Increased focal Kv4.2 channel expression at the plasma membrane is the result of actin depolymerization

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Wang, Zhuren, Jodene R. Eldstrom, Joshua Jantzi, Edwin D. Moore, and David Fedida. Increased focal Kv4.2 channel expression at the plasma membrane is the result of actin depolymerization. Am J Physiol Heart Circ Physiol 286: H749–H759, 2004.—Voltage-dependent potassium channel trafficking and localization are regulated by proteins of the cytoskeleton, but the mechanisms by which these occur are still unclear. Using human embryonic kidney (HEK) cells as a heterologous expression system, we tested the role of the actin cytoskeleton in modulating the function of Kv4.2 channels. Pretreatment (≥1 h) of HEK cells with 5 μM cytochalasin D to disrupt the actin microfilaments greatly augmented whole cell Kv4.2 currents at potentials positive to −20 mV. However, no changes in the voltage dependence of activation and inactivation of macroscopic currents were observed to account for this increase. Similarly, single channel recordings failed to reveal any significant changes in the single channel conductance, open probability, and kinetics. However, the mean patch current was increased from 0.9 ± 0.2 pA in control to 6.7 ± 3.0 pA in the presence of cytochalasin D. Imaging experiments revealed a clear increase in the surface expression of the channels and the appearance of “bright spot” features, suggesting that large numbers of channels were being grouped at specific sites. Our data provide clear evidence that increased numbers and altered distribution of Kv4.2 channels at the cell surface are primarily the result of reorganization of the actin cytoskeleton.

Kv4.2, widely expressed in the brain and heart (33), is rapidly activating and inactivating (12), and this gives it an important role in regulating neuronal excitability and in the rapid repolarization phase of the cardiac action potential. In the cerebellum and cultured hippocampal neurons, filamin, an actin-binding protein that links the Kv4.2 channel protein to the cytoskeleton, changes the subcellular distribution of Kv4.2 channels (34). Expression of filamin is associated with a two- to threefold increased Kv4.2 current density. In the heart, Kv4.2 underlies the transient outward K+ current (Ito) in some mammals and is responsible for the rapid repolarization phase of the cardiac action potential. In normal rat cardiac myocytes, no effects were found on either action potential duration or Kv4.2 current density upon treatment with high concentrations of cytochalasin D (50 μM). However, in the hypertrophied heart, Kv4.2 was downregulated threefold by cytochalasin D treatment in rat myocytes (49).

In the present study, we tested the influence of the cytoskeleton on rat Kv4.2 channels expressed in human embryonic kidney (HEK) cells. Disruption of the actin cytoskeleton with cytochalasin D significantly increased Kv4.2 currents in Kv4.2-expressing cells, as was found for Kv1.5 channels in HEK cells (27). Here, we attempted to distinguish between two possibilities: that this current increase is the result of altered ion channel kinetics at the macroscopic or single channel level or is the result of altered cell surface expression of ion channels. To answer this question, we examined the whole cell and single channel kinetics of Kv4.2-expressing HEK cells before and after pretreatment with cytochalasin D. In addition, we carried out immunofluorescent experiments and utilized bright-field deconvolution techniques to examine the localization of Kv4.2 in transfected HEK cells.

MATERIALS AND METHODS

Cells and solutions. Two forms of rat Kv4.2 were used in the present experiments, wild-type (WT) Kv4.2 in the electrophysiological experiments and T7-tagged Kv4.2 channels for imaging purposes, separately expressed in HEK-293 cells. T7-tagged Kv4.2 was found to respond to cytochalasin D in the same manner as WT channels in whole cell and single channel recordings (data not shown). For whole cell current recordings from HEK cells, patch pipette solutions contained (in mM) 135 KCl, 5 EGTA, 1 MgCl2, and 10 HEPES and were adjusted to pH 7.2 with KOH. The bath solution contained (in mM) 5 KCl, 130 NaCl, 10 HEPES, 1 MgCl2, and 1 CaCl2 and was adjusted to pH 7.4 with NaOH. For cell-attached patch recording, pipette solutions contained (in mM) 5 KNO3, 130 NaNO3, 10 HEPES, 1 MgCl2, 1 MgCl2, and 1 CaCl2 and was adjusted to pH 7.4 with NaOH. The bath solution contained (in mM) 135 KNO3, 10 HEPES, 1 MgCl2, and 1

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CaCl₂ and was adjusted to pH 7.4 with KOH. We used NO₃⁻ to substitute for Cl⁻ to prevent occasional endogenous Cl⁻ channels. All chemicals were from Sigma-Aldrich (Mississauga, Ontario, Canada).

T7-tagged Kv4.2 imaging. HEK 293 cells were transfected with the NH₂-terminus T7-tagged Kv4.2 construct in pcDNA3 using Lipofectamine 2000 (Invitrogen; Carlsbad, CA). For localization experiments, the T7-tagged Kv4.2 cells were rinsed, fixed with 4% paraformaldehyde for 12 min at room temperature, and then quenched in 50 mM glycine (pH 7.4) for 10 min. After three 5-min washes with 1× PBS containing (in mM) 137 NaCl, 2.7 KCl, 4.3 NaHPO₄, and 1.4 KH₂PO₄, cells were incubated in a blocking solution (PBS containing 2% BSA and 0.2% Triton X-100) for 30 min at room temperature. A mouse monoclonal antibody to the T7 tag (1:1,000; Novagen) was diluted in blocking solution and incubated at 4°C overnight or for 2 h at room temperature. Cells were then washed three times for 5 min in PBS on a rotator before incubation with secondary antibody, Alexa 594-conjugated goat anti-mouse IgG antibody (1:1,000; Molecular Probes; Eugene, OR), for 1 h on the rotator at room temperature. For labeling polymerized actin, Alexa 633-conjugated phalloidin was included at the secondary antibody stage (4 U/μl; Molecular Probes). Cells were then incubated in PBS containing 300 nM 4',6'-diamidino-2-phenylindole dihydrochloride to stain the nuclei. Coverslips were once again washed three times with PBS before cells were mounted with 10 μl of a 90% glycerol-2.5% (vol/vol) 1,4-diazobicyclooctane-PBS solution. Images of labeled cells were taken using a Bio-Rad Radiance plus on an inverted Zeiss Axiosvert microscope using Bio-Rad LaserSharp 2000 software. Images were later viewed and prepared using NIH Image and Photoshop software packages. Images collected from Alexa 633-phalloidin-labeled cells were pseudocolored green and Alexa 594-labeled cells red in Photoshop.

Images to be deconvolved were acquired on a Nikon Diaphot 200 equipped for epifluorescence with a ×60/1.4 Planapo objective. The image detector was a theroelectrically cooled charge-coupled device camera with a SiTe S1502AB chip, with a peak quantum efficiency of 80% (Photometrics; Tucson, AZ). A series of two-dimensional images was acquired through the cells at 0.25-μm intervals. The point-spread function of the microscope was measured similarly using 100-nm spheres (Molecular Probes). Images were prepared as previously described (39) and then submitted to an EPR client-server processor for deconvolution (Scanalytics; Billerica, MA) using an algorithm developed by Carrington et al. (8).

Electrophysiological procedures. Coverslips containing cells were removed from the incubator before experiments and placed in a superfusion chamber (volume 250 μl) containing the control bath solution at 22–23°C. Current recording and data analysis were done using an Axopatch 200B amplifier and pCLAMP6 software (Axon Instruments; Foster City, CA). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments). Capacitance and series resistance compensation were routinely used during whole cell current recordings. Data were sampled at 10 kHz and filtered at 2–10 kHz. Leak subtraction was done offline as necessary. Cell-attached single channel recordings were conducted with pipettes resistances between ~10 and ~20 MΩ with the pipette solution. The peak chord conductance (G) was computed as G = I/V, where I is the peak current, Eₘ is the pulse voltage, and E₀ is the reversal potential, which was set as −85 mV under the experimental conditions. Significant differences between groups of data were tested using one-way ANOVA, with pairwise multiple comparisons. A value of P < 0.05 was deemed statistically significant. Data are means ± SE throughout.

Results

Depolymerization of the actin cytoskeleton increases Kv4.2 currents. We tested the effect of 5 μM cytochalasin D on Kv4.2 channels, as shown in Fig. 1. Depolarizing clamp pulses from −80 to 20-mV steps revealed a family of outward currents with rapid decay (Fig. 1, control). HEK cells have a noninactivating endogenous K⁺ current of ~300 pA at +60 mV (see Fig. 4A), which is <5% of the peak current observed here (4.6 ± 0.7 nA, n = 10) at +60 mV. Thus the rapidly inactivating currents reflect Kv4.2 channel activity almost exclusively. The inclusion of 5 μM cytochalasin D in the cell culture medium for 24 h produced a large percentage of rounded cells, suggesting that the cells were less able to maintain their shape after the depolymerization of the actin cytoskeleton. In addition, treatment produced a three- to fourfold increase in the amplitude of Kv4.2 currents, which was further increased by prolongation of cytochalasin D treatment to 48 h (Fig. 1A). This increase was confirmed by current density analysis (Fig. 1C), which showed that the treatment resulted on average in a threefold higher current density than that observed in untreated cells. Inactivation was biexponential and not significantly changed (P > 0.05 in all cases) in the presence of cytochalasin D, with values at +60 mV of 15.4 ± 0.7 and 86.9 ± 4.1 ms (n = 11) for control; 16.5 ± 0.8 and 82.1 ± 4.0 ms (n = 10) at 24 h; and 15.9 ± 1.8 and 84.9 ± 5.3 ms (n = 6) at 48 h of cytochalasin D treatment.

To confirm that the increase in Kv4.2 current in the cytochalasin D-treated cells was caused by depolymerization of the actin cytoskeleton, we tested the effect of phalloidin, an agent that stabilizes polymerized actin (14). Inclusion of 5 μM phalloidin for 5 h in the cell culture medium before treatment with 5 μM cytochalasin D, and throughout the exposure period to cytochalasin D, reduced the increase of the Kv4.2 current amplitude by cytochalasin D at 24 h, and by 48 h, currents from cells pretreated with phalloidin were the same level as control (Fig. 1, B and C). Clearly, these data support the idea that the increase of the Kv4.2 current amplitude results from the depolymerization of actin microfilaments by cytochalasin D.

Increased Kv4.2 currents are not the result of changes in macroscopic channel kinetics. Having seen such a large increase in Kv4.2 current, we tested if the effect of cytochalasin D was voltage dependent and thus was affecting channel gating. Data from experiments such as those shown in Fig. 1 were used to plot the peak current-voltage relations in Fig. 2. The activation threshold (−20 mV) of the Kv4.2 channels was not changed by treatment with cytochalasin D, but the Kv4.2 currents increased at all voltages positive to −20 mV in the drug treated cells. At 24 h (Fig. 2, A and B), phalloidin prevented the effect of cytochalasin D on currents but only partially blocked the increased density of current. At 48 h (Fig. 2, C and D), phalloidin completely prevented the action of cytochalasin D. The partial action of phalloidin at 24 h might reflect some influence of phalloidin on cell volume/surface area. We did not investigate this possibility further in the present study.

Channel conductance was calculated from the peak current amplitude and the reversal potential (−85 mV) and plotted as a function of the membrane potential (Fig. 3A). The conduc-

tance-voltage relations were fitted with a Boltzmann equation of the following form: $G/G_{\text{max}} = 1/1 + \exp[−zF(V − V_{\text{rev}})/RT]$, where $G/G_{\text{max}}$ is the fraction of the maximal macroscopic conductance, $z$ is the apparent valence of the voltage dependence, $F$ is the Faraday constant, $V$ is membrane potential, $V_{\text{rev}}$ is the voltage at which the channels have reached 50% of their maximum open probability, $R$ is the gas constant, and $T$ is temperature. The $V_{\text{rev}}$ values were 16.4 ± 1.2 mV (n = 17) in
cytochalasin D treatment greatly increases Kv4.2 current. A: whole cell recordings of Kv4.2 currents elicited by the pulse protocol shown at the top. The cells were pulsed for 400 ms at 0.2 Hz in 20-mV increments to potentials ranging from −60 to +80 mV. Representative traces of Kv4.2 current in untreated control cells, cells treated for 24 h with 5 μM cytochalasin D, and cells treated for 48 h with cytochalasin D are indicated. Note that the same scale applies to all currents in A. B: attenuation of the cytochalasin D effect by phalloidin. Whole cell current recordings are shown from a control cell (top) and one cell pretreated with 5 μM phalloidin for 5 h before incubation with cytochalasin D for 24 h (bottom). C: summary of Kv4.2 current density in control, cytochalasin D-treated, and phalloidin + cytochalasin D-treated cells. The peak current amplitudes at +60 mV were normalized to cell capacitance. The current densities after 24-h culture are 0.32 ± 0.06 nA/pF (n = 10), 0.57 ± 0.04 nA/pF (n = 4), and 0.97 ± 0.10 nA/pF (n = 16) for control cells, cells in the presence of 5 μM cytochalasin D and phalloidin, and cells in the presence of 5 μM cytochalasin D, respectively; after 48 h, current densities are 0.70 ± 0.17 nA/pF (n = 8), 0.78 ± 0.16 nA/pF (n = 4), and 1.84 ± 0.36 nA/pF (n = 5) for control cells, cells in the presence of 5 μM cytochalasin D and phalloidin, and cells in the presence of 5 μM cytochalasin D, respectively. Significant differences from control are indicated as *P < 0.05 and **P < 0.01. All whole cell experiments were conducted with 135 mM Kᵢ/5 mM Kᵦ, where i and o are used to indicate the internal and external solution throughout. Dotted lines indicate the zero current level.

Kv4.2 channels have a fast inactivation process, as seen by the rapid current decay shown in Fig. 1. To test the effect of cytochalasin D on inactivation kinetics, we used a 1-s prepulse (from −120 to +80 mV) to activate Kv4.2 currents and then a 500-ms pulse to +60 mV to test the fraction of noninactivated channels. The voltage dependence and slope of the inactivation relationship was unchanged (P > 0.05) between the control and cytochalasin D-treated cells (Fig. 3B). The Vᵢ₅₀ values were −66.8 ± 0.4 mV (n = 10) and −63.1 ± 0.7 mV (n = 4) for the control and treated cells, with slope factors of 7.2 ± 0.4 and 7.1 ± 0.6 mV, respectively. It is clear that the severalfold augmentation of the Kv4.2 currents in cytochalasin D-treated cells cannot be explained by a minor change in the macroscopic channel kinetics.

**Short-term effects of cytochalasin D on Kv4.2 currents.** In the above experiments, 24- and 48-h exposures to cytochalasin D were employed to disrupt the actin cytoskeleton. However, in hypertrophied cardiac myocytes (30), a short-term exposure to cytochalasin D (10 min) decreased Iᵦ, and increased the action potential duration. The short-term application (<10–15 min) of cytochalasin D has also been reported to modulate the gating of voltage-dependent Na⁺ channels in the heart. Treatment induced cardiac Na⁺ channels to enter a mode characterized by a lower peak open probability but a greater persistent activity, as if the inactivation rate was slowed (10). In addition, short-term (<40 min) exposure to cytochalasin D has been shown to regulate epithelial Na⁺ channel activity (6). Therefore, the exposure time to cytochalasin D may be an important factor influencing the channel activity and kinetics. To test this possibility, we examined the short-term effects of 5 μM cytochalasin D on Kv4.2 channels. The superfusion of 5 μM cytochalasin D-containing bath solution for 10 min resulted in a modest increase of Kv4.2 currents, but no significant alteration in the shape of the cells could be observed. The density of Kv4.2 currents was increased from 493 ± 76 pA/pF in the control cells (n = 5) to 529 ± 57 pA/pF in the cytochalasin D-treated cells (n = 6, P > 0.05, not significant). The
current decay time constants at +60 mV were 17.3 ± 1.0 and 95.1 ± 3.4 ms (n = 8), which were close to control values (see Fig. 1).

Prolongation of the exposure to cytochalasin D to 1 h produced a significant augmentation in Kv4.2 current. Current density was increased to 1,371 ± 141 pA/pF, which is an approximately threefold increase in current over that measured in the control cells, similar to the effect produced by the long-term exposure (as shown in Figs. 1 and 2). Not only were the Kv4.2 currents increased, but many rounded cells were observed after 1-h treatment with cytochalasin D. The voltage dependence of activation and inactivation of macroscopic Kv4.2 currents after 1-h exposure to cytochalasin D was examined. The conductance-voltage relation fitted with a Boltzmann equation showed a $V_{1/2}$ of 13.5 ± 1.4 mV and a slope factor of 18.3 ± 0.2 (n = 6). The biexponential inactivation was not significantly changed, with time constants of the current decay at +60 mV of 12.3 ± 0.9 and 66.9 ± 2.8 ms (n = 6). The voltage dependence and slope of the inactivation relationship was tested, as shown in Fig. 3B. The $V_{1/2}$ was −59.6 ± 0.4 mV (n = 5), with slope factor of 7.0 ± 0.3 mV. Similar to the long-term treatment, only minor variations of activation and inactivation parameters could be observed in the cells treated with cytochalasin D for 1 h. Clearly, those minor kinetic changes could not explain the huge increase in the Kv4.2 currents induced by 1-h exposure to cytochalasin D.

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**Fig. 2.** Influence of cytochalasin D on Kv4.2 current-voltage relationships. A–D: averaged current-voltage relationships of peak Kv4.2 current amplitude (A and C) or averaged current density-voltage relationships (current density = peak current amplitude/cell capacitance) in control, cytochalasin D-treated, and phalloidin + cytochalasin D treated cells (B and D). Cells were held at −80 mV and pulsed for 400 ms at 0.2 Hz in 10-mV increments to potentials ranging from −60 to +80 mV. Whole cell recordings were obtained with 135 mM K_+H/5 mM K_oH. A and B: after 24-h treatment with cytochalasin D (n = 10 for control, n = 16 for cytochalasin D treatment, and n = 5 for phalloidin + cytochalasin D treatment). C and D: after 48-h treatment with cytochalasin D (n = 7 for control, n = 5 for cytochalasin D treatment, and n = 4 for phalloidin + cytochalasin D treatment).

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**Fig. 3.** Voltage dependence of activation and inactivation of macroscopic Kv4.2 currents. A: activation curves of Kv4.2 currents in control cells (n = 17), cells after 24-h treatment with cytochalasin D (n = 16), and cells after 48-h treatment with cytochalasin D (n = 5). The peak chord conductance (see MATERIALS AND METHODS for the computation of peak chord conductance) was normalized to the maximum at +80 mV and plotted against the membrane potential. The curves are the fits of the Boltzmann equation [see text for the voltages at which the channels have reached 50% of their maximum open probability ($V_{1/2}$) and slope factors]. B: inactivation relationships obtained using the two-pulse protocol described in the text. The peak currents during the test pulses were normalized to the maximum and plotted as a function of the membrane voltage. The curves are fits of the Boltzmann equation (see text for $V_{1/2}$ and slope factors).
Thus we observed that both long- and short-term application of cytochalasin D have a similar influence on Kv4.2 channels, resulting in an increased current density without affecting the channel gating kinetics.

Cytochalasin D had no effect on the single channel behavior of Kv4.2 channels. A possible explanation for the large increase in Kv4.2 current seen during cytochalasin D treatment is a voltage-independent increase in the open probability of Kv4.2 channels. For this reason, we examined the influence of actin depolymerization on Kv4.2 single channel behavior. It has been reported that HEK cells have endogenous K⁺, Cl⁻, and nonselective channels (51, 52). We used Cl⁻-free solution in our experiments and found that this prevented the occasional appearance of large-conductance Cl⁻ channels, probably activated by cell swelling. Whole cell experiments indicated that a small endogenous K⁺ current was present in untransfected cells (Fig. 4A). The most important distinction of this current is that it inactivates only slowly and that its $V_{1/2}$ of inactivation is significantly more positive than that of Kv4.2 (1, 26, 51). As seen in Fig. 4B, when the holding potential was reduced from −80 to −30 mV, little effect could be seen on the amplitude of outward endogenous K⁺ current (Fig. 4B, top trace, compare with A), whereas almost 100% of Kv4.2 channels were inactivated at the −30-mV holding potential (Fig. 4B, bottom trace). On the basis of these observations, we used the two holding potentials to distinguish endogenous K⁺ from Kv4.2 channel activity in our experiments. Our whole cell observations were borne out at the single channel level. Endogenous K⁺ channels were relatively insensitive to the change in holding potential (Fig. 4, C and E), whereas Kv4.2 channels

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**Fig. 4.** Influence of holding potential on Kv4.2 and endogenous K⁺ channels in HEK cells. A: whole cell current recordings obtained from an untransfected (middle) and a Kv4.2 stably transfected HEK cell (bottom) with 135 mM K⁺/5 mM K⁺ using the pulse protocol shown (top). The endogenous K⁺ currents show little inactivation during 400 ms of depolarization. However, Kv4.2 currents inactivate rapidly. B: whole cell current recordings from the same cells as in A elicited by the same protocol except that the holding potential was −30 mV. Note that Kv4.2 currents are inactivated at this holding potential (compare scale bars in A and B), whereas the endogenous K⁺ currents are less affected. C–F: cell-attached recordings from patches with 5 mM [K⁺] pipette solution and 135 mM [K⁺] bath solution to depolarize the cells. Upward indicates outward currents. Data were sampled at 10 kHz and filtered at 2 kHz. Pulses were given at a frequency of 0.2 Hz. C and D: representative sweeps show channel openings during a 400-ms pulse from −100-mV holding potential to 0 mV (pulse protocol shown at C, top) in an untransfected cell and a cell expressing Kv4.2 channels, respectively. Note that openings of Kv4.2 occur mostly at the beginning of the pulse. E and F: representative sweeps from the same patch as in C or D except that the holding potential was reduced to −30 mV. In the Kv4.2 cell, few channel openings could be observed. Ensemble-averaged currents are shown in C–F, bottom, and indicate the average current from the number of sweeps indicated.
were exquisitely sensitive (Fig. 4, D and F). In addition, we noticed other differences that allowed us to clearly distinguish Kv4.2 from endogenous channels. These included a larger conductance (18.3 ± 1.2 pS, n = 8), a transient burst of openings at the start of sweeps, and frequent rapid closings during bursts (Fig. 4D). The transient nature of channel opening resulted in a rapidly decaying ensemble average waveform (Fig. 4D), which, in turn, reflected the macroscopic current waveform (Fig. 4A, bottom trace).

Cytochalasin D pretreatment did significantly affect the distribution of Kv4.2 channels rather than the single channel kinetic properties, as we found that single channel patches were much harder to obtain in the presence of cytochalasin D (Fig. 5). In 48 patches from cells pretreated with cytochalasin D, there were 31 null patches (~65%), 11 multichannel patches (~23%), and 6 single channel patches (~13%), which contrasted with the control cells, where the 66 patches included 20 null patches (~30%), 10 multichannel patches (~15%), and 36 single channel patches (~55%). The mean patch currents elicited by a pulse from −100 to 0 mV were 0.9 ± 0.2 pA (n = 14) for patches from control cells and 6.7 ± 3.0 pA (n = 14) for patches from treated cells, a sevenfold higher current level (P < 0.001). These data suggest that the channels were more closely clustered at the membrane after the disruption of the actin cytoskeleton (see Fig. 7). Even accounting for the frequency of blank sweeps, this large increase in mean patch current suggested an overall increase in channel density at the membrane, sufficient to account for the changes in whole cell current observed (Fig. 1).

There was no clear difference in the amplitude and noise of single channel openings between the treated and untreated groups of cells. The single channel conductance obtained from families of depolarizing pulses from 0 to 30 mV was 18.3 ± 1.2 pS (n = 8) in control and 18.8 ± 0.9 pS (n = 4) after 24-h treatment and 16.9 ± 1.5 pS (n = 5) after 48-h treatment with cytochalasin D. These values were not significantly different from each other (P > 0.05). On the basis of the observations of large numbers of sweeps, the pattern of the ensemble average currents showed the same averaged peak current, and rapid decay of the current amplitude, that was consistent with the whole cell recordings of Kv4.2 currents. This result suggests no obvious changes in the open probability of channels in the presence of cytochalasin D. This was confirmed by mean data, which showed that the mean open probability of the Kv4.2 channels was unchanged (P > 0.05) at 0.25 ± 0.05 in control (n = 4) and 0.26 ± 0.02 (n = 5) at 24 h and 0.28 ± 0.03 (n = 3) at 48 h of cytochalasin D treatment.

We then evaluated the open and closed dwell-time histograms in patches from control and cytochalasin D-treated cells (Fig. 6). Both open and closed dwell-time histograms were fit with double-exponential functions with time constants (τ1 and τ2; n = 4). The mean open times were 0.25 ± 0.07 ms for τ1 and 4.35 ± 1.39 ms (n = 4) for τ2 under control conditions. At 24-h treatment with cytochalasin D in the example shown in Fig. 6, τ2 did increase, but mean values for τ1 and τ2 were 0.31 ± 0.08 and 3.14 ± 0.96 ms (n = 5). At 48 h, τ1 and τ2 were 0.30 ± 0.11 and 4.06 ± 0.79 ms (n = 3; Fig. 6, A–C). Thus, in the presence of cytochalasin D, these mean values were not significantly altered (P > 0.5 for τ1 and P > 0.25 for τ2). Because Kv4.2 channels show rapid inactivation, long silences corresponding to this inactivation in the single channel recordings were expected and observed (Fig. 5). We discarded closed times longer than 4 ms in our analysis because we believe that these reflect long-duration inactivation. This left shorter duration closings reflective of the last closed state in the activation pathway, which has a short dwell time (C1), known to be populated frequently from the open state (18). The closed time histograms were also fit with double-exponential functions (Fig. 6, D–F). There is a trend for the slower closed times to increase with cytochalasin pretreatment, from 1.1 ms in

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**Fig. 5.** Cell-attached patch recordings from HEK cells stably expressing Kv4.2 channels. A, C, and E: representative sweeps in a one-channel patch from a control cell, a cell treated with 5 μM cytochalasin D for 24 h, and a cell treated with 5 μM cytochalasin D for 48 h, respectively. Pipette solution contained 5 mM [K+] and bath solution contained 135 mM [K+] to depolarize the cell. Outward currents are displayed as upward. Channels opened during 400-ms pulses from −100 to 0 mV. Data were sampled at 10 kHz and filtered at 2 kHz. Pulses were given at a frequency of 0.2 Hz. B, D, and F: ensemble averages of 210, 383, and 44 sweeps in A, C, and E, respectively. Dashed lines denote the zero current level. The voltage pulse protocol is shown on the bottom.
control to 5.9 ms after 48 h treatment with cytochalasin D. However, because an increase in channel closings can only reduce the amount of Kv4.2 current observed, and this increase in closed times was calculated from only 250 closing events, we do not believe this difference to be significant. This idea is supported by the mean data. The mean closed times were 0.15 ± 0.01 ms for τ1 and 2.31 ± 0.73 ms (n = 4) for τ2 under control conditions, 0.22 ± 0.04 ms for τ1 and 1.86 ± 0.09 ms (n = 5) for τ2 at 24-h treatment, and 0.29 ± 0.04 ms for τ1 and 4.01 ± 1.33 ms (n = 3) for τ2 at 48-h treatment. The differences are not significant (P > 0.05). In our examination of the single channel properties of Kv4.2 in control and in the presence of cytochalasin D, apart from the increased mean patch current, no alterations of the single channel kinetics of Kv4.2 were observed that could explain the increase in macroscopic current observed in HEK cells expressing Kv4.2 and exposed to cytochalasin D.

Localization of Kv4.2 channels at the HEK cell surface. To determine whether the increase in Kv4.2 current in the cytochalasin D-treated cells resulted from an increased expression of Kv4.2 channels at the HEK cell surface, immunocytochemical experiments were performed. HEK cells expressing T7-tagged Kv4.2 were fixed and labeled with an antibody specific for T7, and images of labeled cells were obtained with a confocal laser scanning microscope to assess the location of the protein (Fig. 7). The specificity of the antibody is demonstrated by the lack of staining in the untransfected HEK cells (Fig. 7B). The images demonstrate that in control cells, Kv4.2 proteins could be seen extensively throughout the interior of the cell and less protein can be seen at the cell surface (Fig. 7, E and F). This is highlighted by the phalloidin staining (Fig. 7D), which labels filamentous actin (11), and shows clearly the majority of the Kv4.2 staining to be interior to the cortical actin cytoskeleton. However, treatment with 5 μM cytochalasin D for 1 h resulted in a significant redistribution of Kv4.2 protein. The amount of protein localized at the cell surface increased significantly (Fig. 7, G–I) and appears to be condensing into clusters, indicated by numerous bright spots in the cell and at the membrane. In the treated cells, the Kv4.2 staining now almost completely overlapped with the F-actin staining.
tochalasins mainly affect actively turning-over actin filaments and thus affect stress fibers more readily than cortical actin fibers (9). This may explain why, in the 1-h-treated cells, we see a largely intact cortical cytoskeleton but the appearance of foci in the interior of cells. In some cells, the 1-h treatment had a more significant effect on the cortical actin cytoskeleton, and in these cells cortical foci were also more evident (not shown). Here, as well, the Kv4.2 staining overlapped the phalloidin staining.

To explore these clusters at slightly higher resolution, wide-field images were taken of control and cytochalasin D-treated cells and then deconvolved (Fig. 8). In both three-dimensional projections (Fig. 8, C and E) and single slices (Fig. 8, D and F) of treated cells, bright spots were found not just at the surface but throughout the cell. These spots are most evident in the 24-h-treated samples (Fig. 8, E and F). It has been suggested that cytochalasin D does not reduce the cytoskeletal content of the cell but induces reorganization toward dense foci (35, 38). Thus our imaging data suggest that Kv4.2 protein was redistributed along with the reorganizing cytoskeleton. The images suggest that many of these bright spots are arranged at the periphery of the cell, and this is consistent with our finding from cell-attached recordings that multichannel patches were often observed and that single channel patches were much harder to obtain than in untreated cell recordings. Because the same kind of bright spots could not be seen in the control cells (Fig. 8, A and B), we believe that cytochalasin D induces a redistribution of Kv4.2 channels from internal pools toward the cell surface. This results in an increased surface membrane expression of Kv4.2 channels and underlies the large increase in current density observed.

**DISCUSSION**

In the present study, we investigated the observation that actin depolymerization in HEK cells results in a significant increase of Kv4.2 current levels. This action of cytochalasin mirrors the effect on other voltage-gated potassium channels, like Kv1.5 (27), human *ether-a-go-go* channels expressed in CHO cells (5), and sodium channels (7, 32). After pretreatment of HEK cells with the cell-permeant toxin cytochalasin D for 24–48 h, the peak amplitude of Kv4.2 currents increased two- to threefold (Figs. 1 and 2). Only the current density seems to be affected by cytochalasin D because significant variation in the activation and inactivation process was observed in addition to the augmentation of the currents. This effect could be completely antagonized by pretreatment with phalloidin (Figs. 1 and 2) and could also be partially reproduced by cytochalasin B (data not shown).

Exposure time seems to be important in the action of cytochalasin D on channel activity and kinetics. Only short-term (<40 min) application of cytochalasin D can regulate the activity of epithelial Na⁺ channels (6), and short-term application (<10–15 min) of cytochalasin D modulates the gating of the cardiac voltage-dependent Na⁺ channel by lowering peak open probability and allowing for persistent activity (10).
Furthermore, in hypertrophied cardiac myocytes (30), a 10-min exposure to cytochalasin D decreased \( I_{\text{to}} \) and, as a result, increased the action potential duration. However, with short-term (from 10 min to 1 h) experiments, we found immediate increases in Kv4.2 current that were close to saturated after 1 h of treatment. As in the long-term experiments, cytochalasin D only increased the current density without altering the activation or inactivation properties of the channels. Our results did not reproduce the downregulation of \( I_{\text{to}} \) seen in the hypertrophied heart by cytochalasin D. However, these effects were not seen in the normal heart, suggesting that they may be specific to pathological states. Overall, all the data support the idea that cytoskeletal remodelling can have profound effects on cell surface channel expression.

Our short-term experiments (from 10 min to 1 h) provided an approximate time course for the effect of cytochalasin D on Kv4.2 channels. After 1-h treatment with cytochalasin D, the Kv4.2 current density increased to that observed after 24 h of cytochalasin D treatment. The fact that the increase of Kv4.2 currents could be induced by the short treatment suggests that an increased insertion of channel proteins into the plasma membrane from internal sites may underlie much of the effect rather than a longer-term change in channel biosynthesis.

This is not the first report showing that cytoskeletal regulation of Kv4.2 can occur. Petrecca et al. (34) have shown that disrupting the binding site on Kv4.2 of an actin-binding protein, filamin, could reduce the current density and alter the subcellular localization of Kv4.2 channels, suggesting that the expression density and subcellular localization of Kv4.2 are dependent on cytoskeletal regulation. Similar to epithelial Na\(^+\) channels (6), disruption of the actin cytoskeleton and disruption of the interaction between Kv4.2 and filamin have different effects on the Kv4.2 channel. As a scaffolding protein, filamin links the Kv4.2 channel protein to the cytoskeleton, and because expression of endogenous filamin has been identified in HEK cells (13, 25), it is very possible that in HEK cells Kv4.2 channels are also linked to the actin cytoskeleton by filamin. Cytochalasin D only reorganizes actin cytoskeletal structure (35, 38) (Fig. 7), so the filamin link between Kv4.2 and actin filaments may remain intact after cytochalasin D treatment and cause the Kv4.2 proteins to be redistributed along with the reorganizing cytoskeleton. This idea is supported by our imaging data, which shows that Kv4.2 proteins were colocalized consistently along with the actin cytoskeleton (Fig. 7). Thus our data showing increased expression of Kv4.2 at the membrane after cytochalasin D treatment likely reflect changes in actin cytoskeletal organization and changes in trafficking or regulatory mechanisms dependent on an intact cytoskeleton.

Another issue that should be noted is that, in the present study, we used a heterologous expression system. The fact that cytochalasin D did not change \( I_{\text{to}} \) in normal cardiac myocytes (30) suggests that the interaction of Kv4.2 channels with the cytoskeleton in HEK cells may be different from that in cardiac myocytes. Extending the studies to cardiac myocytes will be important for further understanding the role played by the actin cytoskeleton in the modulation of channel function.

What is the mechanism for the increase of Kv4.2 currents in cytochalasin D-treated HEK cells? It has been reported that cytochalasin treatment can alter the open probability and avail-
able channel numbers of cardiac Na$^+$ channels (24, 31) and Cl$^-$ channels (44). Cytochalasins can also alter channel kinetics, by influencing inward rectifier K$^+$ channel rectification and substrate expression (28) or by modifying accessory subunit actions on α-subunits of Kv1 channels (15, 23). Alternatively, because microtubules and other elements of the cellular organizational system are involved in the trafficking of ion channels, the increased whole cell current that we observed could also be the result of altered dynamics of this trafficking.

We could not find any significant change in the activation or inactivation kinetics of macroscopic Kv4.2 currents in the treated HEK cells (Fig. 3). The single channel behavior was examined in detail to look for more subtle changes in channel kinetics (Figs. 4–6). It was relatively easy to separate single Kv4.2 channels from the smaller conductance endogenous K$^+$ channel of HEK cells, based on the voltage sensitivity and rapid kinetics of inactivation of Kv4.2 (Fig. 4). The single channel conductance and open probability were not altered significantly by the incubation of cells with cytochalasin D for 24–48 h, as shown in Fig. 5. The ensemble-averaged currents were not increased by cytochalasin pretreatment, which shows that actin depolymerization did not affect the overall function of individual Kv4.2 channels. The single channel kinetics of Kv4.2 are complex, as Kv4 channels show both an open state and a closed state of inactivation (1, 3) and an additional rapid inactivation process that generates long silences in the single channel records (17). As a result of these multiple processes, we found that both open and closed time distributions could be fit best by biexponential relaxations (Fig. 6). However, the single channel kinetics of Kv4.2 were not altered significantly by actin depolymerization with cytochalasin D. There was an increase in the longer closed times at 24- and 48-h treatment, but this was most likely related to the relatively small numbers of these events (<1,000) at 48 h in the example shown and was not borne out by mean data. In any case, an increased number of closings would reduce Kv4.2 current, which was the opposite of the effect observed macroscopically. Effectively, our data exclude the possibility that the increase of Kv4.2 currents by the action of cytochalasin D was the result of an alteration of single channel kinetics.

The number of available channels at the membrane is the other determinant of macroscopic Kv4.2 current amplitude. When we measured the mean patch current from cells treated with cytochalasin D, we did observe a large increase compared with control (6.7 vs. 0.9 pA). This increase in the density of single channel records (17). As a result of these multiple processes, we found that both open and closed time distributions could be fit best by biexponential relaxations (Fig. 6). However, the single channel kinetics of Kv4.2 were not altered significantly by actin depolymerization with cytochalasin D. There was an increase in the longer closed times at 24- and 48-h treatment, but this was most likely related to the relatively small numbers of these events (<1,000) at 48 h in the example shown and was not borne out by mean data. In any case, an increased number of closings would reduce Kv4.2 current, which was the opposite of the effect observed macroscopically. Effectively, our data exclude the possibility that the increase of Kv4.2 currents by the action of cytochalasin D was the result of an alteration of single channel kinetics.

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REFERENCES


REGULATION OF ION CHANNEL SURFACE EXPRESSION BY CYTOSKELETON


