

Chronic Inhibition of Cardiac Kir2.1 and hERG Potassium Channels by Celastrol with Dual Effects on Both Ion Conductivity and Protein Trafficking*[†]

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A high percentage of drugs and drug candidates has been found to cause cardiotoxicity by reducing potassium conductance, more commonly known as QT prolongation. However, some compounds do not show direct block of ionic flow, suggesting that other mechanisms may also lead to reduction of potassium currents. Using the functional recovery after chemobleaching (FRAC) assay, we have examined a collection of drugs and drug-like compounds for potential perturbation of cardiac potassium channel trafficking. Here we report that a significant number of inhibitory compounds displayed effects on channel expression on the cell surface. Further investigation of celastrol (3-hydroxy-24-nor-2-oxo-1 (10),3,5,7-friedelater-29-oic acid), a cell-permeable dienonephenolic triterpene compound, revealed its potent inhibitory activity on both Kir2.1 and hERG potassium channels, causal to QT prolongation. **In addition to acute block of ion conduction, celastrol also alters the rate of ion channel transport and causes a reduction of channel density on the cell surface.** In contrast, celastrol has no effects on trafficking of either CD4 or CD8 membrane proteins. Furthermore, **the potency for reducing surface expression is ~5–10-fold more effective than that for either direct acute inhibition or reported cytoprotectivity via activation of the heat shock transcription factor 1.** Because the reduction of potassium channel activity is a common form of drug-induced cardiotoxicity, the potent inhibition of cell surface expression by celastrol underscores a need to evaluate drug candidates for their chronic effects on biogenesis of potassium channels. Our results suggest that chronic exposure to certain drugs may be an important aspect of acquired QT prolongation.

Potassium channels play important roles in a variety of biological processes ranging from neuronal excitability to tumorigenesis (1–3). Of the more than 400 ion channel genes identified within the human genome, at least 167 are annotated to encode potassium channels (4). Hence, they represent a major repertoire of ion channel proteins. Mutations of potassium channels are causal to a number of human diseases including ataxia, epilepsy, long-QT syndrome, Bartter's syndrome, and familial persistent hyperinsulinaemic hypoglycemia of infancy (5).

In addition to being targeted by therapeutics for treating diabetes,

epilepsy, and neuropathic pain, unintended inhibition of potassium channel activities has become an important concern, especially for the cardiovascular system (6, 7). In human cardiac myocytes, repolarization during an action potential is mediated by a number of potassium channels including Kv4.3, Kv1.4, Kv1.5, Kv2.1, hERG, KvLQT1/mink, and Kir2.x (for reviews, see Refs. 8 and 9). Their activities are temporally involved in different phases of cardiac repolarization. Compound-caused reduction of potassium currents, particularly those mediated by hERG, KvLQT1/mink, and Kir2.1, often slows down the rate of repolarization and hence prolongs the duration of action potential (10). This is also evident in human patients with long-QT syndrome who have genetic mutations in these genes. Both experimental evidence and computational simulation suggest that a small decrease of potassium conductance could significantly prolong the duration of cardiac action potential (11, 12).

Because an unusually high percentage of compounds causing QT prolongation is associated with the reduction of cardiac potassium currents (13), it is important to understand the mechanisms by which these compounds exert the inhibition. A considerable number of compounds are inhibitory to potassium channel current, but their potency of inhibition may not always be explained by acute inhibition of channel conductivity (14). These compounds may exert their effects by alternative mechanisms including chronic mechanism of action. **In general, reduction of potassium current on the cell surface could take place at two levels, ion conduction and channel density.** The effect on channel conductivity can be measured by established techniques such as electrophysiological methods (4). However, changes of channel density are not readily assayed by existing high throughput technologies. Thus, it is not known to what extent the acquired QT prolongation is contributed to by chronic effects independent from direct block among those compounds that have been clinically associated with cardiotoxicity. A critical first step to address this question is to identify compounds that affect the surface expression of cardiac potassium channels.

Recently, we reported the functional recovery after chemobleaching (FRAC)² assay, which measures the rate of protein trafficking with effective throughput (15). This assay system enables an investigation of many compounds in parallel for their roles in regulation of potassium channels, particularly whether and to what extent they affect protein expression on the cell surface. Using this approach, we have screened a compound library of 2000 chemicals including human drugs, natural compounds, and drug-like chemicals, for either acute or chronic effects on cardiac potassium channels. Intriguingly, comparable numbers of compounds have been found to cause acute block or chronic inhibition.

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² The abbreviations used are: FRAC, functional recovery after chemobleaching; hERG, human ether-a-go-go-related gene; MTSET, [2-(trimethylammonium)ethyl]-methanethiosulfonate; BFA, brefeldin A; PBS, phosphate-buffered saline; HA, hemagglutinin; HBSS, Hanks' balanced salts solution.

Inhibition of Potassium Channel Trafficking

One of the identified inhibitors is celastrol, a bioactive compound from the *celastraceae* plant, also called the Chinese Thunder God vine (16, 17). We investigated the effects of celastrol on both channel conduction activity by electrophysiological recording and channel protein trafficking by the FRAC assay (15). Celastrol has an unusual pharmacological profile including both acute and chronic inhibition for Kir2.1 and hERG potassium channels but not for the homologous Kv2.1 potassium channel.

EXPERIMENTAL PROCEDURES

Cell Culture

Cell lines are established by standard protocols using pcDNA3.1. HEK 293 stable cell lines expressing Kir2.1, hERG, and Kv2.1 channel and human neuroblastoma cell line SH-SY5Y were maintained in Dulbecco's modified Eagle's medium/F-12 50/50 medium supplied with 10% fetal bovine serum, penicillin/streptomycin, L-glutamine. The HEK 293 cell lines are supplemented with 500 $\mu\text{g}/\text{ml}$ G418 (complete medium).

Compound Library

The compound library (Spectrum Collection, 2000 compounds, supplied as 10 mM Me_2SO solution) used in this study was purchased from MicroSource Discovery Inc. (Groton, CT). This library consists of mostly human therapeutic drugs or drug-like compounds and natural products. The compounds were diluted with deionized water to reach a concentration of 100 μM with 5% Me_2SO . This was used as the 10 \times compound solution to be introduced to the cell culture.

Compound Screen Using FRAC Assay

HEK 293 cells stably expressing Kir2.1 were plated at $4 \times 10^4/100 \mu\text{l}$ complete medium per well in poly-L-Lysine coated 96-well plates. Three hours later, 50 μl of complete medium containing 15 mM sodium butyrate was added to each well and then incubated at 37 $^\circ\text{C}$ with 5% CO_2 overnight. Thirty microliters of complete medium containing 30 mM RbCl was added to the culture next day and incubated for another 3 h. Cell plating and reagents dispensing were done using a Multidrop 384 dispenser (Thermo Electron Corp., Waltham, MA).

[2-(Trimethylammonium)ethyl]-methanethiosulfonate (MTSET) treatment, compound addition, and the Rb^+ efflux assay were programmed and performed on a Tekbench liquid handling system (Tekcel Inc., Hopkinton, MA). Briefly, after 20 $\mu\text{l}/\text{well}$ of 25 mM (10 \times) MTSET solution was added to the cell culture and incubated at room temperature for 6 min, each cell plate was washed twice and redispensed with 180 $\mu\text{l}/\text{well}$ complete medium containing 5 mM RbCl on an Elx 405 select washer (Bio-Tek Instruments Inc., Winooski, VT). Then 20 $\mu\text{l}/\text{each}$ of the 10 \times compound solution was added to 80 wells of each cell plate. To ensure assay consistency, 20 μl of 5% Me_2SO solution was added to each well in the first column of each cell plate and 20 μl of 10 μM brefeldin A (BFA) in 5% Me_2SO was added to each well in the last column of each cell plate along with the compound solutions; these wells served as negative and positive controls, respectively. Final Me_2SO concentration in the cell medium was kept at or below 0.5% for all cell plates to minimize its toxicity. After adding the compounds, the cells were again incubated at 37 $^\circ\text{C}$ with 5% CO_2 for another 3 h. Then each cell plate was washed twice and redispensed with 200 $\mu\text{l}/\text{well}$ Rb^+ -free complete medium and incubated at room temperature for 15 min before the supernatant was transferred to a new 96-well plate. The cells were then lysed with 200 $\mu\text{l}/\text{well}$ 1% Triton X-100 in PBS. Rb^+ concentrations in both supernatant and cell lysate were measured by an Ion Channel Reader 8000 (Aurora Biomed Inc., Vancouver, Canada).

Kir2.1 IC_{50} Measurement and hERG Rb^+ Flux Assay

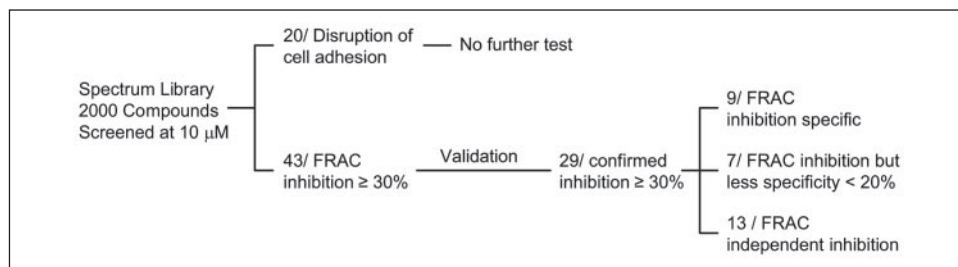
IC_{50} Measurement—A Kir2.1Y stable cell line was plated at $2 \times 10^5/$ well in poly-L-lysine-coated 24-well plates with 5 mM NaBt. Cells were first incubated with complete medium containing 5 mM RbCl for 3 h. Then half of the wells were treated with 2.5 mM MTSET for 5 min, and cells were washed twice with medium containing 5 mM RbCl. Different concentrations of celastrol were added to the cells using 100 \times stock in 40% Me_2SO (final 0.4% Me_2SO), and cells were incubated with the compound for another 3 h. Then the cells were washed twice with 1 ml/well Rb^+ -free medium and incubated in 0.5 ml/well Rb^+ -free medium for 15 min at room temperature. Supernatants were transferred to new 24-well plates. Cells were lysed with 0.5 ml/well 1% Triton X-100 in PBS. Rb^+ concentrations in both supernatants and lysates were measured on an Ion Channel Reader 8000, and Rb^+ efflux was calculated as described previously (15). Each data point was the average from three different wells. IC_{50} measurements for celastrol were affected by assay volume due to the poor solubility of celastrol.

hERG Rb^+ Efflux Assay—A hERG stable cell line was plated at $2 \times 10^5/$ well in a 24-well plate. One hour after plating, different concentrations of celastrol were added to the cell culture from 1000 \times Me_2SO stock. 0.1% Me_2SO and 200 nM BFA were used as negative and positive controls, respectively. After 20-h incubation at 37 $^\circ\text{C}$, culture medium were changed to medium containing the same concentrations of compounds and 5 mM RbCl. After about 24-h incubation with the compounds, cells were washed twice with Rb^+ -free medium, and Rb^+ efflux was initiated by adding 0.5 ml/well medium containing 50 mM KCl. After 10-min incubation, the supernatants were transferred to a new 24-well plate. Cells were lysed with 0.5 ml/well 1% Triton X-100 in PBS. Rb^+ concentrations in both supernatants and lysates were measured on an Ion Channel Reader 8000, and Rb^+ efflux rate was calculated as described previously. Each data point was the average from two different wells.

Electrophysiology

Standard rupture patch was used to record the whole-cell current. During the recording, constant perfusion of extracellular solutions containing (mM): NaCl, 135; KCl, 5; CaCl_2 , 2; MgCl_2 , 1; HEPES, 10; D-glucose, 10 (adjusted to pH 7.4 with NaOH) was maintained by a gravity-driven perfusion system (ALA Scientific Instruments, Inc., Westbury, NY). Pipettes were pulled from borosilicate micropipettes (TW150-4, World Precision Instruments, Sarasota, FL). When filled with the intracellular solution containing (mM): 130, KCl; 1, MgCl_2 ; 5, ATPMg; 5, EGTA; 10, HEPES (adjusted to pH 7.2 with KOH), the pipette resistance was about 3–5 M Ω , which was normally compensated by 60–80%. Voltage-clamped currents were recorded using an Axopatch-200A amplifier, filtered at 1 kHz, and digitized by DigiData 1322A and processed by pClamp 9.2 software (Axon Instruments, Foster City, CA). Membrane voltages were clamped at holding levels and then to different depolarization or hyperpolarization levels for particular types of potassium channels. Corresponding current amplitudes for each type of channel were monitored to evaluate the inhibition caused by the compound. For Kir2.1 channels, membrane voltage was maintained at -40 mV and then stepped into a series of voltage steps ranging from -120 to $+50$ mV. Steady state currents at each voltage step were measured. For hERG channels, the voltage protocol consisted of a pre-pulse of $+60$ mV and then a testing step of either -100 mV or -50 mV to obtain tail currents. Tail currents were fitted by an exponential function, and then the actual amplitudes were measured by extrapolating the fitting curve to the beginning of the testing step. For Kv2.1 channels, steady state currents were measured when membranes were depolarized from -80

FIGURE 1. Schematic diagram of hits identified from different stages of screen and validation. FRAC-dependent inhibition indicates the requirement of MTSET treatment to detect the inhibition.



to 30 mV at a 10-mV step. Patch clamp data were preprocessed using Clampfit 9.2 (Axon Instruments) and then analyzed in Matlab 6.1 (Mathworks, Natick, MA) and Origin 7 (OriginLab, Northampton, MA). Data are presented as means \pm S.E. Significance was estimated using the paired Student's *t* test.

Flow Cytometry

HEK 293 cells were transfected with HA-tagged (between M1 and P-loop) Kir2.1 or CD8 by using FuGENE 6 (Roche Applied Science). At 6 h after transfection, cells were trypsinized and thoroughly resuspended before plating in 6-well plates at 4×10^5 /well. Compounds were added to each well from 500 \times or 1000 \times Me₂SO stocks, and an equal volume of Me₂SO was added to one well used as a negative control. Cells were incubated at 37 °C with compounds for 16–20 h. Cells were washed with 1 \times PBS and harvested by incubation with 0.5 mM EDTA in 1 \times PBS for 5–10 min at room temperature. Cells were then washed twice with Hanks' balanced salts solution (HBSS) plus 5 mM HEPES, pH 7.3, and 2% fetal bovine serum and incubated with mouse anti-HA monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) on ice for 1 h. Cells were then again washed twice with HBSS staining medium and incubated with Alexa Fluor 488-labeled goat anti-mouse antibody (Molecular Probes, Eugene, OR) for 15 min on ice. Finally, the cells were washed twice with HBSS staining medium, and the surface fluorescence was measured by FACSCalibur (BD Biosciences), using CELLQUEST software (BD Biosciences).

Immunoprecipitation and Western Blot

Kir2.1 and hERG stable cell lines were plated in 6-well plates at the density of 5×10^5 /well. The next day, cells were treated with compounds with 0.1% Me₂SO added. For transient transfection experiments, HEK 293 cells were plated at 2×10^5 /well and the next day transfected with C-terminal HA-tagged CD4 or CD8 plasmids. Compounds were added 6 h after transfection. After 24 h of incubation with compounds, cells were washed once with PBS and lysed with 300 μ l/well 1% Nonidet P-40 lysis buffer (150 mM NaCl, 25 mM Tris, pH 7.5, protease inhibitor mixture) at 4 °C for 30 min.

For the Kir2.1 stable line, cell lysates were centrifuged at 14,000 rpm at 4 °C for 20 min, and the supernatants were immunoprecipitated with 1 μ g anti-HA antibody-conjugated protein A-Sepharose beads at 4 °C overnight. The beads were washed three times with 1 ml of lysis buffer and resuspended in 30 μ l of 2 \times SDS sample buffer for 15–20 min at room temperature for elution. The eluates were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in PBS plus 0.1% Tween (PBST) at 4 °C overnight and immunoblotted with rabbit polyclonal anti-Kir2.1 (1:500 dilution, 1 h at room temperature). After twice washing with PBST, the membrane was blotted with horseradish peroxidase-conjugated secondary antibody. Western blots were developed using the ECL kit (Amersham Biosciences) on a Fujifilm Luminescent Image Analyzer LAS 3000 (Fujifilm, Valhalla, NY).

For the hERG stable cell line and HA-tagged CD4, the cell lysate supernatants were incubated with SDS sample buffer for 15–20 min at room temperature and separated on 6% (hERG) or 12% (CD4) SDS-polyacrylamide gel. The protein-transferred membranes were immunoblotted with either rabbit anti-hERG (Alomone Laboratories, Jerusalem, Israel) or anti-HA antibody followed by the corresponding secondary antibodies. Western blots were developed as described above. hERG surface expression levels were quantitated using Multi Gauge v2.3 (Fujifilm) by measuring the band density ratio of the 155-kDa mature form and the 135-kDa immature form, normalized to that of control sample.

SH-SY5Y cells were plated in 60-mm dishes at 1.5×10^6 /dish. On the next day, cells were treated with compounds with 0.1% Me₂SO for 24 h. Cells were then washed once with PBS and lysed with 150 μ l of 1% Nonidet P-40 lysis buffer. Cell lysates were collected and centrifuged at 4 °C. Then 100 μ l of the supernatant was incubated with SDS sample buffer at 75 °C for 5 min and separated on a 6% gel. Membranes were immunoblotted with rabbit anti-hERG or rabbit anti-calreticulin (Affinity BioReagents, Golden, CO) followed by horseradish peroxidase-conjugated secondary antibodies, and the blots were developed by ECL. The band density of the hERG mature form was quantitated and normalized to that of calreticulin as a reference protein.

RESULTS

To identify small molecules that may have acute or chronic effects on Kir2.1 potassium channel activity, we screened a library of 2000 compounds using the FRAC assay (15). The assay takes advantage of Rb⁺ permeability of potassium channels and sensitive quantification of Rb⁺ by atomic absorption spectrometry. Because the methanethiosulfonate (MTS) reagents, such as cell-impermeable MTSET, quantitatively link to the native cysteine (Cys-149) of Kir2.1, which irreversibly nullifies the channel conduction, we are able to probe the rate of channel transport by testing the time to recover after MTSET treatment, *i.e.* measuring the channel functional recovery at different time points after MTSET treatment (15). By applying the compounds before or immediately after the MTSET treatment, one can assess whether a given compound potentiates or inhibits the recovered activity conferred by newly surfaced channel proteins. A compound may exert its effect on vesicular transport and/or conductive activity of newly surfaced channels.

The compound collection used in this analysis consisted of about 1000 human drugs, 700 natural products, and 300 drug-like molecules (see "Experimental Procedures"). A diagram of the screening strategy and summary of the hit compounds is shown in Fig. 1. The compounds were screened initially at 10 μ M using a Kir2.1 channel stable cell line. The activity of Kir2.1 was monitored by the ratio of released Rb⁺ to that of total Rb⁺, determined by atomic absorption spectrometry (15, 18). Twenty compounds caused a more than 50% loss of the total Rb⁺ concentration when compared with controls, indicating that these compounds are toxic to the cells, disrupting cell adhesion for example. Hence, they were not included in the hit selection for the purpose of this

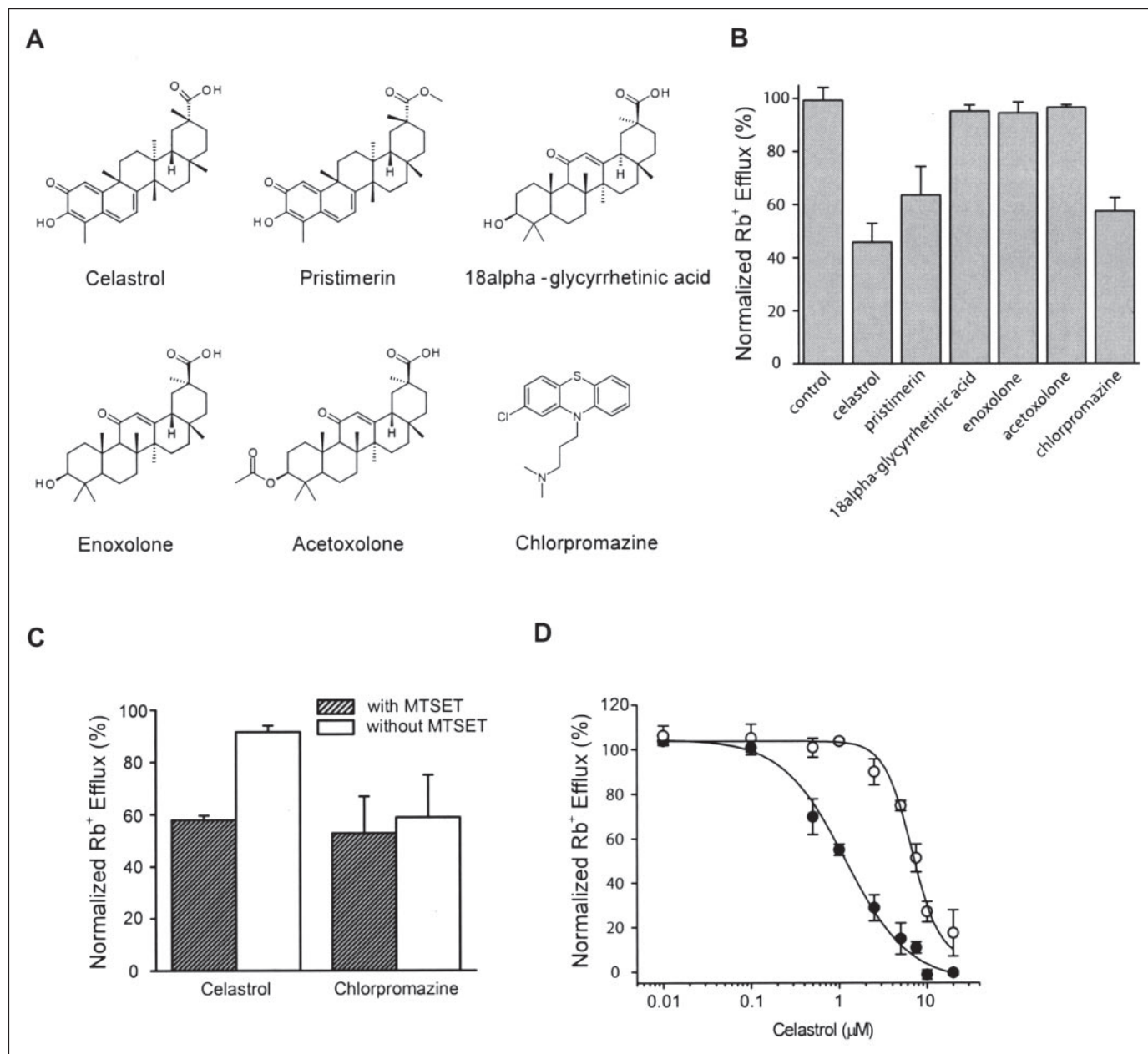


FIGURE 2. Effects on Kir2.1 potassium channel by celastrol and structurally similar compounds in the FRAC screen. *A*, structures and names of celastrol and related compounds; *B*, Rb⁺ efflux comparison by FRAC assay. Normalized Rb⁺ efflux (vertical axis) in the presence of 10 mM of indicated compounds (horizontal axis). *C*, normalized Rb⁺ efflux (vertical axis) in the presence of 10 μM of indicated compounds (horizontal axis). The FRAC assay was performed in a 96-well format following the compound screening protocol (see "Experimental Procedures") with or without MTSET as indicated. *D*, dose-dependent effects by celastrol on Kir2.1 with (closed circles) or without (open circles) MTSET treatment. All experiments were performed either in duplicate or triplicate.

study. Forty-three compounds showed at least 30% inhibition of Kir2.1 channel Rb⁺ efflux and were selected for further analyses. Among these, 30 compounds can be grouped by structure similarity into six different structural classes. The remaining 13 compounds showed no significant structural similarity (Fig. 1).

Reduction of Rb⁺ efflux could be a result of reduced surface expression and/or direct inhibition of ion conduction by the surfaced channels. These two mechanisms of action may be differentiated by testing the compound effects with or without MTSET treatment. Twenty-nine compounds with more than 30% reduction in Rb⁺ efflux were selected for further analyses. To distinguish the two possible mechanisms, we performed the FRAC assay and compared the signals obtained with the MTSET treatment and without the MTSET treatment. Thirteen com-

pounds showed a similar level of inhibition in both conditions, indicating the inhibitory effect is independent of the MTSET treatment. Nine compounds showed more than 30% significant inhibitory effect in MTSET-treated cells but less than 10% reduction in untreated cells, indicating their effect is MTSET-dependent. Seven compounds showed inhibitory effect in both conditions but with a higher inhibition level in the MTSET-treated cells, indicating the compound effects may affect both channel activity and surface expression. These results suggest that a significant percentage of compounds affect channel surface expression in recombinant cell lines.

Celastrol was identified as one of the natural products showing MTSET-dependent inhibition on Kir2.1 channel activity. Because of its therapeutic potential (Ref. 19 and see "Discussion"), celastrol was cho-

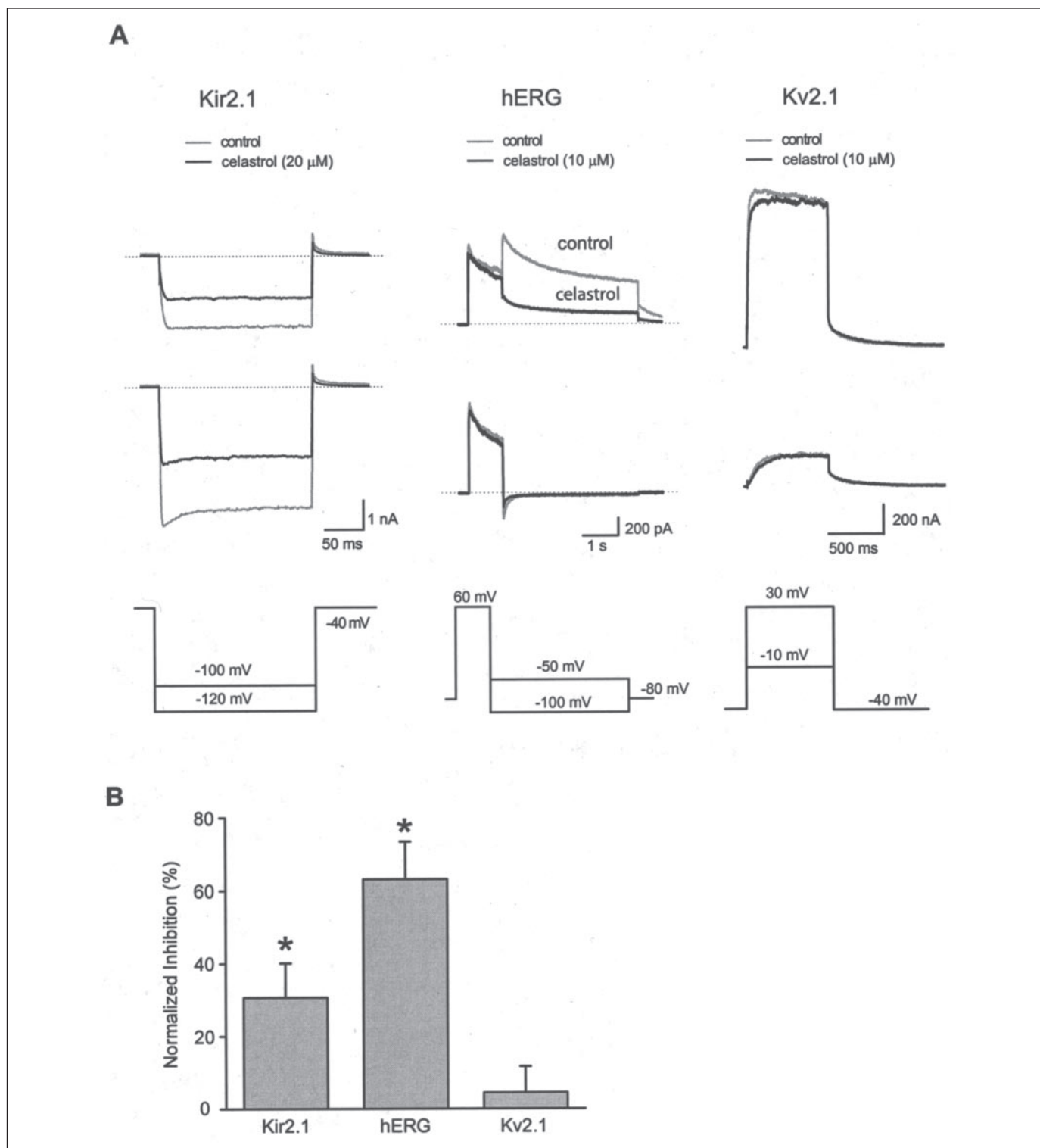


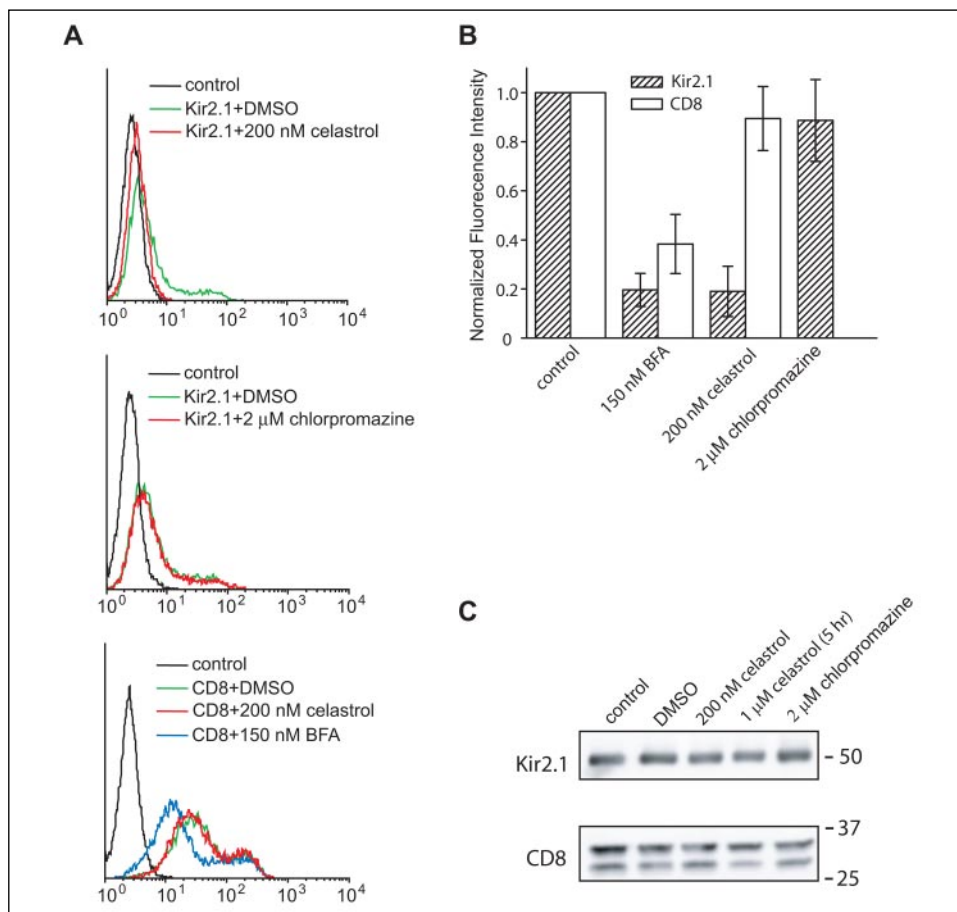
FIGURE 3. **Acute inhibition of celastrol on potassium channels.** *A*, heterologously expressed Kir2.1, hERG, and Kv2.1 currents were recorded by whole-cell voltage clamp before and after extracellular treatment of 10 (hERG and Kv2.1) or 20 μM (Kir2.1) celastrol. The control and treated traces are as indicated. *B*, normalized inhibition by celastrol on Kir2.1, hERG, and Kv2.1 currents is shown in histogram (*, $p < 0.05$).

sen for more detailed analyses. Four additional compounds in the screened library possess structures similar to celastrol (Fig. 2*A*). Among them, only celastrol and its methyl ester pristimerin showed inhibitory effects in FRAC analyses (Fig. 2*B*), while the others, despite the striking structural similarity, had no effects. This indicates that the effects on Kir2.1 channels were chemical structure-specific.

In FRAC analyses, celastrol showed 40% inhibition with MTSET treatment but only 10% inhibition without MTSET treatment (Fig. 2*C*). In contrast, chlorpromazine, a tricyclic antipsychotic drug (Fig. 2*A*), which has been reported to block hERG channels (20), showed a similar level of inhibition independent of MTSET treatment. To measure the potency of the inhibitory effect of celastrol, we performed Rb^+ efflux

Inhibition of Potassium Channel Trafficking

FIGURE 4. Inhibition of Kir2.1 surface expression by celastrol. *A*, histogram of flow cytometry analysis of Kir2.1 (*top and middle panels*) and CD8 (*bottom panel*) surface expression in transiently transfected HEK 293 cells. Treatments with celastrol (200 nM), chlorpromazine (2 μ M), BFA (150 nM), or Me₂SO before antibody staining are as indicated. *B*, histogram display of mean fluorescence intensity of cells treated with celastrol, BFA, or chlorpromazine. The values (*vertical axis*) were normalized against control samples (cells treated with corresponding concentrations of Me₂SO). Each column was the average from two or three experiments. *C*, immunoblot analyses of Kir2.1 and CD8 proteins in the indicated treatments by corresponding antibodies.



assay in the presence of different concentrations of celastrol with or without MTSET treatment (Fig. 2D). The resultant inhibition potency (IC_{50}) is $1.2 \pm 0.1 \mu$ M with MTSET treatment, 5-fold lower (or more potent) than the IC_{50} measured without MTSET treatment ($6.7 \pm 0.9 \mu$ M). These data suggest that celastrol has dual effects on Kir2.1 channel, both blocking the channel activity and inhibiting channel protein expression on the cell surface.

To more precisely define acute block of the Kir2.1 channel activity by celastrol, we measured the Kir2.1 inward currents before and after the extracellular application of 20 μ M celastrol using whole-cell voltage clamp recording (Fig. 3A, left panel). Upon application of 20 μ M celastrol, the whole cell Kir2.1 current was decreased by $30 \pm 9\%$ ($n = 4$). This indicates that celastrol can acutely inhibit the Kir2.1 channel. To test whether the acute inhibition effect of celastrol is general to cardiac potassium channels, we measured the compound effect on the hERG and Kv2.1 channels (Fig. 3A). Celastrol at 10 μ M reduced the hERG peak tail current amplitude by $63 \pm 10\%$ ($n = 4$). However, 10 μ M celastrol showed no effect on Kv2.1 ($n = 4$), a delayed rectifier potassium channel. Together, these data demonstrate the capability and specificity of celastrol in acute inhibition of cardiac Kir2.1 and hERG potassium channels.

To test biochemically for any change at the protein level, the inhibitory effect of celastrol on Kir2.1 channel surface expression was further evaluated by flow cytometry analyses using antibody specific to an extracellular epitope. Kir2.1 with an extracellularly tagged HA epitope was transiently expressed in HEK 293 cells and detected with an anti-HA antibody under non-permeable conditions (see "Experimental Procedures") (21). Overnight incubation (16–18 h) of celastrol significantly reduced Kir2.1 protein on the cell surface compared with cells

incubated with Me₂SO alone as control (Fig. 4A, top panel). In contrast, chlorpromazine, which showed MTSET-independent inhibition in our screen (Fig. 2C), did not change the Kir2.1 surface expression level (Fig. 4A, middle panel). The reduced surface expression level of the Kir2.1 channel could be the consequence of general blockade of vesicular transport or reduction in protein synthesis. We tested celastrol for any effect on the surface expression level of CD8 receptor with considerable O-linked glycosylation. Overnight incubation with either 200 nM celastrol in Me₂SO or Me₂SO alone did not change the surface expression level of CD8 protein (Fig. 4A, bottom panel). In contrast, overnight incubation of BFA, a fungal toxin that inhibits vesicular trafficking by disruption of the Golgi apparatus (22), dramatically reduced CD8 surface expression. Quantification of surface expression by fluorescence intensity showed that celastrol specifically reduced surface expression of the Kir2.1 protein by 80%, with no effect on CD8 (Fig. 4B). In contrast, BFA reduces the surface expression of both Kir2.1 and CD8 by 60–80% (Fig. 4B). Furthermore, immunoprecipitation and Western blot analyses of total Kir2.1 and CD8 proteins indicate that celastrol does not affect the overall protein level after treatment for 18 h at 200 nM (Fig. 4C). These results support the notion that celastrol inhibits transport of Kir2.1 channel to the cell surface.

The acute inhibition by celastrol of both Kir2.1 and hERG potassium currents raises the question whether celastrol also affects the biogenesis of hERG. We therefore examined whether celastrol could also reduce the surface expression of hERG protein. The hERG channel protein is synthesized as a core-glycosylated immature form of 135 kDa and a fully glycosylated mature form of about 160 kDa. It is believed that only the fully glycosylated mature protein can express on the cell surface (23). A stable cell line expressing hERG potassium channel was used (see

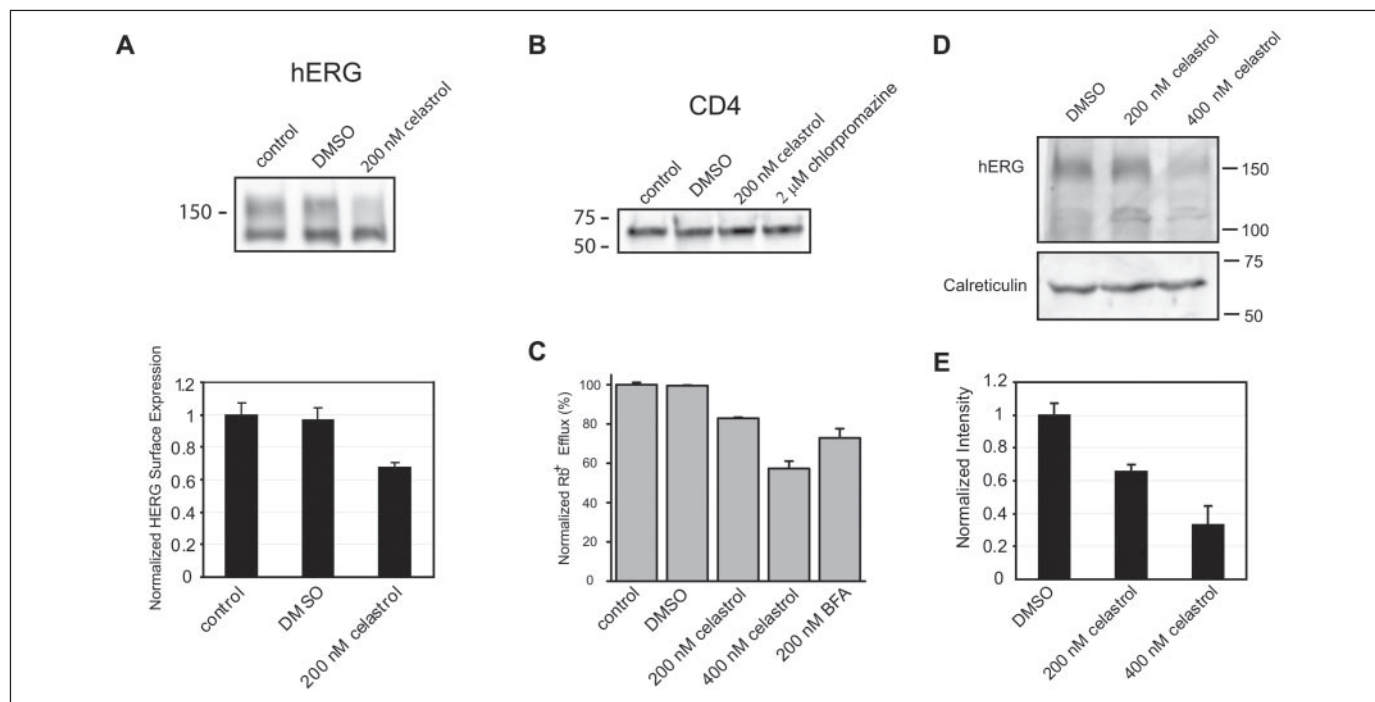


FIGURE 5. Celastrol inhibits hERG surface expression in HEK 293 stable cell line and native cells. *A*, immunoblot analyses of hERG after overnight treatment with the indicated concentration of celastrol or Me₂SO ($n = 3$). The signal intensity of the fully glycosylated form is quantified and shown in the histogram after normalization against control. *B*, immunoblot analyses of CD4 after indicated treatments. *C*, Rb⁺ efflux of hERG channel after incubation for 24 h with indicated compounds. *D*, immunoblot analyses of hERG (*upper panel*) and calreticulin (*lower panel*) from a SH-SY5Y neuroblastoma cell line after overnight treatment with celastrol. *E*, quantification of fully glycosylated hERG protein. The signal intensity is displayed after normalization against signals of calreticulin.

“Experimental Procedures”). Overnight incubation with 200 nM celastrol significantly decreased the amount of the 160-kDa mature protein but had no detectable effect on the 135-kDa immature protein (Fig. 5*A*). Since the hERG protein maturation undergoes *N*-glycosylation, celastrol could potentially exert its effect by inhibiting the *N*-linked glycosylation, despite no effect on *O*-linked glycosylation of CD8. This possibility was tested by examining the amount of CD4 glycoprotein in the presence and absence of celastrol. Celastrol has no detectable effect on the amount or the extent of *N*-linked glycosylation of CD4 (Fig. 5*B*).

To validate our findings obtained from Western blot analysis, we performed an Rb⁺ efflux assay using HEK 293 cells stably expressing hERG channel. This expression system has been shown to express hERG with considerable similarity to Ikr (24). Overnight incubation with celastrol at concentrations of either 200 or 400 nM reduced hERG channel Rb⁺ efflux levels by about 20 and 40%, respectively. Inhibition of surface expression by a potent and generic inhibitor, BFA at 200 nM, displayed about 30% reduction (Fig. 5*C*). Exposure to celastrol at these concentrations did not have an acute effect on hERG potassium current in patch clamp measurements (data not shown). The inhibition of Rb⁺ efflux is in agreement with reduction in signal intensity of immunoblot analyses (Fig. 5*A*).

To evaluate the effect of celastrol on biogenesis of native hERG channel, we used a human neuroblastoma cell line SH-SY5Y, which constitutively expresses hERG potassium current (25). The hERG proteins are detectable as a fully glycosylated form by antibodies (26). Treatment of celastrol at both 200 and 400 nM concentrations for 18 h again induced a reduction in the glycosylated form (Fig. 5*D*). The extent of reduction was quantified by calculating the ratio of signal intensities of hERG to that of calreticulin, a constitutive ER protein. Treatments of 200 and 400 nM celastrol induced ~25 and 50% reduction, respectively, compared with the signal of the control cells treated with Me₂SO alone (Fig. 5*E*).

DISCUSSION

Membrane proteins constitute ~30% of the proteome in both prokaryotes and eukaryotes (27). Different from many diffusible soluble proteins, both spatial localization and molecular density of membrane proteins are critical for physiological functions. Biogenesis of membrane proteins consists of multiple steps of vesicular transport from the site of synthesis in the endoplasmic reticulum to the final functional sites, such as the cell surface. Membrane-permeable small molecules, including drugs and metabolites, may exert their effects by interacting with their targeted membrane proteins at or prior to the site of function. However, little is known about whether and how drugs may exert their effects in early stages of biogenesis, largely because of the lack of effective methodology.

Chronic effects could take place at multiple levels of protein biogenesis. In our FRAC screen, we have chosen a 3-h duration for testing compound effect on recovery. The selective time window minimizes the contribution of some of the other chronic effects including translation and transcription (15). In addition, this is the middle point of recovery, which affords identification of compounds that modulate either potentiation or inhibition. Using this protocol, we have identified and confirmed 29 compounds with more than 30% inhibition of Kir2.1 at 10 μM concentration; 12 of these also displayed strong acute inhibition of hERG activity (Fig. 1). Eleven distinct structures out of 29 displayed stronger effects on Kir2.1 in the presence of MTSET than that in the absence of MTSET. These results reveal that a considerable fraction of compounds in this library are capable of exerting effects on channel expression on the cell surface. Recent reports showed that pentamidine causes QT prolongation but has no detectable acute effects on cardiac potassium channels including hERG, KvLQT1/mink, Kv4.3, or the cardiac sodium channel, SCNA5. Indeed, pentamidine was shown to reduce hERG protein expression on the cell surface (28, 29). Collec-

Inhibition of Potassium Channel Trafficking

tively, these results suggest that chronic effects exerted by certain compounds on channel expression are more widespread, arguing for the need to evaluate compound effects on biogenesis of ion channels.

Celastrol is a potent anti-inflammatory and antioxidant compound extracted from a perennial creeping plant belonging to the Celastraceae family (30). Recent data have shown that celastrol activates heat shock transcription factor 1 in cultured cells, including both HeLa and SH-SY5Y cells (31). This leads to a significant increase of the hsp70 protein. In animal models, celastrol displays cytoprotective activity against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- and 3-nitropropionic acid-induced neurotoxicity (19). The potency of these effects has an EC_{50} of $3 \mu\text{M}$ (31). In our study, celastrol, at a concentration as low as 200 nM, reduces Kir2.1 and hERG expression to a level comparable with that induced by BFA, a general inhibitor for vesicular trafficking (Figs. 4 and 5). A similarly potent effect is seen for native hERG potassium channels as well (Fig. 5). The precise mechanism of action for celastrol to inhibit the surface expression is an intriguing subject for future study. Recent evidence has shown that some forward trafficking motifs are phosphorylation sites (32, 33). It is conceivable that celastrol effects on heat shock response and potassium channels involve two different mechanisms. Because of the neuroprotective effect, celastrol has been investigated for possible therapeutic benefits in treating Parkinson disease (19). It is of importance to evaluate whether celastrol could have any adverse effects on cardiac function in animal models.

In addition to pharmacological action on intended receptors, many drugs of abuse elicit cellular adaptation following prolonged exposure, especially in several G-protein-coupled receptor systems (34). In many of these cases, drug binding causes internalization of receptor proteins, resulting in down-regulation. In the case of nicotinic acetylcholine receptor, nicotine binding up-regulates receptor expression through a mechanism that is consistent with drug-enhanced intracellular maturation (35). These examples highlight that drug binding to targeted receptors plays an important role in surface density, either positively or negatively. Celastrol indeed also displays acute inhibitory effects (Fig. 3), consistent with the ability to directly interact with the channel proteins. For potassium channels, a prominent example of drug-channel interaction is estradiol binding to calcium-activated potassium (BK) channels (36). While the precise role of celastrol remains to be investigated in terms of specific mechanism of action, **our data indicate that there is a clear contribution of chronic effects from drugs as a result of affecting biogenesis of potassium channels.** Thus, chronic exposure to certain drugs may be an important aspect of acquired QT prolongation.

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Chronic Inhibition of Cardiac Kir2.1 and hERG Potassium Channels by Celastrol with Dual Effects on Both Ion Conductivity and Protein Trafficking

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