesicles contained thin layers of the putative pellicle. During the course of ecdysis thin thecal plates apeared within the vesicles. Morrill suggested that those vesicles were precursors of AVs. Wedemayer and Wilcox (1984) reported about the dense vesicles lying beneath AVs in *Peridiniopsis berolinense*. Furthermore, large (ca. 200 nm) and small (ca. 70 nm) dense vesicles were found under the layer, termed pellicle *sensu stricto*, in *N. miliaris* (Melkonian and Höhfeld, 1988). In some cases the large ones fused with IAVM.

Bricheux and colleagues described the tubules, vesicles and irregular structures with electron dense or translucent contents in *G. foliaceum* (Bricheux et al., 1992). Structures with electron dense contents could fuse with the plasmalemma and release the dense material to the outside. Structures with translucent contents were termed amphisome and suggested to be precursors of AVs. Moreover, they proposed that the cortical microtubules may be involved in amphisomal vesicles movement to the plasma membrane after ecdysis.

Sekida and co-workers (2001) studied AVs development in *S. hexapraecingula*. They observed the electron dense vesicles beneath the cortical microtubules just after ecdysis. Soon the vesicles, which suggested being the juvenile AVs, appeared in the space between the new plasmalemma and microtubules. During muturation of the pellicle the number and size of the novel AVs increased. Then the nascent AVs formed network structures within certain regions. The shape and arrangement pattern of these territories were similar to those of mature thecal plates of motile cells.

There are two hypothesis of the AVs contents development. The first hypothesis suggests that the vesicles with electron dense material contain the precursors of the thecal plates and pellicle. These vesicles may transport the precursor materials to

AVs. During ecdysis some of the dense vesicles may become the new AVs (Wetherbee, 1975; Morrill, 1984; Melkonian and Höhfeld, 1988). According to the other hypothesis, AVs originate from the amphisomal vesicles, whereas the AVs contents develop from putative soluble precursors but not from dense vesicle contents. Indeed, the electron dense material was not heavy labled with anti-70 kDa antiserum specific to cell covering elements, in contrast to mature thecal plates and homogeneous layer of the pellicle in *G. foliaceum* (Bricheux et al., 1992). Moreover, these researchers noted that the dense vesicles fused only with the plasma membrane, and therefore these vesicles resembled the dinoflagellate mucocysts. These observations contrast with those by Wetherbee (1975) and Morrill and Loeblich (1984), who showed fusion of the dense vesicles with IAVM.

It should be noted that the chemical composition of thecal plates is still not clear. Using histochemical and enzymatic assays and X-ray diffraction, Nevo and Sharon (1969) concluded that the main component of the thecal plates in *Peridinium westii* is glucan with both β1-3 and β1-4 linkages. However, they did not determine whether these linkages belonged to one or different types of molecules. Although cellulose is a β1-4 glucan, researches made a decision that there was no cellulose in the thecal plates. However, Morrill and Loeblich (1983) determined the presence of cellulose in *H. niei*. Based on X-ray diffraction analysis it was concluded that cellulose was in a low crystalline state (Morrill and Loeblich, 1983). Dodge in his review (1971) also proposed cellulose as the main component of dinoflagellate thecal plates.

Kwok and Wong (2003) investigated coupling of cellulose synthesis to the cell cycle in *Crypthecodinium cohnii* by calcofluor white (CFW) staining and flow cytometry. CFW fluorescense intensity reflects cellulose amount in the cell. Authors reported that cellulose amount per unit of cell surface area peaked by the S phase start, increasing through the G_1 phase. A drop of relative cellulose amount happened in G_2/M phase. Moreover, using 2,6-dichlorobenzonitrile, an inhibitor of cellulose synthesis, researchers showed that cellulose synthesis was required for the cell cycle progression at G_1 phase. Thus, we can assume that morphogenesis of dinoflagellate cell covering is interlinked with the cell cycle.

General conclusions

Based on the works considered above we integrated the data in a generalized scheme of changes in amphiesmal arrangement during ecdysis in armored dinoflagellates. At the motile stage of the life cycle, amphiesma represents a conventional cell covering typical for the group Alveolata (Fig. 5A). Such amphiesma includes continuous OM (the plasma membrane) and AVs beneath which contain thecal plates and pellicular layers (in pelliculate dinoflagellates). There are cortical microtubules under AVs. During the motile stage, the cell stores cellulose in thecal plates. Before the start of ecdysis, the motile cell sheds its flagella and enter the non-motile stage (Fig. 5B). In such cells separated AVs fuse and thus the cell have three continuous membranes: OM, OAVM, and IAVM. At this stage, continuous pellicular layer is formed between OAVM and IAVM under the thecal plates. In the pelliculate organisms, this layer is originated from discontinuous pellicle pieces accumulated in AVs. Apparently at this stage continuous IAVM transforms into the new plasma membrane. Initially the new plasmalemma is underlied by microtubules, but soon the small juvenile AVs apear between these two layers (Fig. 5C). These vesicles concentrate in the territories that correspond to the thecal plates pattern typical for certain species of dinoflagellate. By the start of ecdysis the juvenile AVs may contain thin thecal plates (Fig. 5D). During the course of ecdysis the old plasmalemma (OM), OAVM and thecal plates are shedded and the pellicle thickens by development of several additional layers (Fig. 5E). The novel AVs enlarge by fusion with each other. After the old cell covering shedding the new flagella are formed under the pellicle (Fig. 5F). Then flagellar cell discards the pellicle and becomes motile againg (Fig. 5A).

However, it must be noted that the described scheme is not likely the universal one. First, it excludes unarmoured dinoflagellates genera *Noctiluca* and *Amphidinium*, which probably have another way of pellicle formation as reported by Melkonian and Höhfeld (1984) and Höhfeld and Melkonian (1992). Second, the new question arises: is the pellicle an obligate structure of any ecdysing cell? Future morphological research on broader species diversity will bring the answers to these questions.

Nevertheless, even now based on our generalized scheme we can judge about the cell cycle stages most accessable for the patch-formation. First of all, dinoflagellates have less armored cell covering at the juvenile motile stage. However, patch-clamp cannot be performed on motile cells and therefore cells should be somehow immobilized in advance. One of the ways to immobilize cells is to expose them to stress conditions, such as slight centrifugation. Unfortunately, centrifugation can trigger ecdysis

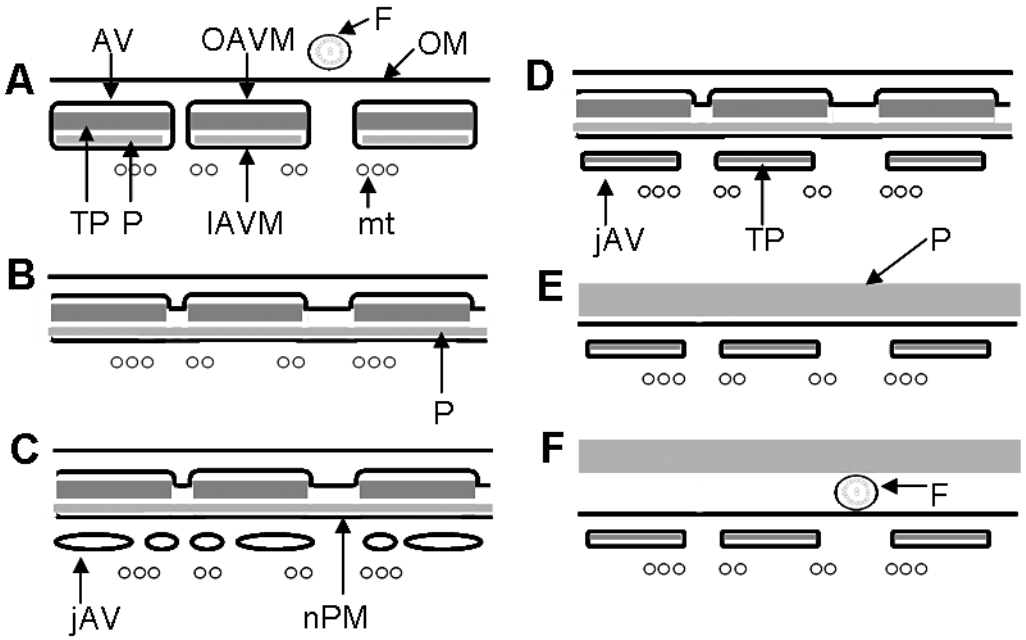

Fig. 5. Generalized scheme of changes in amphiesmal arrangment through the life cycle and ecdysis in armored dinoflagellates. A – Cell covering arrangement at motile stage; B – amphiesma after deflagellation and fusion of the amphiesmal vesicles; C – appearance of the juvenile amphiesmal vesicles; D – thecal plate formation in the juvenile amphiesmal vesicles; E – cell covering in ecdysed organism; F – appearance of a new flagellum. *Abbreviations*: AV – amphiesmal vesicles, F – flagellum, jAV – juvenile AV, IAVM – inner amphiesmal vesicle membrane, mt – microtubule, nPM – new plasma membrane, OAVM – outer amphiesmal vesicle membrane, OM – outermost membrane, P – pellicle, TP – thecal plates.

and the pellicle formation. That is why we argue that the best way to solve this problem is to block thecal plate's synthesis. For example, it can be achieved by application of 2,6-dichlorobenzonitrile, as it was done by Kwog and Wong (2003).

Acknowledgements

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