

Recombinant Fluorescent Ligand of Potassium Kv1.1 and Kv1.3 Channels: Design, Properties and Applications

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Abstract Here we report on the development of the recombinant fluorescent ligand RFP-AgTx2 by fusing tagRFP fluorescent protein through a flexible linker with a peptide blocker of Kv1.x (x = 1, 3) channels, agitoxin 2 (AgTx2). RFP-AgTx2 can be easily produced in *E. coli* cells and purified in the folded functionally active form. The yield of RFP-AgTx2 achieves 100 mg per 1 L of bacterial culture that makes it the cheapest fluorescently labeled ligand of Kv1 channels. It is shown that RFP-AgTx2 binds to chimeric KcsA-Kv1.1 and KcsA-Kv1.3 channels with low nanomolar dissociation constants and is displaced from these complexes with Kv1.x pore blockers such as tetraethylammonium, AgTx2, kaliotoxin and OSK1. RFP-AgTx2 is an advanced fluorescent molecular tool to image Kv1 channels in cells and tissues. We present and discuss the distribution of Kv1 channels highlighted with RFP-AgTx2 in living rat glioma C6 and pheochromocytoma PC12 cells.

1 Introduction

Potassium ion channels constitute one of the most widespread superfamily and are present in all organism from bacteria to mammals [1]. K^+ -channel modulators include metal ions, small organic molecules, venom-derived peptides and antibodies [2]. Peptide blockers from venomous animals (snakes, sea anemones, scorpions and others) are increasingly used in neurobiology due to their high affinity and selectivity. In particular, fluorescently labeled peptide blockers are widely applied for K^+ -channel imaging in cells and tissues.

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Three basic approaches are currently used for fluorescent labeling of peptide blockers. A fluorophore can be attached to the N-terminal NH_2 -group of the peptide at the last step of a solid phase peptide synthesis. Alternatively, peptide can be conjugated with an amino-reactive organic label in solution via any free NH_2 -group (including N-terminal group, amino-groups of Lys and Arg side chains). Pre-modified peptides bearing additional Cys residue or a biotinylated amino acid can be labeled with a thiol-reactive dye or a (strept)avidin-conjugated fluorophore, respectively. Main limitations of these approaches relate to the high cost of a commercial synthesis of a labeled peptide as well as to low reaction yields in the case of labeling in solution. Last limitation becomes a big problem when multiple sites of labeling are present in the peptide, and separation of different variants of labeled peptide is required.

Here we report on the development of a new type of fluorescent probes for the studies of voltage-gated potassium Kv1-channels. The new probe consists of the peptide blocker of Kv1-channels fused by a flexible linker with a fluorescent protein. In our bioengineering construct RFP-AgTx2, a red fluorescent protein TagRFP (RFP) and agitoxin 2 (AgTx2) are used as a fluorophore and a channel blocker, respectively. This recombinant chimeric protein is produced with a high yield in *E. coli* cells, preserves high affinity and specificity to target channels and has a wide spectrum of applications.

2 Materials and Methods

Chimeric RFP-AgTx2 protein was expressed in *E. coli* Rosetta-gami (DE3) pLysS strain transformed with pET23-RFP-AgTx2 plasmid. Recombinant protein was purified from soluble cytoplasmic fraction by Ni-affinity chromatography and further by gel-filtration on a Superdex 75 10/300 GL column.

E. coli BL21 (DE3) cells that express KcsA-Kv1.1 or KcsA-Kv1.3 hybrid channels in the inner membrane were obtained as described earlier [3, 4]. Protocols of cell cultivation and preparation of spheroplasts can be found elsewhere [3, 4]. Spheroplasts staining with RFP-AgTx2 and competitive binding with Kv1.x pore blockers was performed in the buffer: 10 mM Tris-HCl (pH 7.5), 0.1 % bovine serum albumin, 0.25 M sucrose, 10 mM MgCl_2 , 4 mM KCl, 0.3 mM EDTA. Recombinant agitoxin-2 (AgTx2), kalitoxin (KTX) and OSK1 were produced by us and purified as described earlier [5, 6].

Rat pheochromocytoma PC-12 cells was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10 mM HEPES, whereas rat glioma C6 cells were grown in a Dulbecco's minimum essential medium at 5 % CO_2 , 37 °C in a humidified atmosphere (95 %). Both growth media were supplemented with 8 % fetal calf serum (FCS) and 2 mM L-glutamine. The day before microscopic experiments C6 and PC-12 cells were transferred into corresponding culture medium with 2 % FCS but without phenol red (to reduce background fluorescence).

Cells were thoroughly washed with a Hank's balanced salt solution, incubated with RFP-AgTx2, rinsed again and analyzed with confocal laser scanning microscopy (CLSM).

CLSM measurements of spheroplasts were performed with the LSM 710 microscope (Zeiss, Germany) using the α Plan-Apochromat objective (NA 1.46×63) and the 543.5 nm excitation wavelength. Fluorescence of RFP-AgTx2 was registered within the 560–760 nm range. In CLSM of eukaryotic cells, fluorescence of RFP-AgTx2 was excited with the 561 nm wavelength and detected within the 570–650 nm region.

3 Results and Discussion

AgTx2 from *Leiurus quinquestratus* scorpion venoms is a well known high-affinity pore blocker of Kv1.1 and Kv1.3 channels that acts at subnanomolar concentrations. It can be produced as a recombinant molecule in *E. coli* [7]. To obtain recombinant fluorescent AgTx2, the peptide was fused to TagRFP via 50 a.a. linker, which included 6xHis-Tag. TagRFP is a widely used tag for cellular and animal fluorescent microscopy imaging, but it was never fused with peptide pore blockers of K⁺-channels. N-terminal location of TagRFP in the RFP-AgTx2 was dictated by the known structural organization of a blocker-channel complex, in which N-terminus of a peptide is exposed to solvent and not involved in interactions with a channel [7]. Long flexible linker was introduced between TagRFP and AgTx2 to avoid considerable disturbance of blocker-channel interactions.

After careful optimization of cell growth and protein expression, the high yield of recombinant RFP-AgTx2 was achieved (ca. 100 mg per 1 L of a bacterial culture). Fluorescence excitation and emission spectra of RFP-AgTx2 and TagRFP were found to be very similar (not shown) indicating correct folding of a fluorescent protein in RFP-AgTx2. Peptide portion of RFP-AgTx2 was cleaved off with TEV protease, isolated by reverse-phase HPLC and analyzed by MALDI MS. The measured molecular mass of the peptide corresponded to that of native AgTx2, in which three disulfide bonds were formed indicating that the peptide blocker adopted a correctly folded conformation in RFP-AgTx2.

To characterize affinity of RFP-AgTx2 to Kv1-channels, the spheroplasts were used, which expressed KcsA-Kv1.1 or KcsA-Kv1.3 hybrid channels in plasma membrane. As shown with the CLSM-based approach earlier [3–5, 7, 8], such hybrid channels bind specifically with pore blockers of Kv1-channels, and dissociation constants of these complexes correspond to the constants estimated with electrophysiological techniques on native Kv1-channels. As revealed with CLSM, RFP-AgTx2 but not TagRFP binds to KcsA-Kv1.x (x = 1, 3) channels on spheroplast membrane (Fig. 1) and demonstrates concentration-dependent saturation of binding in a low nanomolar range of concentrations (data not shown). RFP-AgTx2 binds very weakly to spheroplasts expressing KcsA and to

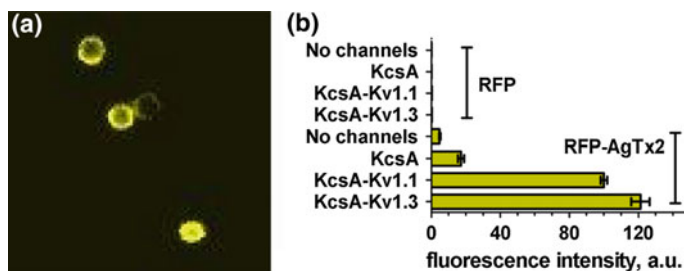


Fig. 1 **a** An example of CLSM images of KcsA-Kv1.3-bearing spheroplasts stained with RFP-AgTx2. Bar is 2 μ m. **b** Comparison of binding of RFP-AgTx2 (10 nM) and TagRFP (10 nM) to KcsA-Kv1.1-, KcsA-Kv1.3- and KcsA-bearing spheroplasts as well as to spheroplasts without recombinant channels (no channels)

spheroplasts without hybrid channels (Fig. 1b). RFP-AgTx2 is replaced from complexes with hybrid channels by Kv1 pore blockers such as tetraethylammonium, AgTx2, KTX and OSK1 (data not shown) that confirms additionally the specific type of interactions between RFP-AgTx2 and KcsA-Kv1.x ($x = 1, 3$). Evidently, RFP-AgTx2 is a reliable alternative to rhodamine labeled AgTx2, which is an essential component of our cellular systems for search and study of Kv1 pore blockers in animal venoms [5, 6, 8, 9].

High affinity of RFP-AgTx2 to Kv1.1 and Kv1.3 opens a possibility to apply fluorescent chimeric proteins for investigation of distribution of Kv1-channels in eukaryotic cells. In cells expressing Kv1-channels endogenously, amount of these channels can be rather low, and analysis of their localization and distribution in situ is a challenging task with any molecular instrument, either fluorescently labeled peptide blockers or antibodies.

C6 cells are known to express Kv1.1 channels [10]. RFP-AgTx2 accumulated in cytoplasm of C6 cells in granular structures (Fig. 2a). No membrane staining with

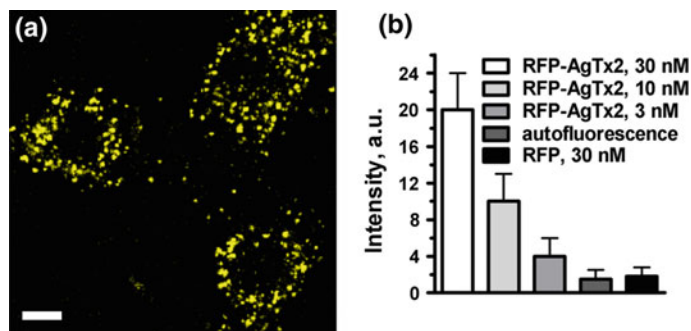


Fig. 2 **a** CLSM analysis of RFP-AgTx2 distribution in living C6 cells. Cells were stained with 30 nM RFP-AgTx2 for 1 h. Bar is 5 μ m. **b** Average fluorescence intensities of RFP-AgTx2 and TagRFP in C6 cells (1 h incubation) as compared to cellular autofluorescence intensity

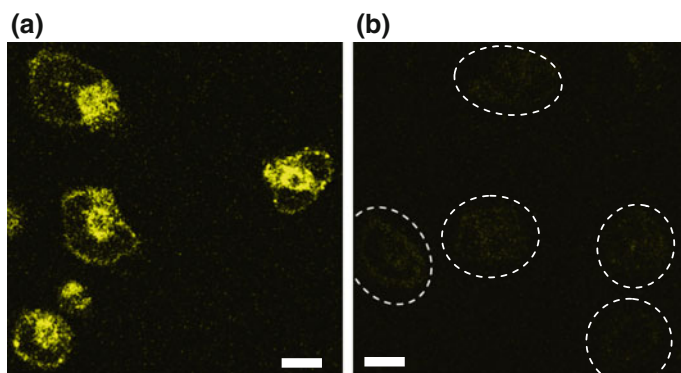


Fig. 3 CLSM analysis of distribution of RFP-AgTx2 (**a**) and TagRFP (**b**) in living PC12 cells. Cells were stained with 30 nM RFP-AgTx2 (**a**) or TagRFP (**b**) for 1 h. Bar is 10 μ m. *Dashed ovals* in panel **b** mark locations of cells

RFP-AgTx2 was detected. This pattern is reproduced at different concentrations of RFP-AgTx2. Intracellular accumulation of RFP-AgTx2 varies as a function of its extracellular concentration (Fig. 1b). One can suppose that RFP-AgTx2 binding to Kv1.1 on membrane of C6 cells is accompanied with fast internalization of complexes, intracellular release of the blocker and recycling of Kv1.1 channels to membrane.

In contrast to C6 cells, complexes of RFP-AgTx2 with Kv1.3 channels in PC12 cells are observed on plasma membrane, and only restricted fraction of RFP-AgTx2 is internalized (Fig. 3a). Distribution of RFP-AgTx2 on plasma membrane is non-uniform: particular bright clusters are distinctly observed. TagRFP does not bind to PC12 cells, and a level of autofluorescence was much lower than the RFP-AgTx2 signal in cells (Fig. 3b). These control measurements as well as data on endogenous expression of Kv1.3 (along with other Kv channels) [11] confirm that RFP-AgTx2 binding to PC12 cells is specific.

4 Conclusions

A novel RFP-tagged peptide blocker of Kv1-channel was obtained by simple protein engineering technique avoiding any chemical modifications. Using membrane-embedded hybrid KcsA-Kv1.1 and KcsA-Kv1.3 channels we confirmed that RFP-AgTx2 retained nanomolar affinity to Kv1 channels. We have demonstrated an applicability of RFP-AgTx2 for optical microscopy investigation of cellular distribution and trafficking of Kv1-channels in different cells as well as for CLSM-based analytical test-systems. We argue that tagged Kv1 pore blockers such as RFP-AgTx2 can be used for localization and imaging of their targets and can efficiently substitute peptide ligands labeled with organic fluorophores in most neurobiology applications.

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