Selective fluorophore-assisted light inactivation of voltage-gated calcium channels

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Abbreviations: ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FALI, fluorophore-assisted light inactivation; FRET, fluorescence resonance energy transfer

Fluorophore-assisted light inactivation (FALI) is an investigative tool to inactivate fluorescently labeled proteins by a mechanism of in situ photodestruction. We found that Ca_{1.2} (L-type) and Ca_{3.1} (T-type) calcium channels, labeled by genetic fusion with GFP derivatives, show differential sensitivity to FALI. Specifically, FALI silences Ca_{1.2} calcium channels containing EYFP-labeled \( \alpha_{\text{1C}} \) subunits but does not affect the EYFP-\( \alpha_{\text{1G}} \) Ca_{3.1} calcium channels or Ca_{1.2} channels containing EYFP-labeled \( \beta \) subunits. Our findings limit the applicability of acceptor photobleaching for the measurements of FRET but open an opportunity to combine the fluorescent imaging of the live cell expressing labeled calcium channels with selective functional inactivation of their specific subsets.

Introduction

Fluorophore-assisted light inactivation (FALI), an investigative tool to inactivate fluorescently labeled proteins by a mechanism of in situ photodestruction, is a potentially important method to study multisubunit complexes, such as voltage-gated calcium channels. Previously, the chromophore-assisted light inactivation (CALI) was demonstrated with the voltage-gated L-type (Ca_{1.2}) calcium channel genetically fused to small tetracysteine motifs that specifically bind in situ the membrane-permeable red biarsenical dye ReAsH. The light-induced inactivation in FALI or CALI with coherent or diffuse UV-light irradiation is based on photooxidation of the protein by singlet oxygen as the mediating reactive species that has a half-maximal radius of damage of ~40 Å. The Aequorea victoria green fluorescent protein (GFP) and its enhanced yellow (EYFP) and cyan (ECFP) variants have become the unique research tools in the functional studies of ion channels. The great advantage is that genetic fusion of these GFP variants to the amino- and/or carboxyl-termini of the Ca_{1.2} \( \alpha_{\text{1C}} \) and \( \beta \) subunits doesn’t compromise the membrane localization and the functional expression of the channel\(^{1,5} \) and requires no pretreatment of the expressing cells with membrane-permeable fluorescent dyes. In this work we compared the sensitivity of the EYFP-labeled Ca_{1.2} (L-type) and Ca_{3.1} (T-type) calcium channels to FALI. Our results show that the EYFP-labeled T- and L-type calcium channels are differentially sensitive to FALI. This property provides an opportunity to combine the fluorescent cell imaging of calcium channels with selective functional inactivation of their specific subsets in the live cell.

Results

Differential sensitivity of the Ca_{1.2} \( \alpha_{\text{1C}} \) and \( \beta \) subunits to FALI. The sensitivity of the EYFP-labeled \( \alpha_{\text{1C}} \) and \( \beta \) subunits to FALI was tested with Ca_{1.2} calcium channels functionally expressed in COS1 cells. These cells, naturally deprived of calcium channels, incorporate recombinant Ca_{1.2} into a fully functional Ca^{2+}-signaling pathway with spatiotemporal organization of CREB-dependent transcriptional signaling characteristic for cardiomyocytes. The whole cell calcium channel peak currents were recorded before, simultaneously with and after the termination of photobleaching of EYFP fused to the N- or C-terminus of the Ca_{1.2} \( \alpha_{\text{1C}} \) subunit. Continuous illumination (13 W/cm²) caused on average 75.0 ± 1.5% (1 min) and 86.5 ± 1.5% (2 min) photobleaching of EYFP (n = 10). The photobleaching of the labeled EYFP-\( \alpha_{\text{1C}} \) channels for 2 min irreversibly inhibited 80.2 ± 2.5% of the EYFP-\( \alpha_{\text{1C}} / \beta_{\text{1a}} \) channel current (n = 8, p < 0.001, Fig. 1A) and 82.1 ± 3.6% of the EYFP-\( \alpha_{\text{1C}} / \beta_{\text{1d}} \) channel current (n = 8, p < 0.001, Fig. 1B). This result shows that Ca_{1.2} channels are almost equally sensitive to FALI under condition when EYFP is present on the amino- or carboxyl-tail of \( \alpha_{\text{1C}} \) subunit. Thus, the region of \( \alpha_{\text{1C}} \)-sensitive to FALI mediated by EYFP is situated in a vicinity of the \( \alpha_{\text{1C}} \)-termini.

Both the amino- and carboxyl-termini of \( \alpha_{\text{1C}} \) point to the cytoplasm. In spite of a large molecular size (2,171 amino acids), there is FRET between EYFP and ECFP fused to the \( \alpha_{\text{1C}} \) amino- and carboxyl-termini indicating that these fluorophores are closer than 11 nm of each other in the functional Ca_{1.2} calcium channel complex. FRET was also observed between EYFP-\( \alpha_{\text{1C}} \) and...
Weak sensitivity of Ca\textsubscript{3.1} to FALI. In contrast to Ca\textsubscript{1.2}, the T-type Ca\textsubscript{3} calcium channels don't require auxiliary subunits to exhibit functional activity. The \(\alpha\) subunits of the T-type calcium channels share structural homology with the pore-forming subunits of the L-type calcium channels. Whether that homology is sufficient to mediate a comparable sensitivity of the Ca\textsubscript{3} calcium channels to FALI is an interesting question to be asked. Here we tested the effect of FALI on the Ca\textsubscript{3.1} channel composed of the N-terminally-labeled EYFP_{N-}\(\alpha\)\textsubscript{IC} (Fig. 1D). The peak T-type calcium current was decreased by 15.3 ± 3.5% (\(n = 8\)) after 3 min of illumination. In control experiments without photobleaching, rundown of the current did not exceed 2%. These data point to a weak sensitivity of Ca\textsubscript{3.1} to FALI, suggesting that the common structural features of the Ca\textsubscript{\(\alpha\)} family of pore-forming proteins, such as the four structural repeats each composed of six transmembrane regions folded around the central pore, is not key to the high sensitivity of \(\alpha\)\textsubscript{IC} to FALI.

Discussion

Our results indicate that EYFP photobleaching robustly accelerates run-down of the Ca\textsubscript{1.2} calcium channel containing the EYFP-labeled \(\alpha\)\textsubscript{IC} subunit, but not EYFP\(_{N-}\)\(\beta\). This may be a result of specific oxygen radical sensitivity of the \(\alpha\)\textsubscript{IC} subunit NH₂- and/or COOH-termini. We previously showed the importance of both \(\alpha\)\textsubscript{IC} termini for the Ca\textsubscript{1.2} calcium channel gating and inactivation.\(^7\) The described selective FALI is a new research tool to selectively inhibit the EYFP-\(\alpha\)\textsubscript{IC}-labeled Ca\textsubscript{1.2} channel subsets in the presence of the fully functional Ca\textsubscript{1.2} containing labeled Ca\textsubscript{\(\beta\)} subunits and/or labeled Ca\textsubscript{3.1}. The ability to manipulate the activity of specific subsets of the channel in situ, combined with quantitative fluorescent cell imaging of L- and T-type calcium channels, sets the stage for an experimental insight into the structure-functional organization of Ca\textsubscript{1.2} clusters\(^8\) aimed at controlling calcium influx through the differentially labeled channels of various types and, more broadly, in optogenetics approaches to the mechanisms of calcium signaling.

Acceptor photobleaching is one of the common FRET tools in cell biology,\(^6\) but our results indicate that acceptor photobleaching method can’t be adequately applied to the measurements of FRET of the Ca\textsubscript{1.2} calcium channel \(\alpha\)\textsubscript{IC} subunits in live cells, because of uncertainty in the functional state of the channel. This conclusion may be generalized to other proteins because acceptor photobleaching may change the conformation of a protein and

the functional Ca\textsubscript{1.2} channels aimed at selective irreversible inactivation of an \(\alpha\)\textsubscript{IC}-labeled subset.

Weak sensitivity of Ca\textsubscript{3.1} to FALI. In contrast to Ca\textsubscript{1.2}, the T-type Ca\textsubscript{3} calcium channels don't require auxiliary subunits to exhibit functional activity. The \(\alpha\) subunits of the T-type calcium channels share structural homology with the pore-forming subunits of the L-type calcium channels. Whether that homology is sufficient to mediate a comparable sensitivity of the Ca\textsubscript{3} calcium channels to FALI is an interesting question to be asked. Here we tested the effect of FALI on the Ca\textsubscript{3.1} channel composed of the N-terminally-labeled EYFP\(_{N-}\)\(\alpha\)\textsubscript{IC} (Fig. 1D). The peak T-type calcium current was decreased by 15.3 ± 3.5% (\(n = 8\)) after 3 min of illumination. In control experiments without photobleaching, rundown of the current did not exceed 2%. These data point to a weak sensitivity of Ca\textsubscript{3.1} to FALI, suggesting that the common structural features of the Ca\textsubscript{\(\alpha\)} family of pore-forming proteins, such as the four structural repeats each composed of six transmembrane regions folded around the central pore, is not key to the high sensitivity of \(\alpha\)\textsubscript{IC} to FALI.

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thus influence the interpretation of FRET data. Thus, conclusions made on results obtained from acceptor photobleaching experiments should require more careful consideration.

**Methods**

**Molecular biology.** In this study we used the following Ca\(_{1,2}\) subunits: α\(_{1C}\) (GenBank ID: z34815), β\(_{1s}\) (M25817) and α\(_{δ-1}\) (U73483). The plasmids coding for N- and C-terminally labeled EYFP\(_{N-α_{1C}}, \) α\(_{1C}-\)EYFP\(_{C}\) and EYFP\(_{N-β_{1s}}\) were constructed as described earlier in reference 4 and 5. EYFP\(_{N-α_{1C}}, \) constructed as follows to fuse EYFP to the N-terminus of rat α\(_{1C}\) the coding sequence of pEYFP vector (Clontech) was amplified by PCR using 5'-ClaI linker and 3'-XhoI linker without stop codon. An

**Electrophysiology.** Patch-clamp recording of the Ba\(^{2+}\) current from COS1 cells was performed at 20–22°C using the Axopatch 200B amplifier (Axon Instruments) 48–72 h after transfection. The extracellular bath solution contained (in mM): 100 NaCl, 20 BaCl\(_2\), 1 MgCl\(_2\), 10 glucose, 10 HEPES, pH 7.4. The electrodes had resistance 3–6 MΩ and were filled with pipette solution containing (in mM): 110 CsCl, 5 MgATP, 10 BAPTA, 20 tetra-ethylammonium chloride, 0.2 cAMP, 20 HEPES, pH 7.4. The whole cell T-type calcium currents were recorded using the following external solution (in mM): 5 CaCl\(_2\), 166 TEA-Cl and 10 HEPES, pH adjusted to 7.4 with TEA-OH. The internal pipette solution contained the following (in mM): 125 CsCl, 10 EGTA, 2 CaCl\(_2\), 1 MgCl\(_2\), 4 Mg-ATP, 0.3 Na\(_2\)GTP and 10 HEPES, pH adjusted to 7.2 with CsOH. Currents were sampled at 2.5–5 kHz and filtered at 1 kHz. Voltage protocols were generated and data were digitized, recorded and analyzed using pClamp 8.1 software (Axon Instruments).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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