Non-hydrolyzable analog of GTP induces activity of Na^+ channels via disassembly of cortical actin cytoskeleton

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Abstract The role of G proteins in regulation of non-voltage-gated Na^+ channels in human myeloid leukemia K562 cells was studied by inside-out patch-clamp method. Na^+ channels were activated by non-hydrolyzable analog of guanosine triphosphate (GTP), GTPγS, known to activate both heterotrimeric and small G proteins. Channel activity was not affected by aluminum fluoride that indiscriminately activates heterotrimeric G proteins. The effect of GTPγS was prevented by phalloidin and by G-actin, both interfering with actin disassembly, which indicates that GTPγS-induced channel activation was likely due to microfilament disruption. GTPγS-activated channels were inactivated by polymerizing actin. These data show, for the first time, that small G proteins can regulate Na^+ channels, and an intracellular mechanism mediating their effect involves actin cytoskeleton rearrangements.

Key words: Patch-clamp; Na^+ channel; G protein; Guanosine 5′-[γ-thio]triphosphate; Actin cytoskeleton; Leukemia cell

1. Introduction

Na-conducting channels fulfill a variety of physiological roles in different cell types. In electrical excitable cells, voltage-gated Na^+ channels contribute to the initiation and propagation of the action potential [1]. Epithelial sodium channels (ENaC) are located in the apical membranes of certain epithelial cells. In addition to epithelial cells, many other cell types have been shown to express epithelial-like channels. Outside of the reabsorbing epithelia, these channels have been described in sensory [2], vascular endothelial [3], smooth muscle [4], human B lymphoid [5], rat basophilic leukemia [6], human myeloid leukemia cells [7] and in rat macrophages [8]. The individual channels have a conductance of 5–30 pS and different sensitivity to diuretic amiloride. Recently ENaC- and ENaC-like channels have been considered to belong to a gene family which also includes *Caenorhabditis elegans* degenerins, proton-gated cation channels (ASIC) that could be involved in pain transduction in the central and peripheral nervous system and FMRFamide-gated channels in snail [9,10]. The extended ENaC family, members of which are clearly but distantly related in primary sequence, shows the common property of being non-voltage-gated Na-selective channels.

Many ion channels are known to be regulated by GTP-binding (G) proteins. Modulation of ion channels by G proteins can be indirect, via second messengers and protein kinases, or direct, via physical interactions between G protein subunits and the channel protein. A direct regulation has been demonstrated for several voltage-dependent Ca^{2+} channels and the G protein-activated K^+ channels [11]. Interactions between epithelial Na^+ channels and G proteins, particularly G_{i3}, have been reported [12,13], and G_{i3} has been shown to form a part of the purified Na^+ channel complex [12]. However, little is known about the physiological significance of these interactions. One possibility is that G proteins mediate the response to an intracellular signal, e.g. aldosterone receptor stimulation [14] and cyclic adenosine monophosphate (cAMP) [15]. A recent study has suggested that in mandibular gland duct cells, G proteins mediate the regulation of ENaC-like channels by intracellular Na^+ and Cl^- [16]. In non-epithelial tissues the G protein-mediated regulation of non-voltage-gated Na^+ channels was shown for rat basophilic [6] and for B lymphoid [17] cells. The molecular mechanisms of this regulation remain unclear.

The non-voltage-gated epithelial-like channels described in human leukemia K562 cells [7] are activated by disruption of membrane-associated actin filaments and inactivated by polymerizing actin [7,18,19]. The modulation of channel activity by actin cytoskeleton has been the only known mechanism of Na^+ channel regulation in K562 cells. In this report we present results of patch-clamp inside-out experiments that assessed the role of G protein-mediated regulation of Na^+ channels in human leukemia K562 cells. Using combinations of treatments we have found that G proteins can regulate Na^+ channels via reorganization of membrane-attached actin filaments. We suggest that uncapping of actin filaments by small G proteins is likely to be involved in this process.

2. Materials and methods

2.1. Cells

Human myeloid leukemia K562 cells (Cell Culture Collection, Institute of Cytology, Russia) were kept in culture as described elsewhere [7]. For patch-clamp experiments cells were plated on coverslips and maintained in culture for 1–3 days before use.

2.2. Electrophysiology

Single channel currents were recorded using standard inside-out configuration of the patch-clamp technique [20]. Pipettes were pulled from soft glass capillaries to a resistance of 10–15 MΩ when filled with external solution. Membrane currents were recorded using a

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home made head stage, based on Burr–Brown operational amplifier OPA-128 with 20 GΩ feedback resistor and a computer controlled set of Bessel filters LM-202 and amplifiers LM-201S (L-Card, Moscow, Russia) for signal conditioning. Data were filtered at 200 Hz (if not otherwise stated) 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 the 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).

2.3. Solutions

Recording pipets 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). The bath cytosol-like solution for inside-out measurements contained 140 mM potassium aspartate, 5 mM NaCl, 1 mM $MgCl_2$ (if not otherwise stated), 20 mM HEPES/KOH (pH 7.3), 2 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and an appropriate amount (0.176 mM) of $CaCl_2$ to establish free ionized calcium concentration at the level of 0.01 μM (*p*Ca 8). HEPES, EGTA, GTPγS (guanosine 5′-[γ-thio]triphosphate), cytochalasin B (CB), phalloidin were from Sigma.

2.4. Proteins

G-actin isolated from rabbit skeletal muscle [21] was stored in a low ionic strength solution (2 mM Tris–HCl, pH 7.5, 0.1 mM $CaCl_2$, 0.2 mM adenosine triphosphate (ATP), 0.02% NaN_3) and used within a week. An aliquot of the G-actin stock solution was added to the bath to a final concentration of 300 μg/ml.

3. Results

To search for the role of G proteins in the regulation of Na^+ channels in K562 cells, we used GTPγS, a non-hydrolyzable analog of GTP, which activates both, heterotrimeric and small G proteins [22], and aluminum fluoride AlF_4^-, which indiscriminately activates heterotrimeric G proteins [23]. In inside-out experiments in a standard cytosol-like solution the activity of non-voltage-gated sodium channels was very low (P_o close to 0). To test the involvement of heterotrimeric G proteins, a routine protocol was used [22]: first the cytosol-like bath solution was complemented with $AlCl_3$ (Al^{3+}, 100 μM) and then in 2–3 min KF (F^-, 10 mM) was added to the bath. We found that AlF_4^- did not change low level of background channel activity ($n = 43$). In contrast, addition of 100 μM GTPγS to the bath resulted in an activation of Na^+ channels in the membrane fragment (Fig. 1). An evident increase of channel open probability (P_o) was developed with rather a long delay – 6–10 min after GTPγS was added to the bath solution; thereafter the channels remained active. Channel activity was not affected by the following wash-out of GTPγS with the control bath solution. Fig. 1A represents typical current records measured in 8 min after GTPγS application. A similar activation of Na^+ channels elicited by GTPγS was observed in 23 out of 67 (34%) inside-out patches. The mean current–voltage relation (Fig. 1B) approximated by linear regression corresponds to a single channel conductance value of about 11 pS and a reversal potential of about 20 mV; the estimation of relative permeability gives the value P_{Na}/P_K of about 3. These parameters are close to those obtained previously for Na^+ channels which were activated by the agents disrupting actin cytoskeleton, such as cytochalasins [7,24] and gelsolin [18].

Fig. 2 compares the effects produced by GTPγS and CB on Na^+ channel activity in inside-out patches. As seen from single channel records and P_o values, the effects are very similar

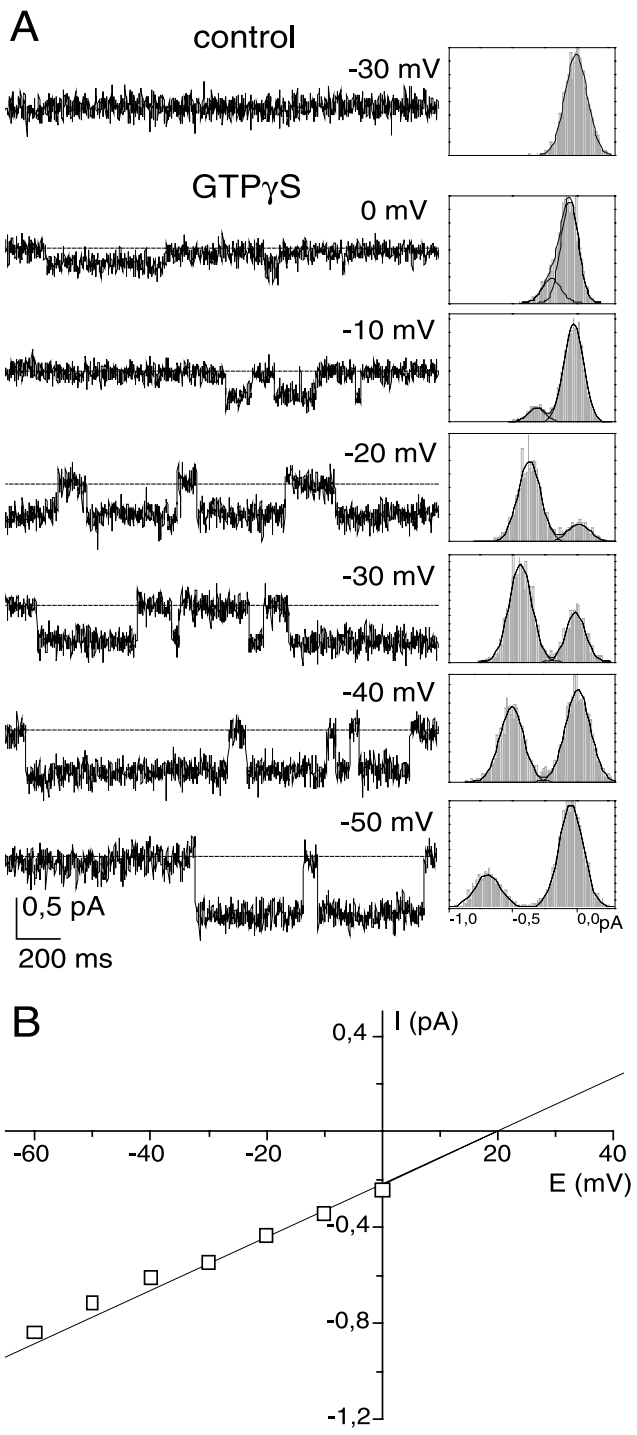

Fig. 1. Application of GTPγS to the intracellular membrane surface resulted in activation of Na^+ channels in excised patches. A: Inside-out current records at different membrane potentials and corresponding amplitude histograms show the activation of Na^+ channels in 8 min after the addition of 100 μM GTPγS to the cytosol-like bath solution. Filter 100 Hz. B: Mean current–voltage relationship of Na^+ channels activated by GTPγS; data are summarized from 15 experiments. Unitary conductance was 11.1 ± 0.6 pS; the reversal potential obtained by extrapolation was 20 mV.

and differ only in the time course of channel activation. The delay between cytochalasin application and channel activation was short (1–3 min), whereas upon addition of GTPγS a 6–10 min lag was typically observed. It turned out that the Na^+

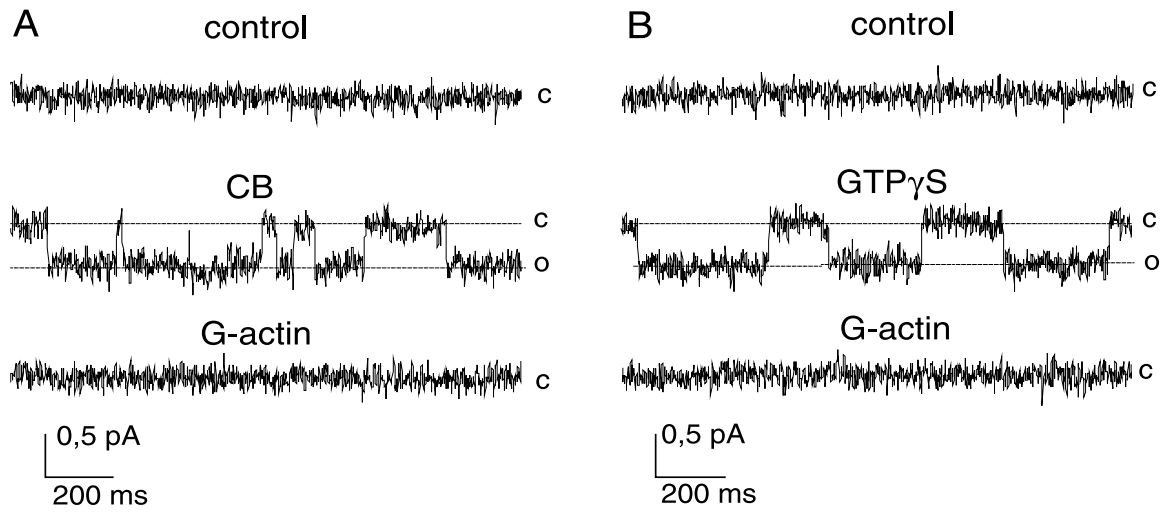

Fig. 2. Na^+ channel activity caused by GTPγS or CB could be abolished in a similar manner as by actin addition to the cytosol-like solution. A: Representative current records show Na^+ channel activity in response to the application of 10 μg/ml CB. Subsequent addition of 300 μg/ml G-actin to the cytosol-like solution resulted in a fast inhibition of Na^+ currents. B: Representative current records show an analogous effect of G-actin addition on Na^+ channel activity induced by 100 μM GTPγS in inside-out patch; currents were recorded at −20 mV.

channel activity elicited by GTPγS can be strongly affected by the following addition of G-actin (300 μg/ml) to the cytosol-like solution. In 1–2 min after injection of an aliquot of G-actin stock solution to the bath the channel activity (and the corresponding P_o values) were decreased to the background level (Fig. 2). This effect was practically indistinguishable from the G-actin-induced inhibition of the channels activated by cytochalasin. Thus, the Na^+ channel activity induced either by GTPγS application or by submembranous actin disassembly can be inhibited by actin in the same manner.

The actin-induced inhibition of Na^+ channel activity elicited by cytochalasin was previously shown to correlate with efficiency of actin polymerization in solution [19]. It is known that actin containing Ca^{2+} as a tightly bound cation (Ca-actin) polymerizes much slower than actin containing Mg^{2+} (Mg-actin) [25]. In order to elucidate whether Na^+ channels activated by GTPγS could be inhibited by polymerizing actin, as it occurs upon cytochalasin-induced channel activity [19], incubation of the membrane fragment with GTPγS was followed by addition of G-actin with or without magnesium ions in the bath (Fig. 3). GTPγS-induced channel activity was not inhibited when magnesium was excluded from the bath solution. However, the Na^+ currents were abolished when, after 5 min of the current recording, Ca-actin was replaced with Mg-actin (Fig. 3), indicating that actin-induced channel inactivation occurs only under conditions promoting fast filament formation.

In the next series of experiments we have shown that no inhibition of the GTPγS-induced channels was observed in response to F-actin polymerized prior to its addition to the bath solution (Fig. 4). In some experiments ($n = 4$) we modulated the length of preformed filaments. To break the long actin filaments, we let F-actin solution pass through a thin needle for 10–15 times. However, independently of the length, preformed actin filaments had no effect on channel activity.

Since the delay in channel activation after application of GTPγS was rather long and since polymerizing actin could inhibit GTPγS-elicited channel activity, we suggested that G protein activated by GTPγS did not directly modulate the activity of Na^+ channels but rather initiates the cascade of reactions that leads to the disruption of membrane-associated actin filaments. This possibility was first tested in the set of experiments with phalloidin, an agent known to stabilize actin filaments and prevent their depolymerization, including that induced by cytochalasins [26]. 12 μM phalloidin was added to the experimental chamber 1 min before addition of 100 μM GTPγS. In 14 out of 14 experiments no channel activity was observed during 10–12 min after GTPγS application in such conditions. When patches were stable ($n = 12$), in 10 min GTPγS was washed-out and 10 μg/ml CB was applied to the cytosol-like bath solution. As expected, no current was evoked by CB, confirming that the phalloidin-stabilized actin filaments could not be disassembled. These findings strongly suggest that GTPγS-induced activation of Na^+ channels is due to disassembly of cortical F-actin.

To prove this suggestion more directly, we examined whether GTPγS could activate the channels in the presence of Ca-actin. Ca-actin polymerizes too slowly to inhibit the channel activity but it can effectively add to the free ends of existing filaments, thus preventing their disassembly [25]. Indeed, when 100 μM GTPγS and 300 μg/ml Ca-actin were applied to the bath solution simultaneously, no channel activation occurred during 9–10 min of the recording ($n = 9$). We have additionally demonstrated that the absence of channel activation was not due to the absence of Na^+ channels in the membrane fragment: in four experiments patches kept stability and in 10 min it was possible to remove the mixture of GTPγS and Ca-actin and to apply 10 μg/ml CB. In two out of four experiments CB evoked the activation of Na^+ channels in 1–2 min after its application, indicating that potentially active channel molecules were present in the membrane fragment.

Taking together, the results of these experiments clearly show that GTPγS initiates the cascade of reactions resulting in a disruption of actin filaments and activation of Na^+ channels in inside-out patches on K562 cells.

4. Discussion

The results presented in this study show that in inside-out patch experiments on leukemia cells GTPγS activates non-vol-

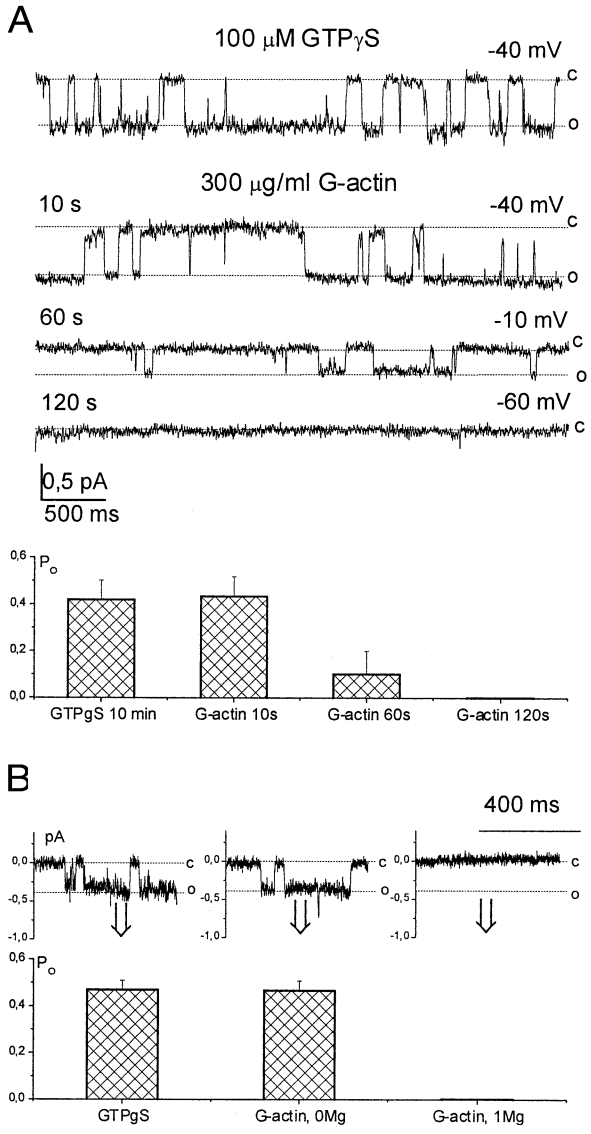

Fig. 3. GTPγS-induced activity of Na^+ channels is abolished by actin only under conditions promoting fast filament formation. A: Current records (above) and correspondent mean P_o values (below) show time course of Na^+ channel inactivation in response to addition of G-actin to the cytosol-like Mg-containing solution. Time intervals after actin addition and holding potentials are indicated. B: Mean P_o values (below) demonstrate that the GTPγS-induced activity of Na^+ channels was not affected by Ca-actin and was inhibited by subsequent addition of Mg-actin to the bath. Correspondent current records at a holding potential of −30 mV are shown above. Filter 100 Hz.

tage-gated Na^+ channels suggesting that G proteins can control Na^+ channel activity in these cells. AlF_4^- that is known to activate heterotrimeric G proteins [23] did not change the channel activity. Involvement of small G proteins in the activation process is, therefore, more plausible. The effect of GTPγS was prevented by phalloidin and by the presence of globular actin. Moreover, the GTPγS-activated channels were inactivated by polymerizing actin in a manner similar to inactivation of these channels upon disruption of cortical actin filaments [19]. These data indicate that G proteins regulate Na^+ channels indirectly via rearrangements of membrane-associated actin filaments. These rearrangements may play a permissive role for channel activation by G protein-dependent or -independent mechanisms.

It is well known that small GTP-binding proteins of the Rho family (Rho, Rac, Cdc42) appear to be key players in controlling the organization of actin cytoskeleton [27]. Rho proteins modulate activity of many actin-binding proteins and, in particular, can promote dissociation of capping proteins from the barbed ends of actin filaments [28]. In permeabilized platelets, Rac increases the availability of free barbed ends, as does a peptide agonist of the thrombin receptor and GTPγS [29]. Similarly, in neutrophils, activation of G proteins with GTPγS could trigger actin polymerization by increasing the number of free barbed ends [30], with the effect being abolished by RhoGDI and *Clostridium difficile* toxin B, which inactivate Rho family proteins [31]. It is possible that disassembly of membrane-associated actin filaments in our experiments was also due to uncapping of the filament barbed ends. In vivo, the liberation of barbed ends leads, as a rule, to the burst polymerization since the pool of G-actin is available in cytoplasm. However, during the experiment on the membrane fragment when monomeric actin is not added to the cytosol-like solution, uncapping of membrane-associated actin filaments can lead to the opposite effect, that is the depolymerization of F-actin. In agreement with this suggestion, GTPγS was unable to induce channel activity when Ca-actin was present in the bath solution. Thus, we speculate that in leukemia cells the activation of small G proteins initiates the cascade of reactions resulting in a liberation of the ends of actin filaments. Free ends of actin filaments are available for addition or loss of actin subunits depending on the presence or absence of the pool of G-actin in the vicinity of channels, respectively. The evidence for a possible participation of capping proteins in channel regulation in K562 cells was obtained recently [24].

The mechanism by which Rho family proteins induce the liberation of free barbed ends can involve increase in phosphorylation of phosphatidylinositol resulting in the formation of phosphatidylinositol 4,5-biphosphate (PIP_2), as it is shown for platelets [29]. PIP_2 is known to inhibit capping activities of gelsolin and capping protein and thus PIP_2 can increase the number of barbed ends either by preventing capping of newly created ends, or by removing a capping protein from existing ends [32]. However, in neutrophils, the response to GTPγS did not correlate with PIP_2 synthesis and was not dependent on the presence of protein kinases and phosphatases, as their inhibitors and removal of ATP did not block the GTPγS-induced actin polymerization [31]. Our study indicates that in leukemia cells the GTPγS-induced depolymerization of actin filaments also occurs independently of PIP_2 synthesis or effect of protein kinases, since the cytosol-like bath solution does not contain ATP or any other sources of phosphate.

It is shown that epithelial channels [12,13] and epithelial-like Na^+ channels in B lymphoid cells [17] can be regulated by heterotrimeric G proteins. In addition, the apical Na^+ channels in mandibular gland duct cells were shown to be regulated by G_o and G_i proteins, which activities were controlled, respectively, by cytosolic Na^+ and Cl^- [16]. There is, however, only one evidence for modulation of epithelial-like Na^+ channels by small G proteins. Specifically, in rat basophilic cells, AlF_4^- activated Na^+ current less efficiently than GTPγS, and, thus, the possible role of small G proteins was proposed [6]. Our data show, for the first time, that small G proteins reg-

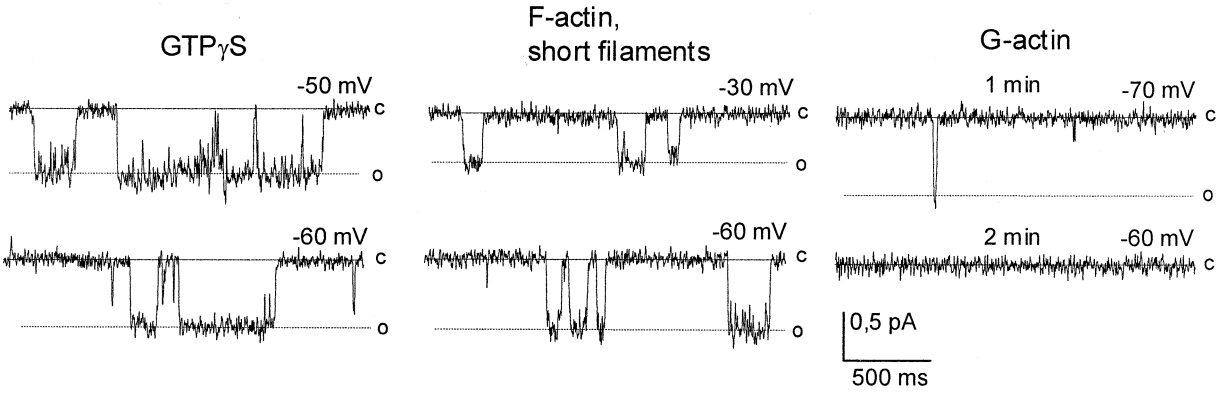

Fig. 4. Preformed actin filaments could not inhibit Na^+ channel activity. Inside-out current records of GTPγS-induced Na^+ currents before (left column) and after (middle column) addition of 300 μg/ml F-actin demonstrate that channel activity is not affected by preformed actin filaments. Subsequent addition of 300 μg/ml G-actin to the bath results in channel inactivation (right column). Time intervals after agent application to the cytosol-like bath solution and holding potentials are indicated.

ulate channel activity and an intracellular mechanism mediating their effect can involve actin cytoskeleton rearrangements.

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