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The effects of reiterated cell photo-stimulation with an azobenzene

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Summary. — Optical stimulation of living cells is an innovative and successful tool, having a significant impact on neuroscience. A recently synthesized amphiphilic azobenzene, named Ziapin2, has been developed exploiting a non-covalent approach for targeting the membrane and achieving cell photostimulation. Here, we investigate, using both experimental data and mathematical modelling, the effect of repetitive light stimulation on the cell response. We discuss some possible explanations for the drop in the stimulation efficiency in reiterated stimulation processes.

1. – Introduction

Since the 18th century, scientists have been studying and controlling living organisms with external stimuli. Today, it is well known that cells can be stimulated by exploiting many strategies. One of these is targeting the plasma membrane [1]. The plasma membrane is, in fact, a barrier that maintains the electrical potential of the cell and controls the transport of chemicals in and out of the cell itself, regulating signalling, replication and metabolism. Therefore, an efficient way to stimulate cells is to perturb the plasma membrane. Usually, electrical techniques are adopted to study and stimulate cells. However, despite high efficiency, the insertion of electrodes and wires greatly limits this approach in real-life applications [2]. For this reason, researchers have focused on other categories of stimuli, with light being one of the most studied tool [3]. Even if most living cells are naturally not sensitive to light, a number of techniques have been developed to render cells responsive to light [4]. The employment in clinical trials of Optogenetics is limited by its invasiveness and ethical implications [5]. Interestingly, cells are responsive to a large variety of other types of stimuli and they can, therefore, be made indirectly light-sensitive by converting the radiation into a thermal, electrical, chemical, or mechanical stimulus [6]. Our approach consists in using a phototransducer which converts light into mechanical stimulation of the cell membrane. Ziapin2 is an amphiphilic azobenzene molecule that positions itself in the plasma membrane, with the two ionic-terminated

alkyl chains aligned with the heads of the polar phospholipids. Ziapin2 is able to switch between two configurations. Visible light absorption (470 nm) can trigger the isomerization from the *trans* to the *cis* isomer, while the reverse transition can either be driven by light or occur spontaneously [7, 8]. In particular, Ziapin2 forms dimer when in its *trans* configuration, causing membrane thinning and consequent increase in membrane capacitance. When exposed to visible light, *trans* to *cis* isomerisation takes place, breaking the dimer and rapidly leading to membrane relaxation and a reduction of the membrane capacitance. The photo-mechanical modulation of the membrane capacitance generates a fast cell hyperpolarization response followed by depolarisation. This mechanism is also able to trigger action potential firing in excitable cells [9,10]. In this paper, we investigate the cell membrane potential variations in response to a series of light pulses, linking the Ziapin2 molecular ensemble dynamics with the cell conduct. We performed experimental measurements through the patch-clamp technique and run mathematical simulation. In the latter, we connect the amount of Ziapin2 in each of the two isomers with the modulation of the membrane capacitance and therefore to the cell membrane potential.

2. – Results and discussion

The effect of Ziapin2 photostimulation on HEK cells has already been reported [7, 9, 11]. Briefly, HEK-293 cell were seeded on glass substrates and treated with Ziapin2 (25 μM for 7 min in physiological conditions). The electrophysiological response of the cells to light stimulation was evaluated through the current-clamp measurements in whole cell configuration. The membrane potential was recorded for 800 ms and during this period of time a light pulse of either 20 ms or 200 ms with power density of 68 mW mm^{-2} was shined on the samples. The 800 ms stimulation protocol was repeated 20 times without any delay between subsequent repetitions.

In patch-clamp measurements, a common procedure consists in acquiring several sweeps and averaging over them in order to increase the signal to noise ratio. This procedure is well accepted, as usually the cell does not change during the measurement stage. Instead, using Ziapin2 as a phototransducer and working with light pulses at 1.25 Hz, we noticed that the signal changes between subsequent sweeps, with the amplitude of the hyperpolarization signal reducing after a few sweeps. We observed that, during the first pulses, there is a decrease in the hyperpolarization peak which reaches a plateau in few pulses. Moreover, the longer the light pulses (20 or 200 ms), the faster the plateau is reached, at around 50% of the initial value. Using 20 ms light pulses, it takes around 10–12 sweeps to reach this plateau, while using 200 ms, 3–4 sweeps are enough (fig. 1). To gain further insights in this phenomena, we adopted the following model to reproduce the modulation of the membrane potential observed in the cells. Passive and active ion transporting mechanisms ensure an electrical potential difference across the cell membrane, with the inside of the cell negative with respect to the outside. The membrane potential is the result of an electrochemical equilibrium of two main factors: the asymmetric distribution of ions between the intracellular and extracellular space, and the selective permeability to ions of the membrane. This condition is modelled by a parallel between a capacitor and a resistor. The different ionic conductances can be grouped into an effective membrane resistance R_m. The resting membrane potential, determined by the Goldman-Huxley-Katz equation, is represented by a voltage source V_r. The insulating lipid bilayer, which separates two conducting media, behaves as a capacitor. Experimental values of the membrane capacitance are in the order of 1 μF cm^{-2}. Moreover, there is an accumulation of charges on the membrane capacitance even if

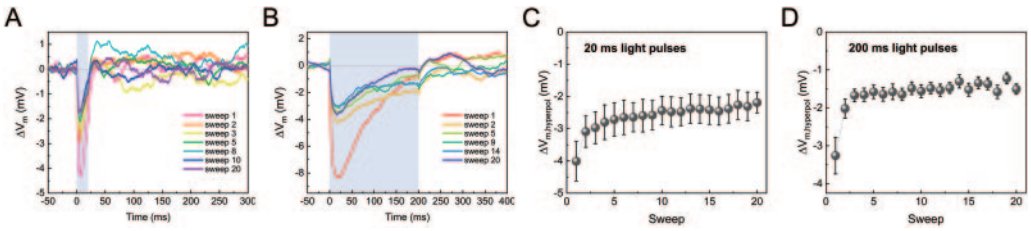

Fig. 1. – Ziapin2-mediated membrane potential photomodulation with 20 ms (A) or 200 ms (B) pulses. Relative hyperpolarization values with 20 ms (C) or 200 ms (D) as a function of the sweep. Error bars represent the SEM, computed over $n > 15$ cells.

the transmembrane potential is kept at zero. This is related to surface charges of the two leaflets of the plasma membrane (the inner side is generally more negative than the outer one) and to the different ionic composition of the extracellular and intracellular compartments, which produces a different distribution of the ions at the two sides of the membrane. This intrinsic potential can be modelled as a voltage generator V_s in series with the capacitance. Translating the exogenous stimulation of the cell into a change in one of the electrical parameters of the RC circuit allows reproducing the consequent membrane potential modulations. In our case, we assume the main effect of the change in thickness of the plasma membrane to be the change of membrane capacitance C_m. A change in capacitance [12,13] induces variations in the membrane potential according to $\frac{\mathrm{d}V_m}{\mathrm{d}t} = -\left(\frac{V_m - V_r}{R_m C_m(t)} + \frac{V_m - V_s}{C_m(t)}\frac{\mathrm{d}C_m}{\mathrm{d}t}\right)$.

As a first approximation, we assume proportionality between membrane capacitance and Ziapin2 fraction molecules in *cis*. The overall population is $n_{trans} + n_{cis} = 1$ where n_{cis} and n_{trans} are the molecules in *cis* and *trans* state, respectively. The population dynamics is described by $\frac{\mathrm{d}n_{cis}}{\mathrm{d}t} = k_{TC} I n_{trans} - (k_{CT} I + \gamma)\, n_{cis} = k_{TC} I - ((k_{CT} + k_{TC})\, I + \gamma)\, n_{cis}$.

Where $k_T C\,(k_C T)$ is the $trans \to cis$ ($cis \to trans$) isomerization rate, normalized by light intensity I, and γ is the thermal relaxation rate from the *cis* to the *trans* isomer. In dark conditions ($I = 0$), the population dynamics reduces to $\frac{\mathrm{d}n_{cis}}{\mathrm{d}t} = -\gamma n_{cis}$.

We hypothesized that there is a fraction of Ziapin2 molecules, for which the relaxation to the *trans* isomer is somehow slowed down. Thus, a certain amount of population in the *cis* state is long living (with a lifetime of the order of few seconds) reducing the amount of molecule available for photoisomerization when a second light pulse comes. There are several phenomena that could explain this. We know that Ziapin2 isomerization photophysics is strongly affected by the different plasma membrane domains [8] resulting in complex dynamics. We speculate that some Ziapin2 molecules end up being "trapped" in membrane domains where the higher viscosity or specific molecular interaction hinders back-isomerization. Another possible conjecture is that, if two molecules in *cis* state are sufficiently close to each other, two apolar heads of Ziapin2 weakly interact, slowing down the thermal relaxation. According to our hypothesis, we introduce two separate Ziapin2 populations that independently evolve with two relaxation rates according to the previous equations. Being α the fraction of molecules belonging to the slow population, the membrane capacitance is given by $C_m(t) = C_{m,0} + \Delta C\,(\alpha \cdot n_{SLOW,cis}(t) + (1-\alpha)\, n_{FAST,cis}(t))$. Exploiting a custom-made Matlab code that solves the set of differential equations described above, we managed to nicely reproduce the experimental data. We used the following parameters, which

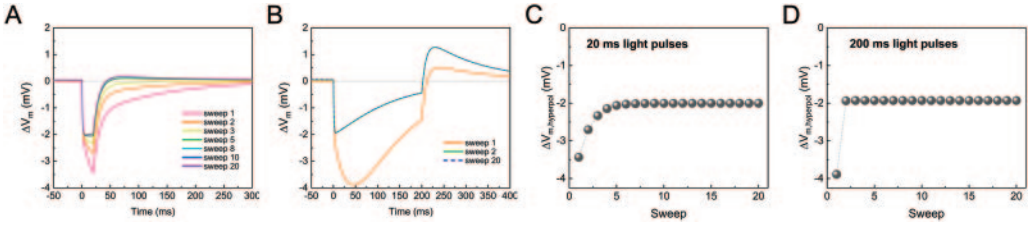

Fig. 2. – Computed membrane potential modulation as a consequence of membrane capacitance variations for 20 ms (A) and 200 ms (B) at different sweeps. Computed maximum cell hyperpolarization ((C), (D)) as a function of the number of sweeps for 20 and 200 ms, respectively.

have been retrieved from previous results [7] or estimated from the electrophysiological measurements: $k_{CT,slow} = 0.004\,\mathrm{mm^2\ ms^{-1}\ mW^{-1}}$; $k_{TC,slow} = 0.01\,\mathrm{mm^2\ ms^{-1}\ mW^{-1}}$; $\gamma_{slow} = 0.04\,\mathrm{ms^{-1}}$; $k_{CT,fast} = 0.05\,\mathrm{mm^2\ s^{-1}\ mW^{-1}}$; $k_{TC,fast} = 0.4\,\mathrm{mm^2\ s^{-1}\ mW^{-1}}$; $\gamma_{fast} = 0.1\,\mathrm{s^{-1}}$; $\alpha = 0.7$; $R_m = 5\,\mathrm{G\Omega}$; $C_{m,0} = 25\,\mathrm{pF}$; $\Delta C_m = -1.8\,\mathrm{pF}$; $V_r = -25\,\mathrm{mV}$; $V_s = 100\,\mathrm{mV}$. The plots in fig. 2 show the results of our simulations. The model replicates the characteristic features of the experimental data. In particular, both using 20 ms and 200 ms light stimulus we obtain a significant reduction of the peak hyperpolarization amplitude due to repetitive stimulation. After 10 and 2 light pulses we reach the plateau value, for the 20 ms and 200 ms stimulations, respectively. The nice agreement between experimental and simulated data supports the presence of two different Ziapin2 molecule populations that recover the *trans* state with different dynamics.

3. – Conclusion

In this work, we investigated the effect of a series of light pulses over the Ziapin2-mediated membrane potential modulation. We developed a custom Matlab code that simulates the molecule ensemble dynamics, the induced capacitance modulation and the effects over the membrane potential dynamics. We highlighted a correlation between the Ziapin2 back-isomerization and the membrane potential modulation. Based on our previous experience, we ascribed this phenomenon to different Ziapin2 populations influenced by different membrane domains.

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