Biochimica et Biophysica Acta 1838 (2014) 1412–1419

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Sodium channels as gateable non-photonic sensors for membrane-delimited reactive species

Navin K. Ojha [a,1], Ehsan Nematian-Ardestani [a,1], Sophie Neugebauer [a], Benjamin Borowski [a], Ahmed El-Hussein [a,b], Toshinori Hoshi [c], Enrico Leipold [a], Stefan H. Heinemann [a,*]

[a] Center for Molecular Biomedicine, Department of Biophysics, Friedrich Schiller University Jena, & Jena University Hospital, Jena, Germany
[b] The National Institute of Laser Enhanced Science, Cairo University, Cairo, Egypt
[c] Department of Physiology, University of Pennsylvania, Philadelphia, USA

ARTICLE INFO

Article history:
Received 17 October 2013
Received in revised form 24 January 2014
Accepted 30 January 2014
Available online 7 February 2014

Keywords:
ROS sensor
Oxidation
Sodium channel
Channel inactivation
Channel gating

ABSTRACT

Reactive oxygen species (ROS) and reactive oxygen intermediates (ROI) play crucial roles in physiological processes. While excessive ROS damages cells, small fluctuations in ROS levels represent physiological signals important for vital functions. Despite the physiological importance of ROS, many fundamental questions remain unanswered, such as which types of ROS occur in cells, how they distribute inside cells, and how long they remain in an active form. The current study presents a ratiometric sensor of intracellular ROS levels based on genetically engineered voltage-gated sodium channels ($roNa_V$). $roNa_V$ can be used for detecting oxidative modification that occurs near the plasma membrane with a sensitivity similar to existing fluorescence-based ROS sensors. Moreover, $roNa_V$ has several advantages over traditional sensors because it does not need excitation light for sensing, and thus, can be used to detect phototoxic cellular modifications. In addition, the ROS dynamic range of $roNa_V$ is easily manipulated in real time by means of the endogenous channel inactivation mechanism. Measurements on ROS liberated from intracellular Lucifer Yellow and genetically encoded KillerRed have revealed an assessment of ROS lifetime in individual mammalian cells. Flashlight-induced ROS concentration decayed with two major time constants of about 10 and 1000 ms.

1. Introduction

Reactive species (RS), such as reactive oxygen species (ROS) and reactive oxygen intermediates (ROI), comprise a family of mostly small molecules capable of oxidatively modifying biomolecules such as nucleic acids, proteins, and lipids. Typical ROS are singlet oxygen ($^1O_2$), superoxide ($O^{\bullet}_2^-$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$) radicals, and hydrogen peroxide (H_2O_2). The major ROS sources endogenous to cells are the mitochondrial respiratory chain and the NADPH oxidases (NOXs). In addition, exogenous sources such as ionizing radiation, UV light, and cigarette smoke contribute to cellular RS exposure. The long-term effects of RS in organisms are often associated with deleterious symptoms such as degenerative diseases and molecular modifications implicated in aging [1,2]. However, RS also serve beneficial roles in the immune system [3] and are now increasingly considered important signaling molecules [4,5].

Given the relevance of RS for numerous physiological and pathophysiological processes, it is essential to understand the molecular mechanisms of their generation, distribution, and function. This, however, requires the precise real-time assessment of their concentrations and/or activities. For this purpose a number of fluorescent dyes have been developed in the past to report on intracellular oxidation events by changing fluorescent properties (for review see [6]). A prominent example is H_2DCF (dihydrodichlorofluorescein), a non-fluorescent low-molecular weight compound that is oxidized to the fluorescent DCF (dichlorofluorescein) when exposed to select reactive species. However, DCF cannot be targeted to specific cellular compartments, and its modification requires a Fenton reaction involving transition metals or an enzymatically driven process [7]. Therefore, genetically encoded RS-sensitive dyes have become very important tools. For example, mutants of the green fluorescent protein (GFP), in which the formation and breakage of a disulfide bridge result in spectral changes (e.g., roGFP2 [8]), bear many advantages such as potentially high sensitivity towards changes in the redox milieu and the option for subcellular targeting. Moreover, owing to two absorption maxima, roGFP2 yields a ratiometric fluorescent signal providing an optical readout that is independent of the concentration of the fluorescent probe itself. The sensitivity and dynamics of roGFP2 have been increased further by fusing it to glutaredoxin-1 allowing real-time imaging of the intracellular redox

* Corresponding author at: Center for Molecular Biomedicine, Department of Biophysics, Friedrich Schiller University Jena & Jena University Hospital, Hans-Knöll-Str. 2, D-07745 Jena, Germany. Tel.: +49 3641 9 39 56 50; fax: +49 3641 9 39 56 52.
E-mail address: Stefan.H.Heinemann@uni-jena.de (S.H. Heinemann).
[1] Both authors contributed equally.

0005-2736/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2014.01.031

potential [9]. Fusion of the bacterial protein OxyR with yellow fluorescent protein has led to the hydrogen peroxide-sensitive fluorescent probe HyPer [10].

Despite these advances, there is still a great need for RS sensors with optimized or specialized features. In the first place, all RS sensors named so far rely on the excitation with light to read-out the fluorescence; cells will be exposed to photons potentially generating RS and, hence, leading to phototoxicity. Intensity-dependent light-induced DNA damage can activate repair, or even apoptotic, pathways that fundamentally alter the cellular context in which an experiment is being performed. During experiments, repeated exposure to even low levels of UV light can affect the cellular system under investigation. Even visible (blue) light can exert deleterious effects as excitation of endogenous flavins may produce RS with adverse consequences [11]. Moreover, even when targeted to the plasma membrane, detection of RS-related processes directly at the membrane is technically very challenging. Currently available molecular RS sensors are not suited for single-molecule measurements and, last but not least, conventional RS sensors are always active, i.e. they cannot easily be employed for complex kinetic analyses.

The present work introduces an RS sensor based on a voltage-gated sodium (Na_V) channel that overcomes some of the limitations listed above. Na_V channels are large membrane proteins that conduct Na^+ upon membrane depolarization, primarily to initiate action potentials in neurons and muscle cells. Voltage-dependent activation (channel opening) occurs in about 100 μs upon membrane depolarization; it is followed by spontaneous closure of the channel within about 1 ms, a process referred to as inactivation. Both processes together yield a transient Na^+ inward current, in which the degree of inactivation after a certain time can be experimentally assessed with electrophysiological methods.

The Na_V channel protein consists of about 2000 amino-acid residues, organized in four homologous domains, each of which has six transmembrane segments (S1–S6) (Fig. 1a). Every domain harbors a pore/gate domain (S5–S6) and a voltage-sensing domain (S1–S4). The cytosolic linker between domains III and IV mediates rapid channel inactivation, where the conserved inactivation motif IFM (isoleucine–phenylalanine–methionine) is of prime importance [12]. Although the structural details are not yet understood, voltage-dependent translocation of the voltage-sensor domains is followed by a conformational change of the DIII–DIV linker with the effect that the IFM motif obstructs the permeation pathway and, hence, terminates Na^+ flow [13–15]. Oxidative modification of the methionine within the IFM motif (e.g., formation of methionine sulfoxide) results in a marked loss of inactivation that can be monitored in real time and with high precision by repeatedly recording currents mediated by Na_V channels using whole-cell patch-clamp technology [16]. Introduction of cysteine residues in this motif strongly enhances the sensitivity towards chemical modification [17,18].

Based on these properties, principally allowing for non-photonic ratiometric determination of oxidative reactions right at the plasma membrane, we equipped Na_V channels with cysteine residues in the inactivation motif to yield membrane-based RS sensors. The engineered Na_V channel most suitable as a cellular RS sensor with the mutated motif IFC ($roNa_V$) is characterized in detail, functionally compared with roGFP2, and used to determine dynamics and lifetime of RS in single living cells.

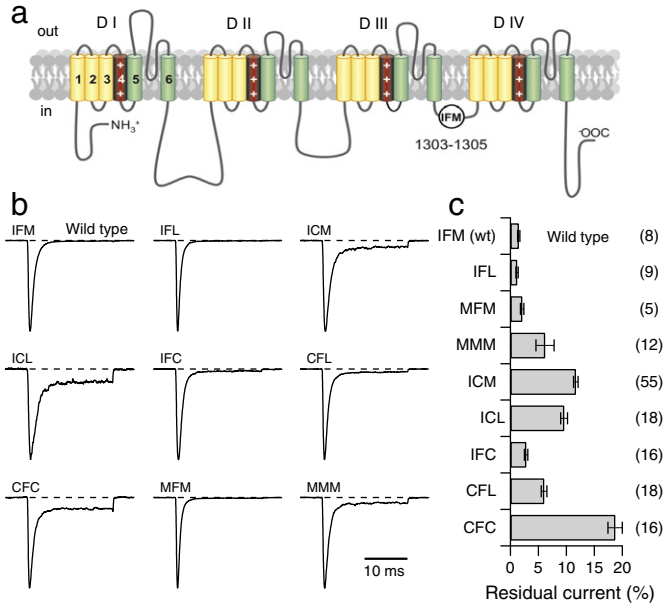

Fig. 1. Properties of point mutants. a) Transmembrane topology of a sodium channel α-subunit highlighting the inactivation motif "IFM". Numbers refer to rat $Na_V1.4$. b) Normalized current responses of $Na_V1.4$ wild type (IFM) and the indicated mutants expressed in HEK 293 cells in response to depolarizations to −20 mV from a holding voltage of −120 mV. c) The residual current at the end of the 20-ms depolarizing pulse relative to the peak current for the indicated mutants. Mean ± SEM (*n* is shown in parentheses).

2. Materials and methods

2.1. Expression plasmids and mutagenesis

The wild-type Na^+ channel construct used in this study was based on rat $Na_V1.4$ (SCN4A, P15390 [19]) in the plasmid vector pcDNA3. Mutants thereof were generated by site-directed mutagenesis and verified by DNA sequencing (also see [16]). Point mutants were introduced into the inactivation motif 1303IFM (Fig. 1a) and thus mutants are termed by this motif only, e.g. "IFC" for mutant M1305C. In addition, all Na_V channel constructs carried mutation M1316L to remove the potentially oxidation sensitive methionine in the inactivation linker. roGFP2 [8] and CD8 were on pcDNA3 plasmids. The genetically encoded photosensitizer KillerRed was from Evrogen (Moscow, Russia).

2.2. Cell culture

HEK 293 cells (CAMR, Porton Down, Salisbury, UK) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) mixed 1:1 with Ham's F12 medium and supplemented with 10% fetal calf serum in a 5% CO_2 incubator. Cells were trypsinized, diluted with culture medium, and grown in 35-mm dishes. Electrophysiological experiments were performed 1–5 days after plating. HEK 293 cells were transfected with the respective plasmids using the Rotifect® transfection reagent (Roth, Karlsruhe, Germany) following the instructions of the supplier. Cells not expressing roGFP2 were cotransfected with CD8 to identify transfected cells by means of anti-CD8-coated beads (Deutsche Dynal GmbH, Hamburg, Germany).

2.3. Electrophysiological measurements

Whole-cell voltage-clamp experiments were performed as described previously [20]. Briefly, patch pipettes with resistances of 0.7–1.5 MΩ were used. The series resistance was compensated for by more than 70% in order to minimize voltage errors. Perforated-patch clamp recordings were performed by adding escin at 1–10 μM [21] to the patch pipette solution yielding a series resistance between 3 and 20 MΩ after about 15 min in the on-cell configuration.

A patch-clamp amplifier EPC9 was operated by PatchMaster software (both HEKA Elektronik, Lambrecht, Germany). Holding potential was −120 mV. Leak and capacitive currents were corrected with a *p*/4 method with a leak holding voltage of −120 mV. Currents were low-pass filtered at 5 kHz and sampled at a rate of 25 kHz. All experiments were performed at a constant temperature of 19–21 °C.