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CELL BIOPHYSICS

The Autowave Electromechanical Activity of the *Physarum polycephalum* Plasmodium

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Abstract—The aim of this work is to clarify the role of the electrical activity of the *Physarum polycephalum* plasmodium in the control of the contractile activity and self-organization of the directed locomotion. This single-celled organism with a non-excitable membrane is a classic object that is used in studies of amoeboid motility. Its patterns of motor behavior and signal systems are common for many tissue cells. The presence of 50 mM KCl in an agar substrate under half of a separate plasmodial strand strongly inhibits the formation of the frontal zone and leads to sharp morphological polarization of the strand, which suggests the involvement of electrical processes in the autowave self-organization of the plasmodial structure. The gigantic sizes of the plasmodium make it possible to record its electrical activity simultaneously at different parts of the cell. It has been established that potentials and currents at parts of the plasmodium that are distant from each other oscillate synchronously and differ only in the shape of the signals, probably due to differences in the phases or the number of excited harmonics. We recorded currents (~50 pA) of single ion channels of the plasmodial membrane using the classical local voltage-clamp method. It has been found that the oscillation spectrum of the current that is generated by the plasmodium has high-frequency fluctuations, which are probably connected with periodic detachments of the membrane from the cytoskeleton during the formation and growth of the pseudopodia. It has been also shown that neomycin, a substrate inhibitor of phospholipase C, prevents oscillations of both the mechanical and electrical activity of the plasmodium. This is consistent with its well-established ability to inhibit mechanosensitive Ca^{2+} channels, which are apparently present in the plasmodial membrane. These data indicate the presence of a general signal system that is linked with the dynamics of the membrane-cytoskeleton association, which could be involved in the galvano- and chemotaxis of amoeboid cells.

Keywords: amoeboid locomotion, auto-oscillations, regulation, contractile and electrical activity, self-organization, *Physarum polycephalum* plasmodium

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The elucidation of the mechanisms that underlie self-organization of directed migration of cells is believed to be of importance due to its determining role in tumor metastasis, wound healing, immunity, and morphogenesis, i.e., in the emergence of highly organized structures during the development of an organism. Impaired capability of normal movements and morphogenesis is one of the typical features of transformed tumor cells. All cells that are involved in these processes are capable of amoeboid locomotion, which is the movement of cells along a solid surface using temporal protrusions that are formed on their surface, so-called pseudopodia. The ability of free-living amoeboid cells for directed movement along gradients of attractants and repellents is critical for their survival. It is well known that amoebas possess galvanotaxis and move to a cathode in a DC field. However, despite the importance of amoeboid locomotion, the mechanisms that underlie all possible types of cellular taxis remain unclear.

A significant element of amoeboid locomotion is an intensive flow of an endoplasm toward the direction of movement. The establishment and maintenance of such flows require the coordination of local contractions all over the body, which leads to the question of what the interactions are that are responsible for this coordination. The plasmodium of the myxomycete *Physarum polycephalum* provides unique opportunities for experimental study of this problem. This unicellular organism with a non-excitable membrane can reach gigantic sizes (up to a thickness of 2 mm and an area of 1 m^2) in the presence of food due to uncoupling of nuclear and cellular division. The

Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate.

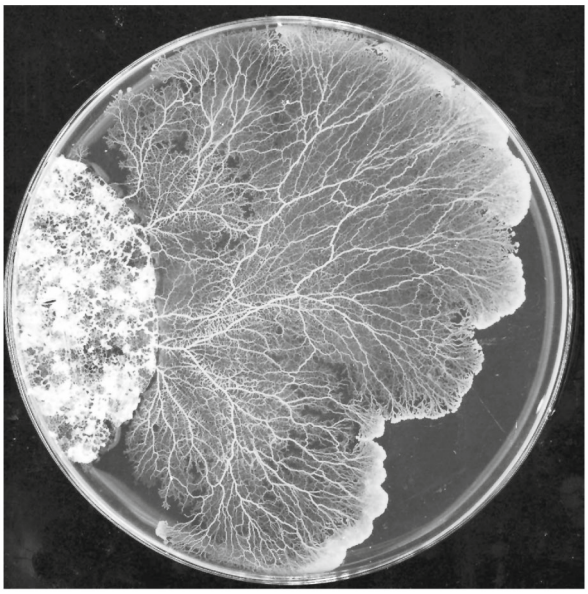

Fig. 1. The typical polar structure of a plasmodium that migrates over an agar surface. The diameter of the Petri dish is 19 cm.

contractile apparatus of this multinucleated cell is a three-dimensional labile network of actin filaments, which is attached to the plasmalemma in the ectoplasm and contains bipolar oligomers of the myosin motor protein. The contraction of this microfilament network is determined by the phosphorylation of light myosin chains by their kinases in the presence of Ca^{2+} ions.

Another important attribute of cellular motility is mechanochemical autooscillations, since no contractile apparatus is capable of providing cell migration over large distances without operating in an oscillatory mode. The plasmodium dynamics manifests in rhythmic pulsations of the body, whose fundamental period varies from 1 to 5 min depending on the physiological state of the cell and changes in response to external chemotactic stimuli [1–4]. The rhythmic contractions are well coordinated along the plasmodium body and induce intensive back-and-forth flows of the endoplasm, which provides not only intracellular stirring, but also cell migration. It starts upon depletion of nutrients in the medium or the occurrence of gradients of external stimuli and results from more intensive or more prolonged protoplasm streaming toward the leading edge. In this case, the cell structure becomes polar and maintains the form of a fan-shaped protoplasmic film at the front that is followed by a tree-shaped network of protoplasmic strands (Fig. 1).

The oscillations of the contractile activity of the plasmodium are accompanied by the synchronous oscillations of the intracellular Ca^{2+} concentration [5, 6], membrane potential [7–11], and a number of other components [12]. The role of Ca^{2+} in the regulation of the contractile activity is well-established; however, the significance of the membrane potential and its oscillations in the coordination of the motor activity of an amoeboid cell has not been experimentally determined as yet. However, it is clear that changes in the Ca^{2+} concentration that regulates the contractile activity occur only due to changes in the conductivity of ion channels, activity of electrogenic ion pumps, and cotransport in the cellular membranes.

The oscillations of the electrical activity of the plasmodium can result from the functioning of an independent membrane oscillator, which in the presence of common components can be synchronized with the intracellular one. However, there are no data on the presence of this oscillator in amoeboid cells. To clarify the mechanism of its autooscillations, it is necessary to determine the current-voltage characteristics of the membrane and reveal nonlinear processes that result in the oscillatory instability of the membrane potential. The cause of the electrical oscillations could be the presence of mechanosensitive ion channels in membranes that are attached to the cytoskeleton. Since their conductivity depends on mechanical stress, the oscillation of the contractile activity could lead to the parametric oscillations of the membrane potential. In any case, it is necessary to note that the plasmalemma has an important function in the modulation of the contractile activity during taxis of an amoeboid cell, since the response of the contractile system to attractants occurs after their reception at the external surface of the membrane.

The main difficulty in electrophysiological studies of the plasmodium of a myxomycete using conventional glass microelectrodes is the rapid formation of a new membrane around the tip of a micropipette. The peak value of the potential that is recorded upon a membrane puncture reaches −130 mV and 5–10 s later it falls to zero, i.e., the electrode is outside the cell. The membrane regeneration and the presence of a renewable mucous cover complicate the study of ion channels by the voltage-clamp method. The ability of the plasmodium to rapidly form a new membrane can be followed by drops of the escaped endoplasm that are formed on the surface of large strands upon their puncture with a glass needle and then are quickly gelatinized [10, 13, 14]. To prevent these processes, the glass electrode is treated with EGTA, which makes it possible to record changes in the membrane potential during different external exposures [15–18]. The transmembrane potential in the plasmodium varies from −60 to −130 mV and is mainly determined by the activity of an electrogenic proton pump that transports H^+ outside the cell, which accounts for the sensitivity of the membrane potential to the pH of the environment [17, 19, 20].

However, it is easy to record the oscillations of the extracellular potential difference in the plasmodium.

The difference of the surface potentials is usually measured by non-polarizable electrodes at two parts of the plasmodium that are connected with each other by a protoplasmic strand and placed onto KCl-containing agar substrates in different sections of a double chamber that are electrically insulated [8]. In the first studies it was already found that the oscillations of the surface voltage correlate with a back-and-forth (shuttle) flow of the endoplasm [7–9]. The frequency of the oscillations coincides with that of the shuttle flow of the endoplasm and the amplitude, which can reach 30 mV, does not always coincide with that of the oscillations of the contractile activity.

In contrast to studies that explored the dynamics of the surface potentials, in [21] using a vibrating glass micropipette [22] it was possible to record extracellular local ion currents, whose density reached 15 $\mu A/cm^2$. In the endoplasm drops that flowed out upon the rupture of large strands, which rapidly form a new membrane, it was found that the current always entered the locations of numerous bulges and flowed out of the regions with a planar surface. In monopodial microplasmodiums that are formed from the drops the current flowed out both of the frontal zone and retractile end of the strand and entered the middle part. These parts of amoeboid cells are usually observed to have noticeable motile activity [2, 4].

However, the most-pronounced manifestation of the autowave electrical activity of the plasmodium is observed in large plasmodia [23, 24]. Surface potentials that are recorded in different parts of the plasmodium demonstrate local synchronous oscillations that are similar to the pulsations of the contractile activity [1, 2, 10, 23]. In contrast to the autowave contractile activity, the mechanisms of the oscillations of the membrane potential in the plasmodium of a myxomycete are still unclear. The purpose of this experimental study is to obtain additional electrophysiological data on the dynamics of the potentials and currents in different parts of the plasmalemma using a voltage-clamp method, which is necessary for the establishment of the mechanism of oscillations of the electrical activity of the plasmodium and elucidation of their role in the regulation of the contractile activity of amoeboid cells and self-organization of their directed locomotion.

MATERIALS AND METHODS

The plasmodium of *Physarum polycephalum* (strain BKM (F) 3283) was grown on 2% agar substrates with rolled oats. In experiments that were carried out at 20–22°C, fragments of protoplasmic strands 4–6 mm in length and 0.4–0.8 mm in diameter that were excised from the plasmodium 12–18 h after the last feeding were used. Any excised fragments restored the integrity of the external membrane during several seconds and behaved as autonomous organisms in 15–30 min, resuming contractile oscillations. The external manifestation of the mechanical activity of the strand is determined by the boundary conditions.

If the strand remains attached to the substrate, which almost excludes its longitudinal strain, a standing wave of radial deformation with antinodes at the end of the strand is formed and an intensive back-and-forth flow of the endoplasm occurs, which has been reproduced well in our distributed mathematical model [25, 26]. If a fragment of the strand is vertically suspended in a humid atmosphere with some load at the end, then longitudinal isotonic auto-oscillations gradually are established there with an amplitude that sometimes reaches 10% of the length. In the case where a fragment of the strand is fixed between the hooks of a strain sensor, it is possible to record the oscillations of the force of the tension of the strand in an isometric mode of contraction, when the distance between the hooks, i.e., the strand length, almost does not change. Both modes of recording these oscillations of the longitudinal dynamics of the isolated strand can even be quantitatively simulated using a mathematical model that we developed [27]. With time, the ends of the strand develop into new frontal regions. Later, one of the competing fronts becomes leading, the second front degenerates and the characteristic polar form of a migrating plasmodium emerges.

In this study, the longitudinal dynamics of the strand were recorded in an isometric mode using a high-sensitivity device [28]. The sensitivity of the force sensor was 0.2 mg; the compliance was 0.1 μm/mg, and the dynamic range was 50 mg. The interface between the sensor and computer consisted of a data acquisition card with an 8-channel 12-bit ADC, 12-bit DAC, and 16 digital input-output channels. Special program software was developed for this system. This device makes it possible to maintain the required time change in either the length or load of an object by measuring the object deformation or its force of tension, respectively, as a response. The strand was joined to tensiometer hooks by warm molten agar and placed into a cuvette with control solution (10 mM HEPES, pH 7.0).

The electrical activity of the plasmodium was recorded using a device; its block diagram is given in Fig. 2. The device makes it possible to measure current that is generated by the plasmodium with a sensitivity of approximately 10 pA and a time resolution 10^{-3} s and the voltage with a sensitivity of 0.1 mV. For this, two electrometric operational K544UD1A amplifiers with high input impedance and a low level of input currents were used. The first current-to-voltage converter amplifier ((*6*) in Fig. 2) was used to measure current; the second voltage repeater amplifier ((*7*) in Fig. 2) recorded the voltage. A constant-voltage source (*8*) was used to compensate for the contact potentials of electrodes and to construct the voltage-current characteristics of the plasmalemma. The

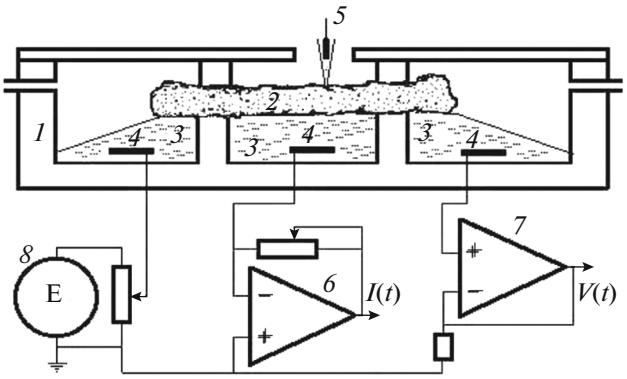

Fig. 2. The block diagram of a device for measuring the electrical activity of a plasmodium: (*1*), experimental chamber; (*2*), plasmodial strand; (*3*), agar; (*4*), non-polarizable electrodes, (*5*), a glass micropipette electrode for recording currents of single ion channels of plasmalemma; (*6*), an operational amplifier to measure current; (*7*), an operational amplifier to measure voltage; (*8*), a constant-voltage source to compensate for electrode potentials and to construct the voltage-current characteristics of a membrane.

device used program software (J. Demster, *Strathclyde Electrophysiological Software W in EDR 3.2.2*); an NI USB-6009 ADC interface was used between the sensors and the computer.

A chamber that has three sections was made ((*1*) in Fig. 2) for the simultaneous recording of the electrical activity of different parts of the plasmodium. The sections were filled with heated 2% agar (*3*), which contained 5 mM KCl so that it covered the non-polarizable silver-chloride electrodes (*4*) that were established in the sections. Narrow (1 mm in width) grooves, which were filled with Vaseline that served as a sealant and electrical insulator, were cut in the partitions between the sections. A plasmodial strand (*2*) was tightly placed into these groves so that it touched the agar in these sections. The resistance between the neighboring sections with the strand was approximately 0.5 MΩ.

Glass micropipette electrodes (*5*) with a tip diameter of approximately 2 μm [29] were used to record the currents of individual channels in voltage-clamp mode. Recording could be performed at any region of the upper surface of the strand by micropipettes in a micromanipulator. Any of the electrodes in the chamber sections could be the reference electrode. EGTA was not added to the micropipettes, since they were not introduced inside the cell and only touched its surface. This system combines the advantages of high spatial resolution and did not result in the disruption of membrane integrity. The micropipettes were filled with heated agar that contained 10 mM KCl. As shown in Fig. 3, this concentration of the electrolyte does not have a strong influence on the plasmodium compared to concentrations of 50 mM and higher. The resistance of the micropipette was approximately 10 MΩ and the potential was smaller than 10 mV.

The HEPES and neomycin sulfate reagents were used in this work (Sigma, United States). During the experiments stock solutions of neomycin and HEPES, pH 7.0, were prepared directly prior to the treatment of the plasmodium due to rapid loss of activity.

RESULTS AND DISCUSSION

As can be seen in the top row of images in Fig. 3, the presence of 50 mM KCl in an agar substrate to the left under half of a separate plasmodial strand strongly inhibits the formation of the frontal zone and leads to sharp morphological polarization of the strand, which suggests the involvement of electrical processes in the regulation of the motility of the plasmodium and its structural self-organization. The pictures were made 3, 4, and 5 h after isolating and placing the strands into a Petri dish perpendicular to the boundary between the left side of the agar, which contained 50 mM KCl (top row) or 10 mM KCl (bottom row) and right side of the agar without KCl. The agar contained 10 mM HEPES, pH 4.6, and 0.1 mM Ca^{2+}. The effect of 10 mM KCl is significantly weaker; therefore, we used this concentration of the electrolyte in glass microelectrodes. The pictures also clearly demonstrate the occurrence of new fronts with the formation of a network of strands and further confluence of the plasmodia, when they meet, which is known to lead to the establishment of a general endoplasm flow that globally coordinates the autowave contractile activity of the plasmodium.

The characteristics of the electrical activity of the plasmodium that are measured vary depending on the state of the plasmodium and experimental technique that is used [30]. However, an extracellular potential measuring method has its advantages, since in this case the membrane is not ruptured by a glass microelectrode and the effect of the ruptures on the functioning of the contractile apparatus can be neglected. At a large input impedance of the recording amplifier, the difference in the surface potentials between different parts of the strand reflects the difference of their transmembrane potentials. The amplitude of the oscillations of this voltage in our experiments reached 30 mM. The gigantic sizes of the plasmodium made it possible for us to simultaneously record the electrical activity in different parts of the cell. A typical example of the simultaneous recording of current and voltage that were generated by the strand in different sections of the triple chamber is given in Fig. 4. In this case, the current kinetics (Fig. 4a) was recorded between the first and the second sections of the triple chamber, while voltages (Fig. 4b) were recorded between the first and the third sections. It can be seen that the oscillations of these parameters in parts of the cell that

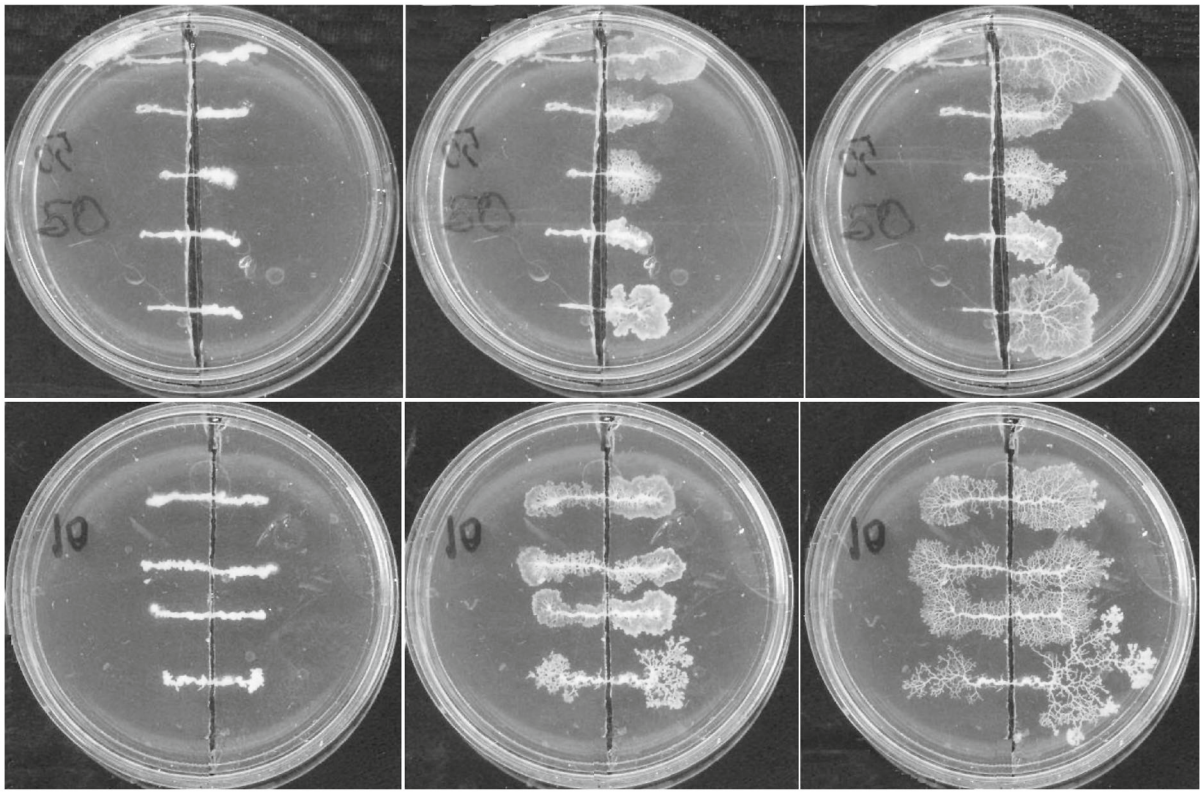

Fig. 3. The structural polarization of fragments of plasmodial strands at different step gradients of KCl. Pictures were made 3, 4, and 5 h after placing isolated strands into a Petri dish perpendicular to the boundary between the left side of the agar that contained 50 mM KCl (a top row) or 10 mM KCl (a bottom row) and the right side of the agar without KCl. Agar contained 10 mM HEPES, pH 4.6, and 0.1 mM Ca^{2+}. The diameter of the Petri dishes is 3.5 cm.

are distant from each others are synchronous, but have different waveforms. This is probably due to the difference in the phase or number of excited higher harmonics. It has been found that the spectrum of oscillations of current that is generated by the plasmodium has high-frequency fluctuations, which are probably connected with the periodic detachment of the membrane from the cytoskeleton at the top of a pseudopodium during its formation and growth as occurs in amoeboid cells during 10 s pulsations [31]. Figure 5 gives a recording of the current of single channels of the plasmalemma of the plasmodium, where two states of the channel (closed with corresponding zero current and opened with a current of approximately ~50 pA) are clearly seen. The channel spontaneously changes its state from time to time from one state to another, with intermediate states being absent.

We previously found that phospholipase C is one of the key regulators of the plasmodial oscillator, as it is in most eukaryotic cells [32]. The hexavalent polycationic aminoglycoside neomycin, which is a substrate inhibitor of phospholipase C, binds phosphatidylinositol 4,5-bisphosphate (PIP_2) with high affinity and forms an electrically neutral complex, which inhibits PIP_2 binding to phospholipase C. It has been shown that PIP_2, which regulates the state of the plasmodium actin [34–36], is a neomycin binding site and the main element that binds the cortical cytoskeleton of the plasmodium and the membrane [37].

Figure 6 shows the cessation of the auto-oscillations of the contractile (a) and electrical (b) activity of the plasmodium, which is induced by 5 mM neomycin at pH 7.0. This corresponds to its well-established ability to inhibit mechanosensitive Ca^{2+} channels. We have previously observed that the effectiveness of neomycin sharply increases upon an increase in the pH of the medium [38], which is probably determined by a strong pH dependence of the membrane potential of the plasmodium, which increases from –50 mV at pH 5.0 to –120 mV at pH 7.0 [39]. The gradient of the membrane potential is certainly important for the penetration of neomycin to the target that is localized on the cytoplasmic surface of a plasmalemma. The neomycin concentration that prevents the auto-oscillations in the plasmodium decreased from 3–5 mM at pH 4.6, which is optimal for the cultivation of the plasmodium [37] to 100 μM at pH 7.0 [38].

The response of the plasmodium to the addition of neomycin is accompanied by the occurrence of hyaloplasm-containing membrane swellings, which is

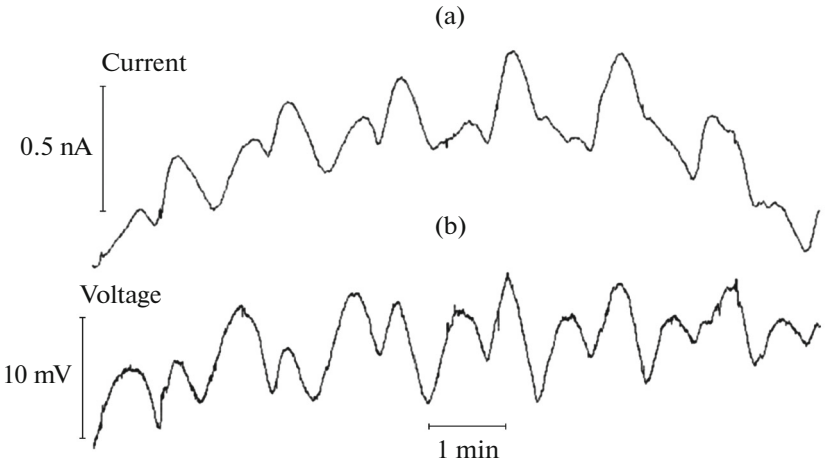

Fig. 4. The simultaneous recording of oscillations of current (a) and voltage (b) that are generated by an isolated strand in different sections of the triple chamber.

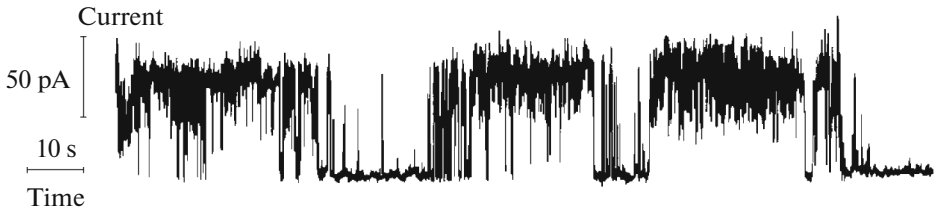

Fig. 5. The recording of the current of a single ion channel of a plasmodial plasmalemma, where its two states are clearly seen: closed (corresponding zero current) and opened (corresponding current of ~50 pA). The channel from time to time spontaneously changes from one state to another; intermediate states are absent.

typical for agents that block PIP_2 and is connected with the local detachments of the membrane from the cortical cytoskeleton. This also points to the fact that PIP_2 is the main target of the inhibitor. After the restoration of auto-oscillations, the membrane bubbles were filled with protoplasm. PIP_2 binding to neomycin stimulates the activity of phosphatidylinositol 5-kinase, an enzyme that catalyzes PIP_2 production, which has been demonstrated in preparations of the sarcolemma of cardiac cells [40]. The results might indicate the presence of mechanosensitive channels in the membrane and make it possible to suggest the presence of general signaling systems that are connected with the functioning of the complex of the cytoskeleton and membrane, which are involved in the galvano- and chemotaxis of amoeboid cells.

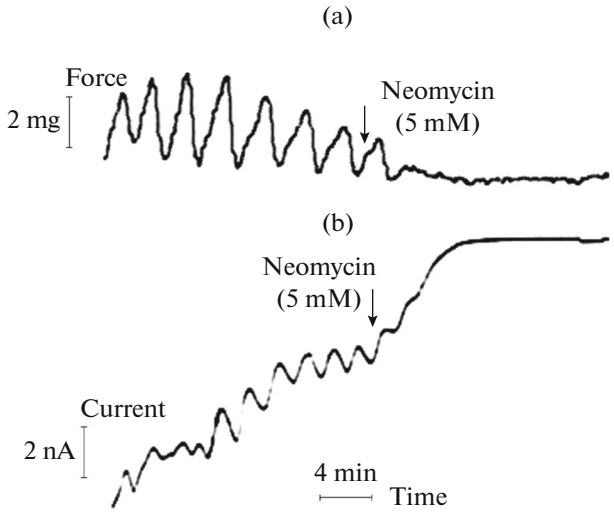

Fig. 6. The effect of 5 mM neomycin at pH 7.0 on the oscillations of isometric force (a) and current (b) that are generated by isolated strands.

The presence of reciprocal connections that regulate the activity of phosphoinositides indicated the possibility of oscillatory changes in the PIP_2 level. PIP_2 and its metabolites in myxomycetes, as in mammalian cells [41], are capable of control of oscillations of the contractile and electrical activity and regulation of directed movement of the cells. In the presence of mechanosensitive ion channels the oscillations of the electrical activity of the plasmodium could be explained within the framework of our mathematical model, which quantitatively describes the endoplasm autowave contractions, as well as its shuttle flows that are observed [25–27, 42, 43].

The main hypothesis of our basic model is the assumption of the presence of a positive feedback between the deformation of the cytoskeleton and entry of Ca^{2+} ions that in turn control active contraction and deformation of the cytoskeleton into an actomyosin network. In this case, changes of the membrane potential are proportional to the deformation of the cell. This is proven by the fact that the amplitude of oscillations of the potentials, like the amplitude of the oscillations of the deformations is significantly greater in the frontal zone of the plasmodium than in its strands. According to this model, the oscillations of the membrane potentials are globally coordinated along the entire plasmodium due to the hydrodynamic interactions; they also could be synchronized by external periodic effects [44]. In a recent study [11], the authors proved by cross-correlation analysis that the spectra of oscillation frequencies of the electrical potential and peristaltic pulsations coincide during their synchronization upon plasmodium spreading. Thus, the correlation between the oscillations of the difference of surface potentials and shuttle endoplasm flow that has been noted in many studies has been proved statistically.

In conclusion, it could be stated that the transplasmodial gradient of the electric potential that maintains the spreading of an electric current through the cytoplasm unites the distributed plasmodium system into a single energy system, where the electrical characteristics of the cytoplasm and external environment are a significant factor that determines its spatial and functional organization. Thus, we believe that the self-organization of the motility of the plasmodium is based on the regularities of autowave processes, with an important role being played by the electrical and hydrodynamic interactions that regulate spontaneously emerging contractions of the cytoskeleton.

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