Particle Movement in Heliozoan Axopods Associated with Lateral Displacement of Highly Ordered Membrane Domains

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Freeze-fracture studies reveal that extrusive organelles displaying saltatory particle movements in centrohelidian axopod are attached to highly ordered domains within the plasma membrane. It is postulated that the motive force for lateral displacement of these membrane domains with the adhering organelle is located immediately underneath the plasma membrane being either part of the peripheral membrane proteins or attached filaments alined parallel to the axopodial microtubules. The attachment domain is interpreted as a "frozen" membrane area preventing untimely organelle discharge by membrane fusion.

Introduction

Among the various models on membrane structure the "Fluid-Mosaic" model proposed by Singer and Nicolson [1] is currently the most favoured one. The model, based on thermodynamic considerations and supported by experiments with spin-labelled membrane lipids [2], the observation of rapid intermixing of fluorescent antibodies in fused cells [3], and other experimental data, implies that both lipids and proteins can diffuse freely in the plane of the membrane thus leading to a more or less random distribution of the membrane components. More recently Jain and White [4] have raised the question whether biological membranes really display such a high degree of disorder. They claim that there is at present no possibility to exclude that the introduction of spin-label from the exterior of the cell or the labelling with fluorescent antibodies perturb a formerly existing order. They place more emphasis on specific intermolecular interaction of the membrane components than Singer did in his model. Jain and White envision a part of the membrane to consist of ordered and rigid membrane domains (endowed with special functions) swimming in a fluid environment – a model which bears some resemblance to the plate-tectonics model for continental drift.

We think that the attachment sites of extrusive organelles in unicellular actinopods can serve as an excellent example for the existence of such highly ordered membrane domains in the plasma membrane.

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Moreover the observations reported here may be of more general interest for they imply that in a certain type of intracellular movement, *i.e.* in saltatory particle movement, membrane-mediated processes are involved.

Material and Methods

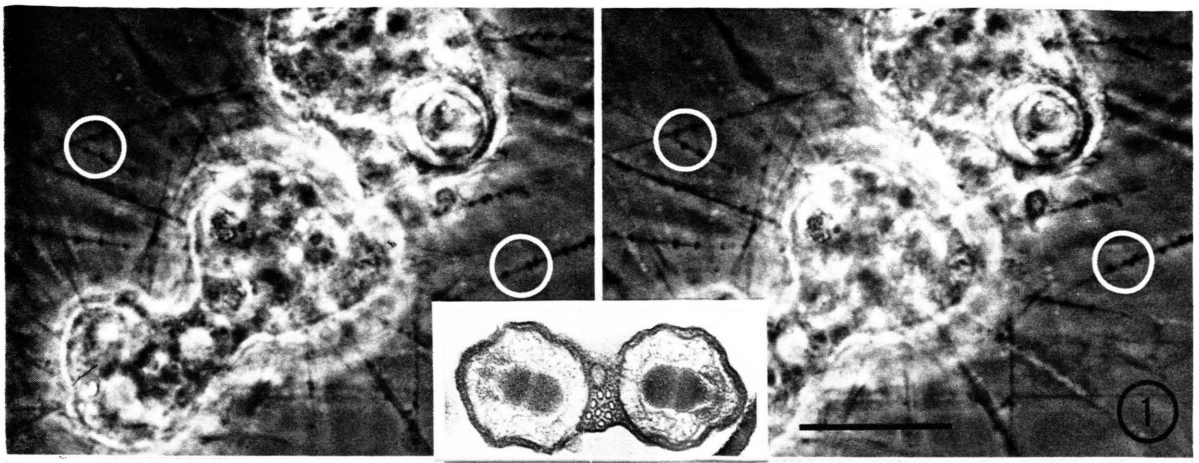
The organisms used for this study, *Acanthocystis erinaceoides* and *Raphidiophrys ambigua*, were collected from a basin in the back-yard of the former Zoological Institute in Tübingen. The cultures were kept in Pringsheim solution and fed with *Tetrahymena pyriformis* and *Chlorogonium elongatum*, respectively. Living organisms were studied with a Zeiss microscope using phase optics and Nomarski differential interference optics. Cinematographic recordings were made at 2 and 12 frames/sec using Kodak Tri-X Reversal film. From selected sequences prints were made for measurements of particle displacement.

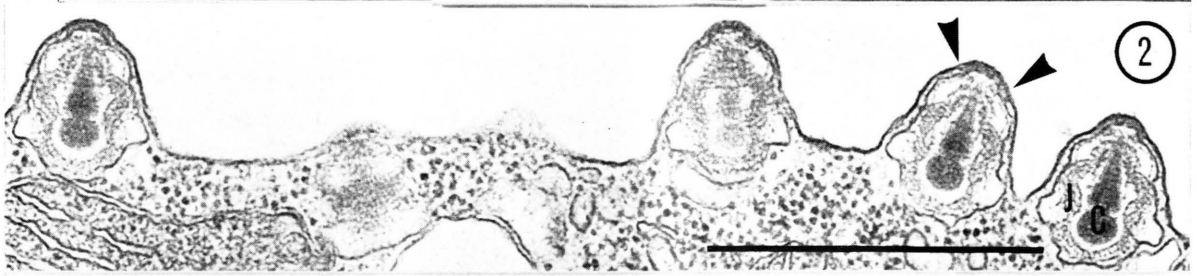
For electron microscopy cells were fixed following the procedure described by Roth *et al.* [5] but using only half the amounts of glutaraldehyde and sucrose than given in the original formula. For the preparation of freeze-fracture replicas cells fixed in 3% glutaraldehyde in 0.015 M phosphate buffer pH 6.6 were used. They were infiltrated with 20% glycerol prior to vitrification in melting nitrogen and fractured in a Balzers BAF 301 apparatus.

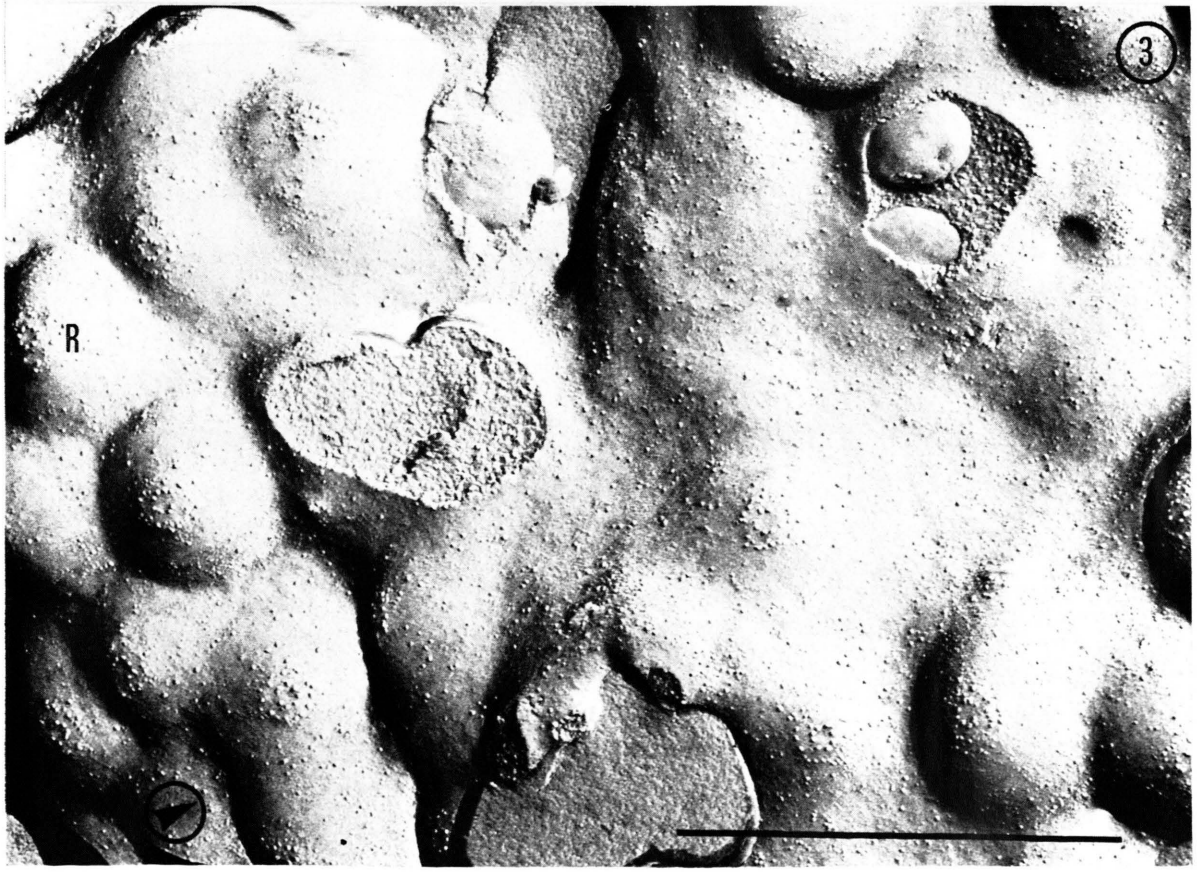
Observations and Interpretations

Phenomenology of granule movement in centrohelidian axopods

Among the Heliozoa, which all have highly ordered microtubule arrays, the Centrohelidia are







Legends to Figs 1—3 see on page 191.

particularly suited for studies on saltatory particle movement for their axopods contain a single class of particles, extrusive organelles known as kinetocysts [6], which fulfil several of the criteria listed by Rebhun [7] for this type of movement.

The refractile granules seen in the axopods in 2 consecutive micrographs in Fig. * 1a and b, taken at an interval of 2 sec perform jerky bidirectional movements with a velocity of $1-5\ \mu$m/sec. The velocity distribution of the granule movements is absolutely discontinuous. A given granule may show no movement for minutes and then suddenly it shoots off for an unpredictable distance, occasionally covering more than $30\ \mu$m at a stretch. During its excursions it will meet with other moving or stationary granules, and it may either pass then without any interference or collide with them. Upon such collisions the granules concerned may swing around the axoneme, move as a tandem in one direction or separate as if repelled and move in opposite direction. The velocity reached and the distance covered by the granules seem to depend to larger extent on the physiological condition of the specimen observed (meaning its degree of "excitement") than on species-specific characters. On the other hand we know from observations in a larger number of stocks and species that distinct differences in axopodial activity exist, which result in a more or less rapid rolling or walking motion of the whole cell. An increased axopodial activity is always accompanied by saltatory movement of the granules. There is one other important and very puzzling peculiarity in kinetocyst behaviour which is not found in other systems showing saltatory particle movement. When the centrohelidians are totally undisturbed and floating freely in the culture medium the granules arrange themselves at very regular distances. Single or in pairs they are then spaced at a distance of $1.5-2.5\ \mu$m, like birds resting upon a perch.

Ultrastructure of the kinetocysts and their possible function

Earlier electron microscopical observation by the author had shown that the refractile granules represented a new type of protozoan organelle bearing some superficial resemblance with the haptocysts in the suctorian tentacle, organelles involved in food capturing [8, 9]. A kinetocyst is a complex polar organelle which measures 380 nm in height and about 300 nm at its largest width. Surrounded by an outer membrane it contains an electron dense bipartite central element enclosed by a jacket of less electron dense material with radial and concentric striations (Fig. 2). The unusual orientation of the polar organelle with its long axis perpendicular to the axis of the axopod is evocative of a streetcar running on microtubular rails powered by overhead wires, the latter represented by the plasma membrane.

In whole-mount preparation of living organisms kinetocyst discharge can be triggered with fumes of formaldehyde, subsequent negative staining has shown that the jacket is expelled from the organelles' membrane. The material of the jacket seems to function as propulsive charge for the central element lies always in front of the burst jacket. These findings together with the observations made on freeze-fractured cells allow to regard the kinetocyst as a special type of compound motile mucocyst which is most likely engaged in food trapping.

Centrohelidian axopods are sticky and have an immobilizing effect on certain other protozoa. If suitable protozoa by chance come into contact with a centrohelidian it is usually their cilia or flagella which touch the axopods first. But because of the minuteness of the organelles involved and the immediate attempt of the prey to escape by violent

* Figs 1–3 see Plate on page 190 b.

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Fig. 1. Two consecutive phase contrast micrographs of the centrohelidian *Raphidiophrys ambigua* showing the displacement of axopodial granules (= kinetocysts) in encircled areas. Scale marker = $20\ \mu$m. Inset shows cross section through distal part of an axopod in *Acanthocystis erinaceoides*. Note size and orientation of the 2 kinetocysts in relation to the microtubular axoneme. 45 000 ×.

Fig. 2. Mature kinetocysts in *R. ambigua* attached to the plasma membrane. Note that the plasma membrane lies close to the organelles over most of their surface leaving only a very narrow space between the 2 membranes. J, jacket material which surrounds the central element, C, of the kinetocyst. Attachment domains shown in Fig. 3 correspond to an area marked by the arrow heads. 45 000 ×. Scale marker = $1\ \mu$m.

Fig. 3. Protoplasmic fracture face of the plasma membrane in *A. erinaceoides* showing numerous kinetocyst attachment domains. A very regular rosette, R, of 8 membrane particles is seen on the left. Direction of shadowing is indicated by the arrow at bottom left. 60 000 ×. Scale marker = $1\ \mu$m.

agitation of their motile organelles it is very difficult to see whether the first contact to the prey is made by a kinetocyst. The struggle of the prey entangles it with further axopods and soon it becomes aligned parallel to them. Transportation towards the cell body is usually accomplished by a food-cup forming pseudopod which grows out at the base of the effective axopods and engulfs the prey. Though it is not easy to follow this sequence in thin sections the involvement of kinetocysts in food capture is further substantiated by the observation of their remains in the early food vacuoles. Discharged kinetocysts are replaced by new ones which originate from vesicles seen first in the vicinity of the dictyosomes. During their development the prekinetocysts migrate to the periphery of the cell where they finally become attached to the plasma membrane.

Structure and function of kinetocyst attachment domain

Freeze-fracture studies on kinetocysts, initiated by Davidson [10], showed that the attachment site of the kinetocyst to the plasma membrane has a particle array very similar to that found at the mucocyst attachment site in *Tetrahymena* so brilliantly studied by B. Satir and co-workers [11]. We remember that freeze-fracture technique produces 2 complementary halves of the membranes. The plane of fracture runs through the middle of the hydrophobic lipid bilayer circumventing integral membrane proteins which show up as particles of variable size while the smooth areas are supposed to represent the lipids [12].

The protoplasmic fracture face of the plasma membrane (the half closest to the cytoplasm) in Fig. 3 shows numerous rosettes of about 8 membrane particles located on top of prominent (about $3/\mu m^2$) bulges. A comparison with Fig. 2 shows that each bulge is produced by a kinetocyst attached to the plasma membrane. It is needless to say that the attachment sites in the axopods look the same, they are only less frequently found on the replicas. The rosettes measure 60 nm in diameter and consist of 6 to 9, most often of 7 to 8 particles, 12 ± 2 nm in diameter. Each rosette is surrounded by an annular area, some 30 nm wide, which shows no particles. Another annulus of less regularly spaced paraicles follows. The particles of the rosette are notably larger than the other particles. Differing from the mucocyst attachment site in *Tetrahymena* there is no particle in the center of the rosette in the Centrohelidia. The other half of the plasma membrane, its extracellular fracture face, contains the corresponding pits which are a little more difficult to see. The protoplasmic fracture face of the kinetocyst membrane has a few particles near the attachment site but they are less prominent than the annulus of particles seen in the mucocyst membrane in *Tetrahymena*.

It has already been noted by Satir and co-workers [11] that the rosette represents a highly ordered inhomogenous arrangement of molecules, probably protein aggregates, in a lipid matrix. They concluded that the rosette aids the mucocyst in the final positioning and that it determines the future fusion site. It has even been suggested that their so-called "fusion rosette" is the initial stage of rearrangement for the actual fusion. Moreover mucocyst discharge served these authors as a model system for membrane fusion in secretory processes in general.

Doubtlessly rosette and particle-free annulus, henceforth called "attachment domain", is a very complex multifunctional membrane specialization. One important and hitherto overlooked function of the attachment domain seems to be to prevent the organelle from being discharged at the wrong time. And we predict that such a "safety catch" will be found in other extrusive organelles in protozoa and lower invertebrates, too. While it has been claimed that membrane fusion demands areas of relatively high fluidity [13], the attachment domain, on the contrary, seems to represent a very stable non-fluid membrane domain. One would of course like to know how this highly ordered arrangement of the membrane components is established. It might be helpful to remember that the attachment domain measures 1000 Å in diameter and covers an area of obout 800000 $Å^2$ compared to 60 $Å^2$ occupied by a lipid molecule. While the rosette particles may result from short range order interaction [1] of two or more subunits, the entire rosette is hardly the result of short range order. Peripheral membrane proteins on the inner surface of the plasma membrane may tie the rosette particles together. And the particle-free annulus, some 40 lipid molecules wide, may be due to lateral phase separation.

We suppose that the development of the attachment domain and the final maturation of the kineto-

cyst are linked very closely to one another. When an approaching kinetocyst comes within a critical distance to the plasma membrane, rearrangements of the membrane components will occur in both the organelle's membrane and in the plasma membrane. At the same time a firm adhesion of the kinetocyst to the attachment domain will be established, perhaps to overcome repulsive forces which may develop once the 2 membrane systems have approached very close. It is only after this attachment to the "frozen" membrane domain that the kinetocyst is supposed to pass the final steps of maturation. The exclusive attachment of mature kinetocysts to the plasma membrane may be part of the safety system for their discharge inside the cell should not occur.

From comparative studies on haptocysts, extrusive organelles in the suctorian tentacle, we know that these haptocysts discharge only upon contact with certain food ciliates. Only the special diet seems to have molecules on its surface which are complementary to receptor sites supposed to be associated with the haptocyst attachment domains. (The latter have a rosette of 12 smaller particles with a larger one in the center and a particle-free annulus, as well.) Recognition of the proper food by specific binding to a receptor molecule may produce a trans-membrane signal, *e.g.* a conformational change in a key molecule of the attachment domain which then may lead to the "liquifying" rearrangements in the attachment domain necessary for membrane fusion.

In Centrohelidia kinetocyst discharge may induce further long range effects in the plasma membrane, *e.g.* changes in ion permeability which alter the microenvironment of the axopodial microtubules. This may result in an almost instantaneous depolymerization of microtubulus registrated as sudden "contraction" of the axopod which is observed in certain food trapping reactions [14].

Possible mechanism of the movement of the attachment domain

So far we have been dealing only with the inner half of the plasma membrane and it might be argued that the attachment domains do not span the whole membrane. Their movement within only the inner half of the plasma membrane is not very likely, it might produce considerable shearing-stress and the supposed receptor molecules would have to penetrate the outer half anyway. Further support for the assumption that the membrane domains span the entire plasma membrane comes from microcinematographic observations on the movement of bacteria on the axopods in a marine centrohelidian. Bacteria are transported with the same characteristics as the kinetocysts [15]. A formerly often hold argument in connection with labelling of the cell surface with exogenous particles was that a perhaps independently moving surface coat might not tell very much about behaviour of the membrane underneath. For centrohelidians this argument is no longer valid. As some kinetocysts remain stationary while others move, it is also conclusive that certain membrane domains move relative to others, which will produce some lateral shearing-stress.

Having now realized that the kinetocyst attachment domains and probably other areas, too, move or are moved within the plasma membrane 2 ques-

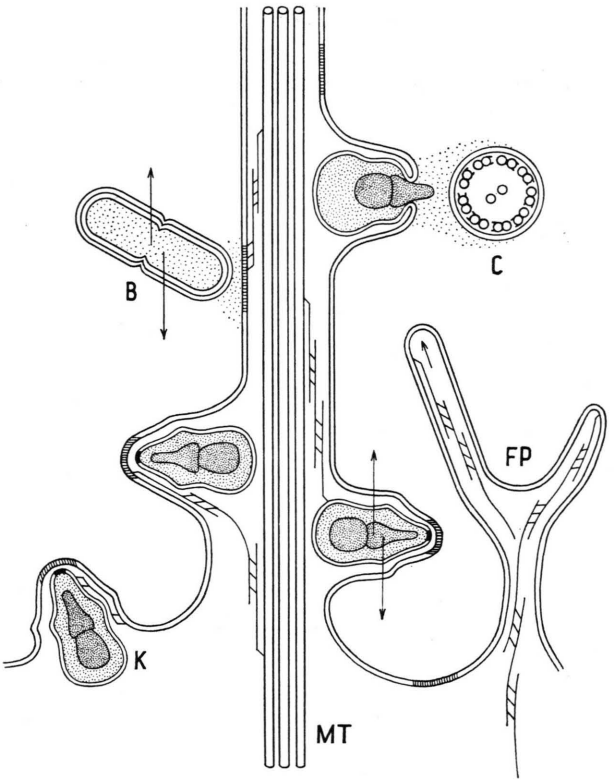

Fig. 4. In centrohelidian axopod kinetocysts, K, attached to highly ordered membrane domains as well as bacteria, B, perform bidirectional movements. The motive force for membrane domain displacement is envisioned in an actomyosin-like system located somewhere in the narrow space between plasma membrane and axopodial microtubules, MT. It is also supposed that discharge of a kinetocyst upon contact with a cilium, C, of a suitable prey has triggered outgrowth of a food-cup forming pseudopod, FP.

tions suggest themselves. On which point does the motive force act and what is its molecular basis?

In the recent past microtubules have often been quoted in connection with particle transport in heliozoan axopods for they are the only linear elements found in the axopods [5, 16]. One might expect to find bridges between the microtubules and the particles transported, but extensive search was unsuccessful so far. Besides the likewise unknown mechanism in other so-called microtubule-associated transport systems reports begin to appear which question the absolute necessity of microtubules for granule movement [17, 18]. Edds found it possible to push a microneedle into and through the cell body of the heliozoan *Echinosphaerium nucleofilum*, and moving out some cortical cytoplasm on the other side of the cell he produced an artificial axopod which even in the presence of colchicine showed some granule movement [18].

We also have no serious reason to suggest that the motive force is located in the integral membrane proteins. What remains is the very narrow space between the plasma membrane and the microtubules. Search for other motile structures, *e.g.* microfilaments in this space which is only some hundred angströms wide, has been unsuccessful so far. Since microfilaments were found in food-cup forming pseudopods of these cells, the difficulty to detect them in the axopods does not seem to be a question of adequate fixation but may rather be a question of aggregate size of the contractile proteins.

Actomyosin-like proteins in close vicinity to the cell surface have been reported from a variety of nonmuscle cells [19]. The best known example is the erythrocyte membrane where the *band 5 protein* seems to have some actin-like function and the spectrin some myosin-like function [20]. While a clear distinction of peripheral membrane proteins from other attached proteins may be a matter of definition, the minimal requirement for a two-membrane system to show motility might be satisfied if *e.g.* the plasma membrane carries a myosin-like protein while the actin is associated with an organelle's membrane. Though we have shown that part of the plasma membrane moves with the kinetocyst, it might be that the other areas of the plasma membrane which are close to the kinetocyst move relative to it. Facts and fancy of axopod behaviour are summarized in Fig. 4.

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