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CELL BIOPHYSICS

In Vitro Fluorescence Assay to Study the Folding of K_v Ion Channels

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Abstract—A fluorescence assay to check the folding of potassium K_v channels expressed in vitro has been developed. For this aim, the fluorescently labeled channel blocker, recombinant agitoxin of yellow scorpion was employed. The level of expression of various K_v channels in vitro has been tested. It has been demonstrated that K_v2 channels form clusters on the cell surface, which are not associated with actin filaments. On the other hand, K_v10 channels form larger clusters, which are associated with actin, indicating the principal differences in the organization of cytoplasmic domains of K_v2 and K_v10 channels.

Keywords: potential-dependent potassium ion channels, neurotoxin, confocal microscopy, cytoplasmic domains

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INTRODUCTION

Ion channels control and regulate the electric activity of the cell [1, 2]. Mutations in genes that are connected with the functioning of voltage-gated ion channels are the cause of such grave hereditary diseases as deafness, epilepsy [3–5], and also cardiac arrhythmia [6], multiple sclerosis [7] and pain syndrome [8]. According to the available data, most of these diseases are caused by mutations in the region of the membrane domain of ion channels (in particular, in the voltage change sensor). In epilepsy there may by truncation of the channel C terminus [9, 10]. It is known also that some ligands/blockers of ion channels may act as medicinal drugs. However only several such molecules have been tested on patients (4-aminopyridine and 3,4-diaminopyridine) [7]. Earlier it has also been shown that dendrotoxin and kaliotoxin may weaken demyelination, blocking the voltage-gated channels in neurons [11].

At present more than 200 genes are known that code for various potassium voltage-gated channels. All these channels are tetramers and carry a homologous segment in the pore region, determining the selectivity for potassium ions [12–14]. Neurotoxin with high specificity binds to the pore region and may present a peculiar structural label permitting determination of the stoichiometric relationship of the subunits of the channel and the structural topology of its extracellular surface [15–17].

The cytoplasmic domains of voltage-gated channels of different families differ in structure. They may be involved in protein–protein interactions regulating the channel conductivity, changing its localization and functioning [18–23]. The cytoplasmic domains mediate reversible interactions of channels with cell enzymes (for example, kinases, phosphatases), which covalently modify the channel and control its localization and functioning [19–24].

In the present work we have developed an in vitro assay for the correctness of folding of voltage-gated ion channels on the basis of fluorescently labeled recombinant channel blocker, scorpion agitoxin. With the use of the developed system we have tested the level of expression of a number of voltage-gated ion channels in vitro and demonstrated the different ability of channels of two families to form clusters on the cell surface.

EXPERIMENTAL

Expression and purification of agitoxin. The plasmid vector pCSP105 containing a hybrid construct of agitoxin 2 was kindly provided by Professor Christopher Miller (Brandeis University, USA). Expression and purification of agitoxin was conducted as described in work [25], with modifications.

Accumulation of plasmid pCSP105 was conducted in *E. coli* strain TG1. For plasmid isolation use was made of a HiSpeed Plazmid Maxi Kit (QIAGEN, Holland). Cells of *E. coli* BL21(DE3) transfected with plasmid pCSP105 were grown in LB medium at 37°C, in the presence of ampicillin (100 μg/mL). Plasmid expression was induced with 0.5 mM IPTG (Helicon, Russia) when the culture OD_{650} reached 0.8–1. In 2 h of incubation the cells were centrifuged, washed and resuspended in buffer containing 10 mM Tris-HCl pH 8.0, 50 mM NaCl and 2 mM Na_2EDTA (35 mL of

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buffer per 1 liter of culture). The cell suspension was incubated in ice in the presence of 10 mg of lysozyme (per 1 L culture), then treated with ultrasound in the presence of protease inhibitors (Roche), 10 μL β-mercaptoethanol, 200 μL PMSF, then centrifuged 30 min at 20000 *g*. All subsequent procedures were conducted on ice. To the cell extract we added 0.1 vol of 30% (wt) solution of streptomycin sulfate, then centrifuged 30 min at 20000 *g*. The supernatant was withdrawn and proteins salted out by adding ammonium sulfate to 50% saturation. After 30 min of stirring the suspension was centrifuged 30 min at 20 000 *g*. The pellet was dissolved in 10 mL of buffer containing 50 mM Tris-HCl, 50 mM NaCl, 5 mM β-mercaptoethanol, pH 7.0, then purified on an anion-exchange column with sorbent DEAE-cellulose (Sigma, USA) equilibrated in the same buffer. Elution using 0.05–0.5 M gradient of NaCl.

The purified fused protein was subjected to cleavage with trypsin (10 μg/mL) in the presence of 1 mM $CaCl_2$ in the course of 30 min. Then the mixture was resolved on a cation-exchange column with SP-Sephadex (Sigma, USA) equilibrated in buffer containing 10 mM HEPES-NaOH, 10 mM NaCl, pH 7.0. Elution using a linear gradient of 0.01–1 M NaCl. The concluding step of purification was conducted by HPLC on a column of RP C18 (ODS-A, 4.6 × 250 mm, YMC, Japan) equilibrated in 0.1% TFA and 5% acetonitrile (Panreac, Spain) in water. Elution with a 5–40% gradient of acetonitrile. Final purification was conducted with the aid of 3YM and 30YM Microcon filters (Millipore).

The mass-spectrometric examination of purified agitoxin 2 was conducted on the basis of the Orekhovich Research Institute of Biomedical Chemistry RAMS by the method described in work [26].

Expression of voltage-gated potassium channels. For expression of potassium channels use was made of a line of eukaryotic green monkey kidney cells (Vero) from the collection of LLC Biolot. Cells were transfected with pMT3 plasmids encoding full-sized channels $K_v2.1$ and $K_v10.2$ with the aid of Metafecten™ PRO (Biontex) according to the manufacturer's manual.

For confocal microscopy the cells were grown on round 24-mm coverslips in 6-well plates (30×10^3 cells per well) for 22–24 h in 2 mL of DMEM high glucose (HyClone, USA) with addition of 10% fetal serum (HyClone, USA) until reaching 50% coverage, after which transfection was conducted (2.5 μL of plasmid DNA and 6 μL of metafectin per 1 well). In 24 h after transfection the nutrient medium was replaced with a fresh one. Testing of channel expression with the use of labeled agitoxin was conducted in 48 h of cell growth after conducting the transfection.

Fluorescence assays. Agitoxin was labeled with a fluorescent dye Cy3 (GE Healthcare) according to manufacturer's recommendations and [27], with small modifications. A weight of Cy3 was dissolved in PBS (pH 8.0), then added to a protein solution and incubated overnight, at +4°C. The excess of unbound dye was removed with the aid of a 3YM Microcon filter (Millipore).

For labeling with a fluorescent dye FITC (Biotium) a dye weight of 0.1 mg was dissolved in 100 μL DMSO. For staining, agitoxin was transferred into PBS using membrane filters 3YM Microcon. To 2 mL of protein solution (of concentration 0.1 mg/mL) we added 200 μL of 1 M carbonate buffer and 70 μL of dye dissolved in DMSO, incubated with stirring for 1 h at 20°C. From excess dye the mixture was purified on membrane filters 3YM Microcon.

For testing channel expression on cell surface, fluorescently labeled agitoxin (10 nM) was added immediately into the nutrient medium with serum, incubated 30 min at +4°C, then washed with medium without serum.

For visualization of lysosomes the cells were stained with a fluorescent dye LysoTracker Green DND-26 (1 μM, 5 min) (Molecular Probes, USA).

Staining of polymeric actin in the cytoplasm was conducted after cell fixation (with paraformaldehyde) with the use of phalloidin bound with red dye Alexa 488.

Confocal microscopy. Images were obtained with the aid of an LSM-510 Meta microscope (Carl Zeiss AG, Germany) equipped with a Plan-Apochromat 10x/1.4 Oil Ph3 objective. Digital images were stored with a resolution of 1024 × 1024 pixel. For excitation of label Cy3 fluorescence we used a helium–neon laser (radiation wavelength 543 nm), for registration of fluorescence use was made of a filter with 565–615 nm bandpass. For excitation of label LysoTracker Green DND-26 fluorescence we used an argon laser (radiation wavelength 488 nm), for registration of fluorescence, a light filter with 500–530 nm bandpass. For simultaneous registration of fluorescence of Cy3 and LysoTracker Green DND-26, data collection over optical sections was actualized in a multipass line-by-line mode. For obtaining images with high resolution the diaphragm value was set according to manufacturer's instructions, for time-resolved filming the diaphragm value was set to 1.74 Airy units to increase the depth of focus. For image processing we used the Zeiss LSM 510Meta software, release 3.2.

RESULTS

Obtaining agitoxin conjugates with fluorescent label. Agitoxin was expressed as a hybrid construct in *E. coli* cells, then purified in several stages. The purity of the preparation at each stage was checked electrophoretically with gel staining with silver nitrate (Fig. 1a). After induction of expression with IPTG upon electrophoresis a broad band is seen with a mass of 50 kDa, corresponding to the mass of the hybrid construct with agitoxin. After salting out of the soluble fraction of proteins with ammonium sulfate the pro-

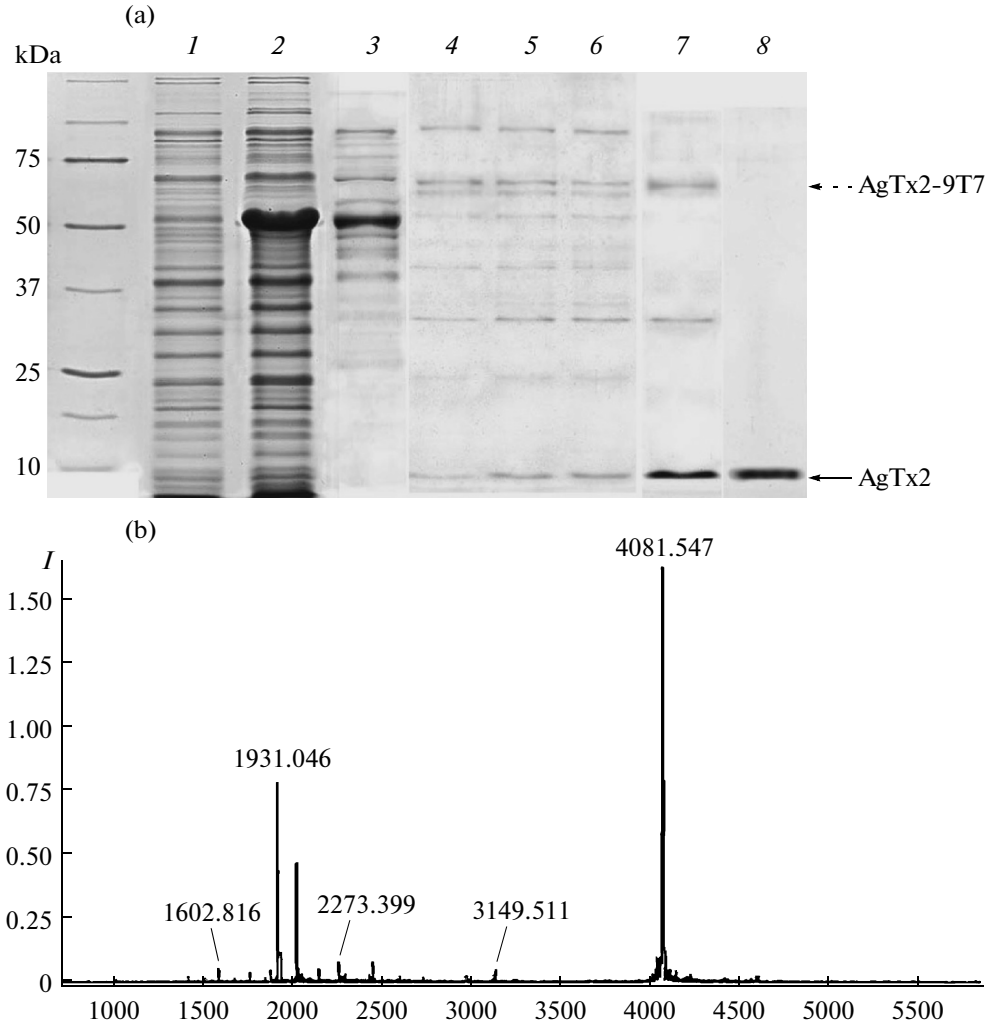

Fig. 1. (a) Electrophoresis of the stages of agitoxin expression and purification: *1*, cell extract of *E. coli* before induction of expression; *2*, cell extract of *E. coli* after induction of expression with IPTG; *3*, fraction containing recombinant protein after purification on DEAE column; *4–6*, kinetics of cleavage of recombinant protein with trypsin (15, 30 and 45 min respectively); *7*, purification on a column with SP-Sephadex; *8*, purification by HPLC on an RP-C18 column. (b) Mass spectrum of an agitoxin sample after purification on a 3YM filter.

tein was purified on an anion-exchange column with DEAE-Sephadex. Fractions containing the 50 kDa band were collected and subjected to trypsinization. Under the action of trypsin the hybrid construct is completely cleaved approximately in 30 min. After this the protein mixture was resolved on a cation-exchange column. The concluding step of purification was conducted by HPLC on a column of RP C18. For acceleration and simplification of the last stage of purification in some experiments instead of purification with the aid of HPLC use was made of purification on membrane filters 3YM and 30YM Microcon. The protein purity in the latter case insignificantly differed. The purified protein was identified with the aid of mass spectrometry (Fig. 1b).

Assessment of expression of ion channels in Vero cells. Cells of the Vero line were transfected with plasmids encoding full-sized ion channels: $K_v1.1$ (*Shaker*), $K_v2.1$ and $K_v10.2$. Cells were grown and transfected immediately on cover round slips for confocal microscopy in six-well plates. The effectiveness of expression of the channel protein was confirmed by immunoblotting (Fig. 2). Staining with agitoxin labeled with Cy3 and FITC was conducted in the medium with serum, to exclude nonspecific binding. In samples subjected to transfection with plasmids encoding $K_v1.1$ channels, we observed intense red fluorescence after staining with Cy3-labeled agitoxin (Fig. 3b), whereas in control cells without transfection fluorescence was not observed (data not shown).

Differences in clustering of ion channels of different families. The fluorescence of labeled agitoxin on the cell surface proved nonuniform, we observed stained clusters from 1 nm to 1 μm in diameter. In order to

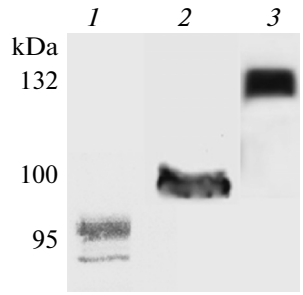

Fig. 2. Western blot with extracts of cells expressing ion channels: *1* – $K_v1.1$; *2* – $K_v2.1$; *3* – $K_v10.2$. In the capacity of primary antibodies use was made of polyclonal antibodies against affinity label 1D4 at the C-end of protein; in the capacity of secondary antibodies use was made of polyclonal goat antirabbit antibodies conjugated with alkaline phosphatase.

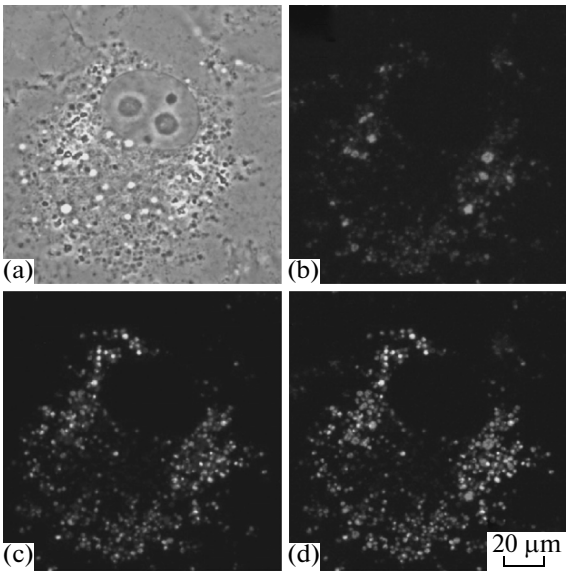

Fig. 3. (a) Vero cell expressing ion channels $K_v1.1$ in transmitted light; (b) Cy3-labeled agitoxin having bound with $K_v1.1$ channels in the same cell; arrows point to channel clusters; (c) lysosome staining with Lyso-Tracker Green DND-26 dye in the same cell; (d) merge of images (b) and (c). Scale bar, 20 μm.

exclude the probability of nonspecific pinocytosis of stained toxin, in cell preparations we stained lysosomes with LysoTracker Green DND-26 dye (Fig. 3c). As a result it has been shown that the regions of fluorescence of Cy3 (red) and LysoTracker (green) do not coincide (Fig. 3d). This result confirms that fluorescing red is the Cy3-labeled agitoxin specifically interacting with tetrameric voltage-gated ion channels $K_v1.1$. We also labeled the toxin with the aid of a green dye FITC (see Experimental section) for studying its interaction with $K_v2.1$ and $K_v10.2$ channels (Fig. 4). With the use of labeled toxin it has been shown that $K_v2.1$ and $K_v10.2$ channels also form clusters on the cell surface and that different dyes do not change the affinity of the toxin to ion channels.

In order to follow whether clustered channels interact with actin filaments, we conducted staining of actin with phalloidin conjugated with Alexa 488. It has been shown that $K_v2.1$ channels united in clusters do

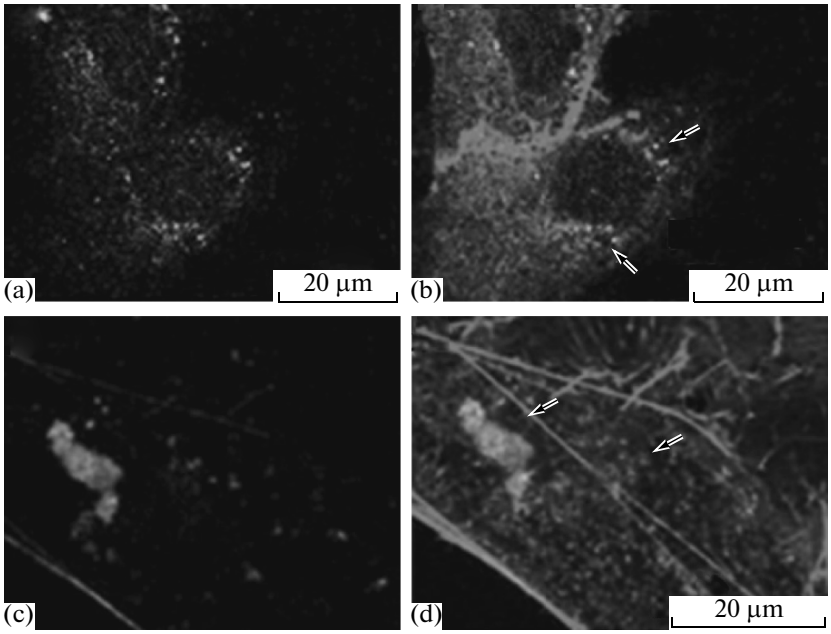

Fig. 4. (b) FITC-labeled agitoxin having bound with $K_v2.1$ channels; (b) superposition of image (a) on an image with Alexa 488-stained actin; arrows point to clusters of $K_v2.1$ channels; (c) FITC-labeled agitoxin having bound with $K_v10.2$ channels; (d) superposition of image (c) on an image with Alexa 488-stained actin; arrows point to clusters of $K_v10.2$ channels. Scale bar, 20 μm.

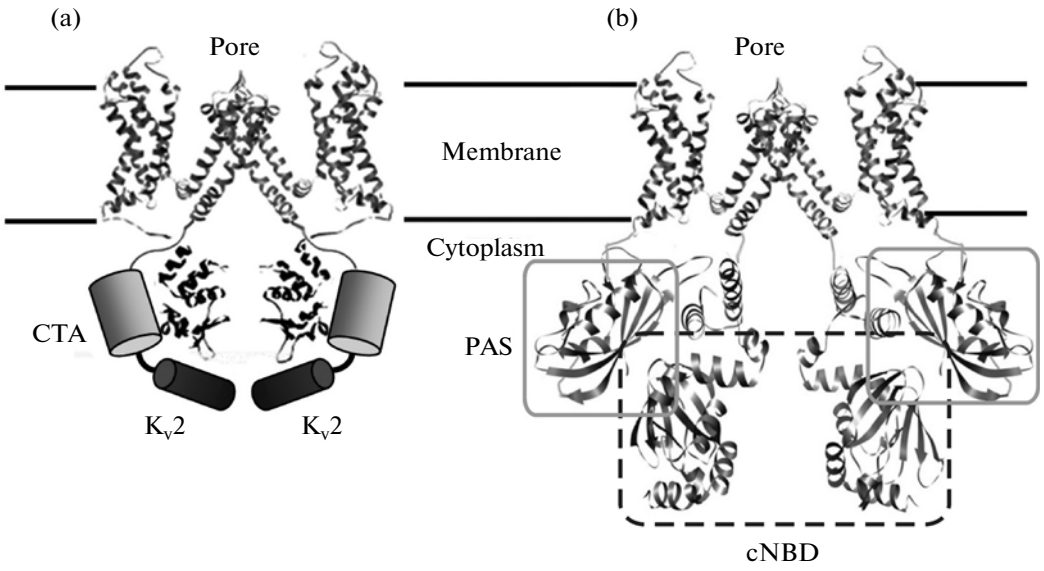

Fig. 5. Schemes of organization of cytoplasmic domains of K_v channels of family $K_v2.1$ (a) and family $K_v10.2$ (b). Only two subunits shown. (a) Hypothetical structure of a $K_v2.x$ channel: K_v2 and CTA, C-terminal domains as predicted in [20]. (b) Hypothetical structure of channel ($K_v10.x$); structure of the PAS domain [47] marked with gray frames; structure of the cNBD domain [48] marked with dashed frame.

not interact with actin filaments, as we did not observe yellow staining (Fig. 4b). At the same time the channels of another family—$K_v10.2$—apparently have contacts with actin, as the regions of their localization coincide (Fig. 4d).

DISCUSSION

The contemporary methods of analysis of ion channel activity can be divided into two main classes—functional assays (patch-clamp, fluorescent dyes sensitive to a change in potential and ionic strength, measurement of ion currents) and estimation of competitive binding.

For the first time the functions of ion channels were investigated with the patch-clamp method more than 50 years ago [28], and up to now this method remains the most informative and exact in estimating the influence of ligand on an ion channel [29]. This method is very laborious, requires relatively expensive equipment and experienced operators, its productivity is extremely low [30]. The most widespread and sensitive express method of evaluating the correctness of folding of voltage-gated channels is based on the use of ligands conjugated with various radioactive isotopes. The main shortcomings of this method are the necessity of synthesizing the radioactively labeled ligand and the work with active isotopes. In this connection, all over the world they gradually switch to the use of fluorescent label. The sensitivity thereof is lower than isotopic one, however with appearance of modern highly sensitive instruments and the use in the capacity of a label of nanoparticles [31, 32] the level of fluorescence detection has significantly improved. This permits using various fluorescent dyes not only for estimating the level of protein expression in the cell [33] but also for studying the interaction of separate domains in protein [34].

The yellow scorpion agitoxin specifically binds only to correctly organized tetrameric voltage-gated ion channels [15]. In our experiments with its help we estimated the level of expression of channels of various families in eukaryotic Vero cells (Figs. 3, 4). The use of different fluorescent labels did not change the affinity of agitoxin to ion channels belonging to different families and standing remote enough from each other in the phylogentic respect [35]. We observed superficial disposition of voltage-gated channels in the form of clusters (Figs. 3b, 4a,b). The sizes of clusters varied from 1 nm in the case of $K_v1.1$ and $K_v2.1$ to 1 μm in the case of $K_v10.2$. It should be noted that up to the present time in the literature no mention has been encountered about formation of clusters by channels of the K_v10 family.

Up to now practically all works on studying channel clustering have been conducted on channels of the K_v2 family [36–40]. The exact mechanism of clustering it still unknown. It is supposed that it may be connected with interaction between the cytoplasmic domains of K_v2 and unknown anchoring supporting proteins that determine the cluster organization of channels [40]. It should be noted that a change in phosphorylation of the C-end of $K_v2.1$ leads to dispersion of surface $K_v2.1$ clusters in vivo and in vitro and to growth of neuronal activity [41–43]. It is known that actin filaments do not take part in the clustering of K_v2 channels [44].

In our experiments we also did not observe association of clusters of the K_v2 channel with actin (Fig. 4b), which was evinced in the absence of coincidence of the regions of fluorescence of agitoxin specifically bound with channels and of phalloidin bound with actin. However this observation has not been confirmed for channels of another family – K_v10. We observed coincidence of the corresponding fluorescence regions (Fig. 4d), which is evidence of that the K_v10 channels are in tight interaction with actin filaments. This unexpected observation may be explained by significant differences in the structure of cytoplasmic domains of the K_v2 and the K_v10 channels (Fig. 5). While in the K_v2 channel the tetramerization domain is found at the N-end of the monomer (Fig. 5a), in K_v10 this function is supposedly performed by the C-terminal cNBD domain [45, 46]. At that outside of the cNBD domain there apparently resides the N-terminal domain PAS [47] (Fig. 5b). To search for sites of binding of the cytoplasmic domains of the K_v10 channel with actin it is necessary to conduct additional investigations.

In this way, the results obtained testify to the basic possibility of using a labeled neurotoxin to study the folding and surface distribution of voltage-gated ion channels in an in vitro system. Further we plan to test the developed method on cells in the norm containing a multiplicity of potassium channels, in particular, on the cells of an isolated nervous ganglion.

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