IL NUOVO CIMENTO **46 C** (2023) 143
DOI 10.1393/ncc/i2023-23143-5

COMMUNICATIONS: SIF Congress 2022

Photostimulation mechanism of an amphiphilic azobenzene

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received 31 January 2023

Summary. — The development of new tools for controlling cell behavior is a hot argument in the scientific community, with important implications in bio-physics, medicine and the advancement of innovative biotechnology. Recently, we proposed an amphiphilic azobenzene, named Ziapin2, able to insert in the plasma membrane and modulate cell capacitance via photoisomerization. This phototransducer allows the use of light for living cell control. Here, we evaluate the dependence of the cell response on the exploited light power density. We enhance our knowledge of the stimulation mechanisms by experimental data and simulation directly correlating the cell response to the Ziapin2 photoisomerization process.

1. – Introduction

Describing and controlling cell behaviour has been an intriguing research goal since the eighteenth century, when Luigi Galvani discovered the possibility of controlling a frog leg twitch by applying an electrical stimulus [1]. This triggered a new research field, broadly known as electro-physiology, where metal electrodes are used to elicit cellular responses and control living organisms [2]. Even though electrical stimulation can be quite effective on a short time scale, the prolonged application of electrodes, their cumbersome wiring and the unavoidable tissue degradation due to heating are important drawbacks of this approach. Furthermore, signal cross talk and current spreading limit the time and space resolution of the stimulation. Aiming at overcoming these drawbacks, scientists started to exploit light as a triggering tool [3,4]. As living cells are not usually sensitive to light, phototransducers are required to absorb light and to transform it into a type of stimulus that cells can process. Several phototransducing mechanisms have been engineered and developed during the last couple of decades, exploiting the conversion of light excitation into electrical, thermal, mechanical or chemical stimuli [5-7]. To this end, different light radiation sources and absorbing materials can be employed, as deeply discussed in a recent perspective [8].

An innovative approach relies on photo-active molecules able to switch between two states due to light interaction [9]. The two states differ in steric hindrance, dipole moment or conjugation length. By targeting a specific cell compartment, the molecular switching can trigger a physiological response. In recently published papers [10, 11], we developed an amphiphilic azobenzene molecule, named Ziapin2, that is able to dwell into the plasma membrane. The presence of the molecule affects the membrane by modulating its thickness [12]. Molecular dynamic simulation, electrophysiological membrane capacitance and neutron scattering measurements showed that, when Ziapin2 is internalized, the membrane becomes thinner. Furthermore, being an azobenzene molecule, Ziapin2 is able to isomerize between two different conformers, *i.e.*, *trans* and *cis* isomers. *Trans* to *cis* isomerization is driven by visible light (the main absorption peak lies at

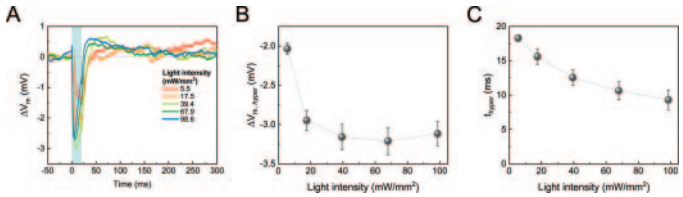

Fig. 1. – Light-induced membrane potential modulation (20 ms pulses) at different light intensities (A). Plots of the peak hyperpolarization changes $\Delta V_{m,hyper}$ (B) and t_{hyper} (C). Hyperpolarization peaks were measured as the minimum voltage. Error bars represent the sem, computed over $n = 14$ cells.

$\lambda = 470\,\text{nm}$), while the reverse transition can occur either thermally or be pushed by light. When photoisomerization takes place within the membrane leaflets, the membrane thickness changes, restoring the initial membrane condition. Photoinduced evolution directly affects the plasma membrane capacitance, inducing a two-step membrane potential modulation. Initially, hyperpolarization occurs as a direct effect of Ziapin2 photoisomerization, and subsequently there is a potential depolarization. This second step has been demonstrated to be able to trigger action potential generation in both neurons and cardiac cells [13, 14].

In this paper, we explore the investigation of the photostimulation mechanism by evaluating the dependence of the peak amplitude and of the time evolution of the membrane potential modulation on light power density used to excite Ziapin2 molecules. Starting from the already available information, we develop a mathematical model that correlates the molecule photoisomerization with the capacitance variations and with the subsequent membrane potential modulation.

2. – Results and discussion

2˙1. *Light intensity dependence.* – Initially, we evaluated the dependence of the membrane potential variations on light power density. These measurements were performed using the patch-clamp technique in a whole cell configuration [15]. This technique allows characterizing cells from an electrical point of view. We used a microelectrode consisting of a glass micropipette whose end is inserted into the cell, and a counter electrode placed in the extracellular medium. At this point, it is possible to record the membrane potential (current-clamp measurement), fixing the current injected into the cell ($I = 0$ in our case). Briefly, glass-seeded HEK-293 cells were incubated with Ziapin2 at 25 μM for 7 min. The samples were illuminated for 20 ms using a cyan LED ($\lambda_{ex} = 470\,\text{nm}$) with intensity from 5 to $100\,\text{mW/mm}^2$ [10, 11]. Figure 1 reports the light-induced membrane potential modulation in HEK-293 cell. In particular, we are interested in the maximum amplitude of hyperpolarization signal $\Delta V_{m,hyperpol}$ and in the time needed to reach this condition (called time to peak, t_{hyper}), as a function of the light power density. As expected, $\Delta V_{m,hyper}$ increases with the applied power density, quickly reaching a saturation value. The saturation of the membrane potential modulation, achieved at $30\,\text{mW/mm}^2$, suggests that the maximum amount of isomerized molecules is obtained below this power density threshold. Differently, the time to peak seems to follow a slightly different trend. Within the light intensity range under examination, we did not reach any saturation value. Our speculation is that by increasing power density, the photostationary state is achieved in a smaller time interval. A faster time induces a steeper capacitance variation producing a higher membrane potential variation. We examine this hypothesis in detail in the following sections by solving a set of differential equations that models this chain of phenomena, from molecule isomerization to membrane potential response.

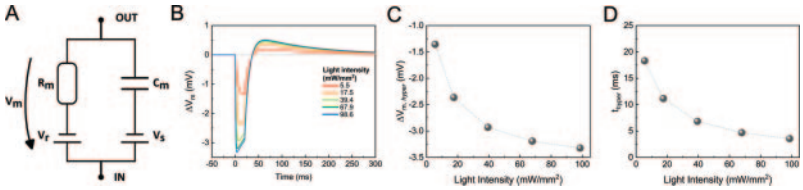

Fig. 2. – (A) Equivalent RC circuit of a cell. (B) Computed maximum cell hyperpolarization (C) and time to peak (D) as a function of the light intensity used for excitation.

2˙2. *Ziapin2 photoisomerization model.* – We know that, under illumination, Ziapin2 photoisomerization reactions take place, and a number of molecules in the thermodynamically stable *trans* state pass to the metastable *cis* state and *vice versa*. Being n_{trans} and n_{cis} the fraction of molecules in the *trans* and *cis* state, respectively, the isomerization process is described by equation $\frac{\mathrm{d}n_{trans}}{\mathrm{d}t} = -k_{TC}I(t)n_{trans}(t) + k_{CT}I(t)n_{cis}(t) + \gamma n_{cis}(t)$. Here, γ is the rate of the *cis* to *trans* thermal relaxation. k_{TC} and k_{CT} are the photoisomerization rates, normalized by light intensity (I), for the *trans* to *cis* and *cis* to *trans* processes, respectively. Considering an ensemble of molecules, all in the *trans* state, subjected to a light stimulus, switched on at time $t = 0$ with intensity I_0 and duration Δt, the population of the two isomers will reach a steady state condition, where the ratio between *cis* and *trans* population is given by $\frac{n_{cis}}{n_{trans}} = \frac{k_{TC}I}{k_{CT}I+\gamma}$. The build-up of the *cis* population will occur with a characteristic time constant $\tau_{build-up} = \frac{1}{(k_{CT}+k_{TC})I_0+\gamma}$, while the relaxation to the stable *trans* state after light off-set has $\tau_{relax} = \gamma^{-1}$ as the characteristic time constant. These two time constants will rule the photostimulation process once integrated into the plasma membrane electrical model. The cell membrane can be modelled using a simple circuit, composed of a parallel circuit between a capacitor (C_m) and a series circuit of a resistance (R_m) and a generator (V_r) (fig. 2(A)). The C_m capacitor accounts for the electrical insulation and charge accumulation provided by the membrane hydrophobic core of the lipid bilayer that lies between the intra and extracellular media, that are two electrolytes. The presence of charged groups in the phospholipids heads produces a surface charge, which generates a surface potential on each side of the membrane. The asymmetry in the composition of the two leaflets of the plasma membrane and in the ionic concentration of the electrolytes are responsible for an asymmetric surface charge distribution, and thus a potential surface difference between the internal and external edges of the membrane, which is represented by battery V_s in the equivalent circuit. Ion channels and pumps together with the intrinsic membrane permeability are represented by resistor R_m in series with a battery, whose potential V_r is given by the reversal potential of the conducting ions [16]. The time evolution of the membrane potential, as a consequence of membrane capacitance variations, is given by $\frac{\mathrm{d}V_m}{\mathrm{d}t} = -\left(\frac{V_m - V_r}{R_m C_m(t)} + \frac{V_m - V_s}{C_m(t)}\frac{\mathrm{d}C_m}{\mathrm{d}t}\right)$. Previously, by molecular dynamics [13], we showed the plasma membrane thinning is induced by two Ziapin2 dimerization processes taking place across the membrane. Light excitation of Ziapin2 breaks these dimers, producing a relaxation of the lipid bilayer to its original thickness. If we model the membrane as a plane capacitor, a change in the bilayer thickness produces a change in the membrane capacitance. Thus, Ziapin2 is able to modify the membrane capacitance, and with the aid of visible light we are able to modulate capacitance, as reported in [11,13]. As first order approximation, we can suppose that the membrane capacitance variation is proportional to the number of molecules in the *cis* state. Thus, membrane capacitance $C_m(t)$ is given by $C_m(t) = C_{m,0} + \Delta C_m(1 - n_{trans})$. We numerically solved the set of equations described above by evaluating the membrane po-

tential variations as a function of the isomerization rates. The azobenzene photoisomerization is usually strongly affected by the environment experienced by the molecule [17]. Moreover, directly measuring the isomerization dynamics in cells is non trivial and beyond the scope of this work. However, we can estimate the characteristic time constants of the photoisomerization based on previous data and looking at the dynamics of the membrane potential. The parameters of the *RC* equivalent circuit are usual values for HEK cells. Membrane potential dynamics (fig. 2) were simulated using the following parameters ($K_{CT} = 0.004\,\mathrm{mm^2\ ms^{-1}mW^{-1}}$; $k_{TC} = 0.01\,\mathrm{mm^2\ ms^{-1}mW^{-1}}$; $\gamma = 0.04\,\mathrm{ms^{-1}}$; $R_m = 5\,\mathrm{G\Omega}$; $C_{m,0} = 25\,\mathrm{pF}$; $\Delta C_m = -1\,\mathrm{pF}$; $V_r = -25\,\mathrm{mV}$ and $V_s = 100\,\mathrm{mV}$. The model is able to reproduce the characteristic features of the experimental data. In detail, increasing light intensity, we reach a sort of plateau in the membrane voltage modulation amplitude as a steady state condition is reached for the number of molecules in the *cis* form. Moreover, by increasing intensity, the time needed to reach the peak reduces. Indeed, this characteristic time is related both to the dynamics of the Ziapin2 population in the two different conformers and to the characteristic time constant of the *RC* circuit, that indicates how fast a cell responds to external stimuli. In this paper, we focused mainly on the *trans*→*cis* process observing a nice correlation between experimental and computed data. We did not investigate in detail the back thermal molecule relaxation process, which could affect both the obtained hyperpolarization and time to peak values.

3. – Conclusion

Ziapin2 is an azobenzene molecule that intercalates within the lipid bilayer and is able to modulate membrane thickness under illumination. We developed a Matlab script able to simulate the cell membrane potential modulation produced by the Ziapin2 photoisomerization inside the plasma membrane. We reproduced the power dependence of the membrane potential modulation starting from the ensemble dynamics of the *trans*→*cis* and *cis*→*trans* process. Even though a wide range of power density was evaluated, both experimentally and by simulation, we highlighted that a saturation of the triggering mechanism take place around $30\,\mathrm{mW\ mm^{-2}}$ suggesting this as the value required to reach steady-state *cis* population, with $n_{cis} = \frac{k_{TC}}{k_{CT}+k_{TC}} \simeq 70\%$.

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