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Cholesterol depletion-induced inhibition of stretch-activated channels is mediated via actin rearrangement

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ABSTRACT

Cholesterol is a critical regulator of lipid bilayer dynamics and plasma membrane organization in eukaryotes. A variety of ion channels have been shown to be modulated by cellular cholesterol and partition into cholesterol-enriched membrane rafts. However, very little is known about functional role of membrane cholesterol in regulation of mechanically gated channels that are ubiquitously present in living cells. In our previous study, the effect of methyl-beta-cyclodextrin (MbCD), cholesterol-sequestering agent, on Ca^{2+}-permeable stretch-activated cation channels (SACs) has been described. Here, cell-attached patch-clamp method was employed to search for the mechanisms of cholesterol-dependent regulation of SACs and to clarify functional contribution of lipid bilayer and submembranous cytoskeleton to channel gating. Cholesterol-depleting treatment with MbCD significantly decreased open probability of SACs whereas alpha-cyclodextrin had no effect. F-actin disassembly fully restored high level of SAC activity in cholesterol-depleted cells. Particularly, treatment with cytochalasin D or latrunculin B abrogated inhibitory effect of MbCD on stretch-activated currents. Single channel analysis and fluorescent imaging methods indicate that inhibition of SACs after cholesterol depletion is mediated via actin remodeling initiated by disruption of lipid rafts. Our data reveal a novel mechanism of channel regulation by membrane cholesterol and lipid rafts.

1. Introduction

Mechanically gated ion channels play an important role in processes of mechanotransduction and calcium signaling in living cells. It is generally accepted that membrane deformability is one of the key determinant in stretch-induced channel activation. Consistently, bilayer models are successfully developed for procaryotic mechanosensitive channels MscL and MscS [1,2]. However, molecular mechanisms underlying stretch sensitivity of membrane channels in eukaryotic cells remain remain undefined [3]. Several lines of evidence have shown that the integrity of actin network is essential for SAC activation and mechanical transduction [5–7, also see Ref. 8]. Particularly, actin filaments are supposed to work as a force-transmitting and force-focusing molecular device to activate mechanosensitive channels [7,9–11]. According to alternative hypothesis, eukaryotic SACs are gated by tension developed purely in lipid bilayer. Intrinsic mechanisms are postulated underlying channel regulation that is unlikely to be linked with submembranous cytoskeleton [12,13]. Nowadays, despite numerous studies, functional contribution of membrane lipids and cortical cytoskeleton to channel mechanosensitivity is still unclear.

It is known that physical properties of the membranes depend on lipid composition; specifically, stiffness and fluidity of the bilayers are essentially determined by sterol content. The presence of sterols in cell membranes is peculiar to eukaryotes. Cholesterol is one of the main lipid components of plasma membranes in mammalian cells, and it plays a pivotal role in lipid organization and lateral heterogeneity of the bilayer [14,15]. Cholesterol-enriched membrane microdomains (lipid rafts) are accepted to concentrate and segregate membrane proteins and may serve as scaffolds for signaling complexes [16,17]. Growing evidence suggests that integrity of lipid rafts is essential for interactions between plasma membrane and cortical cytoskeleton [18–20]. However, an involvement of membrane cholesterol and rafts in cellular mechanosensitivity is not well defined. Lipid rafts are considered to be dispersed when cellular cholesterol is extracted. Therefore, responses to cholesterol depletion are reasonably taken as evidence of a role for lipid rafts in cell function.

Changes in the level of cellular cholesterol content were reported to affect numerous types of ion channels [21]. A variability of cholesterol effects is described whereas specific mechanisms of them are much less understood. Nowadays, little is known about

Abbreviations: SACs, stretch-activated channels; MbCD, methyl-beta-cyclodextrin; alpha-CD, alpha-cyclodextrin; CTB, cholera toxin beta subunit; Cyt D, cytochalasin D; Lat B, latrunculin B; P_o, channel open probability.

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cholesterol sensitivity of SACs that are ubiquitously expressed in a variety of tissues. Earlier, typical stretch-activated Ca^{2+}-permeable channels were identified in plasma membrane of human myeloid leukemia K562 cells [22]. Several studies have argued that K562 cells provide an adequate model to clarify putative impact of cytoskeleton and lipid bilayer on single channel properties in patch clamp experiments [8,23,24]. As we have found previously, treatment with methyl-beta-cyclodextrin (MbCD) affects SAC gating in leukemia cells [25]. The present study was designed to search for the mechanisms of the effect and finally to elucidate SAC regulation in native cells. Using combination of treatments and single channel analysis we found that actin remodeling mediates the inhibitory effect of cholesterol depletion on mechanically gated channels. Our data imply that cholesterol-enriched membrane rafts may affect mechanotransduction and channel behavior indirectly via cytoskeleton rearrangement. We speculate that disruption of lipid rafts increases the stiffness of membrane-cytoskeleton complex in human leukemia cells.

2. Materials and methods

2.1. Cells

Human myeloid leukemia K562 cells (Cell Culture Collection, St.Petersburg, Russia) were grown in RPMI-1640 medium containing 10% fetal bovine serum and 80 μg/ml gentamycin in 5% CO_2 at 37 °C. For patch clamp measurements and fluorescent staining, cells were plated on coverslips, coated with poly-L-lysine. To deplete cholesterol, cells were incubated in serum-free medium with 5 mM MbCD at 37 °C for 60 min according to standard protocol approved for K562 cells [25]; alpha-cyclodextrin (alpha-CD), structural analog having no affinity to sterols, was applied in analogous procedure. Control cells were treated similarly without cyclodextrins. All reagents were purchased from Sigma.

2.2. Electrophysiology

Single channel currents were recorded using cell-attached mode of patch clamp method essentially as described earlier [25,26]. Experiments were performed with HEKA EPC-8 operational amplifier at room temperature (21–23 °C). Pipettes were pulled from borosilicate glass capillaries (BF-150-110-10, Sutter Instruments) to a resistance 5–10 MΩ when filled with normal external solution containing [in mM]: 145 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES/TrisOH. The bath solution for cell-attached measurements contained [in mM]: 145 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/KOH. pH of all solutions was set at 7.3. To analyze stretch-induced channel activation, we used standard method of mechanical stimulation as applied negative (positive) pressure to a patch pipette. Channel open probability (P_o) was determined using following equation: $P_o = I/i*N$, where I is the mean current determined from the amplitude histograms, i is the unitary current amplitude and N is the number of functional channels in the patch. Data are presented as mean ± SEM (n – number of experiments) and compared using unpaired Student's t-test.

2.3. Fluorescence microscopy

For raft labeling, cells were stained with beta subunit of cholera toxin conjugated with FITC (FITC-CTB, Sigma). Control and treated

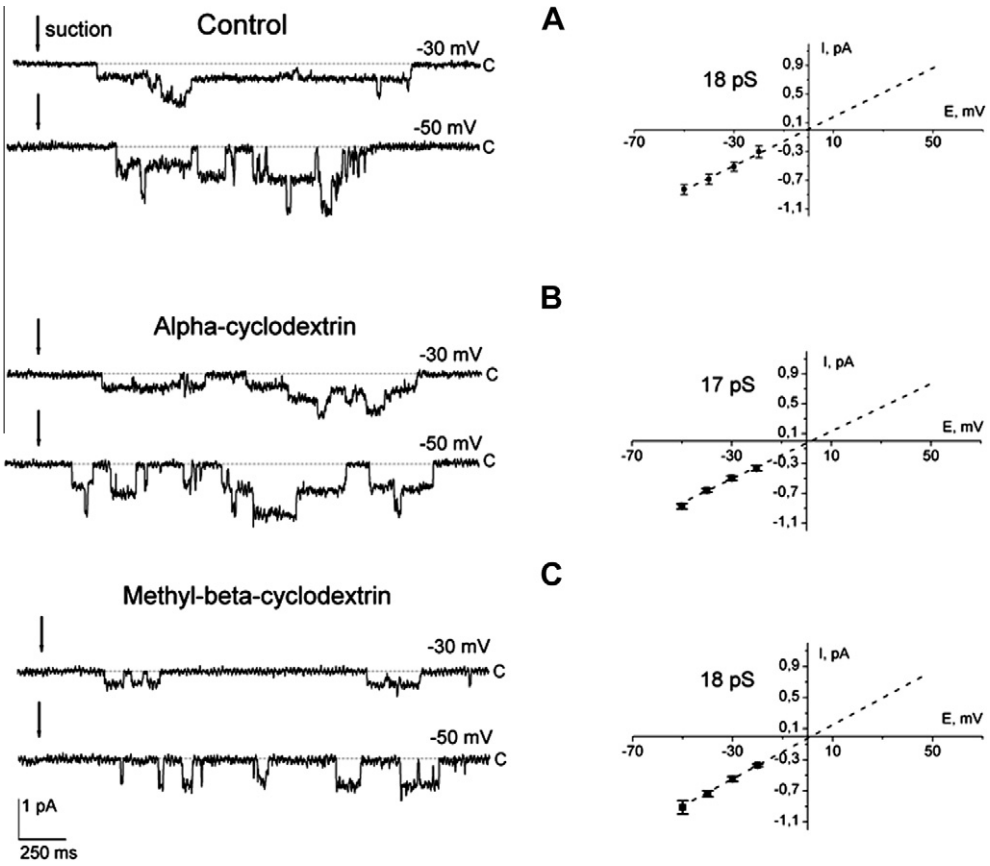

Fig. 1. Cholesterol-depleting treatment with methyl-beta-cyclodextrin suppresses activation of SAC channels in K562 cells whereas alpha-cyclodextrin has no effect. On the left – stretch-activated currents recorded from representative cell-attached patches (A) in control and after incubation (60 min) of cells with 5 mM (B) alpha-CD or (C) MbCD. Holding membrane potentials are indicated near traces. Application of suction is marked by arrows. Closed state is denoted by C. On the right – corresponding single channel current–voltage relationships. Values are means ± SEM of at least six experiments.

cells were fixed in 3.7% paraformaldehyde and incubated with 2.5 μg/ml FITC-CTB for 10 min at 4 °C. Stained cells were mounted on glass slides with Vectashield mounting medium (Vector Laboratories, USA). Samples were analysed using epifluorescence microscope IM 35 (Zeiss Inc.). Fluorescent images were acquired using cooled Alta U2000 CCD camera (Apogee Instruments, Inc) controlled via Maxim DL/CCD software (Diffraction Limited, USA). Images were processed in Maxim DL or in ImageJ (NIH, USA).

3. Results and discussion

In parallel series of experiments, stretch-activated currents were analyzed in cells treated with cyclodextrins – MbCD, cholesterol-sequestering agent, and alpha-CD, structural analog which does not selectively bind sterols [27,28]. Fig. 1 shows representative single currents reversibly activated by suction at two holding membrane potentials. In control experiments on untreated cells, we observed typical stretch-induced activation of the channels in 51% of stable cell-attached patches ($n = 81$). Application of negative pressure of 30–40 mm Hg was sufficient to induce channel activity in control as well as after treatment of cells with alpha-CD (8 out of 18 stable patches). In contrast, after incubation of the cells with MbCD, we did not observe activation of currents in response to equivalent levels of negative pressure in all patches tested. However, application of about 2-fold higher pressure (70–80 mm Hg) induced reversible activation of currents in cell-attached experiments (14 out of 30) on cholesterol-depleted cells. In agreement with earlier results [25], treatment of cells with MbCD resulted in evident decrease of SAC activity as estimated by open state probability: $P_o = 0.08 \pm 0.03$ (in comparison to control $P_o = 0.28 \pm 0.03$). Importantly, after treatment with alpha-CD, the level of stimulus (35–40 mm Hg), as well as the level of channel activity ($P_o = 0.29 \pm 0.04$) were close to control. Thus, treatment with MbCD significantly altered activation parameters of SACs whereas alpha-cyclodextrin had no effect. Both in control and treated cells, single channel conductance was 17–18 pS that is similar to the values reported previously in K562 cells [8,22,26]. Comparative data obtained in experiments with cyclodextrins argue that inhibition of SACs in K562 cells after MbCD treatment is a result of cholesterol sequestration (Fig. 1, see also Fig. 4). In this way, we have to conclude that cholesterol depletion strongly suppressed stretch-induced channel activation. This inhibitory effect indicates an increase of plasma membrane rigidity (stiffness). However, lipid bilayer rigidity should be expected to increase with cholesterol elevation, rather than depletion. Consequently, the inhibition of stretch-induced currents could not be explained by the change of dynamic properties of lipid bilayer according to classical considerations based on model lipid research [29]. It is reasonable to suppose that suppression of SACs in cholesterol-depleted cells may be resulted from disruption of cholesterol-enriched microdomains (rafts) in plasma membrane.

To assess putative changes in lipid raft integrity we stained the cells with fluorescently labeled cholera toxin beta subunit (CTB)

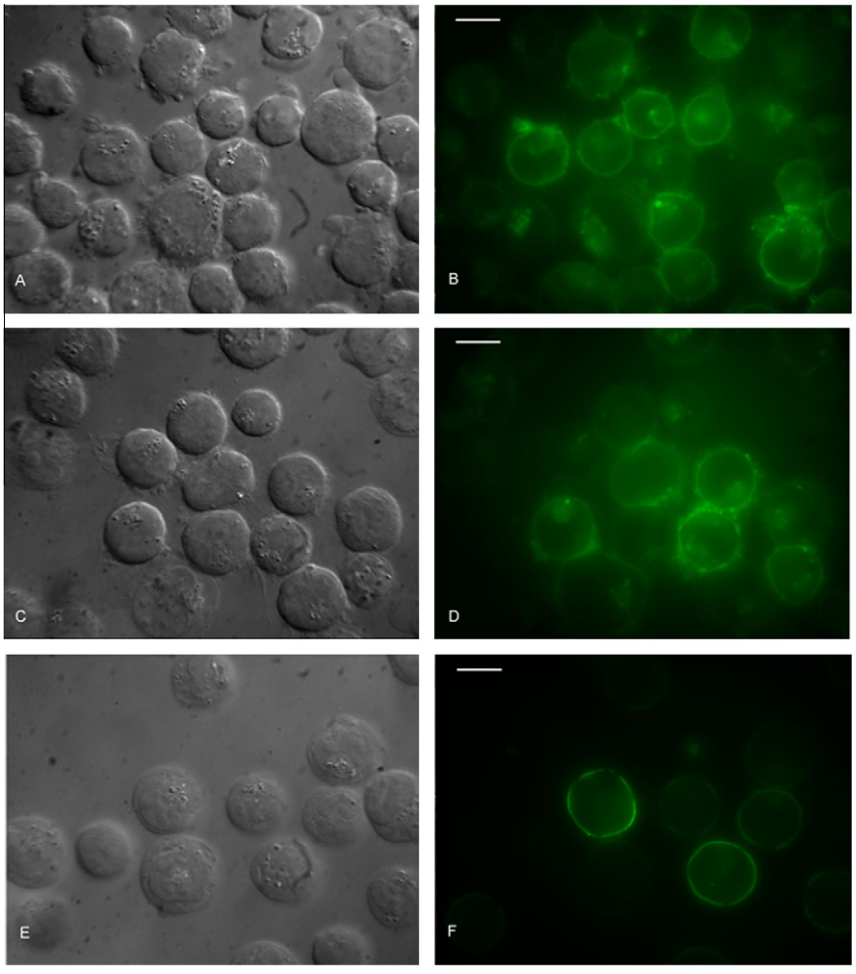

Fig. 2. Visualization of lipid raft marker ganglioside GM1 in plasma membrane of K562 cells by FITC-conjugated CTB. (A and B) – control (untreated) cells, (C and D) – after treatment with 5 mM alpha-CD, (D and E) – 5 mM MbCD. On the right – fluorescence of GM1-bound FITC-CTB; on the left – cells in the same field of view in differential interference contrast (DIC, Nomarsky optics). The images shown are representative of findings in six independent experiments for each condition. Scale bar 10 μm.

that binds specifically to ganglioside GM1, indispensable component of rafts [30,31]. Staining of control and treated K562 cells indicated that exposure to cholesterol-insensitive alpha-CD produced no effect on GM1 distribution in plasma membrane (Fig. 2). In contrast, MbCD treatment results in significant changes in fluorescence that apparently corresponds to lipid raft dispersion in cholesterol-depleted cells. Comparison of fluorescence and differential interference contrast images reveals that cholesterol depletion prevents CTB from binding to a part of cells. Observed reduction of total fluorescence signal is likely to be due to effect of MbCD on bilayer structure, particularly, extraction of cholesterol together with GM1 from rafts [32]. Besides, destruction of rafts itself may also contribute to decrease of fluorescence labeling because of pentameric structure of cholera toxin beta subunit (CTB). Simultaneous interaction with five colocalized GM1 molecules is necessary for FITC-CTB binding to membrane surface [33].

Fluorescent microscopy confirmed that cholesterol depletion-induced suppression of SACs is due to raft destruction. However, channel functions remained principally unchanged thus indicating no specific lipid–protein or protein–protein interactions. Summarizing the results of our patch clamp experiments with cyclodextrins, we have to state that effect of cholesterol depletion on stretch-activated currents looks like "non-specific" shift of activation curve which could not be explained by changes in bilayer elasticity. As known, mechanical properties of multiple cell types are primary dependent on the membrane–cytoskeleton complex. Particularly, stiffness of endothelial cell membranes was demonstrated to increase upon cholesterol depletion and this stiffening effect depended on integrity of F-actin [34]. Earlier, reorganization of cortical actin in K562 leukemia cells after treatment with MbCD was observed [25] and confirmed here using confocal microscopy (Fig. S1). Thus, we can assume that inhibition of SACs is coupled with rearrangement of actin filaments induced by cholesterol depletion and lipid raft perturbation. This hypothesis was checked directly in the next series of electrophysiological experiments.

To search for possible involvement of microfilaments in control of SAC activity, cholesterol-depleted cells were treated with cytochalasin D or latrunculin B, known actin disrupters with different mechanisms of action [35]. Therefore, two protocols have been used: cytochalasin D was applied after MbCD whereas latrunculin B was added to MbCD-containing serum-free medium after half-hour exposure of cells to cyclodextrin. Importantly, we found that in both cases actin-disrupting treatments completely abolished inhibition of mechanosensitive channels after cholesterol depletion (Fig. 3). Cell-attached recordings demonstrated that disruption of F-actin network fully abrogated the effect of MbCD. For comparison, activation parameters (level of stimulus and P_o values) after various treatments are summarized in Fig. 4. High level of SAC activity was restored after drug-induced destruction of actin cytoskeleton. In whole, our data show unequivocally that suppression of SACs in cholesterol-depleted leukemia cells is due to the rearrangement of cytoskeleton.

The results of patch clamp experiments confirmed that lipid raft destruction induced actin remodeling in leukemia K562 cells thus modulating channel activity in plasma membrane. Our data are in principal agreement with the notion that cholesterol depletion affects coupling between cortical cytoskeleton and plasma membrane [34,36]. Interestingly, an accumulation of F-actin throughout the cytoplasm was observed in cholesterol-depleted K562 cells (Fig. S1). We suggest that cholesterol depletion induces actin assembly in leukemia cells due to the pool of cytoplasmic G-actin is available for polymerization. Similarly, the reduction of cellular

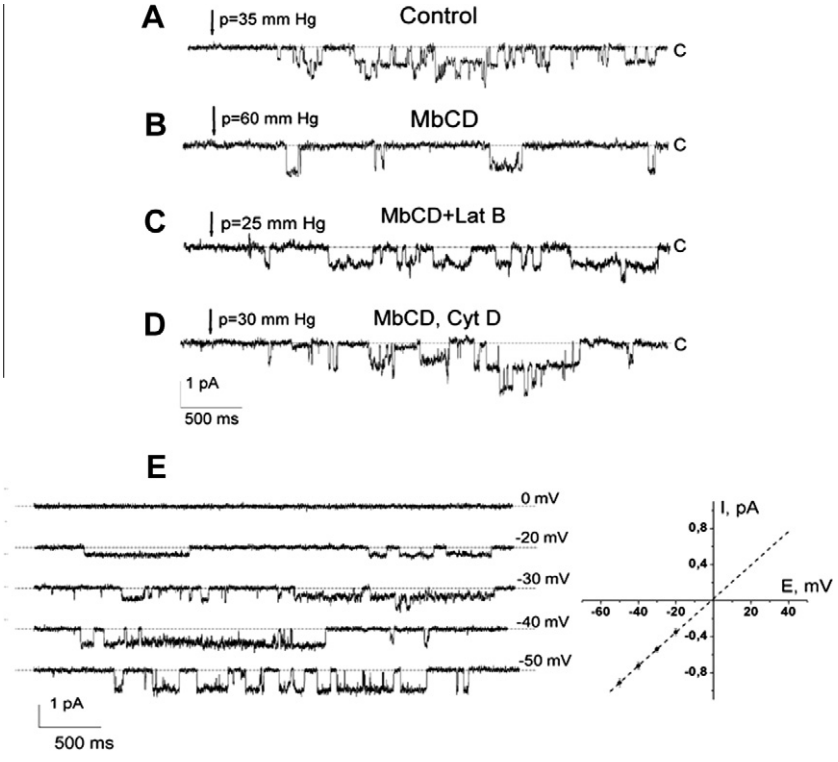

Fig. 3. Actin disassembly restores high level of SAC activity in cholesterol-depleted cells. (A–D) Cell-attached currents recorded in four representative experiments. Membrane potential was −40 mV; closed states are denoted by C. The arrows show suction application. (A) Control. (B) Cholesterol-depleting treatment with 5 mM MbCD, 60 min. (C) Cells were incubated with 5 mM MbCD (30 min), and then with 5 mM MbCD + Lat B (10 μM) for 30 min. (D) Cells were treated with 5 mM MbCD (60 min) and subsequently with Cyt D (10 μg/ml, 10 min). (E) SAC activity after treatment of cells with MbCD and Cyt D. Single currents were recorded at applied negative pressure; membrane potentials are indicated near traces. Current–voltage relationship corresponds to channel conductance of 17.5 pS. Values are means ± SEM of at least six experiments.

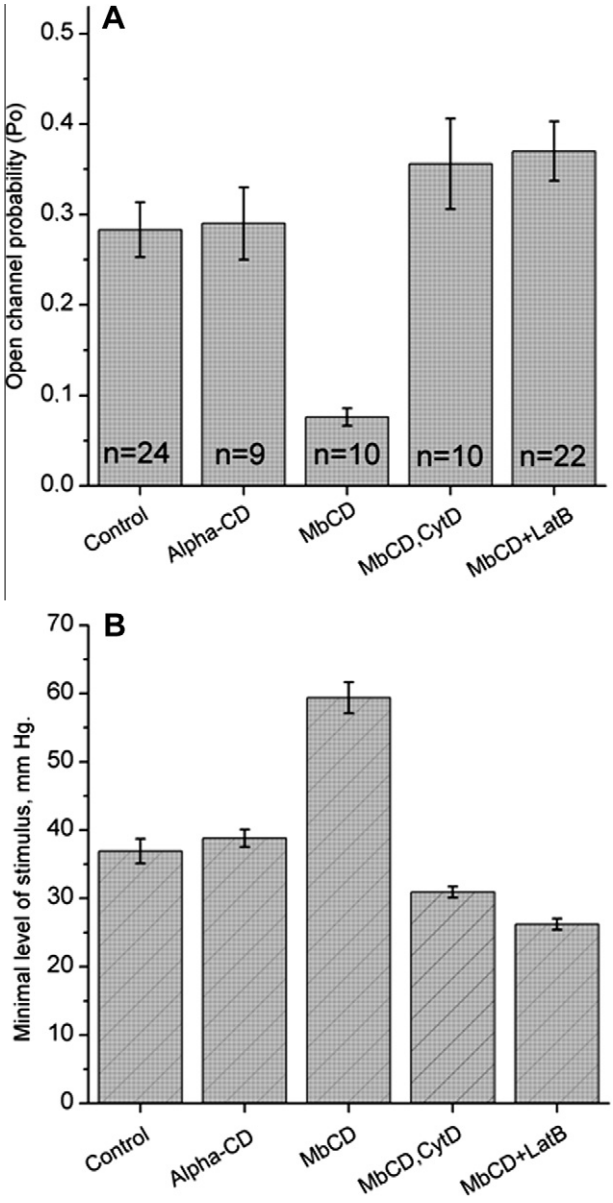

Fig. 4. Summary graphs comparing (A) the open probability values (P_o) and (B) the level of stimulus (“negative” pressure, mm Hg) needed to induce stretch activation of the channels in control and treated K562 cells. Number of observations in each group is shown.

cholesterol level was shown to increase F-actin content in ELA cells [37] and to promote stress fiber formation in mesenchymal and epithelial cell lines [38]. Lipid raft destruction is likely to be a key step in initiation of actin remodeling. How lipid raft disruption initiates reorganization of cytoskeleton still remains elusive. Various pathways have been proposed, specifically, possible involvement of phosphoinositides (PIP_2) [36] and small G-protein-dependent mechanisms has been documented [39]. Recenly, Qi et al. have shown that cholesterol depletion by MbCD induces activation of raft-associated Src kinase, which is lying upstream of WASP/WAVE – Arp2/3 complex – a well-known promoter for actin rearrangement [38].

As it was found in our experiments, SAC functions maintained after drug-induced actin disruption (Figs. 3 and 4). Moreover, typical stretch-activation of single currents was observed in response to both negative and positive pressure (Fig. S2). These observations together with earlier results [8] imply that, in general, modified “bilayer model” is plausible for SAC gating in leukemia cells consistently with the recent study of Sharif-Naeini et al. [40] (see also [41]). The authors demonstrated the interplay between the membrane and the cytoskeleton in the regulation of SAC gating, suggesting a combination of both bilayer and tether models for the gating of SACs.

Taken together, our results allow us to suppose that actin network may indirectly regulate stretch-sensitive channels by affecting membrane deformability in eukaryotic cells. Therefore, in some cells and tissues, cytoskeleton disruption could be expected to increase SAC activity, in agreement with the number of earlier studies (see [8] for Ref.). Single current measurements on K562 leukemia cells show that specific effects of actin disrupters may depend on cytoskeleton-plasma membrane organization and cellular mechanical status. Specifically, in control cells (without cholesterol depletion-induced actin assembly) cytochalasin or latrunculin caused only insignificant increase of the open probability of SACs. In contrast, actin destructors evidently promote SAC activation in cholesterol-depleted cells.

Despite intensive studies, molecular identity of eukaryotic SACs is still problematic [42] thus limiting possible experimental approaches to explore their gating; novel candidates Piezo1 and Piezo2 have just appeared [43]. Putative roles of TRPP and TRPC channels as mechanosensors are currently discussed [4,13,40,42]. Basing on our electrophysiological data, an association of SAC molecules with lipid rafts in leukemia cells seems to be unlikely. Evidently, raft targeting is not always a crucial factor in control of pore-forming membrane proteins and other putative regulatory molecules by lipid environment. Specifically, cholesterol-enriched lipid rafts may affect channel behavior indirectly via actin remodeling.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.046.

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