The actin cytoskeleton has been shown to be involved in the regulation of sodium-selective channels in non-excitable cells. However, the molecular mechanisms underlying the changes in channel function remain to be defined. In the present work, inside-out patch experiments were employed to elucidate the role of submembranous actin dynamics in the control of sodium channels in human myeloid leukemia K562 cells. We found that the application of cytochalasin D to the cytoplasmic surface of membrane fragments resulted in activation of non-voltage-gated sodium channels of 12 pico siemens conductance. Similar effects could be evoked by addition of the actin-severing protein gelsolin to the bath cytosol-like solution containing 1 mM [Ca\(^{2+}\)]. The sodium channel activity induced by disassembly of submembranous microfilaments with cytochalasin D or gelsolin could be abolished by intact actin added to the bath cytosol-like solution in the presence of 1 mM MgCl\(_2\) to induce actin polymerization. In the absence of MgCl\(_2\), addition of intact actin did not abolish the channel activity. Moreover, the sodium currents were unaffected by heat-inactivated actin or by actin whose polymerizability was strongly reduced by cleavage with specific Escherichia coli A2 protease ECP32. Thus, the inhibitory effect of actin on channel activity was observed only under conditions promoting rapid polymerization. Taken together, our data show that sodium channels are directly controlled by dynamic assembly and disassembly of submembranous F-actin.

Functional coupling between channel proteins and the cortical cytoskeleton may play a key role in membrane ion transport and cellular signaling. Involvement of F-actin in ion channel functioning has been established and studied extensively in polarized epithelial cells (1–7). Specifically, several lines of evidence revealed an association between the amiloride-sensitive sodium channels and the actin-based cytoskeleton in renal epithelia. Indirect immunofluorescence and confocal microscopy demonstrated that sodium channels in the apical membrane colocalize with actin, spectrin (fodrin), and ankyrin (5, 6). Electrophysiological studies on epithelial A6 cells showed that disruption of actin microfilament networks by cytochalasin D induced sodium channel activity both in cell-attached and excised patches (1). Similar effects were observed in the presence of actin or actin-gelsolin complexes added to the cytoplasmic side of excised inside-out patches, whereas the actin-DNase I complexes did not activate sodium channels. These results were explained by a model suggesting that the channels are activated by short actin filaments produced either by severing of endogenous long filaments with cytochalasin or by assembly from monomeric actin during spontaneous or gelsolin-mediated polymerization (1). Similar observations were made on planar lipid bilayers containing cloned epithelial sodium channels (4, 7). In addition, interaction of actin with epithelial channels was reported to modulate considerably the intrinsic channel characteristics including conductance and selectivity.

Along with epithelial amiloride-sensitive sodium channels, novel non-voltage-gated sodium-selective channels insensitive to amiloride (up to 100 \(\mu\)M) and to tetrodotoxin have been described in different non-excitable cells, particularly in vascular smooth muscle cells (8), macrophages (9), and carcinoma (10) and leukemia cells (11–13). In these studies, single current measurements in different patch configurations showed that conductance, selectivity, and gating properties of the novel family of sodium channels proved to be very similar to those of the epithelial channels (14). Extremely low sensitivity to amiloride and its derivatives is the major difference between these channels and epithelial sodium channels. This novel family of sodium channels is also clearly distinct from well-known voltage-gated channels typically expressed in excitable membranes (15). The regulatory mechanisms of non-voltage-gated sodium-selective channels partially mediating sodium influx in different non-excitable cells are largely unknown. We have previously found that cytochalasin D treatment of K562 leukemia cells strongly increased the open probability of sodium channels recorded in cell-attached experiments, indicating that the actin cytoskeleton is involved in the control of non-voltage-gated sodium channels (12). Further experiments on leukemia cells showed that activity of sodium channels could be increased by the actin-severing protein gelsolin applied to the cytoplasmic surface of membrane fragments at the micromolar level of [Ca\(^{2+}\)], (16). In the experiments on intact cells, the elevation of [Ca\(^{2+}\)], using the ionophore 4Br-A23187 also resulted in channel activation. Subsequent addition of actin to the cytoplasmic membrane surface reduced sodium currents to the background level (16). This implies that calcium-dependent modulations of the actin cytoskeleton are involved in the regulation of sodium channels. More specifically, these data allowed us to suggest that inhibition of sodium currents by actin may be due to assembly of microfilaments at the cytoplasmic surface of cell membranes, whereas disassembly of submem-
branous F-actin induces channel activation. The aim of the present work was to verify these suggestions and to elucidate intracellular mechanisms underlying activation and inactivation of the novel non-voltage-gated sodium channels.

The data presented here support the putative role of assembly-disassembly of cortical actin microfilaments in the control of sodium channels in leukemia cells. We show that the efficiency of actin in abolishing the sodium channel activity correlates with the kinetics of actin polymerization and that non-polymerizable actin does not affect sodium currents. These data represent the first direct evidence for involvement of actin polymerization in the regulation of non-voltage-gated sodium channels.

EXPERIMENTAL PROCEDURES

Cells—Human myeloid leukemia K562 cells (Cell Culture Collection, Institute of Cytology, St. Petersburg, Russia) were kept in culture as described elsewhere (12). For patch clamp experiments cells were plated on coverslips and maintained in culture for 1–3 days before use.

Electrophysiology—Single channel currents were recorded using the standard inside-out configuration of the patch clamp technique (17). Pipettes were pulled from soft glass capillaries to a resistance of 10–15 megohms when filled with external solution. Membrane currents were recorded using a homemade head stage, based on Burr-Brown operational amplifier OPA-128 with a 20-gigohm feedback resistor and a computer controlled set of Bessels LM-202 filters and L-Card amplifiers (L-Card, Moscow) for signal conditioning. Data were filtered at 200 Hz and sampled at a rate of 1 kHz by 12-bit analog-digital converter for analysis and display. Experiments were performed at room temperature (21–23 °C). Channel open probability (P_o) was determined using the following equation: P_o = I/I, 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. Averaged data are given as the mean ± S.E.

Solutions—Recording pipettes were filled with normal external solution containing 145 mM NaCl, 2 mM CaCl_2, 1 mM MgCl_2, 10 mM HEPES/Tris-OH (pH 7.3). Bath cytosol-like solution for inside-out measurements contained 140 mM potassium aspartate (or glutamate), 5 mM NaCl, 1 mM MgCl_2, (if not otherwise stated), 30 mM HEPES/KOH (pH 7.3), 2 mM EGTA, and an appropriate amount (0.98 mM) of CaCl_2 to establish a free ionized calcium concentration ([Ca^{2+}]_i), at the level of 0.1 μM (pCa 7). In the series of experiments with exogenous gelsolin the bath solutions contained different [Ca^{2+}], varying from 0.01 to 1 μM adjusted with 2 mM EGTA or HEDTA. In some experiments chloride or sulfate were used as a major anion in the cytosol-like solution. HEPES, EGTA, HEDTA, and cytochalasin D were from Sigma.

Proteins—G-actin isolated from rabbit skeletal muscle (18) was stored in a low ionic strength solution (2 mM Tris-HCl, pH 7.5, 0.1 mM CaCl_2, 0.2 mM ATP, 0.02% NaN_3) within a week. An aliquot of G-actin stock solution was added to the bath to the final concentration of 0.3 mg/ml. Inactivated actin was obtained from intact actin by heating for 5 min at 70 °C. Inactivation of the sample was checked using the stock solution was added to the bath to the final concentration of 0.3 mg/ml. Inactivated actin was obtained from intact actin by heating for 5 min at 70 °C. Inactivation of the sample was checked using the fluorescence intensities at 320 and 365 nm, respectively (19). The values of the parameter A were 2.5 and 1.3 for intact and inactivated actin, respectively. Proteolytically cleaved actin was prepared from intact actin by incubation of G-actin (2–3 mg/ml) with Escherichia coli A2 protease ECP 32 (20) at an enzyme/protein mass ratio of 1:100 for 2 h at room temperature. Gelsolin was isolated from pig smooth muscle as described (21) and stored as a pelletted ammonium sulfate precipitate at −70 °C.

Actin Polymerization—Actin polymerization was registered as an increase in intensity of light scattering at 350 nm, at 90 °. The measurements were performed in a Shimadzu PC 5000 fluorometer.

RESULTS

Under the control conditions the activity of non-voltage-gated sodium channels was very low, which is in agreement with our previous data on K562 cells (12, 13, 16). In the first series of experiments we studied the effect of cytochalasin D on single currents in the inside-out patches (Fig. 1). Addition of 10 μg/ml cytochalasin D to the bath cytosol-like solution resulted in an activation of sodium-conducting channels in the membrane fragment (Fig. 1A); well resolved inward currents representing single channel openings were observed at negative membrane potential. Fig. 1B represents typical current records measured within 3 min after cytochalasin D application at different levels of holding potential up to +10 mV. Such recordings were analyzed to obtain current-voltage relation data (Fig. 1C). A similar activation of transmembrane ionic currents elicited by cytochalasin D was observed in 14 inside-out patches. As a rule, an evident increase of channel open probability was observed in 2–3 min after cytochalasin D was added to the bath cytosol-like solution; thereafter the channels remained active. They were not affected by the following wash-out of cytochalasin D with the control bath solution. The amplitude of cytochalasin D-induced channel events did not depend on the change of the major anion in the bath “intracellular” solution, confirming a cationic nature of the currents. The mean current-voltage relation (Fig. 1C) approximated by linear regression corresponds to a single channel conductance value of 12 picosiemens and a reversal potential of 23 mV; the estimation of relative permeability gives the value P_Na/P_K of about 3. These param-
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Fig. 2. Sodium channel activity caused by cytochalasin D or gelsolin could be abolished by addition of actin to the cytoplasmic membrane side. A, the effect of exogenous gelsolin and actin on sodium channel activity in inside-out membrane patches. Representative current records at a holding potential of −30 mV show channel activation in response to the application of 25 μg/ml gelsolin at 1 μm free [Ca^{2+}]. Subsequent addition of 0.3 mg/ml G-actin to the cytosol-like solution resulted in a fast inhibition of the sodium currents. B, representative current records show an analogous effect of G-actin addition on sodium channel activity induced previously by cytochalasin D in an inside-out patch; the holding potential was −30 mV. C, time course of inactivation of cytochalasin-evoked sodium currents in response to addition of G-actin. Currents were recorded at −20 mV at time intervals after the actin addition indicated.

The actin-associated inhibition of sodium channel activity might be due to polymerization of actin induced by the physiological concentration of salts in the bath solution. To test this possibility a special series of patch clamp experiments was carried out in which the known characteristics of actin polymerization in solutions were exploited and in which non-polymerizable actin species were used. It is known that actin containing Mg^{2+} as a tightly bound cation (Mg-actin) polymerizes faster than actin containing tightly bound Ca^{2+} (Ca-actin) (22, 29). Consistent with these data, only a small increase in light scattering intensity characteristic of filament formation was observed within 8–10 min after polymerization of 12 μM Ca-actin was initiated by addition of 0.1 mM KCl (Fig. 3). In contrast, Mg-actin was completely polymerized within 2 min (Fig. 3).

To avoid actin polymerization, we took advantage of the limited proteolysis of the actin polypeptide chain between Gly-42 and Val-43 with E. coli protease ECP32 (24). The cleaved actin cannot polymerize while in the calcium form; ECP32-cleaved Mg-actin polymerizes slowly, and the critical concentration of its polymerization is very high (25). Fig. 3 shows that no polymerization of 12 μM ECP32-cleaved Mg-actin was observed during at least 10 min after addition of 0.1 mM KCl. In addition, heat-inactivated actin, which cannot polymerize under any conditions (19), was used in the following patch lag upon addition of cytochalasin D was typically observed. These data imply that the exact mechanisms of cytochalasin and gelsolin actions may be different. However, both mechanisms seem to involve disassembly of the submembranous actin filament system required for non-voltage-gated sodium channels to be opened.

As shown in Fig. 2, A and B, the sodium channel activity elicited in response to F-actin disassembly by cytochalasin D or gelsolin (at 1 μM [Ca^{2+}]) could be strongly affected by application of G-actin to the cytoplasmic surface of the membrane fragment. Specifically, addition of G-actin to the bath solution reduced the channel activity (and correspondent Po values) to the background level. Fig. 2C shows the kinetics of the inhibitory effect of actin on sodium channels in a typical experiment; the amplitudes of the single currents were not affected, whereas Po significantly decreased. Development of the channel inactivation was observed during a nearly 3 min-period after injection of an aliquot of G-actin stock solution to the bath solution.

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clump experiments.

The results of inside-out experiments with application of different ionic conditions and different actin species are summarized in Fig. 4. The sodium channel activity was induced by cytochalasin D as described above, and then the effect of subsequent G-actin addition was monitored. Stock solutions of G-Ca-actins (see “Experimental Procedures”) were injected into the cytosol-like solution so that the final concentration of actin near the cytoplasmic membrane side was equal to 0.3 mg/ml (7.2 μM), being the same in all experiments. The concentration of free Ca^{2+} in the bath cytosol-like solutions was established at the level of 0.1 μM. Under these conditions, actin either remains in the calcium form or is inactivated due to loss of divalent cation.

We found that addition of intact G-actin to the bath solution in the absence of Mg^{2+} did not affect sodium channel activity; i.e. no inhibition of the cytochalasin D-induced currents was observed during the 6–10-min recordings. The high level of channel activity was also not affected either by inactivated actin or by actin cleaved with protease ECP32. In contrast, sodium currents were rapidly abolished by intact actin when 1 mM MgCl2 was present in the bath solution, promoting a rapid replacement of tightly bound Ca^{2+} by Mg^{2+} and hence the transformation of Ca-actin to Mg-actin (22, 23). Comparison of these results with the kinetics of actin polymerization (Fig. 3) indicates that the “closing” effect of actin correlates with the efficiency of filament formation. However, if actin had been incubated during a 10–30-min period in magnesium-containing cytosol-like solution before being added to the inside-out patch, this polymerized actin did not produce any effect on the channel activity (data not shown). This suggests that actin polymerization at the cytoplasmic surface of the cell membrane may be a factor regulating the activity of the non-voltage-gated sodium channels in leukemia K562 cells.

**DISCUSSION**

The data presented here show that cytochalasin D and gelsolin, known to disassemble the actin filament network, induce activation of non-voltage-gated sodium channels in leukemia cells. On the other hand, addition of actin resulted in inactivation of the channels, and this effect strongly correlated with actin polymerization. The results of inside-out measurements of sodium channels activated by cytochalasin D are in full agreement with the previous data obtained in cell-attached experiments on leukemia K562 cells (12). Earlier, electron microscopy studies of patch-clamped membranes demonstrated that the patch is not a bare bilayer but a membrane-covered bleb of cytoplasm that may include organelles and cytoskeleton (26). The images of excised patches obtained using atomic force microscopy also indicated the presence of cytoskeleton structures forming a strong connection with the membrane (27). Taken together with these morphological data, the similarity of the effects of the F-actin disrupters on the excised membrane fragments with those on native cells implies that after patch excision the part of cortical microfilaments that plays a crucial role in channel regulation does remain attached to the cytoplasmic membrane surface.

The disrupting effect of gelsolin on the cortical cytoskeleton remaining in the excised membrane fragment is in line with the results of gelsolin-produced disassembly of the actin cytoskeleton in permeabilized and microinjected cultured cells (28, 29). This effect may be accounted for by changes that gelsolin generates in the cortical actin network by shortening actin filaments. On the other hand, being in excess, gelsolin can extract most of the actin from the peripheral cytoskeleton by formation of soluble actin-gelsolin 2:1 complexes. Whereas under our experimental conditions, the latter mechanism is likely to take place, both mechanisms may be involved in calcium-dependent rearrangements of the F-actin network and, hence, in regulation of channel activity in living cells.

The novel family of non-voltage-gated amiloride-insensitive sodium channels in non-excitable cells (8–13) has many similarities with epithelial sodium channels (14). Several lines of evidence showed an interaction between the amiloride-sensitive sodium channels and the F-actin network in renal polarized reabsorbing epithelia (1, 2, 4–7). It is reasonable, therefore, to compare the effects of actin rearrangement on non-voltage-gated sodium channels in leukemia cells with those on epithelial sodium channels. In agreement with the fast effects of cytochalasin D on the sodium channels in epithelial cells (1, 2), sodium channels in leukemia cells were also activated by F-actin disrupters. However, in contrast to the epithelial channels, sodium channels in leukemia cells were not inactivated during a prolonged incubation with cytochalasin D (12). Moreover, in variance with epithelial sodium channels, in our experiments, polymerizing actin decreased rather than increased an open probability of the sodium channels in leukemia cells. Therefore, our data do not lead us to assume any special role of short filaments in channel activation. These variances may be due to the difference between epithelial amiloride-sensitive sodium channels (4, 14) and novel amiloride-insensitive sodium channels in non-excitable cells (8–13).

We also did not observe any actin cytoskeleton-dependent modulation of the sodium channel selectivity or conductance reported earlier for interaction of actin with epithelial sodium channels (4, 7). In most experiments, the background activity in the control patches was very low (or close to zero), making difficult a determination of single channel parameters before the channel activation was evoked. Nevertheless, the amplitude of single currents could be reliably monitored during the time course of the actin-induced channel inactivation (Fig. 2C); it remained unchanged in the course of actin assembly.

Inactivation of the amiloride-insensitive sodium channels with polymerizing actin could be due to actin assembly at the cytoplasmic membrane surface. Alternatively, the inactivation could result from interaction of the channels with filaments...
preformed in the cytosol-like solution. It is known that such an actin solution is a heterogeneous population of filaments of different lengths including a high proportion of short filaments (30). In our experiments, addition of F-actin did not inactivate the channels. Therefore, we assume that the mechanism of sodium channel inactivation involves assembly of actin filaments at the membrane surface rather than interaction of the channel with long or short actin filaments.

Thus, our data provide the novel mechanism for regulation of sodium channel activity via actin filament assembly-disassembly. Previously we have found that in intact cells elevation of the intracellular free Ca²⁺ concentration by ionophore 4Br-A23187 also resulted in activation of the sodium channels and that actin reduced this activity to the background level (16). It is plausible, therefore, that a cellular mechanism of sodium channel regulation involves calcium-dependent rearrangement of the cortical cytoskeleton mediated by gelsolin at the disassembly (channel opening) step and can be promoted by actin-nucleating proteins at the assembly (channel closing) step.

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