Na CHANNEL ACTIVITY IN LEUKEMIA CELLS IS DIRECTLY CONTROLLED BY ACTIN POLYMERIZATION.

Yuri A. Negulyaev#, Sofia Y. Khaitlina#, Horst Hinssen§, Ekaterina V. Shumilina#, and Elena A. Vedernikova#

From the #Institute of Cytology RAS, 4 Tikhoretsky Ave., St. Petersburg, 194064, Russia
and the §Biochemical Cell Biology, University of Bielefeld, Bielefeld, Germany

Editorial correspondence to Elena Vedernikova, Institute of Cytology, Russian Academy of Sciences, Tikhoretsky Ave., 4, St.-Petersburg 194064, Russia
Tel: 7-812-2471497; Fax: 7-812-2470341; E-mail: elenmo@link.cytspb.rssi.ru
Abstract. The actin cytoskeleton has been shown to be involved in the regulation of Na-selective channels in non-excitatory 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 Na 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 Na channels of 12 pS conductance. Similar effects could be evoked by addition of the actin-severing protein gelsolin to the bath cytosol-like solution containing 1 μM [Ca^{2+}]_i. The Na 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 Na currents were unaffected by heat-inactivated actin or by actin which polymerizability was strongly reduced by cleavage with specific E.coli A2 protease ECP32. Thus, the inhibitory effect of actin on channel activity was observed only under conditions promoting rapid polymerization. Taking together, our data show that Na channels are directly controlled by dynamic assembly and disassembly of submembranous F-actin.

Key words: Patch clamp, Na channel, Cytoskeleton, Actin polymerization, Leukemia cell, Cytochalasin D, Gelsolin
INTRODUCTION

Functional coupling between channel proteins and the cortical cytoskeleton may play a key role in membrane ion transport and cellular signalling. 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 Na channels and the actin-based cytoskeleton in renal epithelia. Indirect immunofluorescence and confocal microscopy demonstrated that Na 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 Na 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 Na 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 Na 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 Na channels, novel non-voltage-gated Na-selective channels insensitive to amiloride (up to 100 µM) and to tetrodotoxin have been described in different non-excitable cells, particularly in vascular smooth muscle cells (8), macrophages (9), 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 Na 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 of these channels from epithelial Na channels. This novel family of Na channels is also clearly distinct from well-known voltage-gated channels typically expressed in excitable membranes (15). The regulatory mechanisms of non-voltage-gated Na-selective channels partially mediating sodium influx in different nonexcitable cells are largely unknown. We have previously found that cytochalasin D treatment of K562 leukemia cells strongly increased the open probability of Na channels recorded in cell-attached experiments, indicating that actin cytoskeleton is involved in the control of non-voltage-gated Na channels (12). Further experiments on leukemia cells showed that activity of Na channels could be increased by the actin-severing protein gelsolin applied to the cytoplasmic surface of membrane fragments at the micromolar level of \([\text{Ca}^{2+}]_i\) (16). In the experiments on intact cells, the elevation of \([\text{Ca}^{2+}]_i\) using the ionophore 4Br-A23187 also resulted in channel activation. Subsequent addition of actin to the cytoplasmic membrane surface reduced Na currents to the background level (16). This implies that Ca-dependent modulations of the actin cytoskeleton are involved in the regulation of Na channels. More specifically, these data allowed us to suggest that inhibition of Na currents by actin may be due to assembly of microfilaments at the cytoplasmic surface of cell membrane whereas disassembly of submembranous 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 Na channels.

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

EXPERIMENTAL PROCEDURES

Cells

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

Electrophysiology

Single channel currents were recorded using standard inside-out configuration of the patch clamp technique (17). Pipettes were pulled from soft glass capillaries to a resistance 10-15 MΩ when filled with external solution. Membrane currents were recorded using home made head stage, based on Burr-Brown operational amplifier OPA-128 with 20 GΩ feedback resistor and computer controlled set of Bessels filters LM-202 and amplifiers LM-201S (L-Card, Moscow) for signal conditioning. Data were filtered at 200 Hz and sampled at a rate of 1 kHz by 12 bit ADC for analysis and display. Experiments were performed at room temperature (21-23°C). Channel open probability ($P_o$) was determined using following equation: $P_o = I/iN$, 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.m. (number of experiments).
**Solutions**

Recording pipettes were filled with normal external solution containing 145 mM NaCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES/TrisOH (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), 20 mM HEPES/KOH (pH 7.3), 2 mM EGTA and an appropriate amount (0.98 mM) of CaCl$_2$ to establish 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$^{2+}$]$_i$ varying from 0.01 to 1 µM adjusted with 2 mM EGTA or HEDTA. In some experiments chloride or sulphate were used as a major anion in the cytosol-like solution. HEPES, EGTA, HEDTA, 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 parameter A=I$_{320}$/I$_{365}$ of the intrinsic fluorescence spectrum, where I$_{320}$ and I$_{365}$ are 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 pelleted 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 Na 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 Na-conducting channels in the membrane fragment (Fig. 1A); well-resolved inward currents representing single channel openings were observed at negative membrane potential. Figure 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 pS and reversal potential of 23 mV; the estimation of relative permeability gives the value P\textsubscript{Na}/P\textsubscript{K}.
of about 3. These parameters are very similar to those obtained previously for non-voltage-gated Na-permeable channels in human leukemia K562 cells (12,13). Thus, the current-voltage relation data (Fig 1C) allow us to identify reliably the channels activated by cytochalasin D treatment of cytoplasmic surface of membrane fragments; activation of the Na channels seems to be due to the cytochalasin D-induced disassembly of membrane-attached actin filaments.

We have previously shown that gelsolin activates Na channels in a Ca-dependent manner (16). Fig. 2 presents a comparison of the effect produced in excised patches by cytochalasin D with that of exogenous gelsolin. Addition of gelsolin to the bath cytosol-like solution containing 1µM [Ca$^{2+}$]$_i$ caused a significant increase in Na channel activity (Fig. 2A) similarly to the cytochalasin D action (Fig. 2B). The values of the channel open probability after cytochalasin D or gelsolin were similar, $P_o$ varied in the range of 0.3-0.4. Thus the efficiency of these agents towards the Na channel activation was comparable. However, at variance to the effect of gelsolin which was not observed at low concentrations of free calcium (0.01 µM [Ca$^{2+}$]$_i$), the cytochalasin-induced activation of Na channels was Ca-independent. In our previous work we have shown in inside-out experiments that variations in [Ca$^{2+}$]$_i$ in the bath solution did not affect the open probability of non-voltage-gated Na channels in leukemia cells (16), that is these channels are not directly calcium-activated. Thus, the strong calcium dependence of the gelsolin effect suggests that the channel activation is due to a specific severing of actin filaments associated with the membrane fragment. In addition, upon application of gelsolin, open probability $P_o$ reached maximum values without marked delay whereas a 1-3 min 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 submembranous actin filament system required for non-voltage-gated Na channels to be opened.

As shown in Fig 2A,B the Na channel activity elicited in response to F-actin disassembly by cytochalasin D or gelsolin (at \(1 \mu M [Ca^{2+}]_o\)) 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 \(P_o\) values) to the background level. Fig 2C shows the kinetics of the inhibitory effect of actin on Na channels in a typical experiment: the amplitude of the single currents were not affected while \(P_o\) significantly decreased. Development of the channel inactivation was observed during a nearly 3 min-period after injection of an aliquote of G-actin stock solution to the bath solution.

The actin-associated inhibition of Na channel activity might be due to polymerization of actin induced by 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 addition, 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) (22,23). 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 \(\mu M\) Ca-actin was initiated by addition of 0.1 M KCl (Fig. 3). In contrast, Mg-actin was completely polymerized within 2 min (Fig. 3).

To avoid actin polymerization, we took advantage of limited proteolysis of the actin polypeptide chain between Gly42 and Val43 with E.coli protease ECP32 (24).
cleaved actin cannot polymerize while being in the Ca-form; ECP-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 ECP-cleaved Mg-actin was observed during at least 10 min after addition of 0.1M KCl. In addition, heat-inactivated actin which cannot polymerize under any conditions (19) was used in the following patch clamp experiments.

The results of inside-out experiments with application of different ionic conditions and different actin species are summarized in Fig. 4. The Na 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 Materials and Methods) 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 Ca-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 Na channel activity, that is, no inhibition of the cytochalasin-induced currents was observed during the 6-10 min recordings. The high level of channel activity was also not affected by either inactivated actin or by actin cleaved with protease ECP32. In contrast, Na currents were rapidly abolished by intact actin when 1 mM MgCl$_2$ was present in the bath solution promoting a rapid replacement of tightly bound Ca$^{2+}$ by Mg$^{2+}$ and hence, transformation of Ca-actin into 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 10-30 min in Mg-containing cytozol-like solution before it was added to the inside-out patch, this polymerized actin did not produced any effect on the channel activity (not shown). This suggests that actin polymerization at the cytoplasmic surface of cell membrane may be a factor regulating the activity of the non-voltage-gated Na channels in leukemia K562 cells.

**DISCUSSION**

The data presented here show that cytochalasin D and gelsolin known to disassemble actin filament network induce activation of non-voltage-gated Na 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 Na channels activated by cytochalasin D are in a 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 a presence of cytoskeleton structures forming a strong connection with the membrane (27). Taking 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 playing 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 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 one is likely to take place, both mechanisms may be involved in Ca-dependent rearrangements of F-actin network and, hence, in regulation of channel activity in the living cells.

The novel family of non-voltage-gated amiloride-insensitive Na channels in nonexcitable cells (8-13) has many similarities with epithelial Na channels (14). Several lines of evidence showed an interaction between the amiloride-sensitive Na channels and F-actin network in renal polarized readsobring epithelia (1,2,4-7). It is reasonable therefore, to compare the effects of actin rearrangement on non-voltage-gated Na channels in leukemia cells with those on epithelial Na channels. In agreement with the fast effects of cytochalasin D on the Na channels in epithelial cells (1,2), Na channels in leukemia cells were also activated by F-actin disrupters. However, in contrast to the epithelial channels, the latter ones have not been inactivated during a prolonged incubation with cytochalasin D (12). Moreover, in variance with epithelial Na channels, in our experiments, polymerizing actin decreased rather than increased an open probability of the Na 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 Na channels (4,14) and novel amiloride-insensitive Na channels in non-excitable cells (8-13).

We also did not observe any actin cytoskeleton-dependent modulation of the Na channel selectivity or conductance reported earlier for interaction of actin with epithelial
Na 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 Na 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 cytozol-like solution. It is known that such 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 Na 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 Na channel activity via actin filaments assembly-disassembly. Previously we have found that in intact cells elevation of intracellular free Ca$^{2+}$ concentration by ionophore 4Br-A23187 also resulted in activation of the Na channels, and actin reduced this activity to the background level (16). It is plausible, therefore, that a cellular mechanism of Na channel regulation involves Ca-dependent rearrangements of 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.

**Acknowledgements:** The work was supported by the Russian Basic Research Foundation, grants ¹ 98-04-48089, ¹ 99-04-49652, ¹ 99-04-49482 and by the Royal
REFERENCES


**LEGENDS**

**Figure 1.** Cytochalasin D application to the intracellular membrane surface resulted in activation of Na channels in excised patches. A, inside-out current record at holding membrane potential of –50 mV demonstrates a development of the cytochalasin D effect. A fragment of the record in an expanded time scale which shows single open-closed transitions is presented at the bottom. B, current records at different membrane potentials indicated near traces show Na channel activity in 3 min after addition of 10 μg/ml cytochalasin D. C, mean current-voltage relationship of Na channels activated by cytochalasin D; data are summarized from 11 inside-out experiments. Mean unitary conductance was 11.9 ± 0.5 pS; the reversal potential value obtained by extrapolation was 22.8 ± 1.3 mV.

**Figure 2.** Na 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 Na channel activity in inside-out membrane patches. Representative current records at 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 Na currents. B, representative current records show an analogous effect of G-actin addition on Na channel activity induced previously by cytochalasin D in an inside-out patch; holding potential was -30 mV. C, time course of inactivation of cytochalasin-evoked Na currents in response to addition of G-actin. Currents were recorded at –20 mV at time intervals after the actin addition indicated.

**Figure 3.** Increase in light scattering intensity reflected different rates of polymerization of intact and protease ECP32 cleaved Ca- and Mg-actins. At time
indicated by arrow 0.1 MKCl was added to 0.5 mg/ml intact or ECP32 cleaved Ca- or Mg-G-actins. Both intact and ECP32 cleaved Mg actins were obtained from the corresponding Ca actins by a 5-min-incubation with 0.2mM EGTA/0.1mM MgCl₂. Actin polymerization is accompanied by an increase of light scattering intensity which was recorded at 350 nm at 90°.

Figure 4. Application of different actin species indicates that actin polymerization is responsible for Na channel inactivation. A, Representative current records of cytochalasin-activated Na currents before (on the left) and after (on the right) addition of different actin species to the cytoplasmic membrane side of a plasma membrane fragment: IA, inactivated G-actin; EA, G-actin cleaved with protease ECP32; noMg, intact G-actin in the absence of magnesium in the cytosol-like solution, 1Mg; intact G-actin added to the standard Mg-containing cytosol-like solution which induces fast actin polymerization. B, The mean $P_o$ values represent the channel activities after cytochalasin D treatment and after actin addition, respectively.
Fig. 1

A

B

C

I_m, pA

E_m, mV

10 s

200 ms

1 pA

1 s

0 mV

-10 mV

-20 mV

-30 mV

-40 mV

-50 mV

0,5

0,5

-1,5

-1
Fig. 2

A

Control
Gelsolin
G-actin

0.5 pA
400 ms

B

Control
Cytochalasin D
G-actin

0.5 pA
400 ms

C

1 min
1.5 min
2 min
2.5 min
3 min

0.5 pA
400 ms

Fig. 2
Fig. 3

Light scattering intensity, a.u.

Time, min

intact, Mg

intact, Ca

ECP, Mg

ECP, Ca
Cytochalasin D

A

inactivated G-actin

Cytochalasin D

ECP32-cleaved G-actin

Cytochalasin D

intact G-actin, no Mg²⁺

Cytochalasin D

intact G-actin, 1mM Mg²⁺

B

⇒

P₀

0,0

0,4

CD IA

⇒

P₀

0,0

0,4

CD EA

⇒

P₀

0,0

0,4

CD noMg

⇒

P₀

0,0

0,4

CD 1Mg

Fig. 4
Na channel activity in leukemia cells is directly controlled by actin polymerization
Yuri A. Negulyaev, Sofia Y. Khaitlina, Horst Hinssen, Ekaterina V. Shumilina and
Elena A. Vedernikova

J. Biol. Chem. published online October 2, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008219200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2000/10/02/jbc.M008219200.citation.full.html#ref-list-1