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Mechanism of cell protrusion formation in electrical field: the role of actin

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An intense alternating current electrical field that imposes membrane-applied force on the cell surface can induce formation of cell protrusions (Popov, S.V. and Margolis, L.B. (1988) J. Cell Sci. 90, 379–389). This technique has been used to investigate the role of actin in the cell protrusion formation. Platinum replicas of the cytoskeleton were prepared to characterize the organization of the cytoskeleton in external force-induced protrusions. Bundles of microfilaments were found in the processes. A specific inhibitor of actin polymerization, cytochalasin B, as well as inhibitors of ATP synthesis (sodium azide and carbonyl *m*-chlorophenylhydrazone) did not change the morphology of electrical field-generated protrusions, revealed by scanning electron microscopy. However, organization of the cytoskeleton inside the processes changed drastically using these inhibitors. The results of these experiments demonstrate that (i) Membrane-applied force is sufficient to produce native-like cell protrusions, even in conditions where activity of the cytoskeleton is inhibited; (ii) Actin microfilaments can be organized into bundles directly under the action of membrane-applied force. The significance of these observations to cell protrusion formation under normal physiological conditions is discussed.

Introduction

Protrusions determine cell morphology and play an important role in the attachment of cells to the substrate, in the movement of cells and in intracellular recognition [2]. The nature of the forces pushing the membrane outward, as well as factors determining the morphology of the protrusions are unclear.

In normal physiological conditions, bundles of actin microfilaments are found in protrusions. Thus, it has been proposed that the force pushing the membrane outward is generated by the polymerization of actin into microfilaments [3]. An alternative hypothesis considers the protrusions to be induced by forces of other origin (e.g. osmotic) [4,5].

Previously we described a model system, based on the phenomenon of dielectrophoresis [1] to investigate the phenomenon of cell protrusion formation. It was shown that membrane-applied force per se is enough to generate cell-specific processes with normal morphology. Specific inhibitors of cytoskeletal activity or inhibitors of ATP synthesis were not able to prevent protrusion formation when the force was applied to the membrane.

To understand how the deformation of the cell membrane (induced by external force) affects the organization of the cell cytoskeleton, we investigated the internal structure of the protrusions using a platinum replica method. In this report we demostrate that actin microfilaments can passively follow deformation of the plasma membrane and organize into bundles of microfilaments directly due to the action of membrane-applied force.

Materials and Methods

Cells. Mouse embryo fibroblasts (MEF) were prepared according to [6]. Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Sigma Chemical Co) in 5% CO_2 and used after 1–3 passages.

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Electrical treatment of cells. Cells were treated by electrical field as previously described [1]. After trypsinization, the cells were transferred to buffered sucrose (290 mM sucrose, 1 mM Hepes-NaOH (pH 7.4)). 50–100 μl of a cell suspension containing 10^2–10^3 cells were placed on the surface of the coverslip in the gap between electrodes (Fig. 1). The coverslip was mounted on the stage of inverted microscope 'Fluovert' (Leitz). The cells were treated by alternating current electrical field (frequency 1 MHz, voltage 6–8 V with a gap between electrodes of 60–80 μm) at room temperature for 20 s to 5 min. Cells were treated by the field no later than 15 min after replacement of the serum-supplemeted medium with buffered sucrose. The cells were fixed for electron microscopy either during application of the electrical field or after the field was switched off.

Pretreatment of cells. Cytochalasin B (Sigma) was added to the suspension of cells in a serum-supplemented medium at a final concentration of 10 μg/ml. Sodium azide (Sigma) at 1 mg/ml or 10 μM carbonyl *m*-chlorophenylhydrazone (Sigma) were added to the cell suspension for 1 h. In both cases 10 mM 2-deoxy-D-glucose was added in the incubation medium.

Scanning electron microscopy. Cells were fixed for scanning electron microscopy by 2.5% glutaraldehyde (Serva) in PBS or in a sucrose solution. The specimens were dehydrated in acetone, critical point dried and examined using a Hitachi 405A scanning electron microscope.

Transmission electron microscopy. Platinum replicas of the cytoskeleton of MEF were prepared according to [7]. Briefly, Triton X-100-extracted cells were rotary shadowed with platinum and strengthened with carbon. The replicas were examined at 80 kV in a Jeol-100C electron microscope.

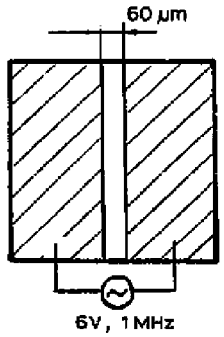

Fig. 1. An experimental chamber. A coverglass covered with aluminium films (shaded areas) of 0.1 μm thick. The films were used as a pair of electrodes. The cells in buffered sucrose were placed in the gap between the electrodes and treated with alternating current electrical field. The average intensity of the field (voltage divided by the distance between electrodes) was 1 kV/cm, the frequency of the field was 1 MHz.

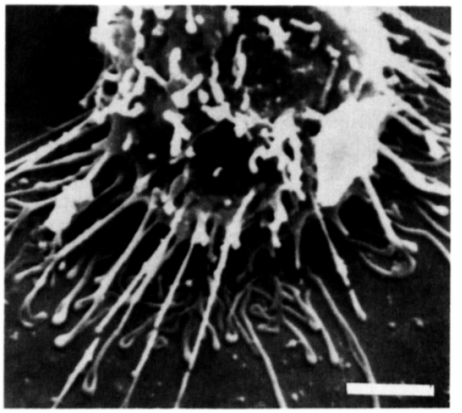

Fig. 2. Scanning electron microphotograph of mouse embryo fibroblast fixed after 5 min application of the electrical field. Electrode occupies the lower part of the photograph. The protrusions are directed from the cell body to the electrode. Bar, 3 μm.

Immunoelectron microscopy of platinum replicas. The cells were prepared for electron microscopy according to [7]. After the field was switched off, cells were extracted with Triton X-100 and fixed with 2.5% glutaraldehyde in PBS. Free aldehyde groups were blocked by sodium borohydride (Sigma) (2 mg/ml, 10 min incubation) and by lysin (0.2 mg/ml, 1 h incubation). Cells were incubated with rabbit anti-actin antibodies [8] for 2 h and with secondary antibodies (goat anti-rabbit antibodies, conjugated with 10 nm gold particles, Sigma) for 2 h. In all cases the treatment was at 37 °C.

Results

Morphology of the protrusions induced by an electrical field.

After the field was switched on, cells were attracted to the edge of the electrode on the surface of the coverslip – to the region of the maximal field strength. This cellular behavior is in full agreement with the theory of the dielectrophoresis and previously reported data [9–11].

During the treatment of cells by an electrical field, cell protrusions directed from the cell body to the edge of the electrode were formed (Fig. 2). The protrusions were formed only on the electrode-facing parts of the membrane.

As described earlier [1] MEF produce 2 types of outgrowths under the action of membrane-applied force: filopodia-like and lamellopodia-like protrusions which were morphologically similar to those formed by MEF during normal physiological spreading.

We investigated organization of the cytoskeleton inside these processes using the method of platinum replicas of the cytoskeleton.

The cells were fixed after a 5 min application of the electrical field and platinum replicas were prepared for electron microscopy. A system of cytoskeletal elements connecting the cell body with the electrode was revealed. This system could be divided into three areas.

(1) Meshwork of filaments (Fig. 3a). This region was close to the cell body. The meshwork was three-dimentional: filaments were located at different levels over the substrate. The proximal part (close to the cell body) of this area was disarranged: there was no preferential direction in the orientation of the filaments. There was a preferential orientation of filaments in the distal part of the meshwork: single filaments and bundles of filaments were oriented mainly from cell body to the electrode, along the axis of the processes (Fig. 3b).

(2) Bundles of filaments (Fig. 3c). Bundles of filaments protruded from the meshwork of filaments and were oriented from the cell body to the electrode, parallel to the long axis of the process. The proximal part of the process was located above the substrate. Single filaments in this region were poorly resolved. The distal part of the bundles was in contact with the substrate. In this region single filaments up to few μm long were easily distinguished. Virtually in 100% of the cells examined microfilaments were oriented along the long axis of the protrusion.

(3) Tips of bundles (Fig. 3d). Two types of structures could be observed at the ends of the bundles. Approx. 50% of the bundle end with the loop of filaments. The characteristic dimension of the loop was 0.5–1.0 μm. In the rest cases bundles end with electron-dense area in which no details can be resolved. As a rule, dimensions of this area were 0.5–1.0 μm.

For identification of the different components of cytoskeleton we used immunoelectron microscopy. Practically all filaments bound anti-actin antibodies (the latter were decorated by secondary antibodies conjugated with 10 nm colloidal gold particles). The filaments were labeled regardless of their position in the cytoskeletal system – in the meshwork, in the bundles or at the tip of the bundles (Fig. 4).

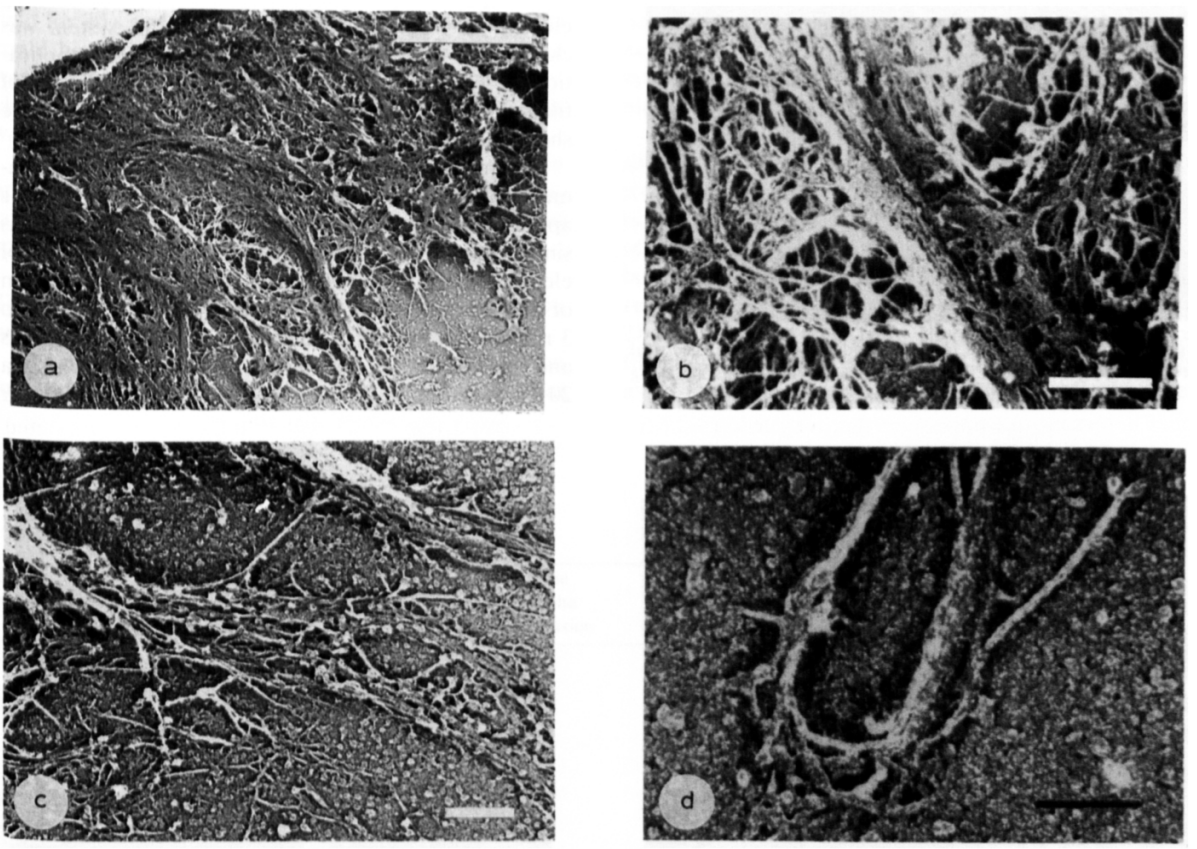

Fig. 3. Platinum replicas of cytoskeleton of mouse embryo fibroblasts. Cells were fixed after 5 min application of electrical field. (a) A meshwork of filaments inside the generated lamella. Bar, 1 μm. (b) A bundle of filaments at the distal part of the lamella. The bundle is directed along the applied force. Bar, 0.2 μm. (c) Bundles of filaments inside the distal processes. Bar, 0.2 μm. (d) Terminal loop of filaments at the distal end of the process. Bar, 0.2 μm.

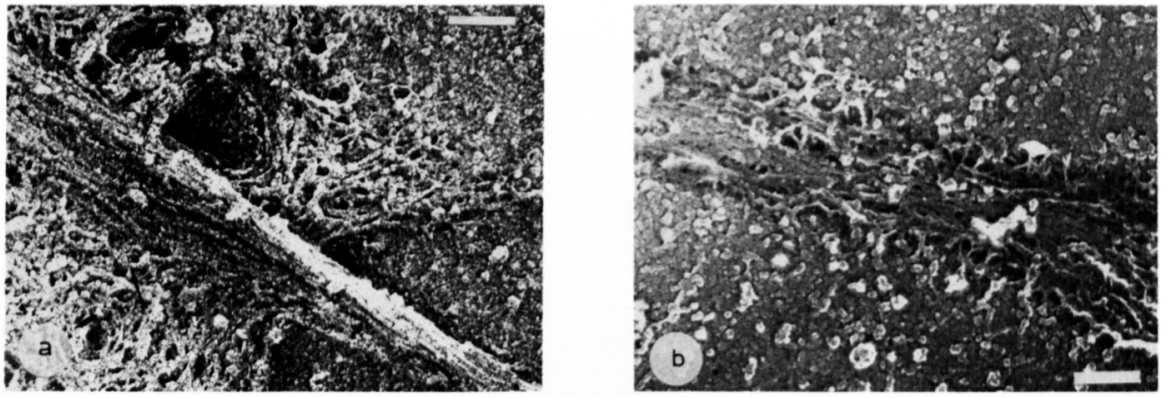

Fig. 4. Immunoelectron microscopy of platinum replicas of mouse embryo fibroblasts, treated with electrical field for 5 min. Arrows indicate anti-actin antibodies, conjugated with 10-nm colloidal gold particles. (a) Bundles of filaments in single protrusions. Bar, 0.2 μm. (b) Terminal loop of filaments at the distal end of the process. Bar, 0.1 μm.

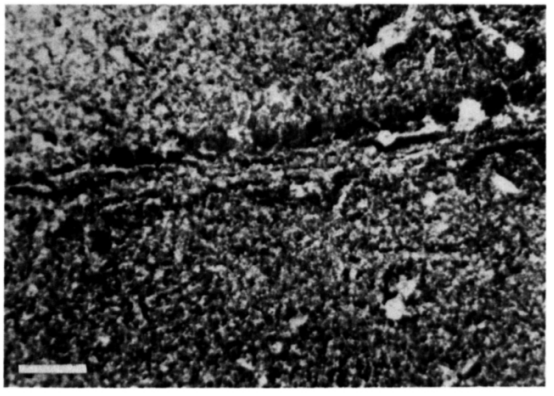

Fig. 5. Bundles of filaments inside filopodia-like process. Electrical field was applied for 20 s. The filaments are oriented mainly along the long axis of the process. Bar, 0.1 μm.

When the time of the electrical-field application was reduced to 20 s, the system of processes connecting the cell body with the electrode was revealed by scanning electron microscopy. Morphologically this system was close to the one formed by MEF after a 5 min application of electrical field. But generally the number of filaments was less and individual filaments were shorter.

Platinum replicas demonstrated a system of filaments inside the processes generated after a 20 s application of electrical field. Morphologically it was similar to the one formed after a 5 min application of electrical field. As for the case of the 5 min application of the electrical field, the system could be divided into 3 regions: meshwork of filaments, bundles of filaments and tips of bundles. The meshwork of filaments after a 20 s application of the electrical field was much shorter

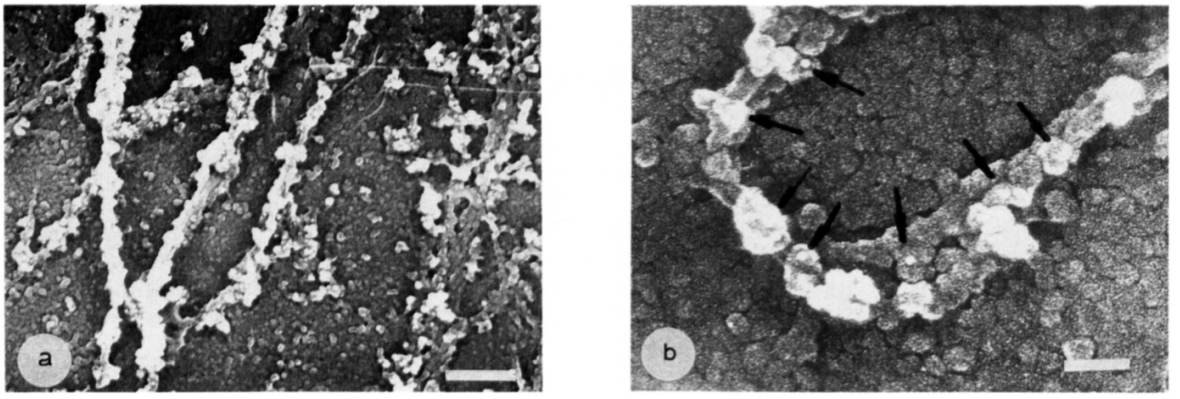

Fig. 6. Cells were fixed after 5 min incubation on the surface of coverglass in normal physiological conditions. No electrical field was applied. (a) A bundle of filaments protruding from the distal meshwork of filaments. Bar, 0.1 μm. (b) A bundle of filaments inside the filopodia. Bar, 0.1 μm.

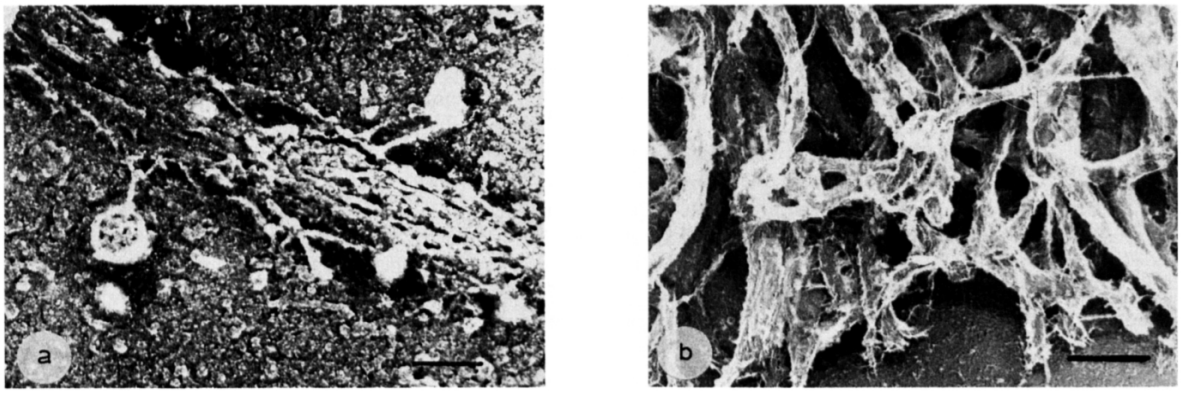

Fig. 7. Platinum replicas of cytoskeleton. Cells were incubated with carbonyl *m*-chlorophenylhydrazone (a) or cytochalasin B (b) for 1 h and treated with electrical field for 5 min. (a) Single filaments could be resolved inside the filopodia-like protrusions. Bar, 0.1 μm. (b) In cytochalasin B-pretreated cells the processes had dense morpholngy. Single filaments could not be resolved inside protrusions. Bar, 1 μm.

(less than 1 μm) than in the case of cells treated by an electrical field for 5 min. Morphology of the bundles of filaments and their tips in cells treated by an electrical field for 20 s and 5 min were the same: single filaments could be found in the bundles (Fig. 5). Usually there were loops of filaments at the end of the bundles. However the number of bundles in 20 s treated cells was less.

The system of actin filaments observed after a 5 min application of electrical field was morphologically similar to the one formed by MEF in the early stages of spreading in normal physiological conditions (Fig. 6).

The influence of inhibitors of actin polymerization on organization of the cytoskeleton in the processes

We investigated the organization of the cytoskeleton in electrical field-generated protrusions of cells that were pretreated by an inhibitor of polymerization of actin, cytochalasin B, and by metabolic poisons sodium azide and carbonyl *m*-chlorophenylhydrazone. Previously we have shown that pretreatment by these drugs neither prevents protrusion formation by electrical field nor changes their morphology as observed with scanning electron microscopy [1].

Cells were incubated with inhibitors of ATP synthesis carbonyl *m*-chlorophenylhydrazone for 1 h and then treated with electrical field. Platinum replicas revealed no cytoskeletal protrusions in 80% of the cells. In 20% of the cells, the cytoskeletal system connecting the cell body with the electrode was found. The meshwork region (zone 1) was absent: bundles of filaments (zone 2) protruded directly from the cell body. Bundles of filaments and tips of bundles had the same morphology as in the case of carbonyl *m*-chlorophenylhydrazone-

TABLE I

Orientation of the filaments in the bundles of filaments

Experimental treatment	Orientation of the filaments along the axis of the protrusion	Number of experiments	Total number of cells observed
5 min electrical field application	+ [a]	38	800
20 s electrical field application	+	17	180
Cyt B + 5 min electrical field application	− [b]	24	480
Sodium azide + 5 min electrical field application	+	20	240
Carbonyl *m*-chlorophenylhydrazone + 5 min electrical field application	+	20	170

[a] +, The filaments are oriented along the long axis of the protrusion.
[b] −, Single filaments are not resolved in the protrusion.

untreated cells. Single filaments were easily resolved in the bundles (Fig. 7a).

Similar results were obtained when the cells were treated with an electrical field after a 1 h incubation with sodium azide.

Platinum replicas of MEF, treated by electrical field after a 1 h incubation with cytochalasin B revealed the system of cytoskeletal elements, directed to the electrode. Bundles of filaments protruded from the cell body; there was no intermediate region of meshwork of filaments. The bundles had dense morphology and no single filaments could be resolved by the platinum replica method (Fig. 7b). The tips of the bundles were usually thicker than the bundles (0.5–1.0 μm).

The influence of different treatments on the orientation of the microfilaments in the bundles of filaments is summarized in Table I.

Discussion

Protrusions of different cells contain actin in the form of microfilaments and actin-associated proteins [12-14]. It was suggested that polymerization of G actin into F-actin can provide a force pushing the membrane outward [3]. A number of experimental facts [15–17] support this hypothesis. It has been alternatively suggested that actin does not push the membrane out, but only passively follows the membrane, deformed by the force of another origin [5,18,19].

To evaluate the latter possibility we developed a new model system to investigate cell process formation. In this system a membrane-applied force was generated due to the treatment of cells by an electrical field of high frequency [9,20,21]. The principle of the force generation is based on the differenet conductivities of the cytoplasm and the extracellular solution. After the electrical field application an undermembrane diffuse layer is formed to compensate the field inside the cell. The thickness of this layer is 10–20 Å [20]. Because of the low conductivity of external solution, during 1 μs (period of electrical field) a diffuse layer on the external surface of the membrane does not form. As a result a force applied to the thin membrane layer and directed outwards is generated. For more details see Refs. 9–11. The action of high frequency electrical field does not have to be confused with the action of direct current field on motility of cells. The latter effect is complex and depends on the type of the cell [22–24]. On the contrary, generation of membrane-applied force in a.c. electrical field is a purely physical phenomenon and does not depend on the nature of the cell.

Previously [1,11] it was shown that in developed experimental system morphologically normal cell processes are formed under the action of membrane-applied force. Pretreatment of cells with cytochalasin, colcemide, sodium azide or incubation at 4 °C did not prevent process formation under the action of membrane-applied force [1].

It was assumed that in this system morphologically normal cell-specific processes are formed directly by the deformation of the membrane induced by external force, without active (ATP-dependent) reorganization of the cytoskeleton. Previously, Margolis and Popov (1988) demonstrated that other possible effects of electrical field – electrical breakdown [25], electrophoresis of receptors on the cell surface [26], heating [21], orientation of bundles of actin filaments in constant electrical field [27] – do not contribute to the observed phenomenon.

The question about inner organization of the processes, however, remained open. Is there actin in the processes and if so, what is the mechanism of formation of this system?

Electron microscopic analysis of platinum replicas of the cytoskeleton revealed a system of cytoskeletal elements inside the processes. Let us discuss the organization of cytoskeleton inside the processes, the nature of the filaments and mechanism of formation of this system.

Comparison of scanning and transmission electron microscopy data showed that there was correspondence between the external and internal organization of the processes.

The diameter of a typical MEF protrusion evaluated using scanning electron microscopy was 0.3–0.5 μm. There was a characteristic thickening at the tip of the process at the region of contact with the substrate. As a rule the process protruded from the lamella region close to the cell body. Three areas could be distinguished in electrical field-treated MEF:

(i) meshwork, corresponding to the lamella region;

(ii) bundles of filaments, corresponding to the cylindrical protrusions;

(iii) tips of bundles ('loops'), corresponding to the thickenings at the tips of the processes in contact with the substrate.

It appears that the cytoskeletal system consists mainly of actin.

(1) The diameter of single filaments was 10 nm, which is close to the diameter of actin filaments (taking into consideration the thickness of the gold cover is 2 nm).

(2) The bundles of filaments revealed at the distal part of the meshwork and in the processes (Figs. 3b, c) were morphologically close to the bundles of actin microfilaments that form at the early stages of spreading in normal physiological conditions (Fig. 6a, b).

(3) According to immunoelecton microscopy practically all filaments were labeled with anti-actin antibodies (Fig. 4).

To understand the mechanism of formation of the

cytoskeletal system in the electrical field-generated processes we used different conditions to inhibit polymerization of G actin into microfilaments.

After incubation of cells with cytochalasins, single filaments could not be resolved in the processes. It seems that the pretreatment of cells by cytochalasin B considerably destroyed actin filaments. Nevertheless, morphology of electrical field-generated processes did not depend on whether the cells were pretreated with cytochalasin B or not [1]. These results provide evidence that actin does not directly generate the force, pushing the membrane outwards in our system.

Two hypothesies can be proposed regarding the mechanism of formation of the actin system inside the processes.

(1) Actin filaments, linked to the plasma membrane, passively follow it and align under the action of membrane-applied force; no ATP is required for this alignment.

(2) Electric field-generated processes are free from the cytoskeleton at the very beginning. Gradually they are filled with actin monomers that ATP-dependently polymerize into microfilaments.

The following experimental data support the first mechanism.

(1) Bundles of actin microfilaments formed in less than 20 s. This time is smaller than the characteristic time of polymerization of actin in normal physiological conditions [16,28].

(2) When polymerization of actin was greatly reduced (the cells were treated with metabolic poisons) the alignment of microfilaments along the direction of the force was still observed (Fig. 7a).

After a 5 min treatment of cells by an electrical field the meshwork region of filaments could be found adjacent to the cell body. In the case of a 20 s treatment of MEF by electrical field this region was either smaller or absent. For the cells treated by sodium azide, carbonyl *m*-chlorophenylhydrazone or cytochalasin B the meshwork region was virtually absent. It seems that the absence of this meshwork reflects disruption of cytoskeletal links between the cortical layer and cytoskeletal structures inside the cytoplasm: deformation of the cell membrane, induced by an electrical field-generated force did not effect cytoskeletal structures in the cytoplasm.

The results described above are compatible with the following model of cytoskeletal reorganization (Fig. 8). In intact cells there is a 'ball' of actin filaments attached at some points to the cell membrane. External force deforms the membrane and generates cell protrusions. Microfilaments, attached to the membrane follow it and organize into bundles of filaments, oriented along the axis of the process. If the attachments are disrupted the processes formed are free from cytoskeleton from the very beginning.

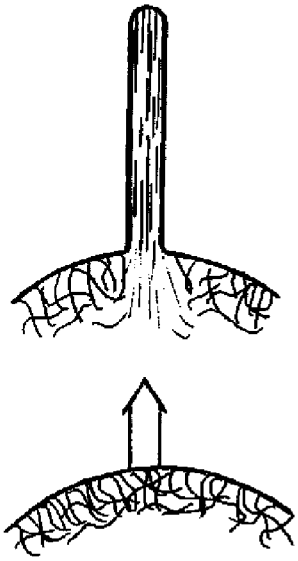

Fig. 8. Membrane-attached filaments follow plasma membrane and organize into bundles of filaments directly under the action of the force. Electrical field-generated force, used in our experiments, mimics membrane-applied cytoplasmic forces.

A similar mechanism of passive ATP-independent orientation of actin filaments under the action of membrane-applied force was demonstated for epithelial cells [29]. It was shown that global deformation of the cell membrane that changes considerably the shape of the cell is accompanied by passive reorientation of the actin filaments in the cortical layer along the direction of the applied force. There were also indirect morphological data on the importance of mechanical tensions in the orientation of stress fibers and actin system as a whole [30–32].

In the developed model system the main role in cell process formation plays not the cytoskeleton but the cell membrane together with the cortical layer. The external membrane-applied force is enough not only to form processes but to organize actin in these processes in the form of bundles of microfilaments. This force pushing the membrane outward is generated in our experiments due to the application of an electrical field. There are no electrical fields of such intensity (1 kV/cm) and frequency (1 MHz) outside cells *in vivo*. But there are forces inside the cytoplasm tending to push the membrane out [5]. Extrapolation of our results to the *in vivo* situation demonstrate that formation of cell processes can take place directly under the action of membrane-applied force, without active reorganizations of the cytoskeleton. This force is enough to pull out cell protrusions of normal morphology and to organize actin in these processes in the 'normal' way.

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