

Phase Response Curve for the Ultradian Rhythm of the Lateral Leaflets of *Desmodium gyrans* Using DC Current Pulses

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In the present study the leaf movement rhythm was perturbed by the application of DC current pulses (15 μ A, 10 seconds, voltage applied: 10 V) to the upper part of the pulvinus, passing through the pulvinus and its stalk. The pulses were applied at four different positions of the leaflets: when the leaves were at the lowermost position, when moving up, at the uppermost position and when moving down. The pre-perturbed and the post-perturbed rhythms were compared. We found that the rhythms were shifted in phase and the phase shifts observed at the four different positions of the leaflets were significantly different in magnitude as well as direction. Furthermore, we could also observe phase advances, which is in contrast to an earlier finding.

A phase response curve (PRC) was constructed to illustrate the sensitivity of the oscillating leaflet system to DC pulses. Substantial delays of about 50 s (as compared to the period of about 200 s) were obtained when pulses were administered at the lowermost position and when leaflet were moving upwards, while advances or no phase shifts were recorded in the uppermost position and when leaflet were moving down respectively.

Introduction

The movements of the lateral leaflets of *Desmodium gyrans*, are generated as a result of rhythmic swelling and shrinking of the motor cells located in the pulvinus of the leaflet system (Engelmann and Antkowiak, 1998). These cells operate in a synchronized manner to generate leaflet movement in the minute range. It has also been reported that electrical potentials across the motor cells oscillate, maintaining a constant phase relationship with the leaflet movement (Antkowiak and Engelmann, 1995). Therefore, it is believed that these electrical potentials play an important role in the generation of ultradian rhythms in leaf movement.

Although the exact physiological mechanism behind the volume changes of the motor cells within the pulvinus is not known in *D. gyrans*, volume changes in all the pulvini investigated so far were found to occur due to uptake and release of large number of ions, especially K^+ and Cl^- (Kumon and Tsurumi, 1984; Starrach and Mayer, 1989; Lowen and Satter, 1989). These ions are

transported between the different parts of the motor cells. In addition to this, a considerable amount of K^+ also shuttles from one part of the pulvinus to the other, acting as a cation reservoir (Freudling *et al.*, 1988; Mayer, 1990). In the case of *D. gyrans*, it was suggested that the depolarization of the membranes of the motor cells causes K^+ and H^+ efflux and that their hyperpolarization causes K^+ and H^+ influx into the cells. The K^+ fluxes were believed to be responsible for the movement of water across the pulvinus, which in turn results in volume changes in the pulvinus and the observable leaflet movement. Any stimulus that can either affect the proton pumps and/or ionic movements across the motor cells or affect the synchrony of the rhythmically oscillating motor cells should in principle be able to influence the leaflet movement.

It has been reported that long term exposure of pulvini to several stimuli can alter the period of the lateral leaflet movements in *Desmodium* (Engelmann and Antkowiak, 1988). The leaflet movement rhythms can also be perturbed by stimuli presented for a brief period of time (of the order

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of seconds) so as to cause phase shifts. When these phase shifts are plotted as a function of phase at which the stimuli were presented, one gets a phase response curve (PRC) (Johnson, 1992). In a previous study, DC currents of 10 to 100 mA strength applied longitudinally through the leaf base for 10 seconds were found to evoke only phase delays in the leaflet movement rhythm (Fostad *et al.*, 1997).

Other electromagnetic stimuli were also found to affect the lateral leaflet rhythm. Electromagnetic fields of 27-MHz frequency can alter the amplitude, period, and the phase of the rhythm. The leaflet movement rhythm could also be stopped by stimuli of DC currents or 27-MHz radio frequencies when used in appropriate strength and at appropriate time (Ellingsrud and Johnsson, 1993; Johnsson *et al.*, 1993; Fostad *et al.*, 1997). Recently, it was also reported that the leaflet movement rhythm slows down in the presence of a static magnetic field (Sharma *et al.*, 2000).

Although in a previous study DC current stimuli were found to cause phase shifts, the phase shifts evoked were always delays (Fostad *et al.*, 1997). In these experiments DC currents were applied through the tip of the leaflets and thus the currents were made to pass the pulvinus longitudinally (perpendicularly to the cross section of the pulvinus). It would therefore be interesting to study the effects of DC currents of comparable strengths as used in the previous study, passing through the pulvinus and the stack. In this situation we expect the current to traverse asymmetrically through the pulvinus and affect the motor cells differently at various phases of its oscillation. In the present paper we report for the first time the PRC due to DC currents for the ultradian leaflet movements in the plant *D. gyrans*.

Materials and Methods

The plants, *Desmodium gyrans* (L.f) DC- also denoted *Codariocalyx motorius*, Houtt, Ohashi- were cultivated under light/dark cycles (12 h light and 12 h darkness) at about 28 °C. The humidity level was about 65%. Further details on the cultivation are given in Johnsson *et al.* (1993).

Leaflets displaying a regular oscillation were cut from the mother plants and were kept in water in an acrylic glass holder. The terminal leaflet was cut off and vaseline applied to the cut surface to

prevent excessive water loss. The leaflets were then placed inside an acrylic glass box to minimize temperature fluctuations (29 ± 0.5 °C). Three light tubes (Osram 40 W/15-1, approximately 20 mm/m²/s) were mounted above an acrylic glass window on top of the box to illuminate the leaflets.

Experimental setup

Leaf movement was recorded by a video camera (FUJITSU TCZ-250E), positioned in front of the box containing leaflets. The video image was digitised in a digitising unit (VIDEO ST 1000) and the digitised image was then processed in an ATARI 1040 ST computer using software ("OXALIS") developed by J. Schuster and Engelmann at the University of Tübingen in Germany. The digitised image is presented in black and white and allows the user to define view fields on the screen where the average horizontal and vertical position of the object within the view field can be recorded. The leaflets were positioned in order to facilitate the recording of both the vertical and horizontal movements. The OXALIS program version 5.1 was used and the setting fields were: FIELDS definition – single, object colour- white, mode- quick, time span- 10 h, record. Time- 5 s, x- coordinate (X), y-coordinate (Y), thus sampling horizontal (x-coordinate) and vertical (y-coordinate) leaflet movement once every five seconds for ten hours.

Direct current (DC) of about 15 mA was applied to oscillating leaflets through the upper surface of the pulvinus and through the stalk. This was done at four different positions; leaflets in the down position (denoted as D), leaflets moving up (denoted as MU), leaflets in the upper position (denoted as U) and leaflets moving down (denoted as MD) for 10 s. The period, phase shifts following perturbations were estimated using two methods:

Method of regression

Period of the rhythmic movement was estimated as the time difference between successive leaf position maxima or minima. The oscillations in the leaf movements were analysed using data recorded for 1000 seconds before and 1000 seconds after the electrical stimulus (1000 seconds of record covers about 5 cycles). A few transient cycles after the stimulus were ignored from the data

series and the maxima/minima of the oscillations both before and after the stimulus were determined after filtering out the noise. The least square fit lines in the plots of the time of the maxima/minima against number of cycle before and after the electric pulse were determined. The time difference between the two least square fitted plots, extrapolated to the cycle of the pulse administration was estimated and gave an objective estimate of the phase shifts immediately after the electric pulse.

Method of curve fitting

The phase shifts were also computed using curve-fitting method (Tablecurve 2D v 4.0, 1996, AISN Software Inc., CA, USA). Also in this method the oscillations in the leaf movements were analysed using data recorded for 1000 seconds before and 1000 seconds after the electrical

stimulus. A few transient cycles after perturbation were ignored while analysing the phase shifts. The data were then fitted to a sinusoid and the rhythm parameters estimated. The phase shifts were estimated as the difference between the phases of pre and post perturbed steady states. The phase shifts results using this method comparable to those obtained in the method described above, and no ambiguities were found. The phase shifts observed at all the four positions of the leaf were compared using Students' *t*-test.

Results

DC currents of 15 mA strength were applied for 10 seconds through the pulvinus of *D. gyrans* when the leaflets were at the four different positions D, MU, U and MD. We found that the phase shifts in the different positions were significantly different in magnitude as well as direction. Fig. 1 illustrates

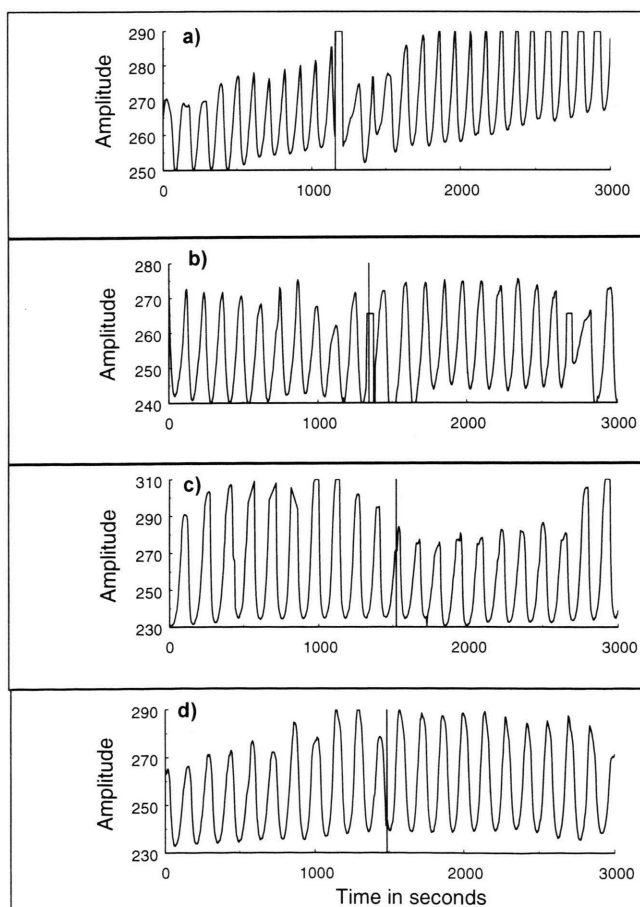


Fig. 1. Four examples of the leaflet oscillations when pulse stimulation was applied. The pulse perturbations were applied at four different positions: D, MU, U and MD as defined in the text. The pulses caused delays (D, MU) and advances (U, MD).

The periods of the leaflet movement rhythms determined before and after the DC current pulses in the examples were (a) 113 s and 110 s, (b) 114 s and 113 s, (c) 146 s and 140 s, and (d) 143 s and 140 s respectively. The phase shifts estimated due to the pulses for the leaflets in the examples were (a) -167 s, (b) -80 s, (c) $+10$ s and (d) $+6$ s respectively.

the regularity of the movements of the leaflets, the transients and phase shifts following perturbation by DC currents at D, MU, U and MD positions of the leaflets.

When the stimuli were applied to the leaflets in the D and MU positions the rhythm was phase delayed whereas stimuli applied at U and MD positions caused either phase advances and minimal responses, respectively. The average phase shifts (mean \pm SE) evoked by the stimuli of direct currents applied at D, MU, U, and MD positions were -54.8 ± 8.53 s, -51.2 ± 7.83 s, 13.4 ± 5.38 s and -1.1 ± 9.52 s respectively. The phase shifts observed at D, MU and U were of fairly large values compared to the period of the rhythm which is of the order of 200 s. The phase shifts observed at the D and MU positions were found to be significantly different from the phase shifts observed in the U and MD positions ($p < 0.001$). However, the phase shifts observed at the D and MU positions were not significantly different between themselves. Fig. 2 summarises these results as a PRC due to DC currents on the ultradian leaflet oscillations of *D. gyrans*.

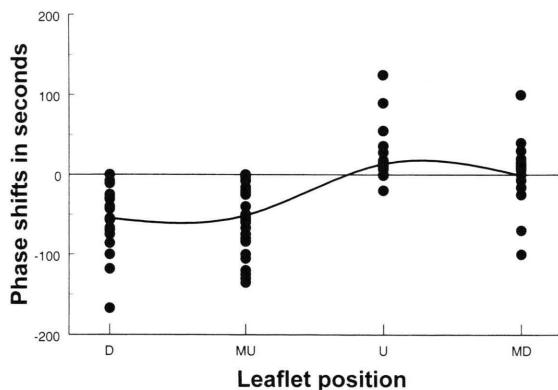


Fig. 2. Phase response curve (PRC) due to DC currents applied to the ultradian leaflet movement of *D. gyrans*. Pulses of approximately 15 μ A DC current were given for 10 sec between upper portion of the pulvinus and the base of the stalk. Four different phases of movements were chosen for the pulses: leaflets in the downward position (denoted as D), leaflets moving up (MU), leaflets in the upper position (U) and leaflets moving down (MD). Delays and advances are plotted in seconds as negative and positive phase shifts. The vertical lines denote the timing of administering DC current pulse to the leaflets.

Discussion

In the experiments described in the present paper we have applied DC currents of 15 mA strength between the outer surface of the upper part of the pulvinus and the base of the stalk for 10 seconds after determining the positions of the lateral leaflets. We demonstrate that the phase resetting due to direct electric current is phase-dependent and the PRC shows significant phase delays and advances.

Electrophysiological studies using microelectrodes inserted into the lateral pulvini and the effects of treatments that are known to influence proton pumps and ion channels suggest that the ultradian oscillations in the motor cells are generated due to its periodically oscillating depolarized and hyperpolarized states (Antkowiak *et al.*, 1991, 1992; Antkowiak and Engelmann, 1995). When microelectrodes were placed on the surface of the lateral pulvinus or within the apoplast, periodic changes in the electrical potentials were observed (Mitsuno and Sibaoka, 1989, Guhathakurta and Dutta, 1961, Antkowiak and Engelmann, 1989, Antkowiak *et al.*, 1991). It was reported that the extracellular potential maintained a stable phase relationship with the leaflet position. Simultaneously recorded abaxial extra- and intra- cellular potentials in the *Desmodium* pulvinus indicated that these changes were generated by changes in the membrane potentials of the abaxial motor cells (Antkowiak and Engelmann, 1995). The membrane potential oscillated between -36 and -136 mV. The extracellular potential and the membrane potentials were 180° out of phase suggesting ionic movements across the motor cells. In our experiments, a current of the order of 10 mA was allowed to pass through the pulvinus and the voltage applied was about 10 V. Estimating the number of pulvinus cells exposed as about 50 the order of magnitude of the voltage along a cell should be about 100 mV. This is certainly of an order that is of physiological interest. The phase shifts observed in the present experiments may, therefore, have been caused due to the effect of the applied DC currents directly on the motor cells or their coupling.

In the literature (Fostad *et al.*, 1997) electrical DC pulses of weaker strength were found to cause phase delays of the lateral leaflet movements,

however, such responses were not found to be phase dependent. In these experiments, the current was applied longitudinally through the pulvinus (perpendicularly to a cross section of the tissue) while in the present experiments the current can be thought of as passing mostly through the upper part of the pulvinus tissue and down the stalk. The shape of the PRC achieved in the present experiments shows that it is possible to get bigger delays than advances for the chosen electric pulse stimulation. This might suggest a possible explanation why Fostad *et al.* (1997) only could record delay phase shifts: When the current is passing the pulvinus in a symmetric way (as in their experiments), cells in different phase positions are stimulated simultaneously independent of the phase of the leaflet movement. Since phase delays are more pronounced, the resulting effect on the

movement will be a delay, independent of the phase position in which the stimuli was given. In fact, this was also reported by Fostad *et al.* while we found delays as well as advances, when the stimuli were applied asymmetrically to the pulvinus.

In the present experiments our main intention was to demonstrate a PRC for an ultradian system, using electrophysiologically relevant stimuli. However, further studies are certainly needed to unravel the underlying mechanisms behind the pulvinus movements in the *Desmodium gyrans*.

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