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The plasmalemma redox system of a fresh-water alga and membrane electrical parameters

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In order to learn more details about the plasmalemma redox system in the alga *Hydrodictyon reticulatum* the electrophysiological characteristics of the plasma membrane as influenced by artificial electron acceptors were estimated. Ferricyanide anion as well as TTF^+ depolarized the membrane potential, the effect being more marked in the dark than in the light. In the presence of ferricyanide the membrane resistance in the light was decreased by about 29% on the average, in the dark it remained unchanged. On the other hand, TTF^+ brought about a large increase in membrane resistance, notwithstanding the light conditions. The results are discussed in view of the impairing influence of both electron acceptors on the active inflow of chloride ions (Rybová, R. et al. (1990) Bot. Acta 103, 404–407). The electrogenic outflow of electrons appears to make the largest contribution to the membrane depolarization. The inhibition of a pumping mechanism coupling the active uptake of Cl^- in some way to the transmembrane electron flow is envisaged as a plausible explanation for membrane resistance increase by TTF^+.

Introduction

A plasma membrane redox system capable of transferring electrons to an external acceptor has been analyzed in the last decade mostly in higher plant cells [1–7]. The system was shown to function also in the charophyte *Lamprothamnium papulosum* [8] and the chlorophyte *Hydrodictyon reticulatum* [9].

Attempts were made to draw useful information concerning the properties of the redox system from electrophysiological measurements. A decrease in the membrane potential [10,11] and also a decrease in the membrane resistance [8,12] were repeatedly reported after application of ferricyanide as an impermeable electron acceptor to plant cells. The membrane depolarization was mostly ascribed to an electrogenic character of electron transfer to the cell exterior (e.g., see Ref. 11). In the depolarized state the transmembrane potential difference of *Lamprothamnium* became more sensitive to changes in K^+ concentration in the surroundings and hence an increase in the potassium conductance was assumed to be the main factor decreasing the membrane resistance [8].

We have shown recently that the tetrathiafulvalene cation radical (TTF^+) can also be used as an electron acceptor and that it is readily reduced in suspensions of *Hydrodictyon reticulatum* cells [13,14]. In the present paper, the effects of ferricyanide and TTF^+ on electrophysiological membrane parameters in this fresh-water alga are compared and from the results conclusions are drawn concerning the possible role of the redox system in the transport of ions.

Methods

The chlorophycean alga *Hydrodictyon reticulatum* was cultivated in a phytotron as described previously [14]. The experimental medium contained 1.0 mM KCl, 0.1 mM NaCl and 0.1 mM $CaCl_2$. When the effect of 0.4 mM $K_3Fe(CN)_6$ was tested, KCl was omitted; the concentration of KCl in the corresponding controls was

Abbreviations: TTF^0, tetrathiafulvalene; TTF^+, tetrathiafulvalene radical cation; TTF^{2+}, tetrathiafulvalene dication.

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increased to 1.2 mM in order to keep the K^+ concentration constant. The 0.4 mM concentration of potassium ferricyanide was chosen because this gave well pronounced effects. The same pattern of responses was exhibited by 0.4 mM sodium ferricyanide in a low K^+ medium, i.e., with 0.1 mM KCl. The high K^+ medium was preferred in electrophysiological studies as it ensures a relatively steady level of potassium at the outer surface. In experiments performed in the light the algae were illuminated by a projector lamp with an intensity 40 W m^{-2}. All treatments were done at room temperature.

Fluxes of chlorides were estimated using the ^{36}Cl isotope. The radioactivity of clumps of algae weighing some 50 mg was extracted for 5 days into 1 ml of 0.01 M H_2SO_4. Aliquots of 0.5 ml were then mixed with 10 ml of a scintillation liquid and the activity measured in a liquid scintillation counter. For experiments in which the chloride efflux was measured, the algae were grown for a week in a medium labelled with ^{36}Cl. The influx of ^{86}Rb was estimated using the low K^+ medium, 0.2 mM rubidium chloride, $Na_3Fe(CN)_6$ as an electron acceptor and the above extraction procedure.

Microelectrodes with tips slightly below 1 μm were pulled from borosilicate capillaries with an inner fibre by a vertical mechanical puller. They were filled with 3 M KCl; the liquid was introduced into the electrode neck by means of a syringe and a thin glass fibre and then allowed to move into the very tip by capillary forces.

Two electrodes were introduced into one cell, one recording voltage, the other serving for current application. For measurement of the membrane voltage, the microelectrodes were connected to a semiconductor electrometer amplifier. Membrane resistance was calculated from the voltage drop caused by injected depolarizing current pulses. Square pulses of 300 ms duration and 10 nA amplitude were applied by a programmable function generator TR-0311 EMG-Budapest. The membrane voltage was recorded on a flat-bed line recorder TZ 4200-L.P. Praha. As only two electrodes were introduced into cylindrical algal cells, parameters like the space and time constants could not be evaluated for calculation of the membrane resistance in Ω cm^2. The membrane resistance is therefore presented in arbitrary units.

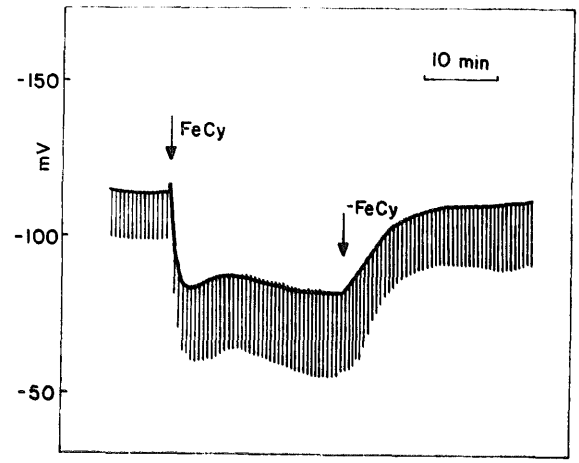

Fig. 1. Depolarization of membrane potential after addition of ferricyanide (0.4 mM) in the light. Arrows mark the addition and removal of electron acceptor.

The EPR spectra were recorded on a JEOL-PE-3X spectrometer provided with an EC-100 computer and an MJ-110R magnetometer Radiopan Poznań. All measurements were performed at 2 mW microwave output and with 100 kHz magnetic modulation using quartz aqueous solution sample tubes JES-LC-01 (effective volume 0.02 ml) at 288 K.

Results

Figs. 1 and 2 show representative responses of the alga membrane potential to additions of ferricyanide and TTF^+. It may be seen that as far as the effect on the membrane potential is considered, the two compounds behave similarly, their application being followed by an immediate depolarization. Table I gives the average decreases of the potential as dependent on the light conditions. The potential did not always remain constant during the period when either ferricyanide or TTF^+ were added to the medium; after an

TABLE I

Changes of membrane potential and resistance caused by ferricyanide (0.4 mM) and TTF^+ (0.05 mM) in the light and in the dark

	Control		Ferricyanide	Control		TTF^+
Light						
E_M (mV)	128.2 ± 4.8	($n = 6$)	117.2 ± 3.6	124.1 ± 4.7	($n = 7$)	103.1 ± 4.1
R_M (a.u.) [a]	1.4 ± 0.2		1.0 ± 0.1	1.5 ± 0.2		3.0 ± 0.6
Dark						
E_M (mV)	125.8 ± 8.2	($n = 9$)	106.9 ± 9.6	121.2 ± 5.7	($n = 5$)	92.8 ± 9.2
R_M (a.u.)	2.9 ± 1.2		2.8 ± 0.9	1.0 ± 0.3		3.0 ± 0.5

[a] a.u. = arbitrary units.

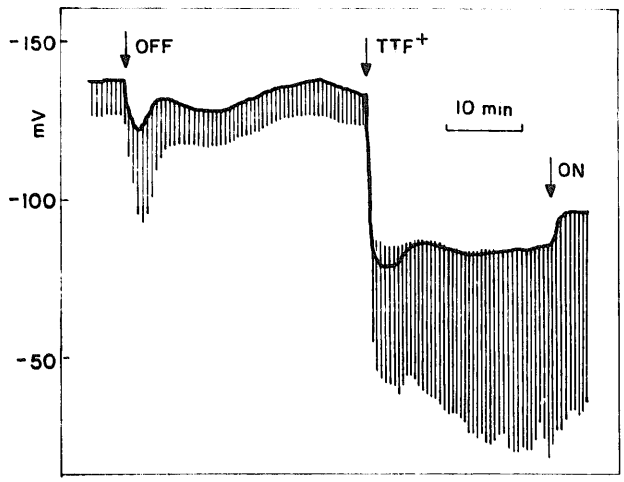

Fig. 2. Depolarization of membrane potential after addition of TTF^+ (0.05 mM) in the dark. Changes in light conditions and addition of TTF^+ are denoted by arrows.

initial decrease the potential often tended to approach the initial level. This occurred most often in the light and with ferricyanide. The maxima of the potential response which appeared during the first 3 min were used for calculations given in Table I.

Two conclusions may be drawn from this table concerning the potential difference. First, in the dark, the effect of both electron acceptors was more marked than in the light and, secondly, 0.05 mM TTF^+ caused a greater voltage drop than 0.4 mM ferricyanide regardless of the light conditions. As for the membrane resistance, it was raised 2-fold in the light and 3-fold in the dark after addition of TTF^+. On the other hand, a 28.6% decrease was observed in the presence of ferricyanide in the light. No significant change in the membrane resistance occurred with ferricyanide in the dark. Such were the results with ferricyanide on the average, in individual experiments either an increase or a decrease in the membrane resistance took place.

While the potential was soon raised to the original level when either ferricyanide or TTF^+ were washed out of the external medium, as demonstrated for ferricyanide in Fig. 1, a different response was obtained for the membrane resistance. Only after withdrawal of ferricyanide the resistance gained rapidly its original value. On the other hand, when TTF^+ was removed from the outer medium, the resistance remained at its higher level and at least some 30 min were necessary before the resistance gradually returned to its original value.

Potassium ferrocyanide at 0.4 mM concentration neither affected the membrane resistance nor depolarized the membrane potential. On the contrary, a meagre increase (3–6 mV) in the potential difference was observed.

We decided to study in more detail why with TTF^+ a substantial increase in membrane resistance was always observed. Fig. 3 demonstrates in two extreme examples how the resistance changed with time after addition of the electron acceptor. This time dependence cannot obviously be correlated with the change in membrane potential which took place during the first 3 min after TTF^+ application.

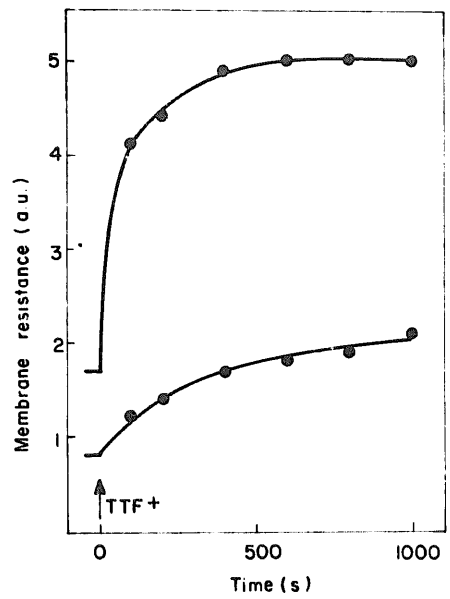

Fig. 3. Time dependence of resistance change after application of TTF^+ (0.05 mM) in the dark (two extreme examples).

The relation between membrane resistance and concentration of TTF^+ added is demonstrated in Fig. 4. We have shown recently [14] that the most remarkable effect of the electron acceptors, whether it is ferricyanide or TTF^+, is the inhibition of the light-activated

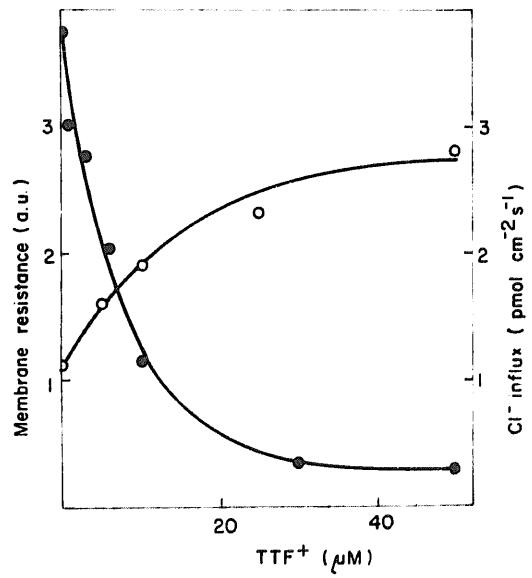

Fig. 4. Changes of membrane resistance with TTF^+ concentration. Empty circles, averages of resistance from four experiments in the light; values taken after a new steady state was established. Full circles, ^{36}Cl uptake calculated from data obtained after a 1 h incubation (see Ref. [14]).

TABLE II

Half-time of $^{36}Cl^-$ efflux from control and TTF^+-treated cells of Hydrodictyon reticulatum in the light

	$t_{1/2}$ (h)	
	control	0.05 mM TTF^+
Exp. 1	17.5	12.2
Exp. 2	10.1	8.1

chloride influx. It may be seen from the Fig. 4 that there exists a correspondence between the decrease of Cl^- uptake and the increase in the membrane resistance.

In our recent papers [13,14] we mentioned the findings that the influxes of other ions including H^+, Na^+ and Ca^{2+} were not affected by the two compounds. Only the uptake of $^{86}Rb^+$ (a marker of potassium ions) was diminished by some 30% in the presence of 0.05 mM TTF^+ and by 10% in the presence of 0.4 mM ferricyanide after the first 60 min of incubation. This inhibitory action was weak when compared with the effect of the above compounds on Cl^- uptake. Under identical conditions, the same concentration brought about a 89% and, respectively, a 45% inhibitions of Cl^- influx.

The possibility that the increase in resistance by TTF^+ is due to inhibition of Cl^- efflux was further tested. The efflux of chloride anions was, however, not found to be impaired by TTF^+. As follows from Table II, TTF^+ rather slightly increased the rate with which the labelled anion was washed out of the cells.

Then we tried to find out whether the potassium conductance was not affected in the presence of electron acceptors. The results presented in Table III show that both compounds decreased the K^+ conductance. The response of membrane potential to a transient rise in K^+ concentration from 1 mmol l^{-1} to 20 mmol l^{-1} was decreased in the dark in the presence of ferricyanide by 17.1% and in the presence of TTF^+ by 39.4%. This change in sensitivity of the membrane potential to potassium ions disappeared completely after withdrawal of ferricyanide or TTF^+ from the medium.

TABLE III

Changes of membrane potential sensitivity to potassium ions in the presence of ferricyanide (0.4 mM) and TTF^+ (0.05 mM)

Figures represent average responses of membrane potent in mV to a 20-fold increase in K^+ concentration.

	Light		Dark
Control	-37.1 ± 3.8	($n = 4$)	-40.3 ± 2.0
Ferricyanide	-31.7 ± 1.7		-33.4 ± 1.3
Control	-38.6 ± 1.1	($n = 5$)	-39.6 ± 1.4
TTF^+	-25.5 ± 2.1		-24.0 ± 0.0

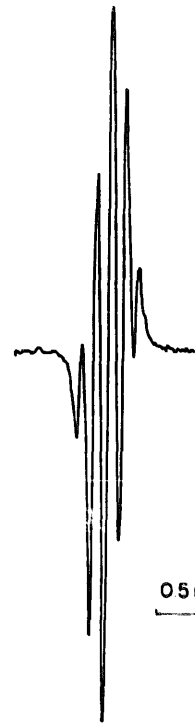

Fig. 5. Electron paramagnetic resonance spectrum of TTF^+ in an algal suspension. Approx. 150 mg of algae were suspended in 5 ml of experimental medium, the concentration of TTF^+ was 0.05 mM. Analysis was performed in aliquots.

EPR spectroscopy was used to find out how the tetrathiafulvalene cation radical behaves in an aqueous solution. When TTF^+ dissolved in a small amount of dimethylformamide (representing a maximum of 1% of the total volume) was added to the artificial pond water, the EPR signal of the radical soon disappeared. The same occurred if TTF^+ was present together with ferricyanide. On the other hand, in the presence of algae, the outer solution with TTF^+ exhibited for some 30 min a quintet EPR spectrum (Fig. 5) indicating an interaction of the unpaired electron with four equivalent protons. The proton splitting constant $a^H = 0.124$ mT as well as the g-factor ($g = 2.0086$) determined were in good agreement with the data obtained by Wudl et al. [15] who analyzed the EPR spectra of TTF^+ in a water-acetonitrile mixture at 298 K. When both electron acceptors were present in the medium together with algae (TTF^+ at 0.05 mM and ferricyanide at 0.4 mM concentrations), the EPR signal of the radical persisted throughout the whole period investigated, i.e., 90 min.

Discussion

We have already reported [14] that TTF^+, which is readily reducible, acts in many respects similarly as ferricyanide: its reduction is accompanied by H^+ extrusion, it brings about an increase in respiration and it does not influence photosynthesis. Moreover, it is more

efficient in influencing the active transport of chloride anion, blocking its light-activated uptake nearly completely at 0.05 mM concentration.

As one may expect that the cation radical of TTF^0 will not be stable in aqueous solutions, we considered it necessary to follow the behaviour of the compound dissolved in artificial pond water. Whereas in the absence of algae the characteristic quintet spectrum of the radical disappeared rapidly, the unpaired electron was detectable for a longer period of time when algae where present and for at least 90 min when both electron acceptors, i.e., TTF^+ and ferricyanide, were added. It appears that, once dissolved, TTF^+ undergoes dismutation yielding the reduced neutral form of TTF^0 and the two-valence cation TTF^{2+}. Since the radical cation is present in media with algae it is evident that also the TTF^{2+} form can accept electrons to give first TTF^+. Moreover, reoxidation of the latter form by ferricyanide has to be proposed for the following reason: we observed that TTF^+ is reduced rapidly during the first 30 min, then a steady state between the reduced and oxidized forms is established, the redox potential of the Pt-calomel electrode system becoming practically unchanged. As in the presence of ferricyanide the radical cation can be still detected after prolonged time periods, reoxidation of the reduced form by ferricyanide must take place.

It may be seen that in principle the light conditions do not influence much the pattern of the effects of the individual electron acceptors, but only their magnitude. With 0.4 mM ferricyanide the average potential decrease was approx. 10 mV in the light and 20 mV in the dark, whereas with 0.05 mM TTF^+ the depolarization represented about 20 and 30 mV, respectively. After washing the electron acceptors out of the medium, the potential returns with a delay of maximally 10 min to the original value. This suggests that the cause of potential decrease disappears with the withdrawal of the electron acceptors. The electrogenicity of the electron transfer to an artificial electron acceptor seems to be, at least for the most part, the cause for membrane depolarization.

Thiel and Kirst [8] observed that in *Lamprothamnium* the potential drop caused by ferricyanide is accompanied by a substantial decrease in membrane resistance. The authors, have brought about persuasive evidence in favour of the view that this decrease corresponds with an increase of potassium conductance, as the membrane potential is switched from the hyperpolarized 'pump' state to a state determined by K^+ permeability. The results obtained with *Hydrodictyon* are different. First, in the presence of ferricyanide the membrane resistance is decreased by only 29% in the light and remains practically unchanged in the dark. The membrane potential sensitivity to K^+, calculated from the difference of response of the potential to a potassium concentration pulse, was, however, lower in the presence of ferricyanide. Moreover, the sensitivity of the membrane to K^+ remains in *Hydrodictyon* considerable even in the hyperpolarized state, i.e., when the potential difference is, e.g., -150 mV, whereas in *Lamprothamnium* the 'pump' state potential is only little affected by potassium ions.

In the presence of 0.05 mM TTF^+ the membrane resistance increased usually more than twice on the average. The rather slow reversal of the membrane resistance after removal of TTF^+ from the medium is probably caused by a direct interaction of the compound with the membrane and, possibly, with a system responsible for the transport of anions, as will follow from conclusions given below; the original value of the resistance is restored as soon as the lipophilic compound is thoroughly washed out of the membrane.

We know already from our earlier results [14] that TTF^+ influences the uptake of cations only slightly, if at all. On the contrary, a strong inhibition of the uptake of anions, i.e., Cl^- and also NO_3^- (unpublished observations), is brought about by the same concentration of TTF^+. Conceivably, if there is a complete inhibition of a one-directional flux of an ion, the corresponding flux in the opposite direction may also be diminished. This in fact happens with a great number of inhibitors [16]. However, the obviously passive outflux of chloride anions is not inhibited by TTF^+; if anything, a slight stimulation results after addition of the electron acceptor.

In order to understand the significance of the rise in membrane resistance by TTF^+, the sensitivity of the membrane potential to K^+ was tested in control and TTF^+ treated algae. The results showed that TTF^+ decreased this sensitivity by about one third. In our opinion the decreased membrane conductance may be only partially ascribed to changes in K^+ conductance, the arguments being as follows.

The results obtained with ferricyanide favour the view that the changes in membrane resistance and in the sensitivity of membrane potential to K^+ cannot be correlated directly. It appears that in the presence of ferricyanide a mechanism tending to increase the membrane resistance prevails in some *Hydrodictyon* specimens, while in others a mechanism with an opposite tendency comes into play. The decrease in membrane resistance may be easily explained if the flow of electrons across the membrane to ferricyanide as an artificial electron acceptor is electrogenic. The inhibitory action of ferricyanide on another source of membrane conductance obviously prevailed in some experimental objects over the latter effect. The fact that ferricyanide blocks the ligh-activated Cl^- uptake by approx. 45% should be recalled.

Our unpublished results showed that 5 mM $BaCl_2$ blocks the K^+ channels effectively since the response

of the membrane potential to changes in the external concentration of potassium ions is then substantially reduced. Nevertheless, the membrane resistance increased less in the presence of $BaCl_2$ than with TTF^+.

It may be concluded that there is another process which is resposible for the increase in membrane resistance in the presence of TTF^+ and, sometimes, with ferricyanide. A plausible conclusion may be that the system transporting anions is not entirely electroneutral.

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