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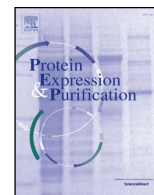

Functional characterization of cell-free expressed $K_v1.3$ channel using a voltage-sensitive fluorescent dye

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ABSTRACT

Using a cell-free expression system, we produced the $K_v1.3$ protein embedded in one step within detergent micelles. The protein was then purified and relipidated into mixed lipid bilayers. These proteoliposomes held an average of 0.8 protein per liposome. We examined channel forming activity using an oxonol VI fluorescent probe and verified its inhibition using margatoxin and ShK toxins. This assay was automatized and optimized so as to get a Z′ statistical factor acceptable for venom fraction screening. We obtained a sensible amount of membrane protein using the cell-free assay, that proved to be active when embedded in liposomes. These findings emphasize the quality of the cell-free produced $K_V1.3$ proteoliposomes and the usefulness of a fluorescent probe. This method can benefit the field of channel characterization, as well as provide tools for the development of new inhibitors, so as to reinforce our therapeutic arsenal against autoimmune diseases.

1. Introduction

Autoimmune diseases, like multiple sclerosis, rheumatoid polyarthritis, type 1 diabetes, and psoriasis, can affect almost any part of the body. In this case, the immune system attacks the body by mistake, provoking positive immune responses to autoantigens. Memory T-cells play a major role in the progression of these diseases, the autoreactive T-cells attacking target organs and causing tissue injury.

However, activation of T lymphocytes also requires continuous calcium influx across the plasma membrane [1,2]. The voltage-gated K^+ channel, $K_V1.3$, and the Ca^{2+}-activated-K^+ channel, KCa3.1, modulate this calcium influx by regulating the transmembrane potential and by providing the electrical driving force needed for continuous calcium entry into T cells. A way of preventing this Ca^{2+} influx in T cells *in vivo* is the blockage of both K^+ channels. This reduces the cytokine production and cell proliferation, which in turn attenuates the immune response. On one hand, the blockage of $K_V1.3$ channels cause a depolarization of the T cells leading to a reduction in intracellular calcium concentration. Several agents can block the pore of this channel, e.g. metal ions, small organic molecules and venom toxins. These latter peptide agents are often blocking the channel more efficiently than small chemical agents. On the other hand, the KCa3.1 blockers have been shown to prevent proliferation in mitogen-activated lymphocytes. Nevertheless, the ratio of $K_V1.3$/KCa3.1 channels present in the T lymphocytes membrane is 250/5 and therefore, targeting the $K_V1.3$ channel is an assumed strategy for remitting autoimmune diseases.

Potassium channels are tetrameric membrane proteins that mediate K^+ efflux to hyperpolarize the cellular membrane. Numbering 78 family members, the K^+ channel family is abundant in our organisms. So far, patch clamping has been used to record electrical signals from those channels. In the past 30 years, hundreds of toxin peptides targeting K^+ channels have been discovered. The toxins contain between 18 and 60 amino acid residues and are assembled with 2–4 disulfide bridges, which make them resistant to denaturation. $K_V1.3$ is notably found in T-cells where it mainly regulates calcium signaling during cell activation. Numerous electrophysiology studies of new synthetic $K_V1.3$-blocking toxin peptides have been reported, like margatoxin, initially identified and isolated from a scorpion venom. In addition, ShK-186, an analog derived from the sea anemone ShK [3] is currently under evaluation in clinical trials, providing a novel possibility for autoimmune disease treatments. Nevertheless the medical field may still benefit from the discovery of new toxins with more affinity and selectivity for $K_V1.3$.

In addition, patch clamping has so far been extensively used to

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record membrane potential in isolated T-cells or in recombinant cells. Nevertheless, it is fastidious and not adapted to screening small amounts of venom. Fluorescent probes able to report the membrane potential have also been described, in particular 3,3-dihexyloxacarbocyanine iodide (DiOC6) [4] and oxonol diBA-C4 [5] using nuclei and cells, respectively. Recently, we published an alternative approach using a voltage-sensitive fluorescent dye, oxonol VI and K_V1.3-proteoliposome in microtiter plates [6]. We exploited the inside-positive membrane potential generated by K^+ fluxes through active K_V channels embedded in an artificial membrane, in the presence of a K^+ concentration gradient across the membrane.

K_V1.3 consists of four α-subunits arranged around a central pore as a homotetramer. Each subunit is made of six transmembrane segments S1–S6 and a pore loop between S5 and S6. When membrane depolarization occurs, the arginine residues present in the S4 segment act as voltage sensors and cause a structural change leading to channel opening. Expression of the channel membrane protein in classical overexpression systems is sometimes difficult due to its toxicity, although this is not the case of K_v1.3. Several studies have been carried out in recombinant cells in order to obtain sufficient amounts of K_V1.3 for functional and structural studies. An attractive alternative for producing proteins, which are difficult to express, is the use of cell-free expression systems [7]. Recently, we published an article showing that a mouse K_V1.3 monomer expressed by cell-free synthesis auto-tetramerizes *in vitro* and can be inserted in a liposome with the margatoxin binding site on the outside (natural conformation). This method allowed us to recover enough of an active K_V1.3 protein embedded into a lipid bilayer to realize several tests and the screening of scorpion venom fractions in two microtiterplates. Membrane proteins can be produced in cell-free expression systems either in a precipitated form without any additives or in a soluble form in the presence of lipids or detergents. We choose the synthesis with detergent which we described in our previous article [6]. In addition, during relipidation, different lipid compositions of the liposomes as well as different detergents can be tested for optimizing yield and protein activity.

In this study, we optimized the *E. coli* cell-free expression system containing a detergent and obtained K_V1.3 relipidation in mixed lipid liposomes. The most commonly used method to analyze ion channel activity is to measure the currents caused by the ion flow using electrophysiology. We chose instead to use the fluorescent oxonol VI probe to record its activity and optimize its use in order to enable high quality tests in microtiter plates. We showed that the channel activity was inhibited by margatoxin and ShK, as predicted. The obtained proteoliposomes represent a great tool for the study of the K_V1.3 channel and the development of new treatments against autoimmune diseases.

2. Material and method

2.1. Liposome preparation

Liposomes composed of 2:1:1:1 (weight ratios) 1,2-dioleoyl-sn-glycero-3-phosphocholine: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine: 1,2-dimyristoyl-sn-glycero-3-phosphate: cholesterol purchased from Avanti Polar Lipids, Inc, and CORDEN Pharma, are solubilized in chloroform at 10 mg/mL. The lipids were mixed in a glass vessel and the chloroform was evaporated using a univapo 150H. The thin lipid film was rehydrated in 50 mM Tris, pH 7.5 by vortexing to obtain a 5 mg/mL lipid slurry. To form liposomes, the suspension was sonicated using a tip sonicator (Branson Digital Sonifier 250), sonicating for 1 min and placing on ice for 1 min so as to avoid excessive heating. After 5 times sonication, the solution became transparent indicating the formation of small unilamellar vesicles. The solution was then filtered with a 0.22 nm PES filter.

2.2. K_V1.3 synthesis with detergent and purification

The expression of the full-length mouse K_V1.3 with N terminal His-Tag was performed using an *E. coli* S30 extract-based cell-free expression system in batch mode as previously reported [6,8]. Detergent (DDM) was directly added into the reaction mixture in order to solubilize the K_V1.3 protein. Expression conditions were 600 rpm for 4 h at 25 °C in 10 mL reaction solution. After expression, the reaction solution was centrifuged at 20,000*g* at 4 °C for 20 min. The supernatant fraction, containing the recombinant K_v1.3-DDM protein, was collected and purified using QIAexpress Ni-NTA Protein Purification System (Qiagen, USA) previously equilibrated with resuspension buffer. After incubation, the resin was washed sequentially with 10 vol of washing buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.98 mM DDM, 10 mM imidazole). The K_v1.3-DDM protein was eluted in two fractions (E1 and E2) after incubating the beads with elution buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.98 mM DDM, 500 mM imidazole) for 2 h. The production level of each reaction was examined by SDS-PAGE, Coomassie Brilliant Blue staining, and Western blotting with HisProbe-HRP (Sigma, USA).

2.3. Reconstitution of K_V1.3 mouse into proteoliposomes

Two low CMC detergents were tested for the liposome destabilization: DDM, weight ratio Detergent/Lipid (=D/L): 0.1/0.3/0.8 and Triton X100 (Sigma, USA), ratio (D/L): 0.3/0.8/2. First 24 mL of L4 liposome at 5 mg/mL were mixed with one of the detergent at the ratio mentioned above and incubated for 1 h at 25 °C in a Thermomix. Second, 5 mL of Ni-NTA-purified K_V1.3-DDM (E1 + E2 at 20 μg/mL) were added and incubated for 1 h at 25 °C at 500 rpm in the Thermomix. Finally, the detergent was removed using 4 successive incubations with Biobeads (Biorad, USA) as recommended by the supplier. The ratio Biobeads/detergent was 10 (weight ratio). The final supernatant was stored at 4 °C.

To purify the proteoliposomes from the precipitated protein, the K_V1.3-liposome mix (detergent having been removed using the Biobeads) was loaded on top of a 3-steps discontinuous sucrose gradient (60%, 30% and 5%) prepared in a 50 mM Tris, pH 7.5 buffer. After centrifugation at 280,000*g* for 2 h at 4 °C, the lipid annulus was pipetted and suspended in a 2 mL 50 mM Tris, pH 7.5 buffer containing 0.02% sodium azide. The proteoliposome solution was then dialysed in 50 mM Tris, pH 7.5 (cutoff 12–14 kDa) and stored at −80 °C using a cryoprotectant solution. This proteoliposome solution was analyzed by Western blotting using a poly-histidine antibody conjugated with a horseradish peroxidase (Sigma-Aldrich, USA) diluted at 1:10,000 in TBS-Tween buffer, 5% nonfat milk.

2.4. Lipid concentration measurement and freezing

Lipid concentration was estimated using 8-Anilino-1-naphthalenesulfonic acid (ANS, Sigma USA) fluorescent dye. Briefly, a calibration curve (from 0 to 0.5 mg/mL) of aliquots of the L4-liposome was first measured using a 1/500 dilution of a 0.23% ANS stock (fluorescence Excitation wavelength 310 ± 8 nm, Emission wavelength 460 ± 8 nm and gain 2000). Then the same 1/500 ANS dilution was applied to the K_V1.3 -liposome and lipid concentration was deduced from the fluorescent calibration curve. For both fluorescent measurements, we have used a black, half area, 96-well microtiter plate Greiner (Dominique Dutscher, France) and a Clariostar spectrophotometer (BMG, USA).

2.5. Dynamic light scattering

The dynamic light scattering (DLS) technique was used to measure the average particle size using a DynaPro NanoStar instrument (Wyatt Technology Corp., USA) on 15 μL samples in cyclic olefin copolymer cuvettes at room temperature. All measurements were taken in

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