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ACTION POTENTIALS ELICITED IN THE LIVERWORT *CONOCEPHALUM CONICUM* (HEPATICAE) WITH DIFFERENT STIMULI

BY

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ABSTRACT

Action potentials elicited in the liverwort *Conocephalum conicum* (Hepaticae) with different stimuli. - APs were detected on thalli of *C. conicum* with intra- and extracellular methods of measurement. APs were obtained by electric, heat and cold shock stimuli, as well as with light during the intracellular measurement. The presence of a threshold level and refractory period were observed as typical characteristics of APs. The duration of APs induced by electricity was shorter in all the cases. As a working hypothesis, we suggest the existence of different molecular/physiological mechanisms depending on the kind of stimulus given.

Keywords: *Conocephalum conicum*; action potentials; electrical, thermal stimulation.

Abbreviations: AP(s), Action potential(s); PAR, photosynthetically active radiation; VP, variation potential.

INTRODUCTION

Conocephalum conicum (L.) Underw. is found from Asia to North America and throughout Europe with small ecotype differences (SCHUSTER, 1992) or unusual variance in expression of chemical components (WOOD *et al.*, 1996; TOYOTA *et al.*, 1996). It abundantly metabolises terpenoids (TORI *et al.*, 1995, MELCHING & KONIG, 1998) and secretes antibiotic agents (CASTALDO-COBIANCHI *et al.*, 1988). In its natural habitat *C. conicum* can keep occasional flooding without damage - this allows its study in immersed conditions for intracellular measurements - or under wet cover for surface potential measurements without excessive environmental stress. PASZEWSKI *et al.* (1982) have initiated electrophysiological studies with this organism. They showed clearly that it was an appropriate plant material for the study of action potentials (APs) since they were easily evoked either by electrical stimulation (DZIUBINSKA *et al.*, 1983), light

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(TREBACZ & ZAWADZKI, 1985; TREBACZ, 1989; TREBACZ *et al.*, 1997a) or mechanical and chemical stimuli (Trebacz *et al.*, 1997b). These authors have well characterised the properties of AP such as chronaxy, rheobase and the refractory period with both electrical (DZIUBINSKA *et al.*, 1983) or light (TREBACZ, 1989) stimuli, using intracellular or extracellular mean of measurement (ZAWADZKI & TREBACZ, 1985).

Our work consisted in studying the response of *C. conicum* to various stimuli including cold and heat treatments, which have been so far, and to our knowledge, not yet been investigated. This paper presents also some aspects of APs elicited with different stimuli in *C. conicum* with intra- and extracellular methods of measurements.

MATERIALS AND METHODS

Intracellular and extracellular measurement of responses under different stimuli were carried out in autumn 1998 at the Biophysics Department of Maria Curie-Sklodowska University, Lublin (Poland).

Plant materials

The original population of *Conocephalum conicum* (L.) Underwood (Conocephalaceae family, order Marchantiales, class Marchantiopsida (Hepaticae), division Hepatophyta (liverwort)) came from the sub-forest near Lublin (Poland). It grows in moist and shady locations on calcareous rocks and soils (BOLD, 1973). The green thallus is anchored to the substrate by rhizoid arising from the lower surface.

Thalli were potted and cultivated in a greenhouse and 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 and washed in order to remove the soil particles from the lower part. Experiments over several days were performed with intact thallus without removing them from their substrate. APs are in general easy to induce in *C. conicum*, however, it may happen that a thallus was poorly or not at all excitable. To test the sensitivity of the thallus under study, a classical light-induced AP was always performed. When an AP didn't appear after 2 to 3 stimulations, the thallus was considered poorly excitable. It thus might react capriciously to excitation and was discarded.

Intracellular measurements

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

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

Membrane potential was registered using a high input impedance (10^{12} Ω) 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 that cut off IR and UV. Photon fluence rate was 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

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. Calomel electrodes ($Hg/HgCl_2$, bridged by 20 mM KCl solution) were used for electrical potential measurement; the symmetrical reference electrode was placed on the thallus away from the measurement site. The electrometer was a high impedance amplifier (VF-4, WPI) and a personal computer stored data on a hard disk after it was digitised by an A/D converter (UMCS, Lublin made). Light was provided by a halogen lamp (11.5 μmol m^{-2} s^{-1} (PAR) at the plant level), IR was filtered out through a water compartment to restrict temperature variation.

Excitation methods

Different kinds of stimulus were used. Electrical stimulation was performed during 5 seconds with two Ag/AgCl tiny wires, which were distant by 5 mm and inserted into the thallus. A 4.5V flat battery was sufficient to elicit APs. A tip of glass was heated to red and brought very close without touching the thallus in order to stimulate cells only by heat. Cold stimulation was obtained by application of a drop of cold water (1°C to 5°C) to the thallus. Light stimulation after at least 5 minutes of dark, was used as control of the thallus excitability only for intracellular experiments. In each case, the stimulated zone was always situated on the distal part of the thallus at about 10-20 mm from the nearest measuring electrode.

RESULTS

Intracellular measurements

The microelectrode technique was used to detect bioelectrical responses with different kinds of stimulus in a single cell (Fig. 1). Pulses of 5 seconds of different voltages were given in the distal part of the thallus (Fig. 1, electricity curve). Voltage of 0.9 and 1 volt resulted only on an artefact of stimulation, but when 1.1 volts was applied an AP appeared with the amplitude of 140 mV. A time period of eight minutes, but not less than four minutes, was necessary to evoke a second AP with the same stimulating voltage.

C. conicum was sensitive to heat. Indeed, heat stimulation generated a characteristic single AP (Fig. 1, heat curve) but subsequent heat stimuli didn't trigger any APs even after 10 minutes. In order to test whether the thallus was still excitable a light stimulus was given. Twenty-five minutes after the heat triggered AP, light applied after eight minutes of darkness was able to generate an AP in the cell. This suggests that the stimulated cell zone couldn't be excited with repetitive heat stimuli, whereas the thallus was still excitable.

Cold stimulation resulted also in an AP (Fig. 1, cold curve). This was not the case with a drop of standard solution at ambient temperature (data not shown). In contrast to heat, cold stimulation was able to elicit again an AP after 8 minutes. Moreover, elicitation of secondary AP with identical amplitude but smaller duration could arise after only 3 or 4 minutes. However, no AP could be elicited after 1 minute (Fig. 1, cold curve, last stimulation).

Mean values of amplitudes for different stimuli were over 100 mV (Tab. I) and were quite similar regardless of stimuli used. In order to obtain a quantification of the AP duration, we evaluated it from the beginning of the phenomena to its maximal hyperpolarisation. AP duration was roughly the same for light, cold or heat stimuli, whereas it was shorter for electrical treatment.

Extracellular measurements

Electrical potential was measured with extracellular electrodes during different treatments aiming at inducing APs (Fig. 2). Pulses of increasing voltage were imposed to the thallus (Fig. 2, electricity curve). They gave only a stimulation artefact at 1.2 volts. Whereas at 1.3 volts an AP was induced and propagated from the stimulating electrode (cathode) to the measuring electrode. After this, several treatments with the same over-threshold voltage at intervals of 4 or 5 minutes resulted only with stimulation artefacts and only the fourth did induce a propagated but smaller response.

First heat stimulation in the vicinity of the reference electrode produced only a stimulation artefact, whereas a second approach did induce an AP which propagated from the reference electrode to the measuring one (Fig. 2, heat curve). AP detected on the reference electrode consisted on two spikes and only single spike appeared on the measuring electrode.

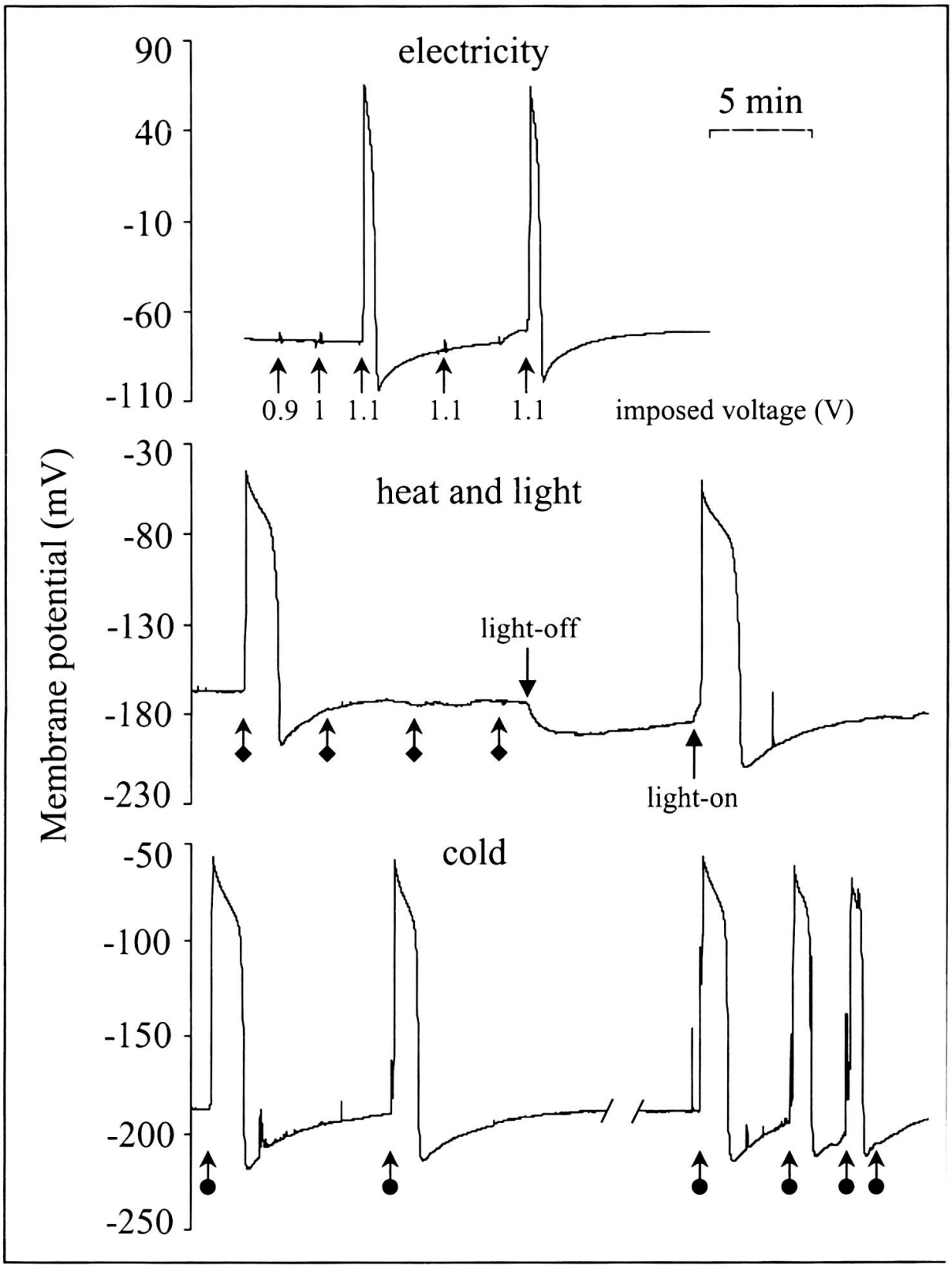

FIG. 1.

Intracellular measurements of *C. conicum* cell showing membrane potential responses during electrical, heat and cold stimulation. Light-off and light-on were given to test the excitability of the cell. Arrows denote instant of stimulation event.

TABLE I.

Amplitude and AP duration mean values of intracellular APs triggered by light, electricity, cold or heat stimuli. Number of AP considered (n).

	Light (n=7)	Electricity (n=5)	Cold (n=6)	Heat (n=2)
Amplitude (mV)	113.4 ± 21.9	134.7 ± 5.3	121.4 ± 15.0	107; 119
AP duration (s)	134 ± 35.5	50.6 ± 8.5	93.4 ±20.2	98; 117

Cold stimulation (Fig. 2, cold curve) immediately generated a small positive spike followed by two rapid negative spikes considered as AP and then a return to the base line with complex variations. Four minutes after the first treatment, a second drop didnít affect the thallus, whereas after 8 minutes it was again possible to induce negative spikes (AP), then the AP at the reference electrode was detected. This latter was composed with a single rapid positive voltage step that slowly returned to the base line. Five minutes later, the biopotential was stabilised and a drop of cold water induced only a small, subthreshold response but finally 4 minutes later, the same treatment induced a negative AP peak without reference signal.

DISCUSSION

Our results showed that increasing the voltage only by 100 mV over a sub-threshold value triggered an AP with a subsequent characteristic refractory period (4 to 8 minutes). This is in agreement with results of the studies on electrical properties of *C. conicum* (e.g. DZIUBINSKA *et al.*, 1983). It is important to underline that threshold voltage, refractory period, constant amplitude and propagation are characteristic properties of true APs, which are present in plants. They differ from variation potentials (VP) (STANKOVIC *et al.*, 1998).

Nevertheless, it could happen that the plant was able to elicit another small AP with the same voltage, but 22 minutes after the first one. This shows the individual susceptibility of the thallus to elicit APs.

The nature of light-induced APs and electrical-induced APs may be identical as the same biochemical agents blocked them (TREBACZ *et al.*, 1997b). However, electrically-induced APs, in intra- and extracellular recordings, are shorter than APs elicited in another way. In this situation, K^+ efflux could be involved to repolarise the membrane potential more rapidly to the resting potential. Thus, the nature of stimulation could implicate different physiological processes (specific receptors, channels?) that promote different responses. Another explanation could be that the difference in duration between light- and electrically triggered APs results, among others, from the way of stimulation itself (KROL & TREBACZ, 1999). In the first case, the whole plant is stimulated simultaneously, and APs of individual cells add up which makes the record broader. Moreover, the cell with a microelectrode inside is directly stimulated by

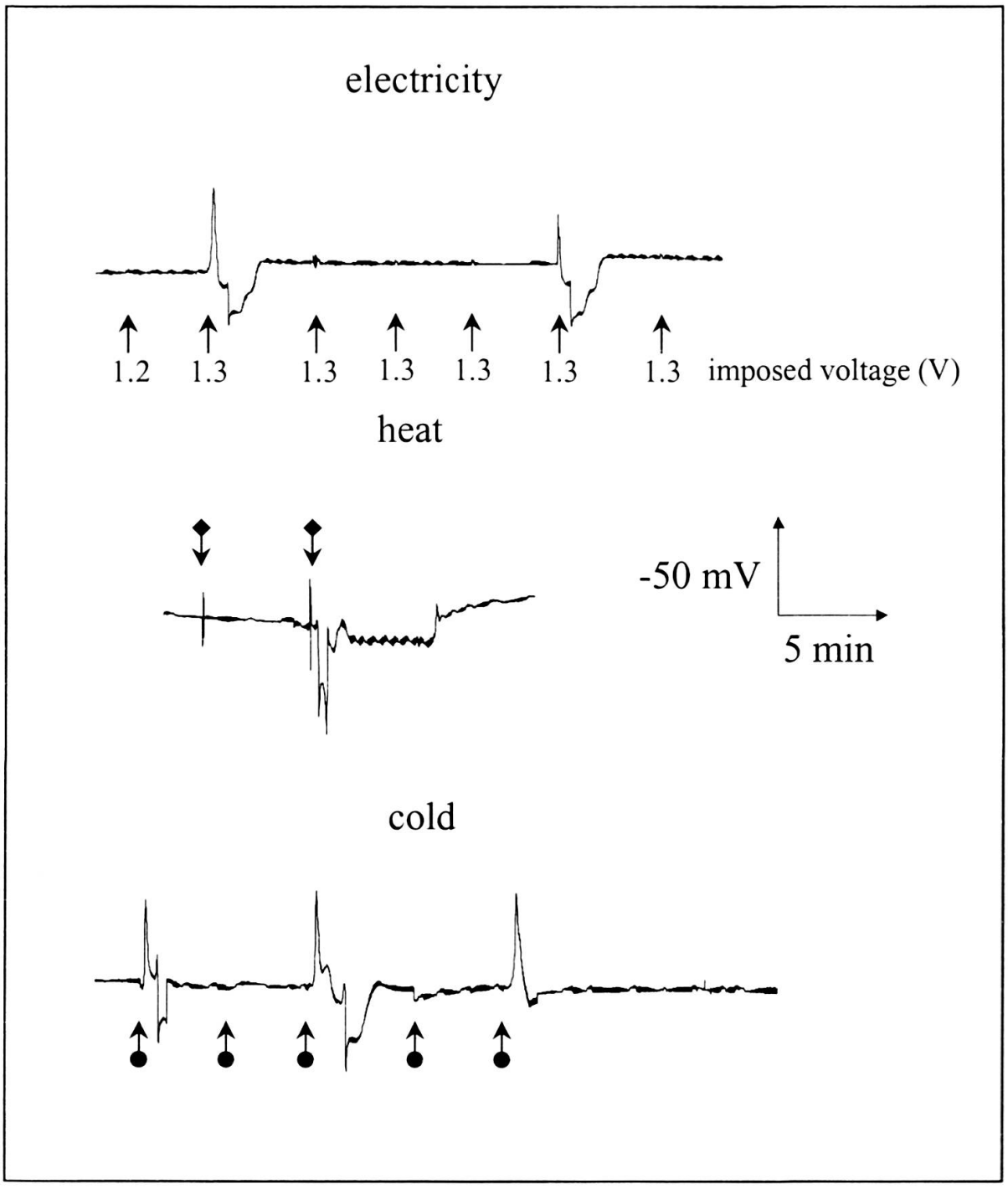

FIG. 2.

Extracellular measurement of *C. conicum* dorsal cells showing biopotential responses during electrical, heat and cold stimulation. Each stimulation is given near the electrode of measurement except for the heat curve where the stimulation is done near the reference electrode. Arrows denote instant of stimulation event.

illumination. Whereas, in the case of electrical stimulation, APs spreads from the cathode, covering on its way subsequent cells until it reaches the cell under study. However, heat and cold stimuli may be broad enough to act as light.

Concerning the temperature stimulation, it is interesting to note that only single APs were observed with heat or cold shocks, whereas wounding stimulation may elicit several APs (repetitive APs reviewed by PICKARD, 1973; PASZEWSKI *et al.*, 1982). Moreover, SINYUKHIN & GORCHAKOV (1966) have observed that heat-induced APs possess a threshold temperature value, assuming the all or nothing law of APs. It might be suggested that a physiological event associated with temperature shocks could involve the opening of ionic channels to trigger an AP. For instance, literature on Ca^{2+} level in cells after heat treatment showed an increase of cytosolic Ca^{2+} provided by intracellular and extracellular pool with a heat-shock refractory period. This is not the case with a cold-shock (GONG *et al.*, 1998). Cold-shock induced the elevation of cytosolic Ca^{2+} from the vacuolar pool (KNIGHT *et al.*, 1996). Note that the influx of Ca^{2+} in cytosolic space is one of the first steps in generating AP (SHIINA & TAZAWA, 1986; THIEL & DITYATEV, 1998; BISKUP *et al.*, 1999) and that Ca^{2+}-voltage dependent channels are involved in the generation of electrically induced APs (TREBACZ *et al.*, 1994; JOHANNES *et al.*, 1998). We might thus suggest the existence of specific cold and heat receptors for calcium influx, which ultimately lead to an AP.

Extracellular APs posses different shapes (Fig. 2) either because of different physiological signalling APs (see above discussion) or APs are a combination (superposition) of the signal detected by the measuring electrode and the reference electrode (the potential difference) with a lag time corresponding to the AP velocity and the distance covered (see also ZAWADZKI & TREBACZ, 1985). In order to explain the heterogeneous AP shapes detected, we schematically represented two electrical potential (EP) traces (A and B) and the difference (A-B). In Fig. 3, the difference between two similar and synchronous EPs (Fig. 3, II) shows a totally annihilated signal. In practice, this could be obtained if the measuring electrode and the reference electrode are very close to each other, or if the same event appears simultaneously in all part of the organism with identical characteristics (e.g. light-on on the whole organism). Subtracting two EP with increasing time lags results in more complex shapes. Extracellular AP elicited by electricity possessed a sufficient lag time to see isolated AP from the measuring electrode and from the reference electrode (Fig. 2 electricity curve; Fig. 3 V). Extracellular heat-elicited AP showed first the AP on the reference electrode and then on the measuring site as the stimulation site was near the reference electrode (Fig. 2 heat curve, Fig. 3 I). With cold stimulation, 3 kinds of AP seemed to be detected, the first one had rapid propagated AP and 2 opposite waves seemed to be fused (Fig. 2 cold curve, Fig. 3 III). The second one was propagating slower and the 2 AP waves were well segregated (Fig. 2 cold curve, Fig. 3 V) and the last one didn't seem to have propagated AP until the reference electrode. It is clear that this aspect could complicate the interpretation of extracellular measuring, nevertheless it could provide some useful information about the propagation of APs within a whole intact organism by a non-invasive method.

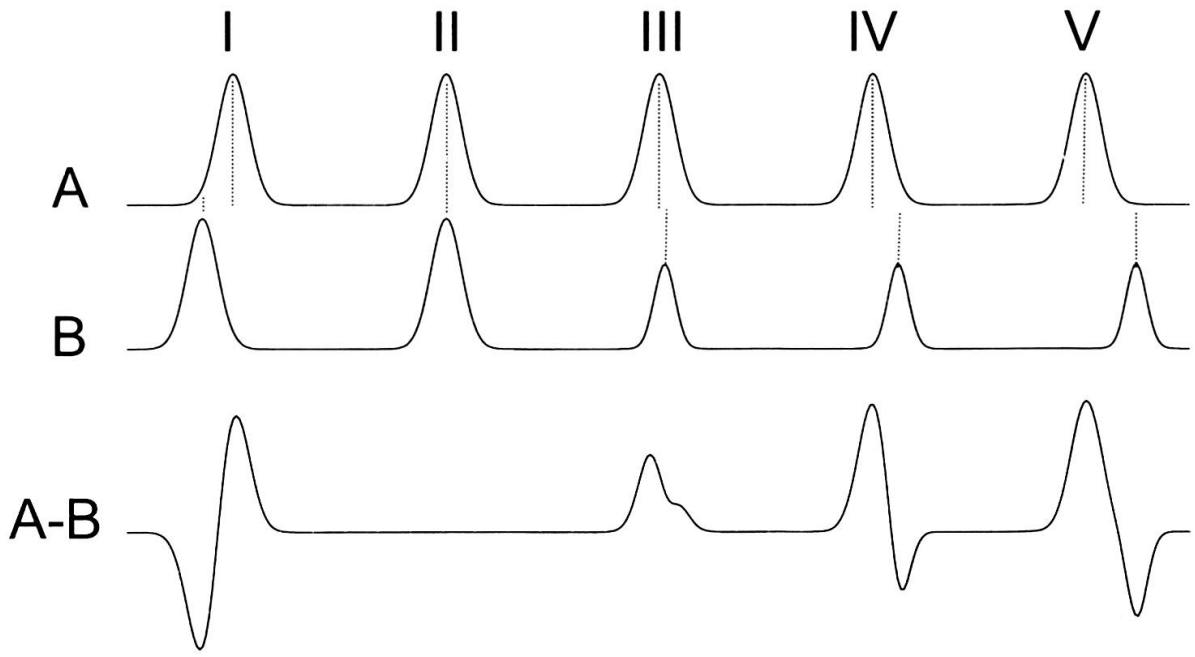

FIG. 3.

Schematic shapes of electrical potential waves (A and B) with different amplitudes and time lags (B) and their difference resulting shapes (A-B).

CONCLUSION

The liverwort *C. conicum* is morphologically and physiologically well adapted for studying APs with intra- or extracellular methods. Both techniques were able to record APs induced by various stimuli, highlighting the sensibility of this plant. It possesses a threshold value and a refractory period, which could change over time, reflecting individual susceptibility of thalli to evoke APs. Some thalli can react capriciously to generate APs. Light, heat and cold elicited APs lasted longer than those electrically induced, suggesting different physiological mechanisms. The elicitation of APs with temperature-shock (heat/cold) in *C. conicum* was not discussed in the literature; it would be interesting to use Peltier elements to determine precisely the threshold stimulating temperature. Cold-shock is an easy means to elicit APs without any cell damages, whereas heat-shock may cause unexpected irreversible effects on the cells. For different stimuli both intra- and extracellular methods were able to detect APs. Each method has its own advantages or disadvantages. However, extracellular measurements were more adapted than intracellular measurement in some experiments involving the whole intact thallus or to perform really non-invasive investigations on very long-term measurements.

RÉSUMÉ

POTENTIELS D'ACTION INDUITS CHEZ L'HÉPATIQUE (*CONOCEPHALUM CONICUM*) AU MOYEN DE DIFFÉRENTS STIMULI

Des Potentiels d'action (PA) ont été détectés sur des thalles isolés de *C. conicum* avec des méthodes de mesures intra- et extracellulaires. Les PA ont été obtenus par des stimuli électriques, des chocs de chaleur ou de froid, ainsi que lors des mesures intracellulaires, par des stimuli de lumière. L'existence du seuil d'excitation et de la période réfractaire ont été mises en évidence en conformité avec les caractéristiques connues des PA. La durée des PA induits par l'électricité était dans tous les cas, plus petite qu'avec les autres types de stimulations. Nous proposons l'existence de mécanismes moléculaires / physiologiques pouvant être spécifiques selon les types de stimuli reçus par l'organisme.

Mots-clés: *Conocephalum conicum*, potentiel d'action, stimulation électrique et thermique.

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