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ORIGINAL ARTICLE

Generation of action potential-type changes in response to darkening and illumination as indication of the plasma membrane proton pump status in *Marchantia polymorpha*

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Abstract The aim of the present study was to characterise bioelectrical changes in the membrane potential of *Marchantia polymorpha* gametophyte cells after light/dark transitions and to determine the role of the proton pump and energy status of the *M. polymorpha* cells in generation of these changes. Darkening caused persistent depolarisation of the resting potential (RP) and generation of short-lasting potential changes that were not uniform among different thalli. In some plants (18%), the changes evoked by darkening were typical action potentials (APs_{dark}), whereas in 69% of the plants, the changes had a form of action potential-like responses (APs_{dark}-like) consisting of a transient depolarisation followed by a plateau phase, whose magnitude and duration were inconstant. The illumination of the *M. polymorpha* always evoked action potentials (APs_{light}) if the thallus was illuminated with light intensity of at least 120 µmol photons m^{-2} s^{-1} after 30-min darkening. To analyse the involvement of H^+-ATPase in formation of the illumination/darkening-induced electrical responses in *M. polymorpha,* the proton pump regulators were used. The proton pump inhibitor (20 µM FCCP) significantly diminished the RP and inhibited dark-induced AP_{dark} and/or AP_{dark}-like responses and illumination-induced APs_{light}. After application of DCMU (20 µM), the RP was strongly depolarised and no response to light/dark was observed. Fusicoccin (20 µM), i.e., an activator of the proton pump, strongly hyperpolarised the membrane potential and blocked dark-induced AP_{dark}/AP_{dark}-like responses and illumination-induced APs_{light}.

Keywords Action potential · Light · Liverwort · Proton pump · Membrane potential

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Introduction

Liverworts belong to the most basal lineage of land plants—embryophytes—located between algae and higher plants. The group is characterised by a gametophyte body as a dominant phase in the life cycle, fast growth, and morphological simplicity. The best molecularly characterised liverwort is *Marchantia polymorpha*, whose nucleus genome is being increasingly sequenced. Molecular analysis of the liverwort organellar genomes has been established with the complete sequence of the chloroplast (Ohyama et al. 1986) and mitochondrial DNAs (Oda et al. 1992; Oldenburg and Bendich 1998). Y-chromosome-specific clones from *M. polymorpha* have also been identified (Okada et al. 2000; Yamato et al. 2007). Moreover, thanks to the easy regeneration of thalli and the ability of young thalli to propagate from gemmae cups (vegetative propagules), *M. polymorpha* is an excellent object for a simple high-throughput production of transformed plants (Ishizaki et al. 2008; Kubota et al. 2013; Takenaka et al. 2000; Tsuboyama-Tanaka and Kodama 2015). Thus, *M. polymorpha* is being developed as a model plant with a critical evolutionary position to study specific molecular and cellular processes in detail.

As a nonvascular plant with the haploid generation as the dominant phase of the life cycle, *M. polymorpha* may serve as a convenient model for electrophysiological measurements, too. However, to date, there has been no

report about the characteristics of electrical signals in *M. polymorpha* gamethophyte cells. Simultaneously, there are reports about intracellular investigations conducted on another liverwort *Conocephalum conicum* (Dziubińska et al. 1983; Favre et al. 1999b; Król et al. 2007; Zawadzki and Trębacz 1985) and on the moss *Physcomitrella patens*, a species that is phylogenetically related to liverworts and regarded as a model system in plant functional genomics (Decker et al. 2006; Reski 1999) and electrophysiology (Ermolayeva et al. 1996, 1997; Koselski et al. 2008, 2013, 2015). *Conocephalum conicum* displays excitability to various stimuli, such as light (Król and Trębacz 1999; Trębacz and Zawadzki 1985; Trębacz et al. 1989a), cold (Król et al. 2003; Kupisz and Trebacz 2011), wounding (Favre et al. 1999a), and direct current (DC) (Dziubińska et al. 1983; Król and Trębacz 1999; Paszewski et al. 1982). Among the stimuli mentioned above, light signals are considered as environmental factors that play a critical role in growth, reproduction, development, and regeneration, distinguishing liverworts, mosses, and other bryophytes from other plant species (Cove and Ashton 1988). At the plasma membrane level, illumination of the *C. conicum* liverwort with low light intensities causes a slow hyperpolarisation of the resting potential (RP), which is gradually overlaid by a faster depolarisation, whose amplitude increases depending on the intensity of the light stimulus (Trębacz et al. 1989a). After crossing the threshold value of light intensity, action potentials (APs) with relatively constant amplitudes occur (Trębacz and Zawadzki 1985). Light–dark transition evokes an opposite response in the range of subthreshold values: the thallus cells respond to darkening by generation of a transient hyperpolarisation of the membrane potential depending on light intensity (Trębacz et al. 1997, 1989a).

Gametophyte cells of *P. patens* generate APs both in response to illumination and darkening (Koselski et al. 2008). The amplitudes of APs evoked by illumination and darkening are similar and relatively constant, but the half-times are different, i.e., light-induced APs are characterised by several times shorter half-time than APs evoked by darkening (Ermolayeva et al. 1996; Koselski et al. 2008). In the range of subthreshold values, electrical responses of the moss cells to light–dark transitions are opposite and symmetrical to the responses evoked by light stimulation, i.e., illumination evokes transient depolarisation first and then long-lasting hyperpolarisation, while darkening causes fast hyperpolarisation followed by long-lasting depolarisation (Ermolayeva et al. 1996; Koselski et al. 2008).

The aim of our study was to examine light- and dark-induced membrane potential changes in gametophytes of *M. polymorpha*. The fact that blue light-absorbing photoreceptor—phototropin (Komatsu et al. 2014) and a single copy of the phytochrome nuclear gene that encodes a photoreceptor protein mediating in developmental responses to red and far red light (Nishihama et al. 2015) have already been established in this liverwort additionally encouraged us to analyse the electrical changes in response to light. Moreover, Okumura et al. (2012) have defined eight isomers of H^+-ATPase in *M. polymorpha*: four are penultimate threonine-containing H^+-ATPases characteristic for vascular plants and four non-penultimate threonine-containing H^+-ATPases such as in green algae. They have established that light causes activation of penultimate threonine-containing H^+-ATPase and its photosynthesis-dependence suggests that the model of the proton pump activation in the *M. polymorpha* liverwort is similar to the one observed in vascular plants (Okumura et al. 2012). Hence, our main task was to investigate the character of the membrane potential changes evoked by light/dark and to analyse the involvement of H^+-ATPase in formation of the light-induced electrical responses in *M. polymorpha*.

Results

General characteristics of darkening-induced membrane potential changes in *Marchantia polymorpha* cells

Microelectrode recordings have shown that 100% of the tested *M. polymorpha* plants ($n = 45$) responded to sudden darkening first by generation of transient hyperpolarisation with relatively constant amplitude of 8.7 ± 0.6 mV and then by persistent depolarisation (Fig. 1). The magnitude of the persistent depolarisation did not depend on the intensity and time of illumination preceding darkening, but it was different among the plants and ranged between 5 and 82 mV. In 87% of the thalli, short-lasting potential changes were recorded against the background of the persistent depolarisation (Fig. 1). The membrane potential at the moment of generation of the short-lasting potential changes was named "initial membrane potential"—$V_{initial}$ (Fig. 1; Table 1) and a part of the persistent depolarisation between the resting potential (RP) recorded shortly before the dark stimulus onset and $V_{initial}$ was defined as "initial depolarisation". The final level of the membrane potential recorded in the plateau phase of the persistent depolarisation was marked out as V_{final} (Fig. 1).

18% of all the tested thalli ($n = 8$) generated the short-lasting potential changes in the form of an action potential (AP_{dark}) (Fig. 1b), whose average amplitude was 147.4 ± 7.4 mV and the half-time ($t_{1/2}$) was 39.8 ± 5.8 s (Table 1). In accordance with the all-or-none law, the amplitude of APs_{dark} did not depend on the stimulus strength, i.e., on the intensity and time of the white light preceding darkening (Fig. 2). Interestingly, in individual

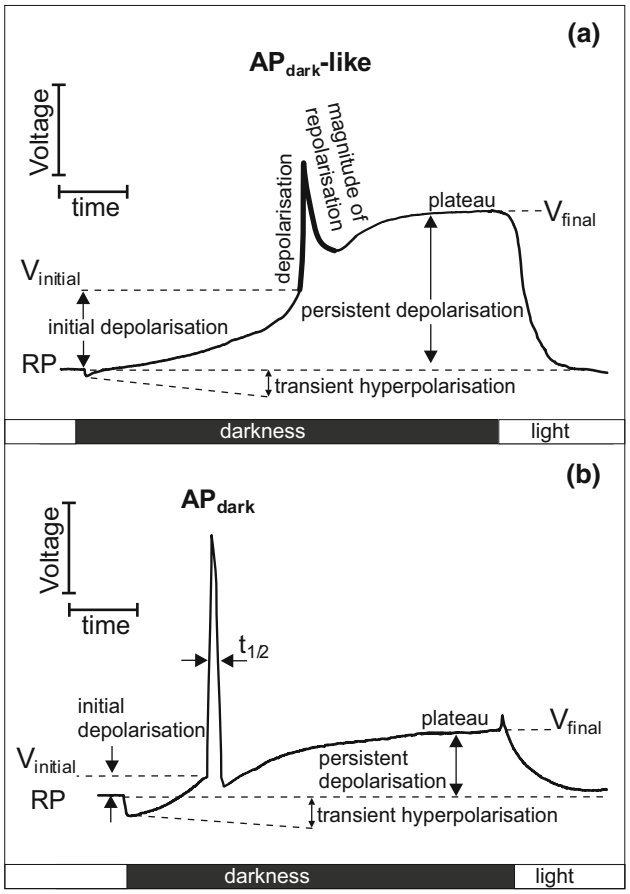

Fig. 1 Scheme of membrane potential changes evoked by darkening. Short-lasting potential change (*bolded*)—a change in a membrane potential in the form of **a** action potential-like response (AP_{dark}-like) or **b** action potential (AP_{dark}). *RP* resting potential—a membrane potential of a thallus cell at rest (under white light of 40 µmol m^{-2} s^{-1}), "transient hyperpolarisation"—a transient change in the membrane potential more negative than the resting potential, "persistent depolarisation"—a long-lasting change in the membrane potential less negative than the resting potential, "depolarisation"—a phase of the short-lasting potential change (AP_{dark} and/or AP_{dark}-like) registered on the background of the persistent depolarisation, defined as a rapid change in a membrane potential more positive than the resting potential, "magnitude of repolarisation"—a magnitude of a change in the membrane potential returning it to a level of the persistent depolarisation, $t_{1/2}$—half-time of AP_{dark} and/or AP_{dark}-like, $V_{initial}$-initial membrane potential—the membrane potential recorded at the moment of generation of the short-lasting potential change, "initial depolarisation"—a part of the persistent depolarisation between the resting potential recorded shortly before the dark stimulus onset, and $V_{initial}$, V_{final}—final membrane potential—a membrane potential recorded in the plateau phase of the persistent depolarisation. *Black bar* indicates dark stimulation; *white bar* indicates illumination

thalli (2 of 45), two or three APs_{dark} (1 of 45) were generated in response to darkening after 2-h illumination with light of 10 µmol photons m^{-2} s^{-1}.

To evoke AP_{dark}, the threshold duration of illumination with 10 µmol photons m^{-2} s^{-1} needs to be longer than 15 min or longer than 5 min in the case of the white light of 300 µmol photons m^{-2} s^{-1} (Fig. 2). The intensity and duration of illumination had no influence on the number of APs_{dark}. In most of the thalli, a single AP_{dark} was generated following 5 min—as well as to 2-h lasting illumination. Similarly, the intensity of light preceding darkening was not a decisive parameter.

In 69% of the tested thalli ($n = 31$), the short-lasting potential changes had a form of action potential-like responses (AP_{dark}-like) (Fig. 1a) with relatively fast transient depolarisation and a long-lasting plateau phase, whose magnitude and delay in respect to the fast depolarisation were not uniform (Fig. 3). The duration of illumination with given intensity influenced the magnitude of the repolarisation phase (Figs. 1a, 3a): the average value of the correlation coefficient ($n = 31$) calculated for the dependence between the magnitude of repolarisation and light duration was 0.98. Moreover, in 29% of plants generating AP_{dark}-like responses ($n = 9$), the magnitude of the repolarisation phase was twice or more times lower than the amplitude of the AP_{dark}-like response, regardless of the stimulus strength (Fig. 3b; Table 1). In such cases, determination of the half-time ($t_{1/2}$) of the AP_{dark}-like was impossible. Therefore, we divided the AP_{dark}-like responses into two main groups for more precise analysis: one with a finite half-time (AP_{dark}-like $t_{1/2}$) and the other one with the infinite half-time (AP_{dark}-like $t^{\infty}_{1/2}$) (Fig. 3). The excitability threshold for the intensity and time of illumination preceding darkening was also hard to establish: AP_{dark}-like responses appeared even after illumination with white light of 1 µmol photons m^{-2} s^{-1} or for 1 min. The average amplitude of AP_{dark}-like $t_{1/2}$ was 81 ± 4.3 and 72.5 ± 4.6 mV for AP_{dark}-like $t^{\infty}_{1/2}$ (Table 1). The duration of illumination with given intensity did not have a strong effect on the amplitude of AP_{dark}-like responses in comparison to the light duration-repolarisation dependence: the mean correlation coefficient ($n = 31$) calculated for the light duration-amplitude dependence was 0.3. The average amplitude of AP_{dark}-like responses differed significantly from the average amplitude of APs_{dark} (Table 1). The difference between the amplitudes was not covered even by the higher magnitude of the initial depolarisation. The initial depolarisation was 53.5 ± 3.6 mV for AP_{dark}-like $t_{1/2}$ and 52.3 ± 4.0 mV for AP_{dark}-like $t^{\infty}_{1/2}$, whereas for AP_{dark}, it was 14.9 ± 2.8 mV. The average lag-time was 8.9 ± 1.6 min for AP_{dark}, 18.1 ± 0.9 min for AP_{dark}-like $t_{1/2}$, and 16.6 ± 2.7 min for AP_{dark}like $t^{\infty}_{1/2}$. The rate of the persistent depolarisation until APs_{dark} generation (or until the beginning of the plateau phase in the case of absence of AP_{dark} and/or AP_{dark}-like) differed statistically from the rate of the persistent depolarisation before AP_{dark}-like responses, too (Table 1). The value of the RP in plants generating different types of the short-lasting potential

Table 1 Average values of parameters characterizing the thalli cells generating AP_{dark}, AP_{dark}-like $t_{1/2}$, and AP_{dark}-like $t_{1/2}^{\infty}$ after dark stimulation following 30 min illumination with 300 μmol photons m^{-2} s^{-1} and AP_{light} after light stimulation with 300 μmol photons m^{-2} s^{-1}

Bioelectrical response	RP (mV)	$V_{initial}$ (mV)	Rate of persistent depolarisation (mV/min)	Amplitude (mV)	$T_{1/2}$ (s)	Magnitude of repolarisation (mV)	V_{final} (mV)
Darkness							
No AP_{dark}/AP_{dark}-like ($n = 6$)	−169.8 ± 11.7	–	0.65 ± 0.07	–	–	–	−147.1 ± 15.6
AP_{dark} ($n = 11$)	−175.1 ± 3.3	−160.2 ± 4.7	0.8 ± 0.2	147.4 ± 7.4	39.8 ± 5.8	146.4 ± 8.6	−128.9 ± 9.5
AP_{dark}-like							
$t_{1/2}$ ($n = 19$)	−177.8 ± 3.7	−124.3 ± 4.4	2.5 ± 0.2[ab]	81.0 ± 4.3[ab]	72 ± 27[b]	45.6 ± 6.0[b]	−65.6 ± 4.0[ab]
$t_{1/2}^{\infty}$ ($n = 17$)	−163.8 ± 5.7	−111.5 ± 4.0[a]	3.5 ± 1.0[ab]	71.3 ± 4.5[b]	–	15.9 ± 4.1[bc]	−48.8 ± 2.6[abc]
Light							
AP_{light} ($n = 33$)	−170.1 ± 4.4	−128.7 ± 5.3	–	103.4 ± 4.3	44.4 ± 3.0	103.4 ± 4.3	−172.5 ± 4.6

RP resting potential of thalli cells recorded after 3-h incubation with light intensity of 40 μmol photons m^{-2} s^{-1}, $V_{initial}$—membrane potential at the moment of AP_{dark}, AP_{dark}-like $t_{1/2}$, AP_{dark}-like $t_{1/2}^{\infty}$, or AP_{light} generation, $t_{1/2}$—half-time of the action potential/action potential-like response, V_{final}—membrane potential in the steady state recorded after generation of AP_{dark} and/or AP_{dark}-like, and n—number of short-lasting potential changes

[a] Statistically significant difference between thalli generating a given type of the short-lasting potential changes and those in which AP_{dark} and/or AP_{dark}-like were not observed

[b] Statistically significant difference between thalli generating AP_{dark}-like responses and those in which AP_{dark} was observed

[c] Statistically significant difference between thalli generating AP_{dark}-like $t_{1/2}^{\infty}$ and those in which AP_{dark}-like $t_{1/2}$ responses were observed, t test, $p < 0.05$. Data show means with the standard errors

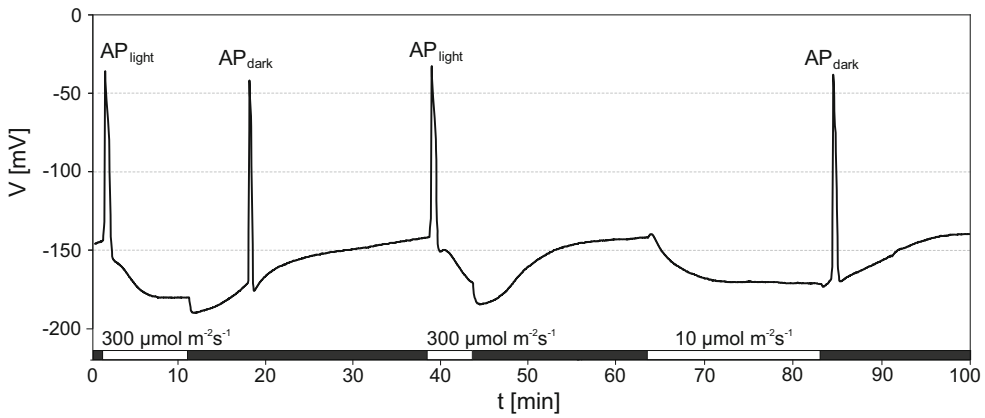

Fig. 2 All-or-none law for action potentials evoked by light/dark in *Marchantia polymorpha* cells immersed in the standard solution containing 1 mM KCl, 0.1 mM $CaCl_2$, 50 mM sorbitol, and Tris/MES (pH 7). Action potentials evoked by darkening (AP_{dark}) and illumination (AP_{light}) are labelled. Darkening (*black bars*) was applied after exposure of the thalli to white light (300 μmol photons m^{-2} s^{-1} or 10 μmol photons m^{-2} s^{-1}, as labelled) for the indicated time of illumination (*white bars*)

changes did not differ statistically and was approx. −170 mV. Simultaneously, the final level of the membrane potential recorded in the plateau phase of the persistent depolarisation (V_{final}) was diverse: thalli generating APs_{dark}-like $t_{1/2}^{\infty}$ responses were characterised by the most depolarised V_{final} (−48.8 ± 2.6 mV), while V_{final} was the lowest (−147.1 ± 15.6 mV) in plants without AP_{dark} and/or AP_{dark}-like (Table 1). Moreover, the higher the rate of the persistent depolarisation, the less negative the value of the V_{final} and the lower the magnitude of the repolarisation phase. The absence of AP_{dark} and/or AP_{dark}-like was noted for thalli ($n = 6$) in which darkening caused depolarisation of the membrane potential with the rate of 0.65 ± 0.07 mV/min or less.

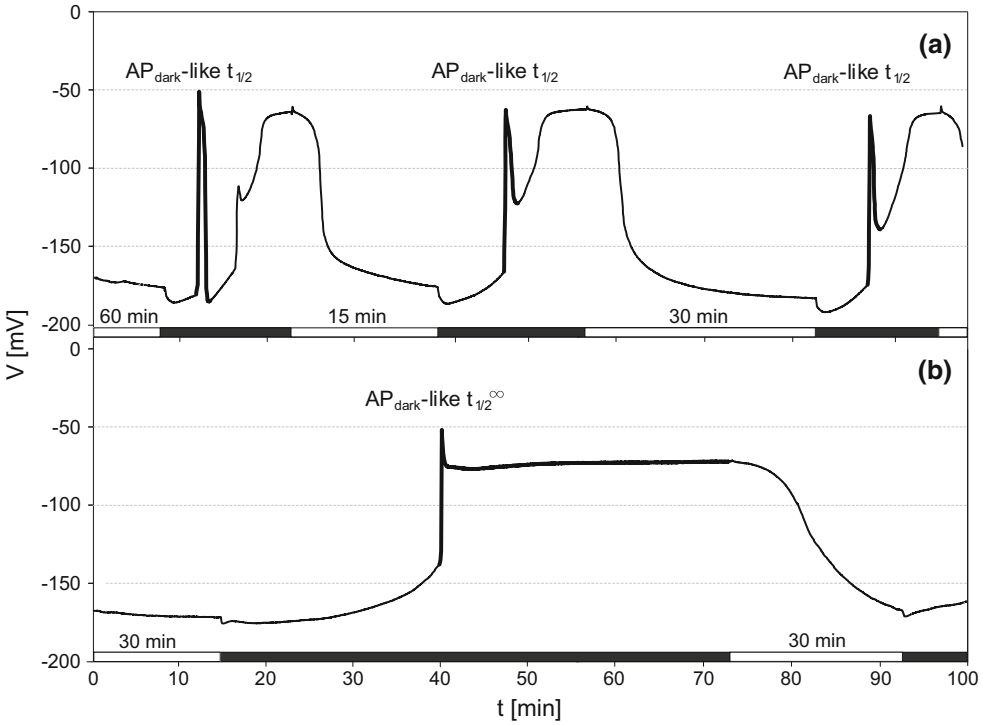

Fig. 3 Examples of traces of action potential-like responses: AP_{dark}-like $t_{1/2}$ (**a**) and AP_{dark}-like $t_{1/2}^{\infty}$ (**b**) in two different thalli evoked by darkening. AP_{dark}-like $t_{1/2}$—action potential-like response with finite half-time, and AP_{dark}-like $t_{1/2}^{\infty}$—action potential-like response with infinite half-time. AP_{dark}-like responses ($t_{1/2}$ and $t_{1/2}^{\infty}$, marked out, respectively) are *bolded*. Dark stimulus was applied after illumination of the thalli with light intensity of 300 µmol photons m^{-2} s^{-1} for the indicated time (*white bars*)

General characteristics of illumination-induced membrane potential changes in *Marchantia polymorpha* cells

Sudden illumination evoked transient depolarisation of the membrane potential that was proportional to the white light intensity and time of darkness preceding illumination. The stimulus strength dependence was registered for the intensities below approx. 120 µmol photons m^{-2} s^{-1}. Above these values, action potentials (AP_{light}) were generated with relatively constant amplitude of 103.4 ± 4.3 mV and half-time of 44.4 ± 3.0 s. Similarly, 17 min of darkness (without AP_{dark}) and 18 min since AP_{dark} (or AP_{dark}-like) generation were the minimal period of time necessary to evoke AP_{light} in response to 300 µmol photons m^{-2} s^{-1} white light stimulation (Fig. 4). *Marchantia polymorpha* cells always generated one or no AP_{light}, depending on the stimulus strength, in agreement with the all-or-none law. Moreover, AP_{light} evoked by the threshold white light intensity of 120 µmol photons m^{-2} s^{-1} or the threshold time of darkness of 17 min were preceded by a generator potential (GP) (Fig. 4, inset). Apart from the strength of the stimulus, the magnitude of the membrane potential seems to play a crucial role in AP_{light} formation; APs_{light} have never been registered for a membrane potential higher than −90 mV.

Role of the proton pump in forming and generation of light-induced membrane potential changes in *Marchantia polymorpha* cells

The important role of the plasma membrane H^+-ATPase in maintenance and post-excitation restoration of the RP is well established (Lopez-Marques et al. 2004; Orlova et al. 1997; Trębacz et al. 1994). Therefore, we used H^+-ATPase modulators to investigate the role of the proton pump in electrogenesis of the RP and formation of AP_{light}, AP_{dark}, and AP_{dark}-like responses. In our studies, application of FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) at a concentration of 20 µM caused substantial depolarisation of the membrane potential in *M. polymorpha* thalli (Fig. 5a). The level of the RP in gametophyte cells after FCCP application depended on the inhibitor concentration; 20 µM FCCP depolarised the RP to −116.3 ± 3.2 mV ($n = 3$), whereas 50 µM FCCP caused depolarisation of the RP to −60.3 ± 5.9 mV ($n = 4$) (Figs. 5a, inset, 6). Moreover, FCCP at 50 µM and higher concentrations evoked short-lasting potential changes that resembled the ones generated by the thalli in response to darkening. In all cases ($n = 7$), the alternate light–dark and dark–light transitions caused only slow responses (depolarisation/hyperpolarisation for darkening/illumination, respectively), pointing to loss of plant excitability

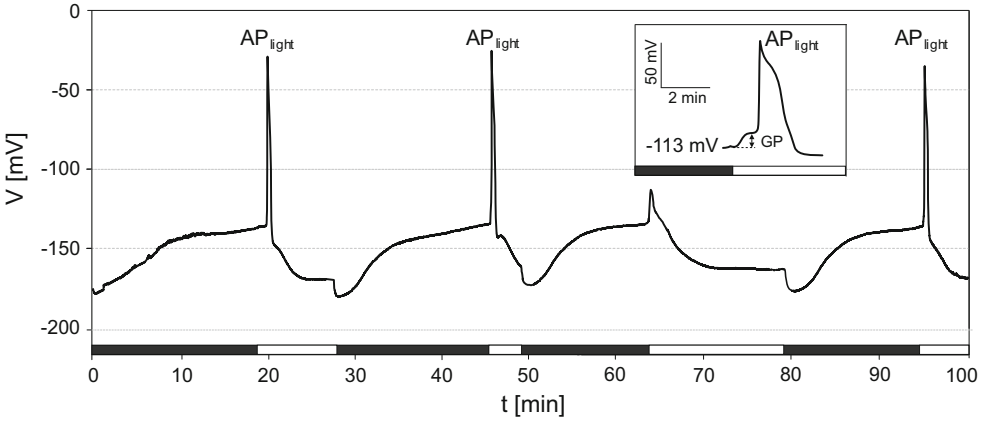

Fig. 4 All-or-none law for illumination-induced action potentials AP_{light} in *Marchantia polymorpha* cells immersed in the standard solution. White light of 300 µmol photons m^{-2} s^{-1} was applied after the indicated time in darkness before illumination (*black bars*). The *inset* shows AP_{light} preceded by GP (generator potential) induced by 17-min threshold time of darkness

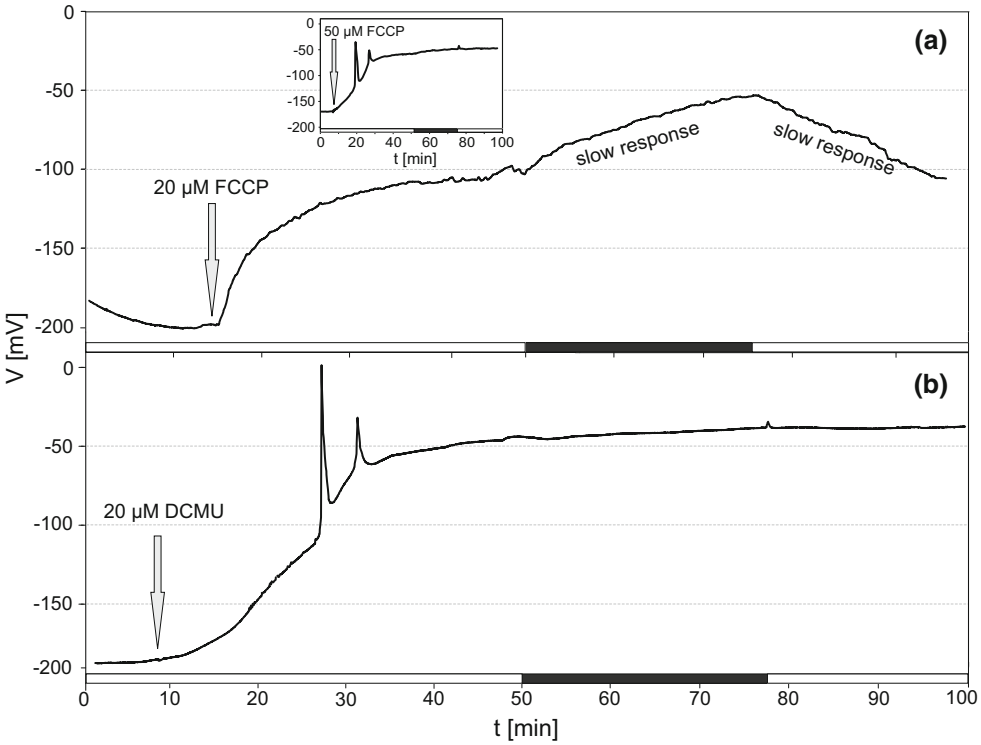

Fig. 5 Light- and dark-induced membrane potential changes in *Marchantia polymorpha* gametophyte cells immersed in the standard solution supplemented with **a** 20 µM FCCP or **b** 20 µM DCMU. The *inset* in **a** presents membrane potential changes in the cells treated with 50 µM FCCP

(Fig. 5a). The magnitude of the slow responses depended on the inhibitor concentration; it was 50.0 ± 8.2 mV for 20 µM FCCP and 19.6 ± 3.7 mV ($n = 4$) for 50 µM FCCP.

To analyse the involvement of photosynthesis in the light-induced electrical reaction, we blocked the photosynthetic electron transport chain with (3-(3,4-dichlorophenyl)-1,1-dimethylurea) DCMU. The addition of 20 µM DCMU to the standard medium caused depolarisation of the RP to −59.3 ± 1.2 mV ($n = 3$) (Fig. 6) and generation of short-lasting potential changes (Fig. 5b). As in the case of FCCP, the plants lost their excitability after application of 20 µM DCMU. Moreover, darkening evoked only slight slow responses with a magnitude of 6.3 ± 2.0 mV ($n = 3$), while illumination did not evoke any changes in the membrane potential. In all cases, dark stimulation in the inhibitor-treated plants caused depolarisation of the membrane potential to the level close to V_{final} of plants generated AP_{dark}-like responses in the standard medium (Fig. 6).

Fungal toxin fusicoccin (FC) was used as an H^+-ATPase activator. In our study, 30-min plant incubation in the standard solution supplemented with 20 µM fusicoccin was sufficient to hyperpolarise the RP to the average value of −203.3 ± 4.3 mV ($n = 6$) (Figs. 6, 7). Moreover, AP_{light}, AP_{dark}, and AP_{dark}-like were not observed in *M. polymorpha* cells that generated a given type of response before fusicoccin supplementation. Interestingly, in the thalli that

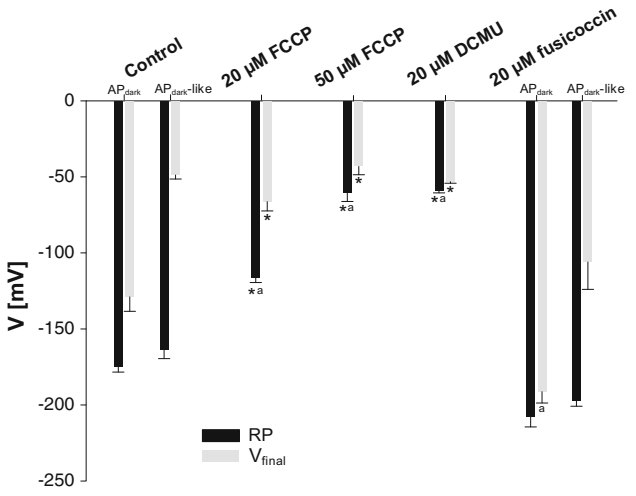

Fig. 6 Effects of proton pump modulators on the *M. polymorpha* resting potential recorded shortly before darkening (RP, *black bars*) and on the membrane potential established in the darkness after dark stimulation (V_{final}, *grey bars*). RP and V_{final} have been detailed for the thalli generating two types of darkening-induced membrane potential changes (AP_{dark} or AP_{dark}-like) in the standard solution (control) and for the thalli immersed in the standard solution supplemented with 20 μM fusicoccin, which generated AP_{dark} or AP_{dark}-like responses (marked out, respectively) before the activator application. The number of each modulator-treated plants was not lower than 3. Data show means with the standard errors. *—Statistically significant difference between the respective treatments and the standard solution in which AP_{dark} was observed, *a*—statistically significant difference between the respective treatments and the standard solution in which AP_{dark}-like response was observed, *t* test, $p < 0.05$

had generated AP_{dark}-like responses, darkening evoked significant depolarisation of 88.3 ± 16.9 mV ($n = 3$), which was not observed in the thalli generating AP_{dark} or AP_{light} before the activator application (Fig. 7). However, V_{final} in plants assigning to AP_{dark}-like group did not differ statistically from the one obtained in the control solution (Fig. 6).

Discussion

It is well established that plants react to light by generation of membrane potential changes (Elzenga et al. 1995; Mimura and Tazawa 1986; Plieth et al. 1998; Szarek and Trebacz 1999). In the present study, we have demonstrated that darkening-induced membrane potential changes generated by the *M. polymorpha* liverwort are characterised by transient hyperpolarisation followed by persistent depolarisation and the short-lasting potential changes generated against the background of the depolarisation. The experiments have revealed the existence of two types of the short-lasting potential changes induced by darkening. The first type—AP_{dark}—was characterised by features typical for action potentials: it fulfilled the all-or-none law, according to which the maximum spike is reached once a stimulus is given and increasing the intensity of the stimulus does not increase the response amplitude. It possesses a refractory period, too. The second type of the short-lasting potential changes—AP_{dark}-like—was the membrane potential change that resembled an action potential mainly in the kinetics of the transient depolarisation phase. The repolarisation phase of the AP_{dark}-like responses possessed a lower magnitude than the depolarisation phase. Moreover, the existence of a strong dependence between the magnitude of the repolarisation phase and the stimulus strength within one thallus (correlation coefficient = 0.98) suggests that transporters driving K^+ and/or H^+ ions that are postulated to be engaged in the repolarisation phase of APs (Trębacz et al. 1994) did not function properly during darkness in those thalli. In extreme cases, the plateau phase of the AP_{dark}-like responses lasted until the moment of illumination that was necessary to induce the repolarisation of the membrane potential and the return to the level before the stimulation. Taking into account the repolarisation features mentioned above and the fact that the threshold value of the intensity and time of illumination preceding darkening (refractory period) was hard to establish, we regarded AP_{dark}-like responses as not being typical action potentials. Interestingly, generation of AP_{dark} and/or AP_{dark}-like after darkening and persistent depolarisation was observed in the moss *P. patens* (Koselski et al. 2008), but not in the liverwort *C. conicum* (Trębacz and Zawadzki 1985). The dark-induced AP_{dark} in *M. polymorpha* was characterised by a several times shorter half-time (10-s range) than AP_{dark} registered in *P. patens* (10-min range) (Koselski et al. 2008). In *M. polymorpha* cells, illumination, in contrast to darkening, always evoked typical action potentials for the threshold and higher light intensities or generator potentials (GPs) for subthreshold light stimuli depending on the light intensity values. This rule is typical for *C. conicum* (Trębacz et al. 1989a) as well as for *P. patens* (Koselski et al. 2008). In *M. polymorpha*, no AP_{light} was recorded after application of the proton pump inhibitors. This observation is characteristic of *C. conicum* (Trębacz et al. 1989b) but not of *P. patens* (Koselski et al. 2008). Moreover, AP_{light}-like responses after light stimulation were never recorded in all the tested *M. polymorpha* thalli, irrespective of the stimulus strength. This rule points to the different mechanisms engaged in the formation of AP_{light} and AP_{dark} or AP_{dark}-like responses. However, in experiments performed on the moss *P. patens*, Koselski et al. (2008) did not observe differences between membrane potential changes evoked by illumination and darkening, except for having different kinetics.

The results from the experiments performed with the use of the proton pump inhibitors FCCP or DCMU imply that

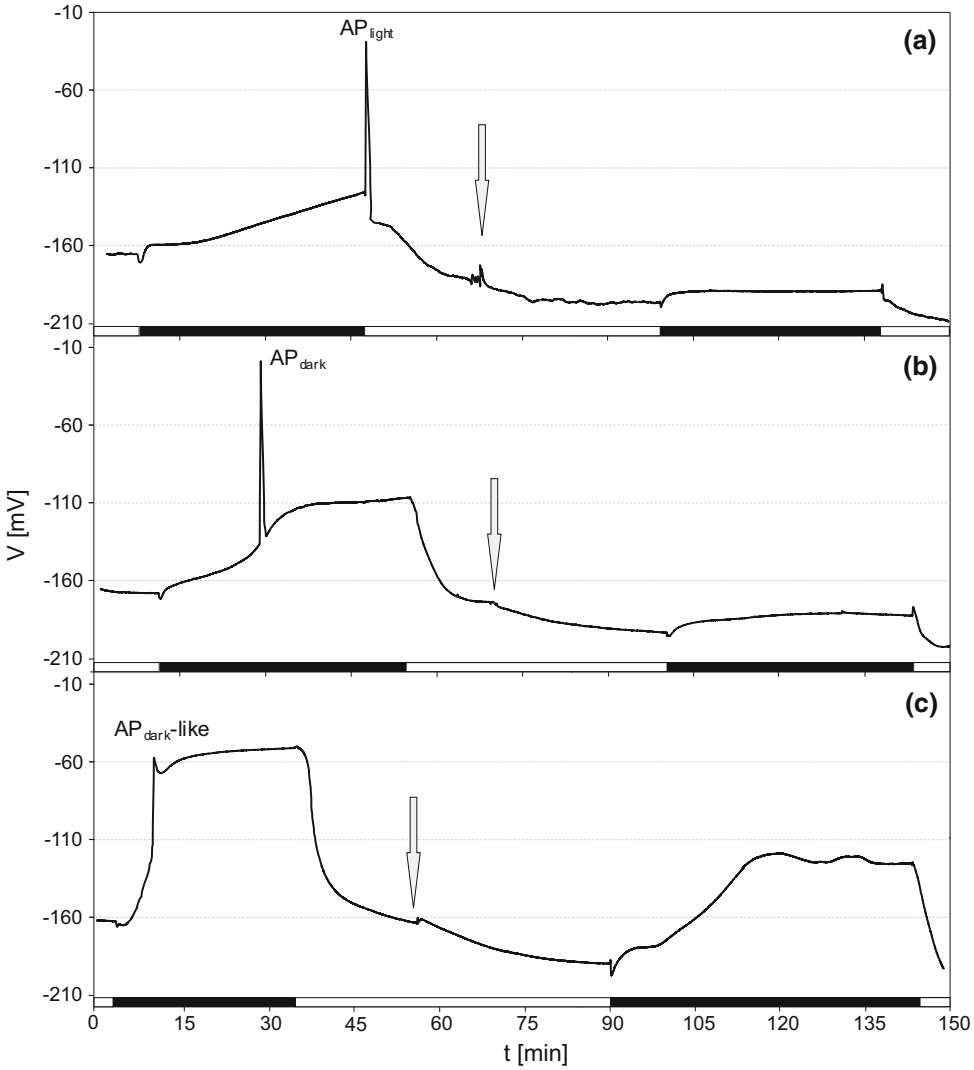

Fig. 7 Light- and dark-induced membrane potential changes in *Marchantia polymorpha* gametophyte cells treated with 20 μM fusicoccin. The *arrow* points to the moment of the activator application. The panels show examples of traces of **a** AP_{light}, **b** AP_{dark}, and **c** AP_{dark}-like responses recorded before fusicoccin application

the main cause of AP_{dark} and AP_{dark}-like generation is disturbance in functioning of the electrogenic ion pump, i.e., plasma membrane H^+-ATPase. FCCP is regarded as an uncoupling agent, because it disrupts ATP synthesis (Benz and McLaughlin 1983), while DCMU is a specific and sensitive inhibitor of photosynthesis that blocks electron transport from PSII to PSI (Trebst 2007). In our experiments, the application of the proton pump inhibitors (FCCP or DCMU) evoked membrane potential changes and persistent depolarisation that resemble electrical responses recorded after dark stimulation in *Marchantia*. This may be one of the indirect indications that the turning off the light has an inhibitory effect on the plasma membrane H^+-ATPase. In addition, when FCCP or DCMU was used at a concentration that evoked maximal inhibition (at least 50 μM FCCP or 20 μM DCMU), neither AP_{dark} nor AP_{dark}-like responses and persistent depolarisation after darkening were observed. This general rule also points to the inhibitory effect of the darkening on the proton pump; it was not possible to block the pump that had already been deactivated chemically. In plants incubated with the lower concentration of FCCP (20 μM), darkening evoked only slow membrane potential responses with magnitude depending on the inhibitor concentration. This suggests that, in plants in which FCCP blocked the proton pump only partially, it was possible to deactivate the H^+-ATPase completely by turning off the light.

Okumura et al. (2012) have revealed that activation of the proton pump in *M. polymorpha* is a consequence of phosphorylation of penultimate threonine-containing H^+-ATPase after illumination, whereas darkening evokes gradual dephosphorylation of the H^+-ATPase (Okumura et al. 2012). Therefore, inhibition AP_{dark} and/or AP_{dark}-like by DCMU may point to dark-induced stabilization of the dephosphorylated (inactivated) state of the H^+-ATPase in *M. polymorpha* cells manifested by the persistent depolarisation. Simultaneously, the absence of AP_{dark}/AP_{dark}-like after the treatment with the inhibitors is hard to explain

as the rate of the slow responses is comparable to the rate of initial depolarisation preceding generation of the AP_{dark} or AP_{dark}-like responses in the control plants. Hence, we conclude that not only the rate of the persistent depolarisation, but also the level of the membrane potential of the cells at the moment of turning off the light determines the appearance of the AP_{dark} and/or AP_{dark}-like. Moreover, it is worth emphasizing that plants generating AP_{dark} and/or AP_{dark}-like exhibited a similar level of the resting potential (~ -170 mV). Hence, one can claim that light-induced electrical responses are a more reliable indication of the H^+-ATPase status than the RP level. Simultaneously, the high value of V_{final} in the thalli generating AP_{dark}-like $t^{\infty}_{1/2}$ responses (-48.8 ± 2.6 mV) suggests that the K^+ and H^+ efflux cannot balance the Ca^{2+} influx and the $C1^-$ efflux from an *M. polymorpha* cell (Trębacz et al. 1994).

In our experiments performed with fusicoccin, i.e., a compound that is regarded to increase acidification of the apoplast by activation of the H^+-ATPase (Johansson et al. 1993), we have observed neither AP_{dark} nor AP_{dark}-like responses. Moreover, in plants generating AP_{dark} or AP_{light} but not AP_{dark}-like responses before the activator application, the persistent depolarisation was also abolished (Fig. 5). This phenomenon points to more potent fusicoccin activation than darkening-induced inactivation of the H^+-ATPase in those thalli. However, from the existence of the persistent depolarisation in plants generating AP_{dark}-like responses in the control solution, it can be deduced that 20 μM fusicoccin is not sufficient to activate H^+-ATPase if the pump is too "exhausted" to repolarise the membrane potential. AP_{light} and GPs were not registered in plants treated with fusicoccin, most probably as a result of a more negative value of the membrane potential than in the control condition. Moreover, taking into account that AP_{light} have never been recorded for the membrane potential above -90 mV, we suppose that ion channels engaged in AP_{light} formation are these activated in a limited range of membrane potentials, with a specific window of activation. Thus, the mechanism of their activation would resemble the mechanism characteristic for animal thermoreceptors and assume allosteric interaction between voltage and temperature (Voets et al. 2004).

A question can be asked: why do plants generate two types of electrical responses—AP_{dark} (single or multiple) or AP_{dark}-like response after darkening? A straightforward answer to this question is difficult, but it can be supposed that the type of the short-lasting potential changes is depended on the scale of disturbances, most probably at the metabolic level, and its influence on H^+-ATPase operation. Plants generating AP_{dark}-like responses are characterised by metabolic disturbances that cannot be compensated by chemical activation of the proton pump in contrast to slight disorders in plants generating APs_{dark}. However, it is hard to determine a distinct limit between AP_{dark} and AP_{dark}-like responses. In our experiments, respiration is the only ATP-producing process during darkening. We postulate that the darkening-induced disturbances in respiration mainly result in lowering of the cytoplasmic ATP level and, in consequence, gradual inhibition of H^+-ATPase manifested by the membrane potential depolarisation. Tiwari et al. (2002) have demonstrated that the rate of ATP production by mitochondria in cells that do not possess functional chloroplasts depends in inverse proportion on the rate of H_2O_2 generation (Tiwari et al. 2002). Moreover, a direct effect of reactive oxygen species (ROS) on the membrane potential has been postulated by Miller et al. (2009). They have revealed that in *Arabidopsis thaliana* cells, propagation of rapid systemic signals evoked by excess light is accompanied by the accumulation of ROS (Miller et al. 2009). Thus, we suppose that generation of AP_{dark} and/or AP_{dark}-like after darkening is an evidence of a decrease in the ATP level in *M. polymorpha* cells caused, most probably, by oxidative stress evoked by oxygen deficiency in experimental conditions. On the other hand, the plants generated different types of electrical responses, although all measurements were performed in the same arrangement (see "Materials and methods"). The rate of the initial depolarisation may reflect the rate of the H^+-ATPase inactivation and seems to be a crucial parameter determining the type of the response, i.e., the faster the persistent depolarisation, the higher the probability of generation of AP_{dark}-like response (Table 1). Hence, it can be claimed that the slower rate and the smaller magnitude of the persistent depolarisation ensure the better H^+-ATPase condition resulting from the higher accessibility of ATP that may provide evidence for efficient respiration.

Conclusions

Our experiments demonstrate that *M. polymorpha* cells respond to illumination and darkening by generating membrane potential changes. The initial phase of the light-induced potential changes exhibited differences in polarity, kinetics, and shapes depending on the light–dark or dark–light transitions. We conclude that the mechanism engaged in formation of light-induced APs is different from that engaged in formation of the short-lasting potential changes evoked by darkening: AP_{dark} and/or AP_{dark}-like. H^+-ATPase probably plays a key role in generation of dark-induced the short-lasting potential changes. Hence, AP_{dark} and/or AP_{dark}-like more suitably indicate the metabolic condition of *M. polymorpha* cells than the action potentials evoked by illumination. The absence of AP_{dark} and/or

AP_{dark}-like probably reflects a high level of ATP and good metabolic condition of the plant cells. However, the biological function of the short-lasting potential changes evoked by darkening needs to be elucidated in the future.

Materials and methods

Plant material and culture conditions

Thalli of *M. polymorpha* were collected in a residential area of Lublin city. Gemmae were taken from a male plant from gemmae cups and cultured on peat pellets in a vegetative chamber under white light of 40 μmol m^{-2} s^{-1} under a 16:8 h light:dark photoperiod. The vegetative chamber was air-conditioned; the temperature of 22 °C and humidity of 50–70% was maintained. After 4–5 weeks of culture, thalli that were about 10–15 mm in diameter were used for electrophysiological experiments.

Electrophysiological measurements

The thalli were collected with their rhizoids, and the plants were mounted in a bath chamber. Incubation in a standard solution containing 1 mM KCl, 0.1 mM $CaCl_2$, 50 mM sorbitol, and Tris/MES (pH 7) performed under white light (40 μmol m^{-2} s^{-1}) lasted 3 h. Sorbitol prevented hypoosmotic shock during the long-lasting immersion and did not influence the resting potential level. Simultaneously, Okumura et al. (2012) have revealed that osmotic shock-depended phosphorylation of H^+-ATPase in *M. polymorpha* cells requires over 100 mM mannitol (Okumura et al. 2012). Electrophysiological experiments were performed as described previously (Król and Trębacz 1999). The membrane potential changes were measured with a 100-mM KCl-filled glass microelectrode inserted into individual cells of the thallus and a reference electrode of Ag/AgCl placed in a bath. Both electrodes were connected to the amplifier Electrometer Duo 773 (World Precision Instruments, Sarasota, FL, USA). The output signals were digitised by an A/D converter Lab-Trax-4/16 (World Precision Instruments) and recorded with the DATA-TRAX3 software. For the insertion of the microelectrode into a plant cell, a micromanipulator (DC-3K; Märzhäuser Wetzlar GmbH & Co.KG, Wetzlar, Germany) was used. The precise localization of the microelectrode tip was not examined; a sudden drop of the membrane potential to the negative value was an indication of the tip location inside the cell.

To analyse the influence of the proton pump regulators on the membrane potential of the *M. polymorpha* cells, the standard solution was additionally supplemented with 20 μM FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) or 20 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) or 20 μM fusicoccin. All proton pump regulators were prepared as a 20-mM stock solution in ethanol. The final ethanol concentration did not exceed 0.3%. It was checked that ethanol in a concentration up to 1% evoked neither APs nor depolarisation of the membrane potential in any of the four attempts.

Author contribution statement K. Kupisz planned and performed most of the experiments, interpreted the results, and wrote the draft of the manuscript. H. Dziubińska performed part of the experiments. K. Trębacz verified the paper.

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