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REPETITIVE ACTION POTENTIALS INDUCED IN THE LIVERWORT *CONOCEPHALUM CONICUM* (L.)

BY

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ABSTRACT

Repetitive action potentials induced in the liverwort *Conocephalum conicum* (L.). – Action potentials (APs) were detected in *C. conicum* with intracellular and extracellular electrodes. We could occasionally observe repetitive action potentials (RAPs) induced by light, burning or pricking stimulation's. Sequences from two to seven action potentials were observed. A treatment with 1M KCl, combined with prior pricking, allowed a reproducible induction of RAPs in this organism.

Key-words: Repetitive action potentials, *Conocephalum conicum*, wounding stimuli, KCl treatment.

Abbreviations: AP, Action potential; A/D, Analog to digital; PAR, Photosynthetic active radiation; RAP, Repetitive action potentials.

INTRODUCTION

APs in plants were first observed in *Dionaea muscipula* by BURDON-SANDERSON (1873). In this respect, it is interesting to mention that in a seminal paper by HODGKIN (1939) on the discovery of APs in animals, plants APs in *Chara corallina* were cited as reference! Since then, they were abundantly studied in this and other similar organisms (e.g. ODA, 1976; TRONTELJ *et al.*, 1994; JOHANNES *et al.*, 1998; PLIETH *et al.*, 1998; THIEL & DITYATEV, 1998; BISKUP *et al.*, 1999). The interested reader will find useful reviews of APs in plants in PICKARD (1973) and SIMONS (1981).

Many kind of stimulation's can evoke APs in plants (see e.g. FAVRE *et al.*, 1999 and literature cited therein), but more often variation potentials are observed instead. However, these latter though being also a bioelectric phenomena, are of a different nature (STANKOVIC *et al.*, 1998). In general, APs are not easily observed in plants. Among exceptions to this are the above mentioned organisms (algae in particular) and some plants with "fast" movements (seismonastic plants, see e.g.: SIBAOKA, 1969, 1979;

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TREBACZ & SIEVERS, 1998; TREBACZ *et al.*, 1996; WILLIAMS & PICKARD, 1979) where there exists links between APs and movements (SIMONS, 1981). In nonseismonastic plants, APs have been observed, among others (see also the review literature cited above), in *Helianthus annuus* (ZAWADZKI *et al.*, 1991; ZAWADZKI *et al.*, 1995), *Lupinus angustifolius* (PASZEWSKI & ZAWADZKI, 1976; ZAWADZKI, 1979; ZAWADZKI & TREBACZ, 1982), *Luffa cylindrica* (SHIINA & TAZAWA, 1986a), *Salix viminalis* (FROMM & SPANSWICK, 1993), *Zea mays* (FROMM & BAUER, 1994) and *Conocephalum conicum* (DZIUBINSKA *et al.*, 1983, TREBACZ *et al.*, 1989b).

        C. conicum, a desiccated-intolerant bryophyte (DELTORO *et al.*, 1998), is a liverwort which does not have many differentiated tissues, but is genetically complex (SCHUSTER, 1992). It has been the subject of many successful bioelectrical investigations (PAZEWSKI *et al.*, 1982; DZIUBINSKA *et al.*, 1983; ZAWADSKI & TREBACZ, 1985; TREBACZ, 1989) and is well suited for biopotential measurements as well by extracellular as by intracellular methods (see also FAVRE *et al.*, 1999).

        Despite the previously cited observations of APs in plants, repetitive action potentials (RAPs) have been less commonly described. In *C. conicum* they have been occasionally observed by PASZEWSKI *et al.* (1982) with single cuttings. Reliable methods of stimulation have not yet been reported. In this paper, we will present some observations of RAPs in *C. conicum* with intra- and extracellular electrode measurements, using mechanical and chemical methods of stimulation. A method to reproducibly obtain RAPs in *C. conicum* is also presented.

MATERIALS AND METHODS

Plant materials

        The original population of *Conocephalum conicum* (L.) Underwood came from the sub-forest near Lublin (Poland) as already reported in FAVRE *et al.* (1999). It grows in moist and shady locations on calcareous rocks and soils (BOLD, 1973). The green thallus (about 3-8 cm long) is anchored to the substrate by rhizoids arising from the lower surface. Thalli were potted and cultivated in a greenhouse. New thalloids grew in a few days from thallus fragments allowing fast vegetative propagation. After propagation, they were transferred to a vegetation chamber under 16:8 (L:D). A thallus piece 30-40 mm long was delicately cut from the organism, washed in order to remove the soil particles from the lower part. Experiments were carried out in autumn 1998 at the Biophysics Department of Maria Curie-Sklodowska University of Lublin (Poland). Long term (3 days) extracellular measurements were realized in Spring 1999 at the University of Geneva (Switzerland) with the same *C. conicum* ecotype from Poland cultivated as follows: Thalli of *C. conicum* were cultivated on soil in 12:12 L:D at 27 μmol m^{-2} s^{-1} (PAR). For experiments the liverworts were transferred in the measuring chamber (Faraday's cage) with 12:12 L:D, but at a higher light intensity (130 μmol m^{-2} s^{-1}). In both cases light was provided by fluorescent tubes (Sylvania, 18W Standard).

Intracellular measurements

Part of the thallus was placed between two thin flat sheets of Plexiglas pierced with 2 mm diameter holes. Then, it was transferred to an experimental chamber (Plexiglas block, UMCS Lublin made) and immersed in a standard solution medium (SSM) containing: 1 mM KCl; 0.1 mM $CaCl_2$; 50 mM sorbitol. The reference electrode Ag/AgCl (3 M KCl) was positioned in one of the 2 mm diameter holes, as close as possible to the measuring cell. The experimental chamber was then installed inside a Faraday's cage.

Micropipettes were prepared from borosilicate glass (Hilgerberg) using a vertical micropipette puller (MI, Industrial Science Associates Inc.), then filled with 3 M KCl solution using a thin needle (PolyFil™, WPI). The microelectrode was inserted into cells by means of a motorized micromanipulator (MS 314, WPI) under a microscopic and voltage observation control. Indeed, when the tip of the microelectrode is either in the water or apoplastic compartments the electrical potential is near 0 mV (TREBACZ *et al.*, 1994). Only when the tip is in the cytoplasm or in the vacuole the potential drops down very quickly to negative values. In *C. conicum*, the cytoplasm occupies about 20% of the total cell volume (TREBACZ *et al.*, 1994), which greatly facilitates membrane potential measurements.

Membrane potential was registered using a high input impedance ($>10^{12}\Omega$) amplifier (VF–4, WPI) and an A/D converter, which stored data on a personal computer hard disk through viewer software (UMCS Lublin made). Light provided by xenon lamp (XBO 101 Wetron) passed through water and interference (Caflex C, Balzers) filters which cut off IR and UV. Photon flux density equaled 47 μmol m^{-2} s^{-1} (PAR) at the plant level. Experiments started 2 h after general installation and the plant was under continuous standard solution flow.

Extracellular measurements:

Short-term measurements: A thallus of 40-50 mm with two thalloid lobes was placed on a wet (with standard solution medium) paper filter in a Petri dish and installed in the experimental set-up within a Faraday's cage. Three kinds of measuring electrodes were used. The first was a fine rod Ag/AgCl electrode impaled through the thallus, the second one was cotton imbibed with a SSM around an Ag/AgCl fine rod (approx. 4 mm diameter of contact surface), and the third one was a calomel electrode, $Hg/HgCl_2$, bridged by 20 mM KCl solution. The impaled electrode remained positioned between the cotton surface and calomel electrodes throughout the experiment. The reference electrode (calomel) was placed on the thallus away from the measurement site. All of them were placed close together to measure if possible the same event. Calomel electrodes were frequently used for extracellular reference potential and surface biopotential measurements (PASZEWSKI & ZAWADZKI, 1973; PASZEWSKI *et al.*, 1982; ZAWADZKI & TREBACZ, 1985). Impaled electrodes were adopted for measuring electrochemical responses in *Helianthus annuus* (ZAWADZKI *et al.*, 1991, 1995). We also used cotton

surface electrode (Ag/AgCl electrode with some cotton imbibed with SSM), which was not injurious and gave a stable registration of the electrical signal as each experiment took 2 or 4 h and evaporation of standard solution was negligible. It seemed also to be more sensitive than the other kinds of electrodes.

The electrometer was a high impedance amplifier (VF-4, WPI) and the electrical potential data was stored on a hard disk of a personal computer after being digitized by an A/D converter (UMCS Lublin made). A halogen lamp provided 11.5 μmol m^{-2} s^{-1} (PAR) of light at the plant level, IRs were filtered out through a water compartment to restrict temperature variation.

Long-term measurements: Long-term studies of biopotential of *C. conicum* were performed with Ag/AgCl electrodes. A tip of cotton permanently imbibed with SSM provided thallus interface for surface potential detection. Symmetrical reference electrodes were placed on the soil near the electrodes of measurement. The electrometer was a high impedance amplifier (INA-116U, Burr-Brown, 10^{15} Ω). Computer stored data on its hard disk after being digitized by an A/D converter (ACL-812PG, ADclone Inc., Schaitt, CH) according to FAVRE *et al.* (1998).

Long-term measurements consisted in 1 day without treatment. On the 2nd day, one hour after the light-on, thalli were pricked on a distal part. Ten min later, a very small drop (5μl) of KCl 1M was applied to the wound. The stimulated zone was always on the distal part of the thallus about 10-20 mm from the nearest measuring electrode.

RESULTS

The microelectrode technique was used to detect bioelectrical responses to light and dark stimuli in single cells. The cell membrane was at a resting potential (RP) of -166 mV (Fig. 1A). Thirty min of light were applied before turning it off (Fig. 1A, (1)). This induced a cell membrane potential (CMP) hyperpolarization by approx. 15 mV. After 4 min darkness, turning light on induced depolarization of CMP by approx. 18 mV. This depolarization is called the generator potential (GP) (TREBACZ & ZAWADZKI, 1985). Four min after light stimulation, came a dark period of 8 min (Fig. 1A, (3) - (4)), which caused hyperpolarization of the CMP by 18 mV. Then, the light was switched on (Fig. 1A, (4)) which induced first a depolarization (GP) (asterisk in Fig. 1A) and then a fast potential shift was observed (AP). Its with amplitude was 125 mV. During repolarization the intracellular potential decreased primarily very fast, then slowly and then continued to decrease rapidly. The after-hyperpolarization was approx. 17 mV and took approx. 4.5 min to come back to the original baseline value.

Figure 1B illustrates another experiment with the same thallus. Thirteen min separated the light-off and light-on (Fig. 1B, (1) and (2)). Light-off caused the same effects as presented in figure 1A, but the light-on didn't affect the membrane potential with exception of a small transient potential variation. The cell didn't respond with a classical GP but after approx. 16 s illumination, an AP appeared with an amplitude of 114 mV.
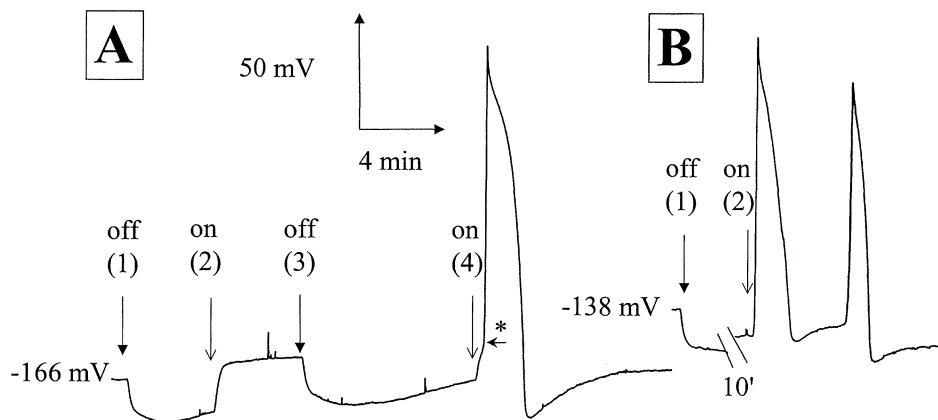
Fig. 1.

Membrane potential of a *Conocephalum conicum* cell measured by intracellular microelectrodes during dark to light transitions. Bold arrows represent light-off, empty ones light-on. Events are noted and numbered. Horizontal arrow with a star indicates the starting point of the action potential (AP). A: Sequences of light-on and light-off. First sequence is 4 min long and produces a generator potential (GP), the 2nd one is 8 min long and produces a GP followed by an AP. B:†After 13 min dark, light-on induces an AP without GP followed by a subsequent AP (repetitive AP, RAP).

Four min 20 s later a second self-elicited AP (RAP) of 92 mV amplitude appeared. The duration of the single light-induced AP (Fig. 1A) was longer than both APs in figure 1B.

Surface biopotential detection was obtained with three different kinds of electrodes with standard electrophysiological apparatus. Figure 2 shows an extracellular response elicited by wounding; the potential is represented with the conventional reverse sign (negative on top). When a red-hot glass tip was approached (without contact) for 3 s to the thallus (dashed zone in a scheme of *C. conicum*, Fig. 2) 10 mm from the measuring point, an AP was triggered approx. 14 s after the stimulation. The records had different amplitudes depending on the kind of electrodes. The AP detected by the reference electrode (stars in Fig. 2) had mainly the same shape in all recordings. Its maximum occurred synchronously approx. 42 s after the stimulation. Since the distance between measuring and the reference electrodes could be estimated as ~30 mm (shortest distance) or 45 mm (longest), we could approximate the AP velocity between 0.8 and 1.4 mm s^{-1}. Two min and 40 s after the first AP, a second AP appeared without a detectable corresponding reference signal (Fig. 2). A third AP appeared approx. 8 min after the first one. Its amplitude was lower than the first one.

Figure 3 shows the results obtained after a different wounding stimulation: traumatic and long-lasting (15 s) puncturing with a fine wire thread near the cotton surface electrode (Fig. 3, pricking zone). The cotton surface electrode measured a series of self-generated APs, which can be grouped depending on their shape. Some APs appeared as
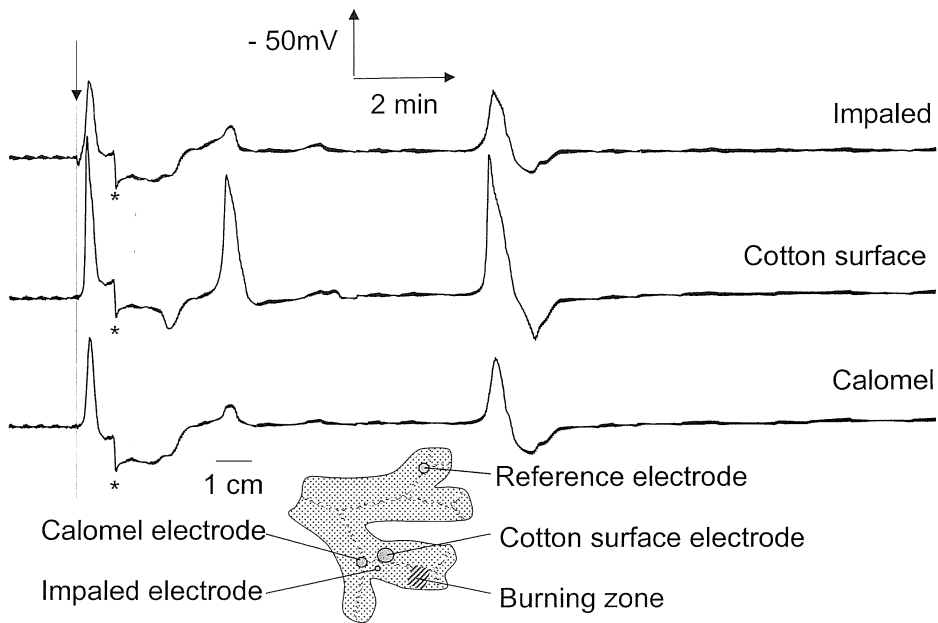
Fig. 2.

Extracellular potential recordings in *Conocephalum conicum* with three kinds of electrodes. The impaled electrode, the cotton surface electrode and the calomel electrode are located in the same area. Circles on the *C. conicum* thallus picture are proportional to the surface electrode occupation. The reference is a calomel electrode and placed as far as possible from the measuring electrodes. The arrow represents the burning event applied on the striated square zone of the *C. conicum* picture. Stars marked are an example of an AP detection by the reference electrode. RAPs can be observed in all cases.

"shark tooth" (Fig. 3, n°2, 6 and 7), different APs had a negative polarization followed by a positive polarization (Fig. 3, n°1, 3 and 5) and a third form only produced a negative polarization (Fig. 3, n°4).

Long-term detection of APs elicited by a single stimulus

To demonstrate the ability of *C. conicum* to generate RAPs, a set of thalli were exposed to 12:12 L:D at a higher light intensity than the cultivating conditions and pricked one hour after the light-on of the 2nd day (Fig. 4, double arrow). Then, a drop of 1M KCl was applied to the wound of test thalli (Fig. 4, A), whereas control thalli received a drop of standard solution (Fig. 4, B). The mean number of RAPs elicited in one hour in different thalli is represented in figure 4A' for KCl treated and in figure 4B' for the controls. Exposure of *C. conicum* to a higher light intensity induced a series of spontaneous APs during daylight and almost none during the night, excepted at the beginning and at the end (Fig. 4, first day). Thalli were more sensitive during the second day of higher light, some APs reached a 30 mV amplitude. All thalli generated AP when
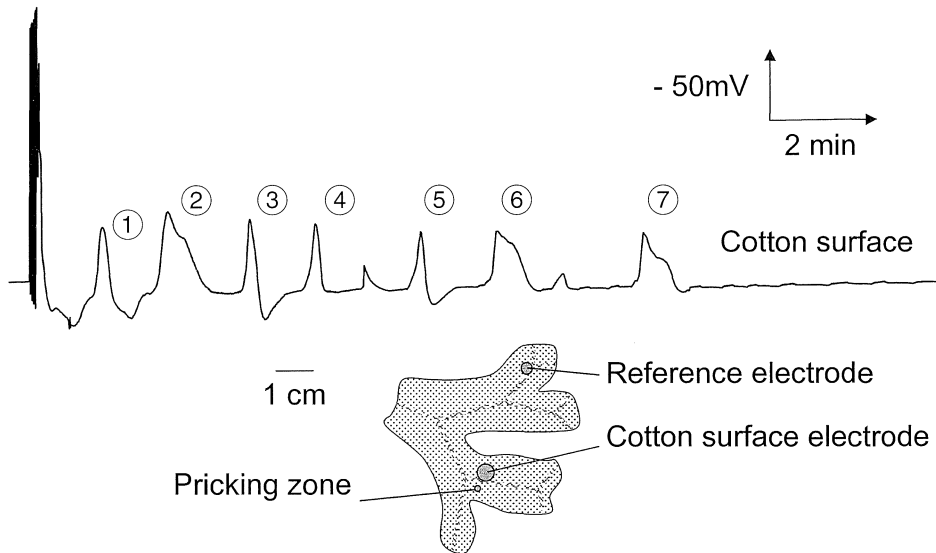
Extracellular potential recording in *Conocephalum conicum* with cotton surface electrode, after pricking stimulation. Repetitive APs are produced. These As are numerated for better understanding. Picture of *C. conicum* shows placement of electrodes and pricking zone.

they were pricked, but when KCl solution was applied on the wound site, thalli clearly produced a higher number of RAPs as compared to the control treatment in the 2nd hour after the light-on. Then APs continued to be generated even during the night, but with a decreasing frequency.

DISCUSSION

In *C. conicum* light-on generally produces a depolarization, defined as generator potential (GP) (TREBACZ & ZAWADZKI, 1985), which is detected with intracellular measurements. Investigations of GPs in *C. conicum* with different light qualities (action spectra) and quantities showed that there are two physiological mechanisms involving photosynthetic processes, one weak and hyperpolarizing, and the other strong and depolarizing (TREBACZ *et al.*, 1989a). When light intensity was strong enough but not too high (to avoid AP generation), then only depolarization was detected. In the case of sufficient light intensity (rheobase at 22 W·m^{-2}, TREBACZ & ZAWADZKI, 1985), the GP led to a light-elicited AP. This in turn has the same properties as the electrically-elicited AP: presence of a threshold and existence of a refractory period. Thus, if *C. conicum* was under absolute refractory period, light stimulation would lead only to a GP without AP. In our situation (Fig. 1) the plant was kept in constant light for (30 min without stimulation, followed by a 4 min dark period, after which the light was switched on. However, this did not evoke any APs, only a GP. This result was a little surprising since
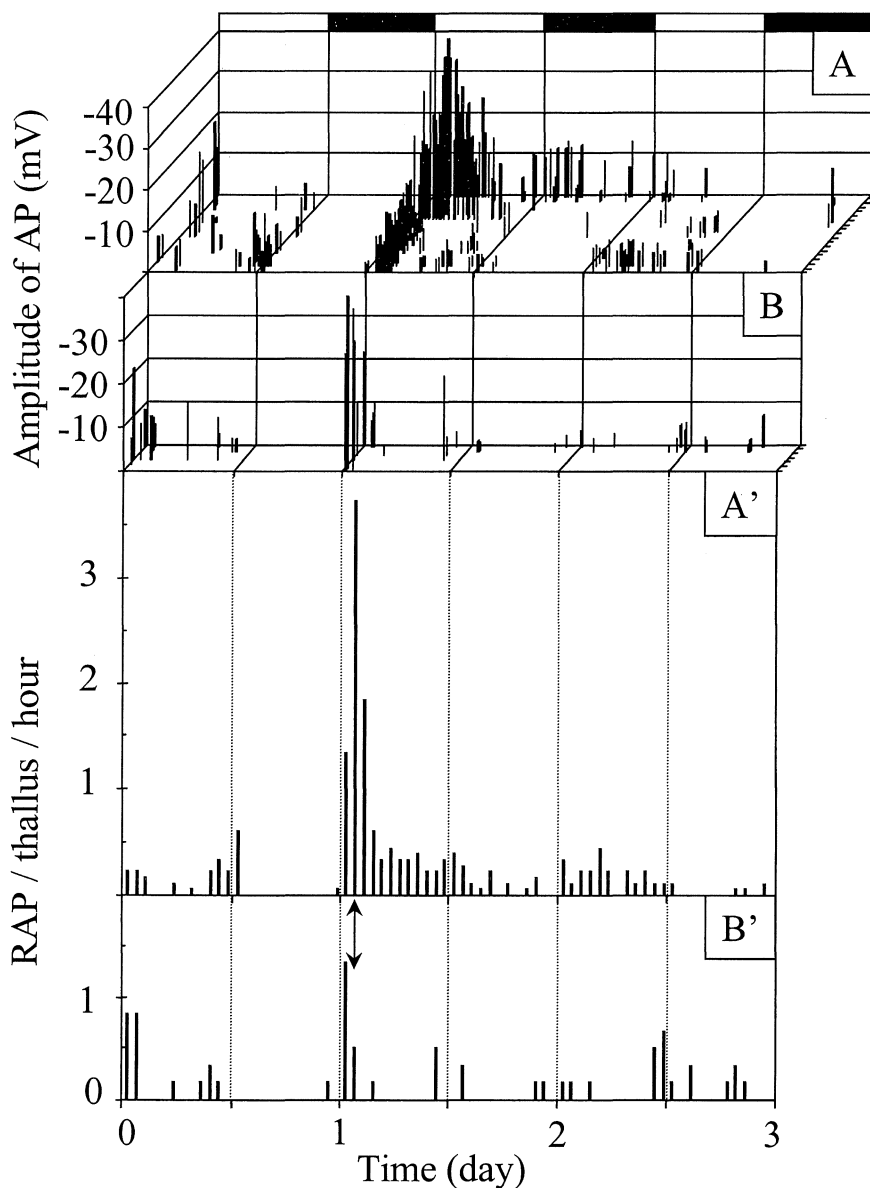
FIG. 4.

Extracellular AP detection on *Conocephalum conicum* intact thalli during 3 days of high light intensity (high light intensity is with respect to the preceding cultivating light conditions, i.e. 27 vs. 130 µmol m^{-2} s^{-1}). One hour after the 2nd day (double arrow), each thalli was pricked and 10 min later a drop of KCl solution (diagram A and A') or a drop of standard solution (diagram B and B') were applied. A' and B': mean number of RAP by plant in one hour corresponding to KCl or SSM treated thalli of A and B respectively. The depth axis in A and B represents individual experiments.

the plant was not in a refractory period state. Perhaps the thallus was unable to elicit AP. Indeed, it has been reported that there are poorly excitable thalli, (DZIUBINSKA *et al.*, 1989). This is not the present case, since thallus generated a single and a double AP after the next light-on stimulation (Fig. 1B). We could suggest then, that the duration of darkness might play a role in the AP burst induced by light (KROL & TREBACZ, 1999).

In the case of double APs (Fig.1B), no GP was induced after turning on the light, but an AP was elicited. Perhaps the membrane potential was naturally very close to the threshold value. This particular membrane state could persist and explain the next self-generated AP after termination of the absolute refractory period. Indeed, it has been reported that a series of APs (RAP) could be obtained in *C. conicum* by producing permanent depolarization of the membrane potential with chemicals (TREBACZ *et al.*, 1989b). We have been able to elicit RAPs, measured by extracellular electrodes, (Figs. 2 and 3) either by heating or pricking. Other stimuli have also been used, such as cutting (PASZEWSKI *et al.*, 1982, DZIUBINSKA *et al.*, 1989), chemicals: 2,4-dinitrophenol, NH_4Cl (TREBACZ *et al.*, 1989b) or by pollination in Brassica (WEDZONY & FILEK, 1998).

Estimation of the AP propagation velocity in of *C. conicum* was in the range given in the literature (ZAWADZKI & TREBACZ, 1985; TREBACZ & ZAWADZKI, 1985). All secondary APs showed smaller amplitude than the original one. Secondary APs could be elicited within a relative refractory period as we presume that the membrane potential is above the threshold value and can initiate an AP when cells excitability is high enough. This was supported by the fact that shortest relative refractory period was about 1 min 30 s to 8 min (DZIUBINSKA *et al.*, 1983) and in our experiment the time period between two APs was between 1 min 15 s and 5 min 20 s.

Eliciting of several APs with 1M KCl has already been observed when applied to the roots of nonseismonastic plants (SINYUKHIN & GORCHAKOV, 1966; PYATYGIN *et al.*, 1999). In our case, KCl solution was perhaps in contact with the rhizoids, indeed the pricking hole where the solution drop was placed crossed the entire thallus. Nevertheless, the application of a single drop of KCl solution on the wound made the thallus more excitable than control plants without KCl. The first day of measurements showed that light promotes spontaneous APs favorably a few hours after and before the light-off period but some appeared during the night. Light at threshold intensity triggers an AP in *C. conicum* (TREBACZ & ZAWADZKI, 1985). The intensity of light given in our experiment was largely over this threshold. This light intensity could increase the excitability of the thalli in a circadian manner, which promotes spontaneous AP in favor of light transition's hours.

Wound-induced RAP might be necessary to increase the systemic response (WILDON *et al.*, 1989). As a working hypothesis we suggest that "AP rhythms" (RAP) could result primarily from biochemical substances that maintain cell polarization over the threshold limit. These substances could be ions or chemicals (TREBACZ *et al.*, 1989b) or may be unknown species-specific agents (SIBAOKA, 1997). Secondarily, the frequency of AP burst could be due to phosphorylation/dephosphorylation of Cl^- channels, that could regulate duration of the refractory period (JOHANNES *et al.*, 1998) and on Ca^{2+}

channels, which could control the membrane excitability (Sнииnа & Tazawa, 1986b, 1987). These processes could be necessary to produce RAP in *C. conicum* and to propagate signal for inducing a systemic response. It is interesting to note that frequency of APs in nerves is well known to code signals. Moreover, in mycelium of fungi the frequency increase of spontaneous AP is correlated to a wood contact (Olsson & Hansson, 1995). In our case, the information may be reinforced by the repetition of propagated APs.

CONCLUSION

APs were detected both with intracellular and extracellular measurements in response to light or wounding stimulation. We have observed the RAPs elicited by a single stimulus and the direct effect of the absolute refractory period in the delay between APs. Calculated values of the AP velocities are similar to ones given by the literature. We observed also that extracellular APs are in a reverse sign of intracellular AP. Despite the fact that *C. conicum* is more or less susceptible to generate APs with light or electrical stimuli, it is always possible to induce a single AP with wound stimuli. When the plant is highly excitable, series of APs can occasionally be evoked. Thallus excitability probably depends on external conditions and on physiological plant conditions, like the age of the tissue or cell states. Induced-stimulation leads in most cases to a single AP and sometimes to RAPs, but application of method with KCl is an easy way to elicit RAPs for a relatively long time. This method may allow a better study of the role of RAP in *C. conicum* and perhaps also in other plants.

ACKNOWLEDGEMENT

RÉSUMÉ

INDUCTION DE POTENTIELS D'ACTION RÉITÉRÉS CHEZ L'HÉPATIQUE *CONOCEPHALUM CONICUM* (L.)

Des potentiels d'action (AP) ont été détectés chez *C. conicum* avec des électrodes intracellulaires et extracellulaires. Nous avons pu occasionnellement observer des potentiels d'action réitérés (RAP) induits par des stimulations de lumière, de brûlure ou de piqûre. Des séquences comprenant de 2 à 7 répétitions de potentiels d'action réitérés ont été observées. Un traitement au KCl 1M, combiné avec un piqûre préalable, et permettant une meilleure reproductibilité des RAP est également présenté.

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