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Excitability and propagation of the electrical impulse in Venus flytrap; a comparative electrophysiological study of unipolar electrograms with myocardial tissue

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ABSTRACT

Mammalian heart cells and cells of leaves of *Dionaea muscipula* share the ability to generate propagated action potentials, because the excitable cells are electrically coupled. In the heart the propagated action potential causes synchronized contraction of the heart muscle after automatic generation of the impulse in the sinus node. In *Dionaea* propagation results in closure of the trap after activation of trigger hairs by an insect. The electrical activity can be recorded in the extracellular space as an extracellular electrogram, resulting from transmembrane currents. Although the underlying physiological mechanism that causes the electrogram is similar for heart and *Dionaea* cells, the contribution of the various ions to the transmembrane current is different. We recorded extracellular electrograms from *Dionaea* leaves and compared the recorded signals with those known from the heart. The morphology of the electrograms differed considerably. In comparison to activation in mammalian myocardium, electrograms of *Dionaea* are more temporally and spatially variable. Whereas electrograms in healthy myocardium recorded at some distance from the site of activation reveal a simple biphasic pattern, *Dionaea* activation showed positive, negative or biphasic deflections. Comparison of patch clamp data from plant cells and cardiomyocytes suggests a role of temperature and ion concentrations in extracellular space for the diversity of morphologies of the *Dionaea* electrograms.

1. Introduction

Electrical activity in plants provides a system to transmit information over long distances. This is probably related to respond its need to environmental stress factors. In species like *Dionaea muscipula* and *Aldrovanda* spread of activation is fast and used to activate a mechanism to catch prey. In plants three types of electrical signals have been reported, action potentials, variation potentials and system potentials. Action potentials are related to rapid propagating electrical impulses that travel at a constant velocity with a constant amplitude [1]. Variation potentials vary with the intensity of the stimulus and amplitude and speed decrease with increasing distance from the site of activation [2,3]. System potentials have been proposed by Zimmerman et al. and are mainly wound induced [4].

Cells of leaves of *Dionaea muscipula* (*Venus flytrap*) and mammalian cardiac cells are both excitable and have a transmembrane potential caused by differences in ion concentrations in the fluid at either side of the cell membrane and the ambient temperature. In the two cell types the resting transmembrane potential is negative, although their values differ. In *Dionaea* and heart cells alike an action potential can be generated if the membrane potential becomes less negative and exceeds a threshold value. Plant cells and cardiomyocytes are electrically coupled by desmoplasmata and connexins respectively, which allows the action potential to propagate through the tissue. Propagation of the impulse is important for a coordinated action that follows electrical activation (closure of the *Venus fly* trap, contraction of the heart). These data show that there is certain similarity between the electrical activity of the *Dionaea* trap lobe and cardiac tissue.

A propagated action potential generates a potential field in extracellular space, and gives rise to an electrogram that can be recorded by an extracellular electrode. If the signal is recorded with respect to a site with zero potential (as a site at a large distance from the tissue) a unipolar electrogram arises, that can be used to analyse the propagation of the electrical impulse. In cardiology, the electrograms (and their derived signals recorded from the body surface, the electrocardiogram or ECG) are often used for diagnostic purposes. Heart diseases often alter the morphology

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of electrograms, which provides information about the underlying (patho)physiology. *Dionaea muscipula* uses spread of activation for trap closure to capture insects [5].

Their leaves contain glands that secrete enzymes to digest captured insects in order to obtain nutrients that are not available in the soil of the swampy areas they live in. Insects initiate activation of the trap by touching trigger hairs at the inside of the leaves (usually 3 hairs are present on each leaf half). *Dionaea muscipula* can, however, also generate propagated action potentials without trap closure. This is the case if a trigger hair is only touched once within a time interval of 20 s, which avoids ineffective closure of the trap and saves energy [6].

1.1. The action potential of cardiomyocytes

In cardiomyocytes, the ionic current responsible for depolarization is carried by sodium ions and generates the fast upstroke of the action potential (from −90 mV to +20 mV for normal working myocardial cells). The sodium current flows into the cell and generates a negative extracellular deflection in the unipolar electrogram. The plateau phase of the cardiac action potential is maintained by an inward calcium current. During this phase the contribution of the transmembrane current to the extracellular electrogram is small. The action potential duration is approximately 200 ms. During the following repolarization phase potassium is the major ionic current, which flows out of the cell, and generates a positive deflection. The potassium current brings the action potential back to its resting value [7].

1.2. Morphology of the cardiac electrogram

In healthy myocardium three types of morphology for the extracellular electrogram during depolarization can be distinguished [8]:

1. At recording sites where activation is passing the electrode a biphasic complex arises during depolarization, a positive deflection followed by a negative one (electrogram at site B in Fig. 1). The initially positive deflection is caused by the capacitive current, which is followed after a short delay by a negative deflection due to the sodium current (blue and green tracings, respectively in the lower panel at site B).
2. At a site where activation arises, a negative deflection only appears (electrogram at site A in Fig. 1). The negative deflection occurs because the capacitive current is virtually zero (as there is no previous activation front) and only the ionic current (flowing into the cell) contributes to the transmembrane current.
3. At sites where activation comes to an end a positive deflection only is observed. This can be at a border site of myocardial tissue or a site where activation fronts collide (electrogram at recording site C in Fig. 1).

During repolarization morphology is less straightforward. T waves that occur if activation passes an electrode can be positive, negative, biphasic or virtually absent. The reason is that also in healthy myocardium the action potential duration is not the same throughout the heart. Duration at the apex of the ventricle is for instance shorter than at the base due to differences in potassium channel expression at those sites [9]. If the action potential duration were everywhere the same, one would expect a biphasic extracellular deflection, which is inverse compared to the biphasic deflection during depolarization. The capacitive current during repolarization then flows inward, which results in a negative deflection and is followed by an outward ionic current carried by K^+ ions that generate a positive deflection. The resulting extracellular deflection during repolarization is therefore biphasic (a negative deflection followed by a positive deflection as illustrated in Fig. 2A).

1.3. The action potential of Dionaea muscipula Ellis

The same biophysical principles of electrogenesis in mammalian myocardium apply to that of plants. The action potential morphology and polarity, the membrane capacitance and the ionic and leak currents, therefore, determine the timing and shape of the electrograms recorded from the *Venus flytrap*.

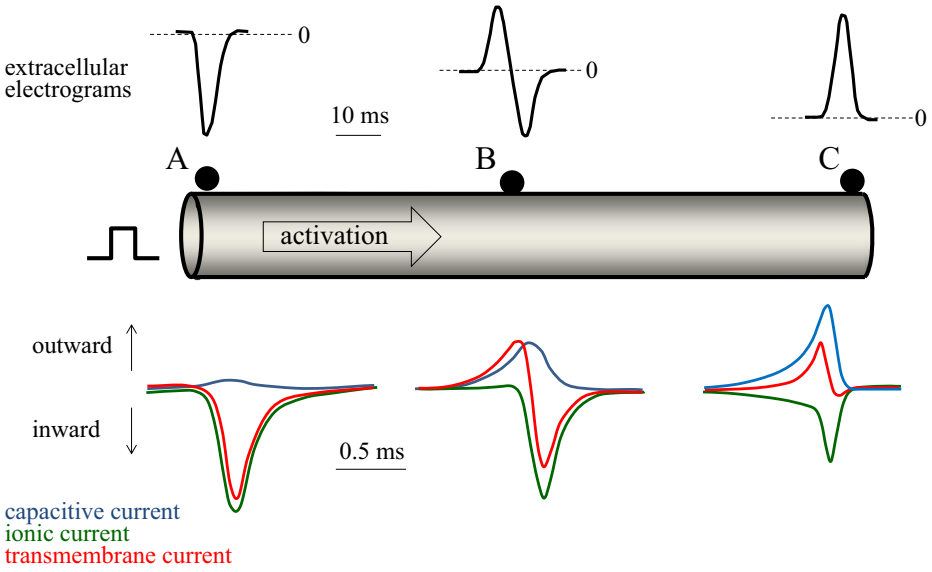

Fig. 1. Upper tracings show morphology of extracellular electrograms of a cardiac fibre during depolarization at a site where activation arises (A), a site where activation passes the recording electrode (B) and a site where activation comes to an end (C). Lower traces are the capacitive (blue), ionic (green) and transmembrane (red) currents that flow at the three recording sites. The transmembrane current, which is the sum of the capacitive and ionic current, mimics in morphology the extracellular electrograms at the 3 recording sites. Electrograms and transmembrane currents are shown for depolarization only. Note the different time scale for the transmembrane currents and the extracellular electrograms.

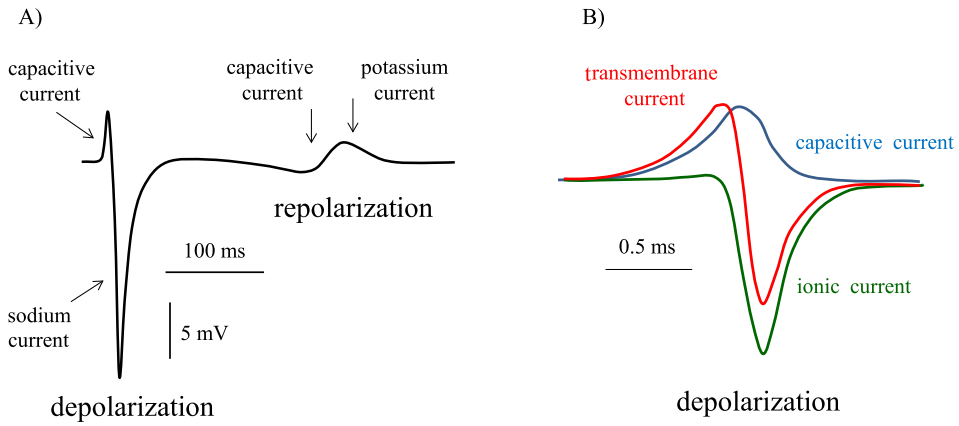

Fig. 2. Deflections of a unipolar extracellular cardiac electrogram **A**). Schematic drawing showing the transmembrane current during depolarization as the sum of the capacitive and ionic current during depolarization **B**). Panel **B**) is adapted from Gray et al. [28].

Hodick and Sievers recorded the action potential of *Dionaea muscipula* in different isolated cell types (sensory cells, upper and lower epidermis, swelling tissue and parenchyma) and at different extracellular concentrations of calcium and magnesium [10]. They showed that not only the sensory cells, but cells from all the major tissues of the trap lobes are excitable, giving rise to action potentials with pronounced after-hyperpolarization before the resting value is reached. The resting membrane potential of sensory cells was −161 mV, similar to that of non-mechanosensitive mesophyll cells (−155 mV). In winter, the resting membrane potential was less negative (about −127 mV) than in summer. Because intramembrane particles are similar between the cell types, the authors suggest that mechanosensitivity of sensory hairs results from its anatomy and not a specialized perception mechanism. Thus, action potentials were virtually the same for the various plant cell types. Action potentials are biphasic with a depolarization duration of 1–3 s followed by an after-hyperpolarization of variable magnitude and duration. The afterhyperpolarizations can exceed the resting membrane potential by 10 to 80 mV (becoming 10 to 80 mV more negative) and is also seen in extracellular signals. The peak of the action potential ranges from −25 mV to +20 mV depending on the extracellular calcium concentration (0.1–10 mM, respectively). The resting membrane potential is independent of calcium concentrations in this range.

1.4. Characteristics of the extracellular electrogram of Dionaea muscipula

Extracellular electrograms recorded from the leaf of the *Venus flytrap* are quite different from those recorded from myocardial tissue. The first extracellular recordings of *Dionaea muscipula* were published in 1888 by Burdon-Sanderson [11]. The recordings were made with a Lippmann capillary electrometer. The investigator reported already in 1876 about the electrical disturbances during excitation of the leaf of *Dionaea muscipula*, but recordings were not shown in that publication [12]. The recordings of 1888 were made with the electrodes applied to opposite points on the upper and lower surfaces of the lamina, and the difference of potential was compensated. Leaves were mechanically activated by touching a trigger hair or by electrical stimulation of the leaf. This recording is in fact a bipolar recording and resulted in electrograms that were initially negative for about 0.8 s and followed by a small positive deflection.

Volkov made electrical recordings of *Dionaea* in 2019 in unipolar mode with electrodes inserted in the leaves or attached to them with a conductive gel [13]. A reference electrode was inserted into the soil. Mechanical stimulation of trigger hairs was applied to activate the leaves. The electrograms were initially negative with a duration of about 0.8 s. The amplitude of the electrograms depended on the electrode mode used (inserted or attached). Electrograms recorded with inserted electrodes revealed amplitudes of about 30 mV, whereas those obtained with the attached electrodes were a factor of 2 lower in amplitude. Values of the amplitude of electrograms of *Dionaea* described in other publications ranged from 14 mV to 200 mV [13]. Other investigators who recorded extracellular electrograms in a similar way, observed, however, electrograms that were mainly positive [14]. It has not been established why differences in extracellular electrogram morphology of *Dionaea* arise.

1.5. Objective

The objective of this study was to explore the various morphologies of unipolar extracellular electrograms of *Venus flytrap* leaves to aid in the interpretation of these signals. In addition, knowledge of the extracellular electrograms of these plants could be helpful to analyse complex electrograms that occur in heart disease. We therefore studied the occurrence of unipolar electrograms of *Dionaea muscipula* in more detail and compared their characteristics with those of myocardial tissue. Leaves of *Dionaea muscipula* are attractive for this purpose, because of the electrical similarities outlined before and because the syncytium seems to be comparable to that of myocardial tissue. Cells are electrically coupled by protein channels (plamodesmata in plants, connexins in mammalians) and conduction velocity of spread of activation is high. In addition, the leaves are well accessible for multi-channel plaque electrodes. Multichannel mapping is needed to construct isochronal maps. These maps are necessary to determine conduction velocity accurately. For the construction of these maps activation times have to be estimated from the extracellular electrograms. For this purpose we used the Laplacian technique that preferentially detects local events and suppresses signals generated by remote electrical events.

2. Theoretical background

2.1. Origin of the extracellular electrogram

The extracellular electrogram arises because of transmembrane currents flowing between intra and extracellular space dependent on the potential differences set up by the activation and repolarization processes. Because extracellular space has finite resistance, a

potential field is generated by the transmembrane currents. The transmembrane current I_m that flows during propagation of the action potential consists of a capacitive component I_c, which is determined by:

$$I_c = C_m \, dV_m/dt \quad (1)$$

and an ionic component I_{ion} (Fig. 2). The transmembrane current I_m is given by:

$$I_m = I_c + I_{ion} \quad (2)$$

C_m is the membrane capacitance of a cell, which is similar (about 1 μF/cm^2) for both heart cells and plant cells [15,16]. dV_m/dt is the first derivative of the membrane potential during an action potential.

2.2. Space constant and conduction velocity

Although propagation of the electrical impulse in *Dionaea muscipula* leaves follows similar principles as propagation of the action potential between cardiac cells, other ions are involved. Conduction velocity for electrical activation in the *Venus flytrap* has been reported to range from 0.06 m/s to 10 m/s, although most reported values are between 0.06 and 0.20 m/s [17]. The high conduction velocity reported by Volkov et al. [13] may have different causes: 1. Stimulation of the leave was done with deposition of 10 μl of 0.01 M HNO_3, whereas other investigators used mechanical and/or electrical stimulation; 2. The propagation path was not known; 3. A large space constant, which is found in some plant cells. A large space constant (lambda) allows electrotonic spread of the transmembrane voltage over a larger distance. The space constant λ is defined by:

$$\lambda = \sqrt{(r_m/(r_i + r_o))} \quad (3)$$

where r_m is the resistance of a unit length of the membrane (plasmalemma) in Ω.cm, r_i is the intracellular resistance per unit length in Ω/cm and r_o is the interstitial resistance per unit length in Ω/cm. The space constant of *Nitella translucens* (*translucent stonewort*) is 1.5–5.7 cm (mean 2.6 cm) [18]. The space constant of a mammalian nerve fibre with a diameter of 1 μm is 0.2 mm, whereas Weidmann reported a space constant of 0.88 mm for a ventricular trabecula [15].

2.3. Depolarization

Although the morphology of action potentials of heart and *Dionaea* cells are both characterized by a fast decrease and inversion of the transmembrane voltage (depolarization, the upstroke) followed by a slower return to the resting value, there are large differences in resting value, duration and upstroke. The maximal upstroke velocity of the action potential of a cardiomyocyte of the human working myocardium has been shown to be 297 V/s [19]. In contrast, the upstroke velocity of the action potential of *Dionaea muscipula* is about 0.5 V/s (derived from reference [20]). The related (Eq. (1)) capacitive current I_c flows out of the cell (outward current) during the depolarization phase, and generates a positive deflection in the extracellular electrogram. Because the upstroke velocity of *Dionaea* cells is a factor of 200 lower than that of cardiac cells, the capacitive current for cells of *Dionaea* is small in comparison.

The ionic component of the transmembrane current is caused by the combined opening and closing of various voltage gated ion channels and by a leak current. The latter component is relatively insignificant in both cell types. In heart cells, a large transmembrane inward current is present during the upstroke of the action potential. It is carried by the sodium current and flows for a few milliseconds. A transmembrane inward calcium current flows during the plateau phase of the action potential, but its amplitude is much lower than that of the sodium (and potassium) current. Outward directed potassium currents cause repolarization. Conduction velocity of the action potential in cardiac tissue is about 0.6 m/s for propagation parallel to the fibre direction and 0.3 m/s for spread of activation perpendicular to the fibres.

2.4. Repolarization

A capacitive current arises because the membrane capacitance is charged (or discharged) during propagation of an action potential. This is due to potential differences at sites in front and behind the activation front. During depolarization the capacitive current is outward. During repolarization the capacitive current can be outward or inward depending on the duration of the action potential at different sites of the myocardium. Therefore, the cardiac electrogram during repolarization is more complex (variable) than during depolarization. Both the capacitive current and ionic current during repolarization are much smaller (about a factor 100) than during depolarization, resulting in a deflection (T-wave) with lower amplitude. The T-wave is nonetheless visible. This is due to the fact that the extracellular signal is related to the summation of transmembrane currents over a distance determined by the duration of the repolarization phase. Duration of the repolarization phase is much longer than that of depolarization [21]. In *Dionaea* cells repolarization is also carried by potassium ions, but conductance of chloride channels remains high for a few seconds after depolarization. This suggests that both ions may play a role for the transmembrane current during a large part of the repolarization process.

3. Materials and methods

Venus flytraps were obtained from Carni Flora nursery, Aalsmeer, the Netherlands. Electrograms were taken from leaves of 3 plants at ambient temperature (about 20 °C, unless stated otherwise). Recordings were made with an NI USB-6009 data acquisition system (1 or 2 kHz sampling rate) provided with a buffer amplifier or a Biosemi active two data acquisition system (2 or 4 kHz sampling rate). With the NI USB-6009 system electrograms were recorded with one or two silver wires with a diameter of 0.2 mm. Wires were electrically insulated except at the end, which was positioned on the leaves via a conductive gel. For the simultaneous recording from two electrodes, one electrode was positioned near the activated trigger hair (at 1–2 mm distance) and the other at 1 cm distance at a line parallel to the midrib of the lobe. If one electrode was used, it was positioned at 1 cm distance from the activated trigger hair.

With the Biosemi Active Two system signals were recorded simultaneously with a 19 by 13 grid electrode (diameter of the electrode terminals, 0.1 mm, interelectrode distance 0.3 mm). The multi-electrode was positioned near the trigger hair that was closest to the petiole. Recordings from the multi-electrode array allow the construction of activation maps to assess spread of activation over the lobe. From activation maps the conduction velocity can be determined reliable, because the direction of activation is known. The multi-electrode was used to determine Laplacian signals. Laplacian signals allow detection of the moment of local activation by suppressing the effect of remote activity and repolarization that often disturb local electrograms. An accurate detection of activation times is necessary to construct reliable activation maps from which conduction velocity can be assessed. The advantage of the Laplacian signal over bipolar recordings is that the Laplacian is independent of the direction of the activation front. The (surface) Laplacian at a recording site is determined from

unipolar electrograms recorded at 8 surrounding electrode sites. The Laplacian is the second spatial derivative of the extracellular potential function, which can numerically be determined from the 9 electrograms. In cardiac tissue, the morphology of the surface Laplacian is similar to the unipolar electrogram for plane surface wave fronts, but deflections are much sharper and reflect the transmembrane current [22–25]. The surface Laplacian as determined in our study is not the real transmembrane current, because a number of requirements (such as interelectrode distance and isotropy of tissue) are not met. Our Laplacian procedure, however, delivers electrograms reflecting local activation and provides information about the direction of the transmembrane currents (in or outward).

Leaves were activated mechanically by touching the trigger hair with a small wooden stick with a diameter of 1 mm tapered at the end. Electrograms were recorded from the inside of the leaf, where the trigger hairs were present or from the outside of the leaf. A silver/silver chloride reference electrode was placed in the soil. Signals were considered to be biphasic if the maximal positive and negative deflection differed less than a factor of 2 in amplitude. Recordings were preferentially made after the first triggering. For the measurements made to assess the effect of an increase in ambient temperature a second trigger was necessary.

4. Results

4.1. Morphology of the unipolar electrograms of Dionaea muscipula

Compared to extracellular electrograms generated by activation in myocardial tissue, extracellular electrograms recorded from leaves of *Dionaea* are more complex and variable. With the solitary electrodes we recorded electrograms at 31 recording sites of 22 leaves. Unipolar recordings made simultaneously with two electrodes showed that signals recorded close to the site of activation (near the trigger hair) and at some distance (1 cm) had similar shapes. The only difference was an initially small negative deflection that was present at the distant recording site but absent in electrograms recorded close to the activated trigger hair. Main morphology of electrograms was either negative, positive or biphasic (Fig. 3). Electrogram differences were observed between plants, but also in the same plant when recordings were made at different times. Amplitude of the electrograms (peak-to-peak values) and duration ranged from 26 to 134 mV and 0.45 to 2.5 s respectively (n = 22). Values of amplitude and duration of the different morphologies are shown in table 1.

Despite the differences in electrogram morphology we found a rather consistent sequence of deflections, which was usually as follows:

Table 1
Morphology of extracellular electrograms, amplitude and duration of complexes. Values for the amplitude are peak-to-peak.

Morphology	Positive	Negative	Biphasic
Number	9	3	10
Amplitude/mv	76 ± 25	62 ± 18	56 ± 15
Duration/s	0.9 ± 0.4	1.4 ± 0.7	1.3 ± 0.4

1. An initial negative deflection marked by 1 in Figs. 3 and 5. The initial deflection at sites distal to the activated trigger hair is always negative, albeit of low amplitude compared to the main deflection (Fig. 4, blue tracing). Close to the trigger hair this negative deflection is absent or hardly visible (Fig. 4, red tracing)
2. A subsequent positive deflection of variable amplitude marked 2 in Figs. 3 and 5. In Fig. 3A and 3C the initial negative deflection is followed by a positive one of low amplitude. In Fig. 5A the positive deflection is larger in amplitude than in Fig. 3, but the positive deflection may also be long lasting, as shown in Fig. 5B. In a number of cases a small positive deflection precedes the broad positive one as illustrated in Fig. 5C. The tip of the broad positive deflection can be smooth as shown in Fig. 5B or multifaceted (Fig. 5C).
3. A terminal negative deflection marked 3 in Figs. 3 and 5. This negativity can be prominent even if the positive deflection is small (Fig. 3A). If the positive deflection is prominent the following negative deflection is usually longer in duration and of low amplitude (Fig. 3C and 5C).

4.2. Electrogram morphology of Dionaea muscipula at different temperatures

The variability in extracellular deflections as observed in our study might be due to the fact that the action potential amplitude depends on ion concentrations in extracellular space and/or the temperature [26]. Fig. 6A and B show electrograms from the same spot of a leaf at two different times. The time difference between recordings was 14 min. During this interval room temperature increased from 20 (panel A) to 22 (panel B) °C. The increase in temperature was due to an increase in ambient temperature that was monitored during the experiment. Other parameters were kept constant; no additional activation and the container of the plant was placed in a dish with rainwater to prevent drying out of the soil. The fast positive deflection was decreased in amplitude during the second recording. In panels C and D electrograms were recorded from another leaf of the same plant. Recordings are from the same spot. The main (fast) response of tracing C is positive and

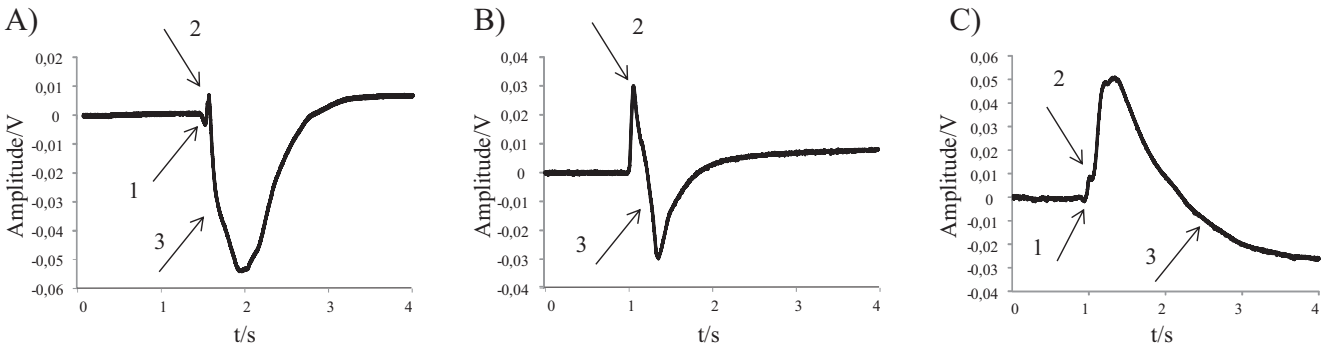

Fig. 3. Different morphologies of extracellular electrograms recorded from the upper leaf epidermis of *Dionaea muscipula* leaves after mechanical activation of a trigger hair and propagation of the action potential. As shown, electrograms can be mainly negative **A**), biphasic **B**) or mainly positive **C**). Initially negative deflections are indicated by arrow 1, the following positive deflection(s) by arrow 2 and 2′. In panel **B**) an initially negative deflection is virtually absent.

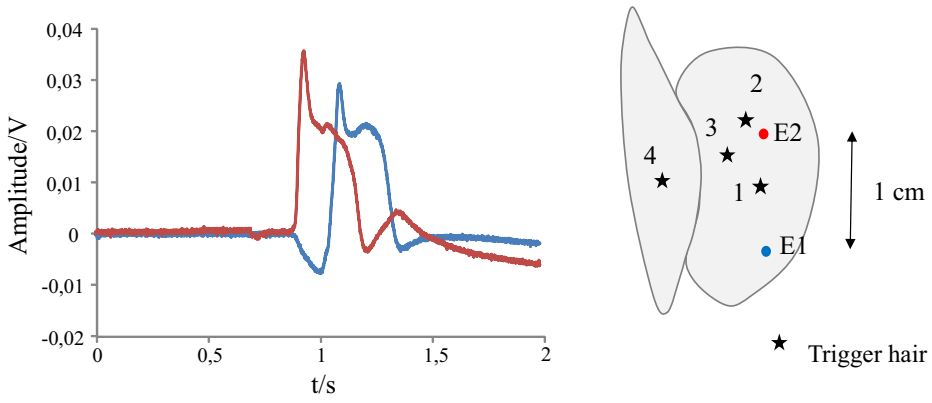

Fig. 4. Extracellular electrograms of *Dionaea muscipula* recorded at the sites indicated in the right panel (red and blue dot). Asterisks mark trigger hairs. The blue electrogram at E1 is recorded about 1 cm away from trigger hair 2 (asterisk marked 2) that was activated mechanically. The initial deflection at site E1 is negative. This initial negative deflection is missing in the signal at site E2 (red tracing) close to the activated trigger hair. Main morphology is, however, similar (mainly positive) at the two sites.

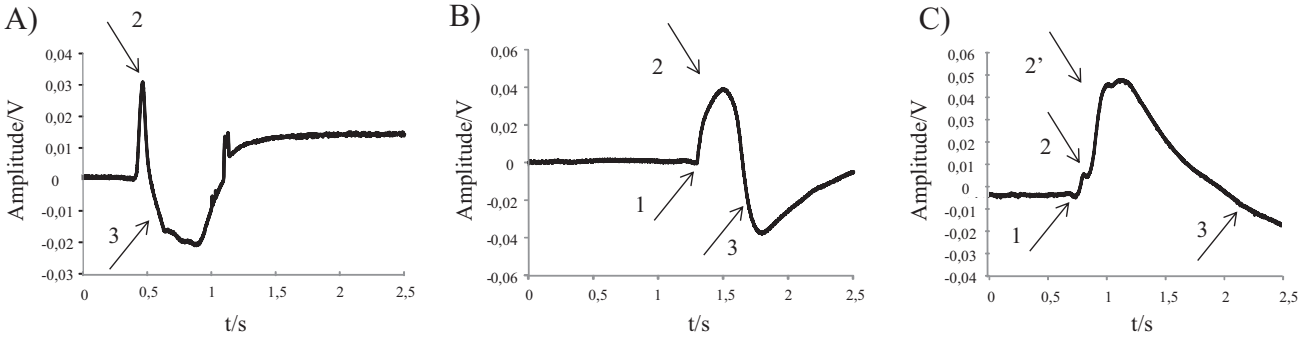

Fig. 5. Initially positive deflection at sites close to the activated trigger hairs (different leaves). A prominent positive deflection of short duration (arrow 2) occurs in the electrogram of panel **A).** In panel **B)** duration of the positive deflection is longer. Panel **C)** reveals an initially negative deflection (arrow 1) followed by two positive ones (arrows 2 and 2′). The tip of the positive deflection is multi in this signal.

followed by a negative slow response. Two hours later the electrogram at the same spot shows a negative fast response, followed by a slow response that was positive. Room temperature rose from 22 to 25 °C during this time interval. The fast positive deflection changed in a negative deflection. Although these data suggest that the difference we observed was due to the change in temperature, we cannot rule out a time effect [27].

4.3. Laplacian signals

A Laplacian transformation of the signals allows the accurate detection of local transmembrane current. Laplacian signals were determined from electrical activity of 3 *Dionaea* leaves where recordings were made with the multi-electrode.

Fig. 7 shows the Laplacian signal at two sites revealing different unipolar extracellular electrograms, one with a prominent positive (panel A) and the other with a notorious negative deflection (panel B). The initial deflection of the Laplacian signal in panel A) is more drastic than the initial phase of the unipolar extracellular electrogram. The Laplacian signal starts with a tiny positive deflection, which likely is caused by the capacitive current (see theoretical background). The following negative deflection(s) is most likely caused by the calcium (and chloride) current (initial ionic currents during activation in *Dionaea* cells). These deflections are nearly absent (invisible) in the unipolar extracellular electrogram.

In panel B), the unipolar extracellular electrogram is mainly negative. The morphology of the Laplacian signal is roughly similar to that of the unipolar electrogram. The unipolar electrogram starts with a tiny positive deflection (arrow) that is most likely caused by touching the trigger hair, because it was present at the same time in virtually all signals. The following positive deflection in both tracings is tailed by a large negative one. The positive deflection might be caused by a capacitive current. The negative deflection must be generated by the calcium and/or chloride current. In the Laplacian signal the negative deflection has a clear inflection point, which may mark the transition from calcium to chloride current.

The lower tracing of Fig. 8 shows an extracellular electrogram comprising 2 complexes with mainly positive deflections. The corresponding Laplacian signal is shown by the upper tracing. The first complex shows the deflection due to activation that did not lead to trap closure. Because the trigger hair was activated twice within a short time, the second activation led to closure of the trap. The initially negative deflection in the Laplacian signal is larger and steeper for the second deflection, after which the trap closes. This indicates that this time enough current is generated to close the trap. Trap activation is reflected in the unipolar electrogram by the steeper upstroke of the electrogram.

4.4. Activation time

Activation time is defined as the time cells underneath a recording electrode are activated (depolarise). For cardiomyocytes the activation time can be determined from the extracellular electrogram. It has been shown that the point of steepest down stroke in the unipolar extracellular cardiac electrogram corresponds with the point of fastest upstroke of the action potential, which can be considered as the time of activation [28].

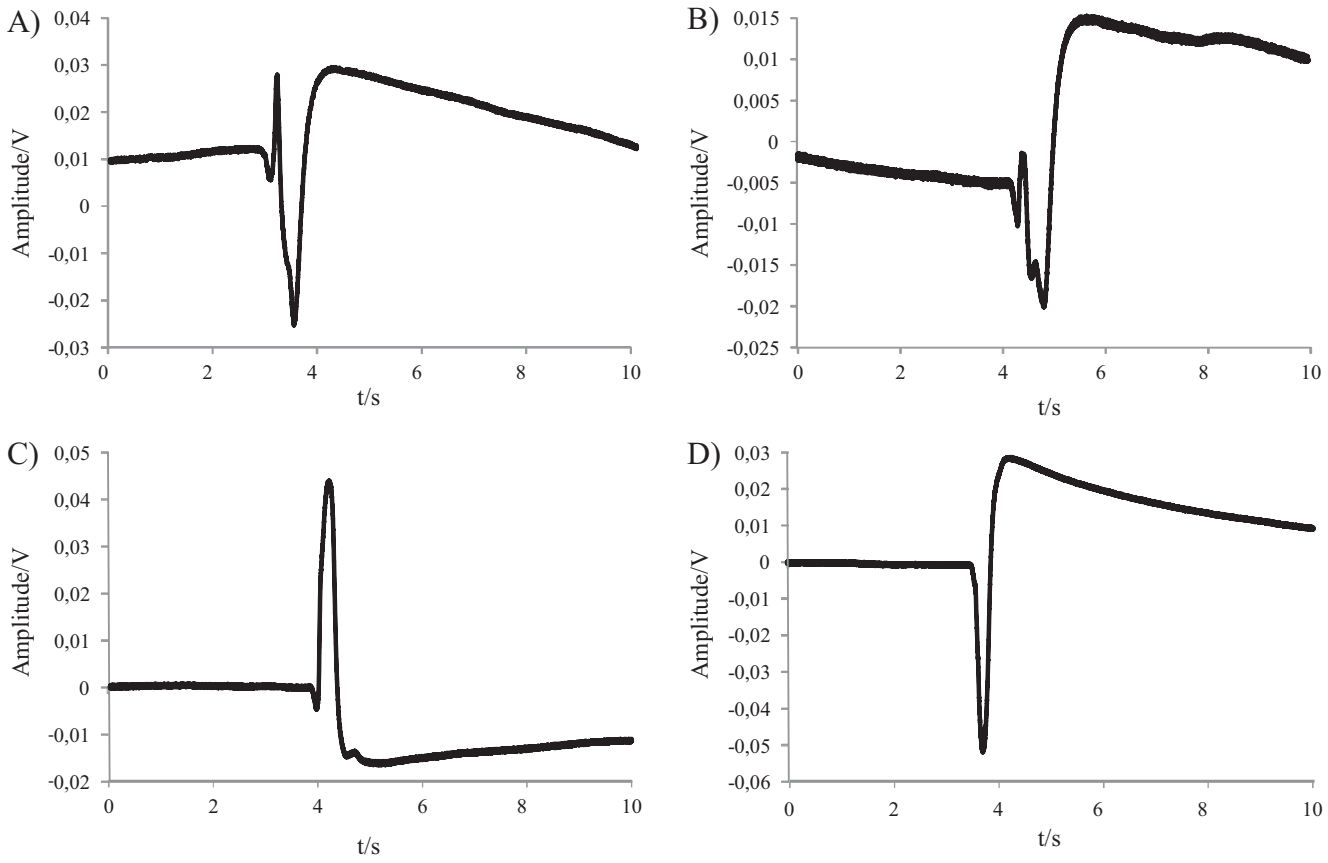

Fig. 6. Changes in electrogram morphology of signals recorded at the same spot of a *Dionaea* leaf over a time interval of 14 min (panels **A)** and **B)**). Temperature increased from 20 to 22 °C from **A)** to **B)**. Panels **C)** and **D)** are recordings from another leaf of the same plant. Change in electrogram morphology for recordings from the same spot after 2 h during which temperature increased 3 °C (from 22 to 25 °C).

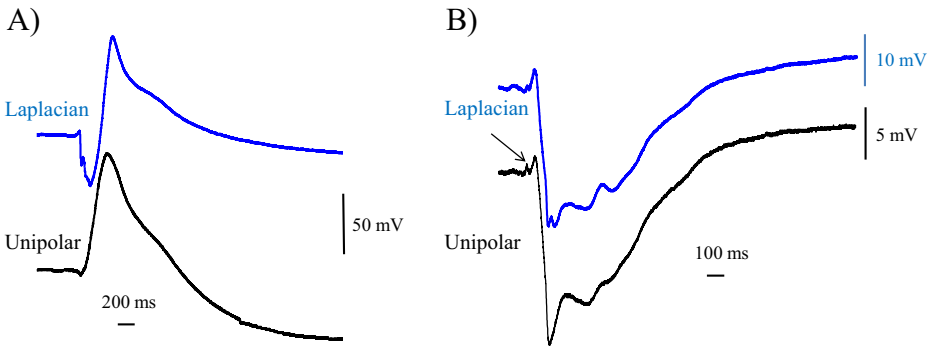

Fig. 7. Laplacian signals (blue) and corresponding unipolar extracellular electrograms (black) from recordings sites of the upper leaf epidermis of *Dionaea muscipula* of different plants, **A)** and **B)**.

Determining the activation time from the extracellular electrogram in *Dionaea* cells is more complex, the more so because electrograms are very variable (positive, negative or biphasic for passing wave fronts). Because calcium is the initiator of activation in *Dionaea muscipula,* the time of transmembrane calcium current might be a good indicator of the activation time. The deflection caused by the calcium current is often not well discernible in the unipolar electrogram. To determine the transmembrane calcium current the Laplacian signal is helpful. Because calcium ions provide an inward current, the early negative deflection in the Laplacian signal is an attractive marker for the activation time (arrow in Fig. 8). The point of fasted down stroke of this negative deflection was used as activation time.

Data from the recording made with a sample frequency of 4 kHz was used to assess conduction velocity. The highest sampling rate was chosen because literature suggests that conduction velocity can be high in comparison to impulse propagation in myocardial tissue where a sampling rate of 1 kHz is usually appropriate. Because of the sample frequency of 4 kHz our mapping system could only acquire 128 of the 247 signals from the multi-electrode grid. Activation times were derived from constructed Laplacian signals. Just 60 electrograms were used to determine Laplacian signals, because the Laplacian algorithm is rather sensitive to artefacts. From these signals the activation map of Fig. 9 was derived. It represents the recoding electrode grid of 3.6 × 5. 4 mm. The unused grid electrodes are located in the gray area.

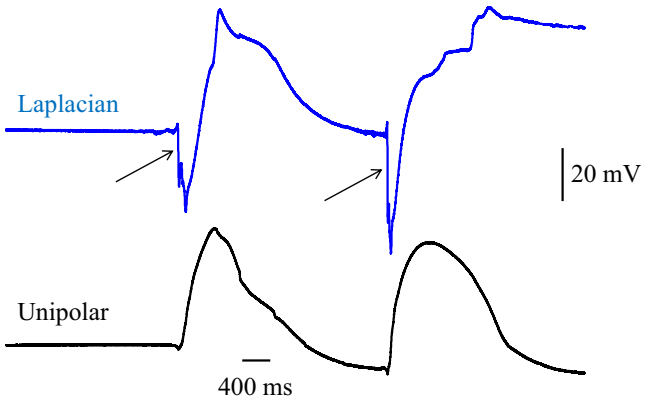

Fig. 8. Unipolar electrogram (black) and corresponding Laplacian signal (blue) of a recording from the upper leaf epidermis of a *Dionaea* leaf. Activation was initiated by touching the trigger hair twice within a short time interval. Closure of the flytrap occurred during the second complex. The inward transmembrane current (arrow), which corresponds with the initial negative deflection, is greatest during the second activation.

The white area represents the location of the 60 recording electrodes. The lines represent isochrones of incremental 1 ms classes. A conduction velocity of 0.85 m/s was determined for activation that did not result in trap closure. This is slightly higher than the highest conduction velocity (parallel to the fibre direction) in myocardial tissue. Activation that resulted in trap closure was higher and about 1.6 m/s.

5. Interpretation and discussion

We have shown that in comparison to cardiac electrogram morphology, unipolar electrograms of *Dionaea* are variable, more complex and less consistent. Although the gross morphology is similar at different sites of a *Dionaea* leaf, there is a slight difference in shape between sites close to the activated trigger hair and more distant sites. *Dionaea* electrograms revealed usually 3 phases, that are related to the sequence of ionic currents. The electrograms are of high amplitude and have a long duration compared to signals from myocardial tissue. Variability in morphology of plant unipolar electrograms are dependent on ionic concentrations of the soil, temperature and time (previous activations). Summation of inward current by a second trigger is related to trap closure. Conduction velocity in *Dionaea* leaves at room temperature as we determined from Laplacian generated activation maps did not differ markedly from that in mammalian myocardium at 37 °C. Conduction velocity associated with trap closure is about two times faster than subthreshold conduction velocity.

5.1. Ion channels in plants

We based our hypothesis about the relationship between the extracellular deflections and the transmembrane ionic current components in Fig. 10 on data obtained from *Characea* algae [29,30]. We realize that the use of *Nitellopsis obtuse* data is not optimal because of the lower order of the algae, but the ionic mechanism of action potentials in higher plants relies on data obtained from giant cells of *Characea* [3]. In these cells onset of depolarization arises via calcium influx. The enhanced intracellular calcium activates chloride channels. This subsequently leads to an increased depolarizing phase through the efflux of chloride ions. The following efflux of potassium ions via voltage dependent potassium channels finally results in repolarization [31]. Results from data derived from higher plant cells indicate that the mechanism of action potential generation is fundamentally similar in higher plants and *Characean* algae [3]. However, other data indicate that reversible inactivation of H^+-ATPase is probably involved in higher plants as well, which is related to a second phase of repolarization [32,33]. During depolarization H^+-ATPase is suppressed by the calcium entering the cell. K^+ efflux starts as early as the depolarization and initiates the first step of repolarization [3]. If the potassium equilibrium potential is reached, H^+-ATPase is made active, resulting in an activated H^+ pump that starts a second phase of repolarization [3]. Vodeneev at al. indicate that H^+-ATPase inactivation does hardly contribute to the initial phase of the action

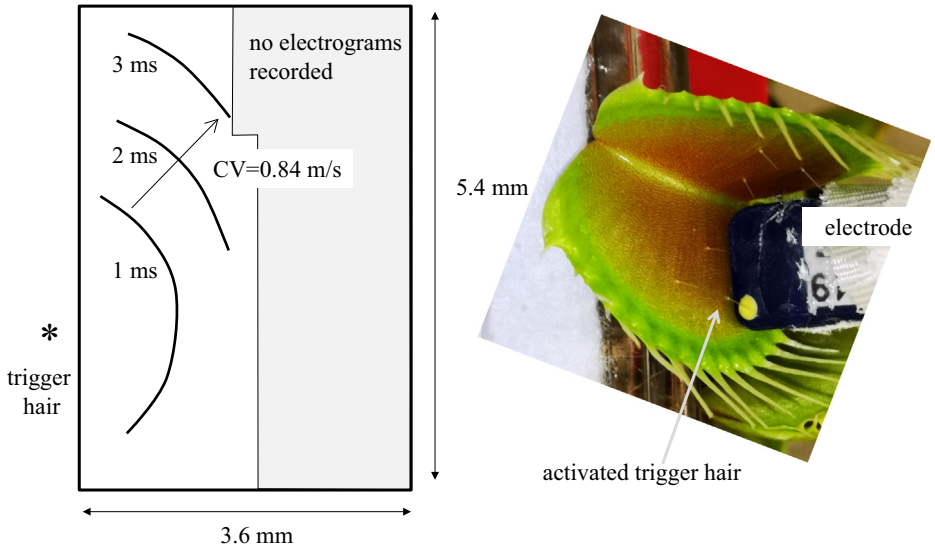

Fig. 9. Left panel: Isochronal map of electrical activation of the upper leaf epidermis of a *Dionaea* leaf. The position of the electrode on the leaf is shown in the right panel. Stimulation was initiated by mechanical activation of a trigger hair (asterisk). The gray area indicates the zone of the grid electrode from which no signals were recorded because a sample frequency of 4 kHz was used. Earliest activation within the grid was taken as activation time zero. Activation times were derived from constructed Laplacian signals. As activation time we used the point of steepest down stroke of the initial negative deflection of the Laplacian signal that is supposed to be caused by the calcium current.

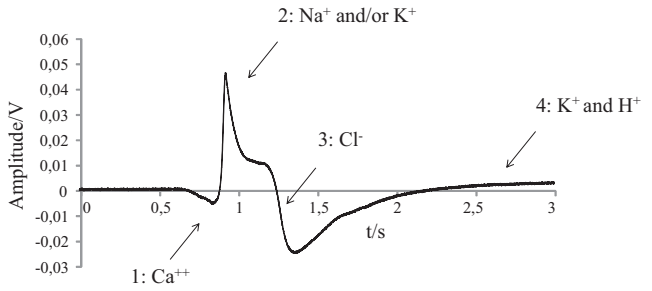

Fig. 10. Extracellular recording from the upper leaf epidermis of a *Dionaea muscipula* leaf after mechanical activation of a trigger hair. The recording site was at 1 cm distance from the trigger hair. Purported ions involved in the generation of the different deflections are indicated.

potential. Inhibition of the enzyme reduces action potential amplitude by about 10%. A comparison between action potentials obtained from a model of *Characea* cells (without H^+-ATPase) and higher plant cells (with H^+-ATPase) shows that the upstrokes of the action potentials are very similar, but that the repolarization phase in the higher plant cells reveal a second delayed component [3]. These data suggest that H^+-ATPase activity may indeed play a role and that our hypothesis for the later phase of the extracellular electrogram must involve an H^+ component as well. Although both potassium and H^+ATPase play a role in repolarization, the resting membrane potential of the plasma membrane of higher plant cells is dominated by the electrogenic H^+ATPase pump. Ion diffusion processes play a predominant role during the action potential [32].

5.2. Propagation of action potentials in Dionaea leaf

Vascular bundles are suggested to be the main propagating pathways for electrical activity in higher plants [34–38]. Hedrich et al suggest that in the *Venus flytrap* conduction also occurs in the phloem network [39]. The lobes of the *Venus flytrap* contain an extensive venation grid. This phloem network allows conduction of electrical signals as in a cable and as a consequence the general cable equation applies to the phloem [39,36]. Although action potentials are the most likely electrical signals for trap closure in *Dionaea*, other types of electrical signals (variation and system potentials) cannot be excluded. They are, however, less likely because variation potentials and system potentials are initiated by chemical agents, mechanical wounding, heating and burning, different from the trigger in *Dionaea* [36].

Spread of activation in the *Dionaea* leaf might, however, be more complex than activation via vascular bundles only. Histology of a cross section of the *Dionaea* leaf shows several cell layers, a layer of inner and outer epidermal cells and a middle layer of parenchyma and swelling cells [10]. Vascular bundles are embedded in the middle layer of the swelling cells. Action potentials can be derived from all these cell types and have been shown to be similar [10]. Data from *Aldrovanda*, an aquatic carnivorous plant similar to *Dionaea* in the structure of its traps, indicate electrical coupling between the different cell types [40]. These data suggest a 3D excitable tissue structure for the *Dionaea* leaf as well. The central portion of the leaves of *Aldrovanda* is composed of three cell layers an inner and an outer epidermis that encloses a middle layer of larger cells. Plasmadesmata are present at the end and lateral walls of the epidermal cells as well as in the cells in the layer in between [40]. Plasmadesmata are only sporadically present in the walls between the epidermal and middle cells. This is similar to the coupling between different cell layers in ventricular myocardium, where coupling in the plane is stronger (in the fibre direction) than perpendicular to it. Activation initiated at the epidermis can spread to deeper layers, but conduction in the plane is faster by the tight electrical coupling between the cells. Because unipolar electrograms reveal both local and remote activation, electrical activity in all layers will contribute to the signal derived from the epidermis. The Laplacian signal only reveals local activity, thus mainly activity from the activated epidermal layer. Activation through a 2D model of excitable plant cells, where cells are electrically coupled with 4 neighbours, has been investigated by Sukhov et al. [36]. The investigators showed that increased cell-to-cell coupling increased conduction velocity of the action potential, similar to myocardial tissue. These data are compatible with the concept that conduction in the leaf of *Dionaea* is similar to that in a sheet of myocardial tissue.

5.3. Changes in electrogram morphology

The observation that the extracellular electrogram may start with an initial positive or negative deflection might be related to the fact that the potassium flux starts as early as the depolarizing phase [3]. If the balance between the outward calcium and inward potassium current shifts, the initial electrogram may reverse. Under variable environmental circumstances propagation of the action potential and/or physiological conditions may alter [31]. The reversal potential associated with the early transmembrane current changes with ionic concentration, as shown by the patch clamp studies of Lunevsky et al. [29].

By computational modelling of plant action potentials Cuin et al showed that potassium activity in *Arabidopsis thaliana* not only determines the length of an action potential but also the steepness of its rise and the maximal amplitude. They conclude that outward-rectifying potassium channels contribute to both the repolarization phase and the initial depolarization phase of the signal [41].

Changes of H^+-ATPase sensitivity to Ca^{++} may also alter the action potential morphology, although such changes will effect mainly the later phase of the action potential [36]. In the model of Sukhov at al. a profound difference was observed between cells with and without H^+-ATPase. Lohse and Hedrich demonstrated with the patch-clamp technique that auxins induced a shift of the voltage dependence of the anion channel to hyperpolarized potentials. The shift was attenuated during an increase in the extracellular chloride concentration, indicating that chloride shields the hormone-binding site [42].

These data indicate that changes in external circumstances may well alter the extracellular electrogram. In addition, unipolar electrograms recorded from the epidermis will also detect activation from activation in deeper layers (parenchyma, swelling and vascular cells). The remote signals generated by activation in the deeper layers may differ and mask the deflection of the electrogram at the epidermis. With the Laplacian this problem is circumvented, because this technique mainly records local activity.

5.4. Anisotropy

Although in heart muscle anisotropy has an important effect on conduction velocity (about a factor of 2 difference for propagation parallel and perpendicular to the fibre orientation), the effect of anisotropy is less outspoken for the extracellular electrogram. Cell cultures of neonatal rat cardiomyocytes (where cells are coupled by connexins after a few days of culture) show that the connexins are homogeneously distributed along the cell walls. In comparison, in adult cardiac tissue connexins are preferentially present at the long ends of the cells. This distribution in adult cells is responsible for the higher conduction velocity in the fibre direction. The morphology of the extracellular electrogram (as illustrated in Fig. 1) for

propagation in and perpendicular to the fibre direction is similar; there is mainly a difference in amplitude. Extracellular electrograms derived from cell cultures of cardiomyocytes with the evenly distributed connexins along the cell walls show a similar morphology as in adult myocardial tissue. The ion channel expression of the various ions that determine depolarization and repolarization differ markedly between neonatal and adult cardiomyocytes. In neonatal cells calcium ions play for instance an important role for depolarization. Based on these observations in heart tissue we feel that the possible anisotropic characteristics of the Dionaea cells as well as the fact that depolarization is based on calcium ions only play a minor role for the morphology of the extracellular electrogram.

5.5. Interpretation of the morphology of the unipolar electrogram in Dionaea muscipula

Fig. 10 summarizes our observations regarding electrogram morphology. It shows an extracellular electrogram recorded at a distance of 1 cm from a trigger hair that was activated mechanically. Four deflections are present that are marked 1 to 4. The initially negative deflection points to an inward current. Activating the trigger hair initiates a receptor potential that causes calcium ions to move from extracellular space into the cell comparable to the initial sodium current in cardiomyocytes [43]. Because activation passes underneath the electrode, one would expect an initially positive deflection caused by a capacitive current as present in cardiomyocytes. The upstroke velocity of the action potential of a *Dionaea* cell is, however, less steep than that of a cardiac action potential (0.5 V/s versus 100 V/s, respectively). Because the membrane capacity of heart and plant cells are similar (1 $\mu F/cm^2$), the capacitive current in *Dionaea* cells will be a factor of 200 smaller and, therefore, virtually indiscernible. Thus, the initial deflection in *Dionaea* cells is a direct reflection of the activating ionic current.

The initially negative deflection caused by the inward calcium current at locations close to the activated trigger hair is small (see Fig. 3A), because only a small number of cells are involved at this early stage of activation (size of the activation front has to build up). At a distance, the number of cells involved increases and therefore the ionic current increases as well. This results in a larger negative deflection as shown in Fig. 10 (arrow 1). For cardiomyocytes the same reasoning is valid, although the initial negative deflection close to the site of onset of activation is typically large. The reason is that the sodium current during activation is large in cardiomyocytes. Further away from the site of activation the negative deflection also increases in amplitude in myocardial tissue [44]. The subsequent positive deflection in *Dionaea* (marked 2 in Fig. 10) is caused by a net outward current. This can be a potassium current that starts already early after activation in plant cells, but sodium current is a good option as well [45]. At rest, potassium channels support inward current because the membrane potential is more negative than the Nernst potential for K^+-ions [33]. However, if the membrane potential reaches the reversal potential (between −20 and −60 mV) the membrane potential is less negative than the equilibrium potential for K^+ and the potassium current may become outward generating a positive deflection for the extracellular electrogram.

The next negative deflection 3 is possibly caused by the activated chloride current. Calcium entrance activates chloride channels in plant cells, which results in an inward current because the chloride ion is negative. Intracellular chloride concentration is high compared to the extracellular concentration (22 versus 0.4 mol/m^3). Therefore, the negative chloride ions move outward, which means that the resulting inward current results in a negative potential. Finally, a small positive hump arises caused by potassium ions flowing out of the cell during repolarization (arrow 4), but H^+-ATPase may be involved as well as shown before.

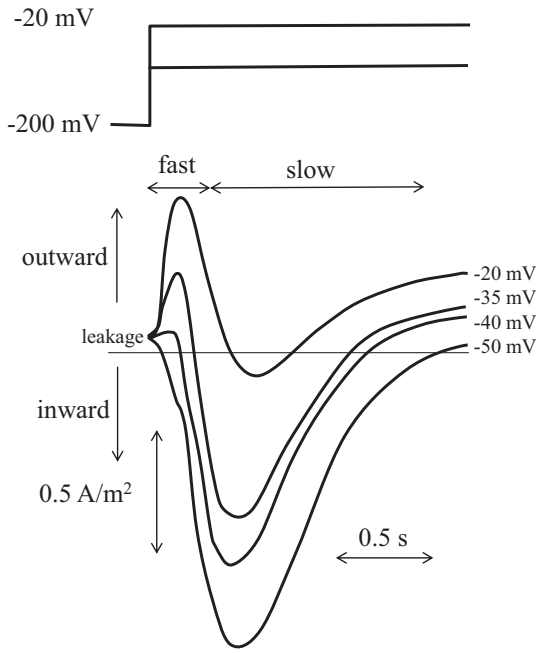

Fig. 11. Lower panel: Transmembrane current tracings in a cell from *Nitellopsis obtusa* after applying Heaviside voltage steps across the membrane. Curves reveal a fast and slow transient response and a leakage current. Numbers next to the curves indicate the step voltage. The fast component changes from inward to outward at a voltage of −45 mV, the slow component changes direction at a voltage of −40 mV. Adapted from Lunevsky et al. [29].

Fig. 11 shows voltage clamp data of a *Characean* internodal cell carried out by Lunevsky et al. [29]. The voltage clamp method measures the ion currents through the membrane after a change in the voltage over the plasma membrane (step function). The upper tracings show the step changes starting from a holding voltage of −200 mV. The lower tracings show the current density through the plasma membrane for different step values.

In case the peak potential of the action potential amplitude is less negative than −45 mV the initial ionic current is outward and the extracellular signal starts with a positive deflection. The outward current can only be generated by sodium or potassium ions flowing out of the cell and this current seems to overrule the inward current caused by the calcium ions. It has been shown that an increase in calcium concentration in the plant cell activates potassium channels to open [46,47]. Nevertheless, one would expect an initially inward current if calcium is the initiator of depolarization. A problem with the transmembrane currents in Fig. 11 is the fact that a Heaviside (step) function for the voltage clamp is used. In reality the action potential voltage (and time) determines the transmembrane currents and the upstroke of the action potential is less steep than that of the step function.

5.6. Why are there pronounced differences in the morphology of the extracellular electrograms of Dionaea muscipula?

As shown, there are large *differences* in the morphology of the unipolar extracellular electrograms of the *Venus flytrap*, depending on temperature, calcium concentration and recording site. The origin of these differences is not quite clear. In comparison, the cardiac unipolar electrograms are spatially and temporally much more consistent.

The differences can be explained by the observation that electrophysiology of plant cells is more complex compared to car-

diomyocytes amongst others because of the presence of prominent vacuoles. Vacuoles are, as cells, surrounded by a plasma membrane. Differences in ion concentration inside and outside the vacuole give rise to a vacuolar membrane potential. Data about total transmembrane current during voltage clamp of *Dionaea* are not known to us, but electrophysiological data of *Characean* cells that have been studied with voltage patch-clamp by Lunevsky et al. may throw some light on the observed differences in electrogram morphology [29]. Lunevsky et al. recorded transmembrane plasmalemma ionic currents in *Characean* internodal cells by applying step changes in plasmalemma voltage from a resting membrane potential of −200 mV to a value up to −20 mV. Results showed that the transmembrane currents exhibited 2 components of a transient current, a fast current followed by a slow current and a leakage current. At step voltages more negative than −45 mV, the fast current was inward, whereas this current was outward for step voltages that are less negative than −45 mV (Fig. 11). They also showed that the slow transient current has its reversal potential at −40 mV; for step voltages more negative than −40 mV, the slow transient current was inward, whereas it was outward for values more positive than −40 mV. Thus, if the maximum action potential voltage changes around −45 mV, the transient fast transmembrane current may change direction and consequently the extracellular deflection will change from positive to negative or vice versa.

As shown by Hodick and Sievers, the maximal action potential amplitude of *Dionaea* muscipula highly depends on the extracellular calcium concentration. There is a shift of approximately 25 mV per tenfold increase in concentration [12]. Changes in calcium concentration do not significantly alter the resting membrane potential. In addition, temperature affects action potential parameters. As temperature increases, the amplitude of action potential is decreased and duration reduced [48,49]. These effects may explain the change in morphology of the extracellular electrogram as observed in Fig. 6. Because temperature increases over the interval it is suggestive that temperature is one of the factors that influence the morphology of *Dionaea*. This could well fit with the observation that at higher temperatures the action potential amplitude decreases. As illustrated in Fig. 11, a membrane voltage of the step function from −20 mV to −50 mV changes the polarity of the fast transient response from positive to negative. Temperature will likely not be the only factor, because moisture of soil may change with time and alter extracellular concentration of ions. Alternatively, time may play an additional role if a leaf is activated at different periods. Fig. 8 shows an example of this time dependence in which a double activation caused trap closure but a single not.

5.7. Laplacian signals

The extracellular electrogram is a reflection of the transmembrane current; it is the weighted summation of transmembrane currents from all cells around the recording spot. A marker of the local transmembrane current is the Laplacian signal [22–25]. This signal only reveals potentials generated by local electrical activity; deflections generated by distant electrical events are not present. For activation in cardiac tissue the Laplacian signal mimics the extracellular electrogram. However, deflections of Laplacian signals are sharper and remote deflections are virtually absent. The contribution of transmembrane currents to the extracellular electrogram will be smaller the further the contributing site is away from the site where the recording electrode is positioned. Summation (integration) of the transmembrane currents makes the signal broader and less steep than that of the local transmembrane current, the reason that the unipolar electrogram is less sharp. We have applied the Laplacian technique to activation maps form which conduction velocity could be reliably determined.

6. Conclusions

In this study we showed that the extracellular electrogram from activation in *Dionaea muscipula* leaves differs from electrograms recorded in mammalian myocardium. Whereas the extracellular electrogram recorded in heart tissue at sites where activation passes is mainly biphasic, the electrogram recorded from *Dionaea* leaves may reveal three different morphologies: purely positive, negative or biphasic. Based on our data and literature we provided a possible explanation for the variability in the extracellular electrograms of *Dionaea*. This could be due to alterations in the amplitude of the action potential, which can arise because of temperature changes or variations in ion concentration in extracellular space. Although determining the activation time from myocardial electrogram is straightforward in healthy tissue, its assessment is difficult in *Dionaea* signals. Because Laplacian signals are a better marker for local activation and the initial activation in *Dionaea* cells are initiated by calcium current, it is suggestive that the Laplacian signal can be used preferentially to determine activation times and conduction velocity. We determined conduction velocity reliably from isochronal maps and documented that conduction velocity is faster in association with trap closure than without trap closure. These specific conditions observed in *Dionaea* permit a rapid propagation of the impulse leading to rapid closure of the trap despite the inherent slow ionic processes relative to the cardiac muscle.

The analysis of plant electrograms may be helpful for analysis of complex electrograms in heart tissue that occur under pathological conditions. Ion currents may change and for instance calcium current may play a more prominent role for instance during ischemia. Knowledge about the electrograms in *Dionaea* might be helpful in analysing such more complex electrograms derived from heart tissue.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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