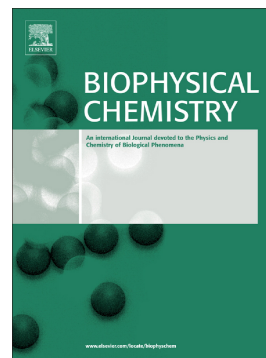


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Electron current recordings in living cells

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Running title: Electron currents in living cells

Key words

cytochrome *b561*, *Xenopus* oocytes, ascorbate, iron

Highlights

- living cells use electricity for a plethora of physiological processes;
- ion currents in channels and transporters are very well investigated by electrophysiological techniques;
- it is equally possible to record electron currents mediated by specialized redox proteins.

Abstract

Living cells exploit the electrical properties of matter for a multitude of fundamental physiological processes, such as accumulation of nutrients, cellular homeostasis, signal transmission. While ion channels and transporters (able to couple ions to various substrates) have been extensively studied, direct measurements of electron currents mediated by specific proteins are just at the beginning. Here, we present the various electrophysiological approaches that have allowed recordings of electron currents and highlight the future potential of such experiments.

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1. Introduction

Electrical phenomena are governed by the general principle of electroneutrality: each negative charge must be associated with a positive charge (for a critical view see [1]). Therefore, to separate two charges of opposite sign, physical work must be employed. Because the electric field is conservative, this work is returned when the system is allowed to go back to equilibrium. Living cells exploit this law in various ways. Specialized proteins capable of separating ionic charges at the edges of the plasma membrane create a membrane potential. In the case of animal cells, this task is performed by the sodium/potassium antiporter, which creates a potassium gradient with the cytosolic K^+ concentration (about 100 mM) being higher than the external K^+ concentration (about

5 mM). The opening of potassium channels drives the membrane voltage to hyperpolarised values close to the equilibrium (Nernst) voltage of potassium, i.e. about -70 mV [2]. In plant cells, the membrane voltage has a different origin, can reach more negative values of up to -200 mV and is created by the combined action of H⁺-ATPases and potassium channels [3].

1.1. Ion and gating currents mediated by ion-selective channels

A very important class of membrane proteins able to detect variations in membrane potential is represented by voltage-gated cation channels. They have a tetrameric structure [4]; the single monomer is equipped with a voltage sensor, an alpha helix called S4 segment, able to perform significant movements upon physiological changes of the membrane potential. The topological variations of the S4 segment are then relayed to different parts of the protein (segments S5 and S6), which allow the opening and closing of the ion permeation pathway of the channel, a mechanism generally called gating [5]. In voltage-dependent channels, gating and selectivity, i.e. the ability of the channel to discriminate between ionic species, are mediated by different structural parts, the latter being determined by the precise assembly of four short segments (P-loops). In voltage-gated proteins, two distinct types of currents can be measured: 1) currents associated with the movement of the S4 segments, the so-called *gating currents*. This type of current is intrinsically transient, since the voltage sensor is anchored to the membrane, and difficult to detect, given the small amount of charge involved [6]; 2) currents associated with ion permeation, which have been investigated in cells of many organisms, including, for example, sponges [7,8] and mussels [9], marine [10,11] and aquatic [12] plants. These currents are modulated by a multitude of factors, i.e. oxidizing and reducing agents [13–16], polyunsaturated fatty acids [17], antibiotics [18], divalent ions [19–21], accessory proteins [22], in some cases even by differences in the voltage stimulation protocol [23]. The composition of the channel can also play an important role; for example, plant potassium channels are tetramers, which can be homomeric or heteromeric, likely according to the status of the plant, with different functional properties in potassium uptake [24–27]. Finally, fine structural differences can result in opposite functional properties such as inward or outward rectification [28]. Recently, an increasing interest in intracellular channels, namely the channels localised in compartments and organelles inside the cell, has also emerged and novel approaches to study their biophysical properties have been developed (see for example [29–32]).

1.2. Ion transporters

Ion transporters have also been intensively investigated. Differently to ion channels, in which the permeating ions simply follow their electrochemical potential, transporters employ a coupling mechanism between an ion and a specific substrate, which allows the substrate to move against its electrochemical gradient at the expense of the electrochemical potential of the coupled ion. For example, the plant proton/sucrose symporter, essential for phloem loading of sucrose (in special

cases also for the unloading), is able, to accumulate up to 1 M sucrose inside the phloem from an external concentration of few millimolar, by using the strong electrochemical gradient for protons (with pH 5.5 in the apoplast and pH 7.2 in the symplast, together with about -200 mV of membrane voltage) [33]. In transporters, the coupling mechanism is achieved through a conformational change of the protein [34], causing the turnover rate to be usually much lower compared to the values found in ion channels. An ion channel with a single channel conductance of 10 pS, stimulated by a 10-mV potential difference, allows the movement of about 600.000 ions per second, while the turnover rate of the proton/sucrose cotransporter has been estimated to be 500 ions per second [35]. Therefore, the currents mediated by transporters are generally small, even though their expression levels are usually higher than that of ion channels. In transporters, currents also exhibit two components: a transient component, called presteady-state current (Figure 1a, b), and a stationary phase associated with the ion/substrate cotransport (named transport-associated current, I_{tr}) The presteady-state current usually occurs in the absence of the substrate to which the ion is coupled. The Peres group has proposed an interesting hypothesis about their origin: they would be the manifestation of the ion movement trapped within the carrier in the absence of the substrate; following the application of the substrate the ion would be unlocked and contribute to the transport-associated current [36]. This hypothesis, which arises from the surprising finding that transport-associated current amplitudes in the GAT1 sodium/GABA cotransporter can be accurately predicted from the mere knowledge of the presteady-state current amplitude [37], was also confirmed in the plant proton/sucrose symporter [35] (Figure 1c).

2. Direct recordings of electron currents

In plants and animals, electron transfer reactions play a major role in fundamental physiological functions like photosynthesis and respiration. However, the first example of a three-dimensional molecular structure of a photosynthetic reaction centre was from the purple bacterium *Rhodospseudomonas viridis* [38] and, after this discovery, theoretical models explaining the primary charge separation process were developed (see [39] as an example). In plants and animals, protein complexes like cytochrome c oxidase in mitochondria and photosystem I in chloroplasts transfer electrons from an electron donor on one side of the membrane to an acceptor on the other side. Few other systems exist that perform trans-membrane electron transfer reactions in other membranes, like plasma membranes or vacuolar membranes. These include b-type cytochromes of the NADPH-oxidase and Cytochrome b561 families. However, in spite of the multitude recordings of currents mediated by ion channels and transporters, there are only two examples of electron current recordings.

2.1 NADPH oxidase

The first electron current recording was performed by the Krause group in 1998 [40], by using the patch-clamp technique applied on human eosinophils. They were able to provide strong experimental evidence of electron transfer from cytosolic NADPH to extracellular oxygen mediated by NADPH oxidase. This process reduces extracellular oxygen to superoxide, which, in turn, rapidly dismutates into hydrogen peroxide; the latter is mostly converted by myeloperoxidase [41] into hypochlorous acid, which is very effective to attack invading bacteria [42]. NADPH oxidase is an enzyme complex formed by the assembly of two membrane proteins with four cytosolic proteins, and involved in innate immunity through ROS (Reactive Oxygen Species) production. Mutations in one of its components, either altering or eliminating its function, gives rise to hereditary chronic granulomatous disease (CGD), in which patients have infections of various kinds [43]. An excellent recent review by Prof. DeCoursey [44] summarizes the electron current recordings mediated by NADPH oxidase in various cell types. Intriguingly, to remove protons released by cytosolic NADPH and to limit the depolarisation caused by the NADPH oxidase activity (both mechanisms inhibiting NADPH oxidase itself) a voltage-gated proton channel is working in parallel to NADPH oxidase [44]. Due to the multimeric nature of NADPH oxidase, which makes its expression in classical heterologous systems such as *Xenopus* oocytes demanding, and to the difficulties related to the handling of its canonical acceptor, molecular oxygen, a full functional characterisation of NADPH oxidase is lacking.

2.2 Cytochrome b561

Cytochrome b561, whose name derives from the characteristic reduced-minus-oxidised absorption band, are simple membrane proteins constituted by a single polypeptide with two heme groups facing both sides of the membrane [45]. By using intracellular ascorbate as an electron donor, they are able to reduce extracellular ferrichelates; the mouse cytochrome b561 (*dcytb*), for example, has been proposed to play a role in dietary iron absorption in the duodenum [46]. When expressed in *Xenopus* oocytes, these proteins show Fe^{3+} reductase activity, as evidenced by an appropriate colorimetric test [46]. Recordings using the double microelectrode voltage-clamp technique on *Drosophila* or soybean cytochrome b561 cRNA-injected oocytes have shown authentic electron currents [47,48]; these currents are generated by electrons derived from endogenous cytosolic ascorbate (Asc) and can be amplified by microinjection of exogenous Asc, as shown in Fig. 2a. An appropriate electron acceptor must be present in the extracellular bath solution to elicit measurable currents: ferricyanide proved to be the most efficient acceptor tested, even though ferrichelates such as ferric nitrilotriacetate (FeNTA) also induced detectable currents [47,48]. In accordance with the electronic nature of the currents, the exchange of any ionic species in the extracellular bath solution had no effect on the current magnitude. In soybean CYBDOM, mutation of the histidine in position 249 (into leucine), known to coordinate the heme group at the cytoplasmic side and to be conserved in all cytochromes b561, completely abolishes the ferricyanide elicited current without

modifying the protein expression level [48], providing strong evidence for electron movements involving the cytochrome's heme groups.

The oocyte system offers ideal experimental conditions to perform a detailed biophysical investigation, since it is possible to control all relevant parameters such as the membrane voltage as well as the cytosolic donor and extracellular acceptor concentrations. The transport mechanism of cytochromes b561 could be described by an extension of the Michaelis-Menten equation: $I = I_{\max} / (K_D / [D] + K_A / [A] + 1)$, where $[D]$ and $[A]$ are the donor and acceptor concentrations, respectively, K_D and K_A are two voltage-dependent constants linked to the apparent affinity constants of the donor and the acceptor, respectively, and I_{\max} is the voltage-dependent current obtained at saturating donor and acceptor concentrations [47]. Despite its simplicity, this equation was able to predict complex experimental responses of the cytochrome, such as the voltage dependence of the apparent affinity constants for the donor and the acceptor (see [47] for details). If this equation can be similarly applied to further members of the cytochrome family, is an interesting question for future research. It is noteworthy that cytochromes b561, similarly to ion channels and transporters, show presteady-state currents, whose molecular origin still needs to be clarified [47].

3. Conclusion

Electron current measurements mediated by specific transporters are only at the beginning. However, considering the existence of integral membrane proteins capable of trans-membrane electron transport and their general importance for the physiology of living organisms, the possibility to directly measure electron currents, together with the recent determination of high-resolution 3-D structures [49], likely indicate significant developments in this field of science in the near future.

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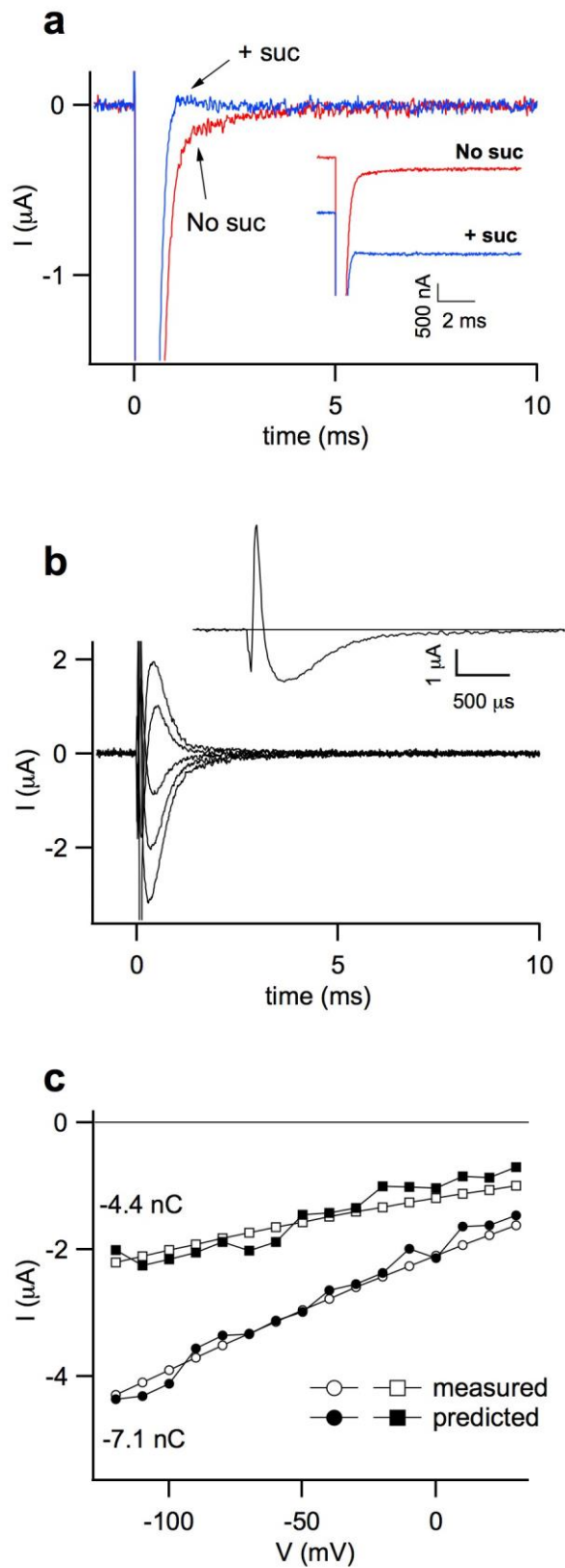
Figure legends

Figure 1: Stationary and presteady-state currents in the proton/sucrose symporter ZmSUT1.

a. In the inset, proton currents recorded in a ZmSUT1-expressing *Xenopus* oocyte, in the absence of sucrose (No suc) and in the presence of a saturating sucrose concentration (+suc) are shown. The same currents are displayed in the main panel after forcing their stationary level to zero. Holding and pulse voltages were -20 and -80 mV, respectively. b. Presteady-state currents vs time were obtained after subtracting the stationary currents in the absence and in the presence of saturating sucrose. Holding voltage -20 mV, voltage pulses from +40 mV to -120 mV (step -40 mV). In the inset: presteady-state current elicited by a voltage of -80 mV. c Measured (empty symbols) and predicted (filled symbols) currents displayed versus voltage for two oocytes with different ZmSUT1 expression levels. From Carpaneto et al. (2010), PLoS One, 5:e12605.

Figure 2: Cytochrome b561 currents recorded in *Xenopus* oocytes

a. Membrane currents recorded in a *Xenopus* oocyte expressing the soybean cytochrome CYBDOM, elicited by application of ferricyanide concentrations (arrowhead) ranging from 10 to 500 μ M, at a holding voltage of 20 mV. Black traces were recorded under baseline conditions using the endogenous pool of cytosolic ascorbate, red traces were recorded from the same oocyte after injection of 10 mM ascorbate. b. Dose-response curves showing the dependence of stationary currents in a on the applied ferricyanide (FeCN) under baseline conditions (black symbols) and after injection of 10 mM ascorbate (red symbols). Data were fitted with a Michaelis-Menten function (continuous lines). c. and d. Summary plots of the apparent maximum current at saturating [FeCN] (I_{max} ; c) and the apparent affinity constant for FeCN (K_{FeCN} ; d) using the endogenous pool of cytosolic ascorbate (Asc [cyt]) and after injection of 10 mM ascorbate (Asc [+10 mM]). From Picco et al. (2015), Plant Physiology, 169:986-95, copyright American Society of Plant Biologists, www.plantphysiol.org.



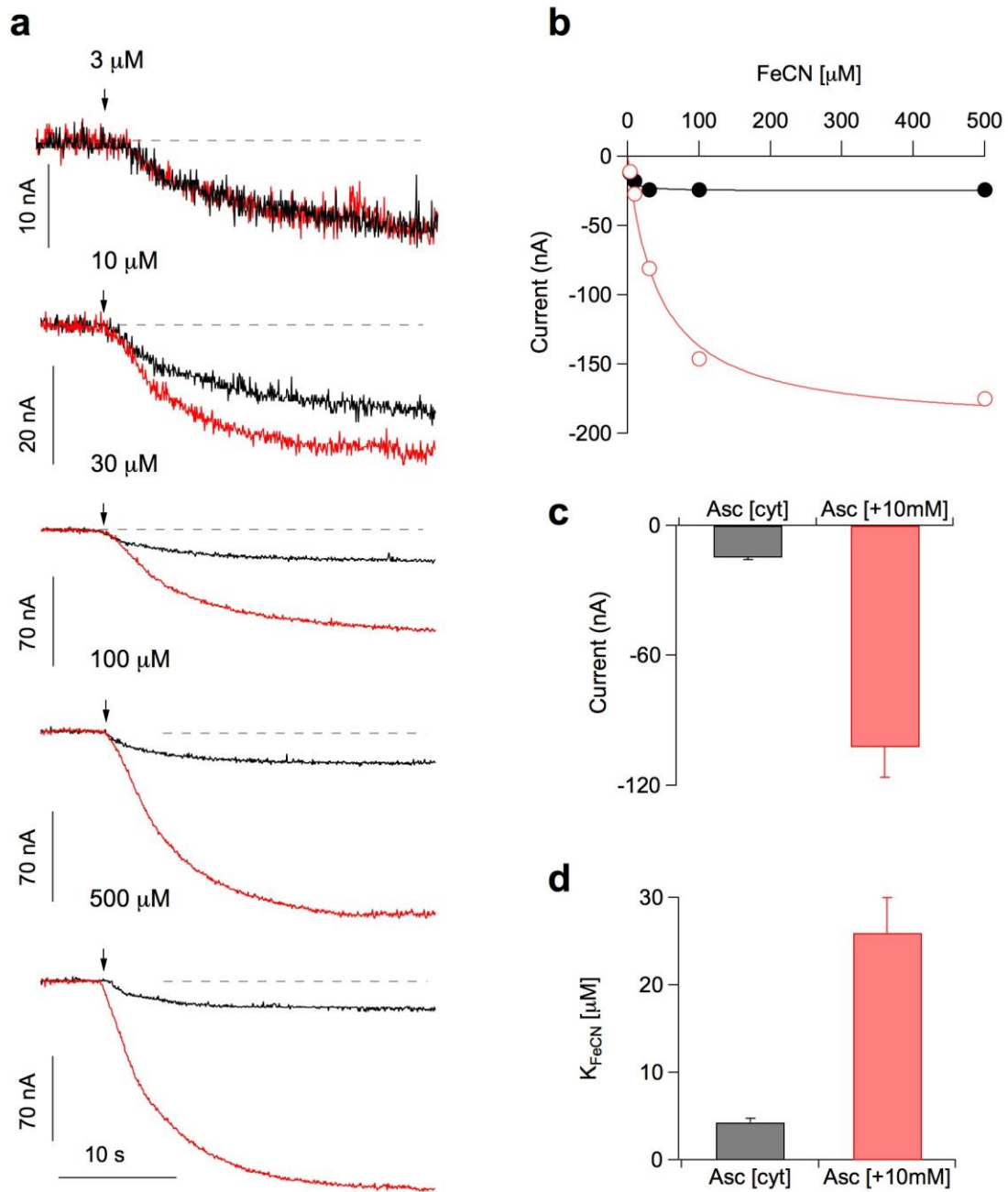
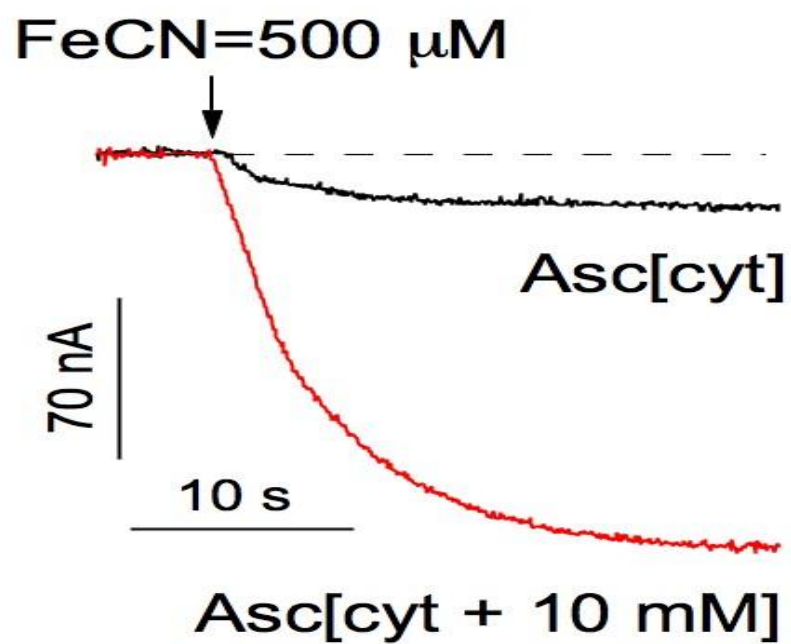


Fig. 2

A

Graphical abstract



ACCEPTED MANUSCRIPT