Potent Activation of Large-Conductance Ca²⁺-Activated K⁺ Channels by the Diphenylurea 1,3-Bis-[2-hydroxy-5-(trifluoromethyl)phenyl]urea (NS1643) in Pituitary Tumor (GH₃) Cells[S]

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ABSTRACT

1,3-Bis-[2-hydroxy-5-(trifluoromethyl)phenyl]urea (NS1643) is reported to be an activator of human *ether-à-go-go*-related gene current. However, it remains unknown whether it has any effects on other types of ion channels. The effects of NS1643 on ion currents and membrane potential were investigated in this study. NS1643 stimulated Ca²⁺-activated K⁺ current $[I_{K(Ca)}]$ in a concentration-dependent manner with an EC_{50} value of 1.8 μM in pituitary tumor (GH₃) cells. In inside-out recordings, this compound applied to the intracellular side of the detached channels stimulated large-conductance Ca²⁺-activated K⁺ (BK$_{Ca}$) channels with no change in single-channel conductance. It shifted the activation curve of BK$_{Ca}$ channels to less depolarized voltages without altering the gating charge of the channels. NS1643-stimulated channel activity depended on intracellular Ca²⁺, and mean closed time during exposure to NS1643 was reduced. NS1643 (3 μM) had little or no effect on peak amplitude of *ether-à-go-go*-related gene-mediated K⁺ current evoked by membrane hyperpolarization, although it increased the amplitude of late-sustained components of K⁺ inward current, which was suppressed by paxilline but not by azimilide. NS1643 (3 μM) had no effect on L-type Ca²⁺ current. This compound reduced repetitive firing of action potentials, and further application of paxilline attenuated its decrease in firing rate. In addition, NS1643 enhanced BK$_{Ca}$-channel activity in human embryonic kidney 293T cells expressing α-*hSlo*. In summary, we clearly show that NS1643 interacts directly with the BK$_{Ca}$ channel to increase the amplitude of $I_{K(Ca)}$ in pituitary tumor (GH₃) cells. The α-subunit of the channel may be a target for the action of this small compound.

NS1643 (Fig. 1) is a novel small-molecule compound that was reported to activate HERG K⁺ channels expressed in *Xenopus laevis* oocytes and in mammalian HEK293 cells (Casis et al., 2006; Diness et al., 2006; Hansen et al., 2006; Lu et al., 2008). This compound has also been shown to affect

spontaneous rhythmic activity in the epididymal duct via the activation of *erg* current (Mewe et al., 2008). Earlier studies have demonstrated that NS1608, NS1619, and NS11021 (Fig. 1), which are chemical structurally related compounds of NS1643, can modulate the activity of BK$_{Ca}$ channels (Holland et al., 1996; Strøbaek et al., 1996; Bentzen et al., 2007). Whether NS1643 can affect other types of ion channels has not been clearly investigated, although its activation of hKCNQ1 channels was shown (Diness et al., 2006).

The BK$_{Ca}$ channel, which is the product of a nearly ubiquitous, alternatively spliced gene (KCa1.1, *Slo1*, or *KCNMA1*), represents a functional subtype of K⁺ channels, which play important roles in the regulation of neuronal excitability and hormonal secretion (Ghatta et al., 2006).

ABBREVIATIONS: AP, action potential; BK$_{Ca}$, large-conductance Ca²⁺-activated K⁺; *erg*, *ether-à-go-go* related gene; HEK, human embryonic kidney; HERG, human *ether-à-go-go*-related gene; *hSlo*, human slowpoke; $I_{K(Ca)}$, Ca²⁺-activated K⁺ current; $I_{K(erg)}$, *ether-à-go-go* related gene-mediated K⁺ current; NS1608, *N*-(3-(trifluoromethyl)phenyl)-*N′*-(2-hydroxy-5-chlorophenyl)urea; PEI, polyethylenimine.

This channel is believed to share many of the common structural features of homotetrameric voltage-gated K$^+$ channels, including an ion-selective pore formed by transmembrane segments S5 and S6 and a voltage-sensing module formed by transmembrane segments S1 to S4. The channel is unique in that it can be dually activated by membrane depolarization and elevations in intracellular Ca^{2+} concentrations. Organic compounds with varying structures capable of activating BK$_{Ca}$ channels have been reported previously (Calderone, 2002; Wu et al., 2006; Nardi and Olesen, 2008). Because of their unique high conductance ($\sim$200 pS), even at low probability of opening, sufficient current could flow, thereby altering the membrane potential of excitable cells. On the other hand, the modulators of BK$_{Ca}$ channels represent a potentially therapeutic approach to a wide variety of diseases (Nardi and Olesen, 2008).

In this study, we found that NS1643 can be effective in activating BK$_{Ca}$ channels in pituitary tumor (GH$_3$) cells and in HEK293T cells expressing α-hSlo. The presence of this compound caused a shift in the voltage-dependence of BK$_{Ca}$ channels to less depolarized voltages accompanied by a decrease in the mean closed time of the channel. Our results clearly show that in addition to stimulation of the HERG channel, NS1643 is a potent BK$_{Ca}$ channel activator.

Materials and Methods

Cell Culture. GH$_3$, a clonal cell line derived from a rat prolactin-secreting pituitary tumor, was obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan), and the detailed methodology has been described previously (Wu et al., 2000). In brief, cells were cultured in Ham's F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 15% (v/v) heat-inactivated horse serum, 2.5% (v/v) fetal calf serum, and 2 mM L-glutamine (Invitrogen) in a humidified environment of 5% CO$_2$/95% air. The culture medium was changed every 2 to 3 days, and cells were passaged when they reached confluence. Cell viability was often evaluated using WST-1 assay (Roche Diagnostics, Taipei, Taiwan). To promote cell differentiation, GH$_3$ cells were transferred to a serum-free, Ca^{2+}-free medium. Under these conditions, cells remained 80 to 90% viable for at least 2 weeks. The experiments were performed 5 or 6 days after cells had been cultured (60–80% confluence).

Human embryonic kidney (HEK) 293T cells were obtained from the American Type Culture Collection (Manassas, VA). They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO$_2$ atmosphere. For transfection of HEK293T cells, cells numbering 2 to 6×10^4 were seeded on the 24-well culture plate for 24 h before transfection. All culture media and supplements were obtained from Invitrogen (Carlsbad, CA).

Transfection. The expression vector pCMV6-XL4 containing a human voltage-dependent and Ca^{2+}-activated K$^+$ channel pore-forming α-subunit (α-hSlo; NM_002247.1) was purchased from Origene Technologies (Rockville, MD). The α-hSlo gene is known to encode a functional BK$_{Ca}$ channel. The expression plasmid was subsequently transfected into HEK293T cells for transient expression. In brief, the plasmid was prepared in 150 mM NaCl as a diluent solution. PEI (ExGen 500; MBI Fermentas, Hanover, MD) and plasmid were mixed together and incubated for 10 min at room temperature to allow for adequate binding of the plasmid to the PEI. Plasmid-PEI mixture solution was added to the 24-well plate and centrifuged at 280g for 5 min. After centrifugation, transfected cells were incubated at 37°C for an additional 48 h. The functional expression of α-hSlo channels was determined by electrophysiological experiments.

Electrophysiological Measurements. Before the experiments, GH$_3$ or HEK293T cells were dissociated with 1% trypsin/EDTA solution, and an aliquot of cell suspension was transferred to a recording chamber affixed to the stage of a DM-IL inverted microscope (Leica Microsystems, Wetzlar, Germany). Cells were bathed at room temperature (20–25°C) in normal Tyrode's solution containing 1.8 mM CaCl$_2$. Patch pipettes were made from Kmax-51 capillaries (Kimble Glass, Vineland, NJ) using a PP-830 puller (Narishige, Tokyo, Japan), and their tips were fire-polished with an MF-83 microforge (Narishige). When filled with pipette solution, their resistance ranged between 3 and 5 MΩ. Patch-clamp recordings were obtained in cell-attached, inside-out, or whole-cell configurations using an RK-400 (Bio-Logic, Claix, France) or Axopatch-200B (Molecular Devices, Sunnyvale, CA) amplifier.

Data Recordings and Analyses. The signals consisting of voltage and current tracings were simultaneously displayed with a digital oscilloscope (Gould, Chandler, AZ) and a liquid crystal display projector (ViewSonic, Walnut, CA). The data were stored online in a Slimnote Vx3 computer (Lemel, Taipei, Taiwan) via a universal serial bus port through a Digidata 1322A interface (Molecular Devices), which was controlled by pCLAMP 9.0 (Molecular Devices). Currents were low-pass-filtered at 3 kHz. All single-channel recordings were digitized at 10 kHz. Ion currents recorded during whole-cell or single-channel experiments were stored and analyzed using pCLAMP 9.0 (Molecular Devices), Origin 7.5 (OriginLab Corp, Northampton, MA), or custom-made macros built in Excel 2007 spreadsheet running on Windows Vista (Microsoft, Redmond, WA). The pCLAMP-generated voltage-step protocols were used to investigate I-V relationships for ion currents [e.g., I$_{K(Ca)}$].

Single BK$_{Ca}$-channel amplitudes were determined by fitting Gaussian distributions to the amplitude histograms of the closed and open state, respectively. The channel activity in a patch was expressed as NP$_o$, which is estimated using the following equation:

$$NP_o = \frac{(A_1 + 2A_2 + 3A_3 + \ldots + nA_n)}{(A_o + A_1 + A_2 + A_3 + \ldots + A_n)} \quad (1)$$
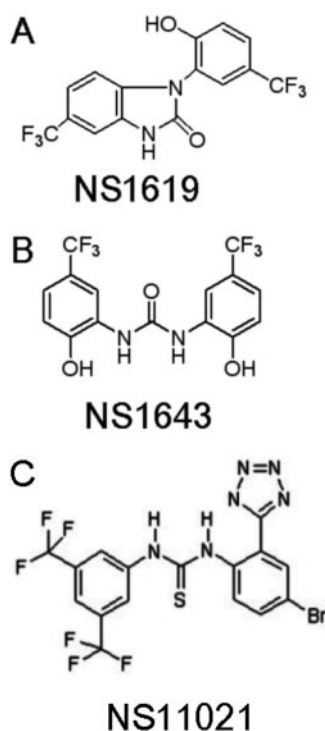

Fig. 1. Chemical structures of NS1619 (A), NS1643 (B), and NS11021 (C).

where N is the number of active channels in the patch, A_0 is the area under the curve of an all-points histogram corresponding to the closed state, and $A_1, \ldots A_n$ represent the histogram areas reflecting the levels of distinct open state for 1 to n channels in the patch. Open- or closed-time distributions were fitted with logarithmically scaled bin width using the method of McManus et al. (1987).

To calculate the percentage stimulation of NS1643 on $I_{K(Ca)}$, the cells were bathed in normal Tyrode's solution, which contained 1.8 mM $CaCl_2$. Each cell was depolarized from 0 to +50 mV at a rate of 0.1 Hz, and the amplitude of $I_{K(Ca)}$ was measured at the end of the depolarizing pulses. The amplitude of $I_{K(Ca)}$ in the presence of 30 μM NS1643 was taken as 100%, and those during exposure to different concentrations (0.3–10 μM) of NS1643 were then compared. The concentration-response relationship for NS1643-induced activation of $I_{K(Ca)}$ in GH_3 cells was determined by the Hill equation using a nonlinear regression analysis, that is:

$$\% \text{ increase} = \frac{E_{\max} \times [C]^{n_n}}{EC_{50} + [C]^{n_n}} \quad (2)$$

where [C] is the NS1643 concentration, EC_{50} and n_H are the half-maximal concentration of NS1643 and the Hill coefficient, respectively, and $E_{\max}$ is the maximal activation of $I_{K(Ca)}$ induced by this compound.

To determine the effect of NS1643 on the activation curve of BK_{Ca} channels, the ramp pulses from 0 up to +90 mV over a 1-s period were applied to the detached patch. The activation curve was calculated by averaging current traces in response to 20 voltage ramps and dividing each point of the mean current by single-channel amplitude for each potential, after leakage currents were corrected (Carl and Sanders, 1990; Wu et al., 2000). The relationships between the membrane potentials and the relative open probability obtained before and after the application of NS1643 (3 μM) were then fitted with a Boltzmann function of the following form:

$$\text{Relative open probability} = \frac{P_{\max}}{1 + \exp\left[\frac{-(V - V_{1/2})qF}{RT}\right]} \quad (3)$$

where $P_{\max}$ is the maximal open probability of channel openings during exposure to NS1643 (3 μM) at +90 mV, $V_{1/2}$ is the voltage at

which there is half-maximal activation, q is the apparent gating charge, F is Faraday's constant, R is the universal gas constant, and T is the absolute temperature. Curve-fitting to data sets presented here was performed with the aid of Excel 2007 (Microsoft) and Origin 7.5 (OriginLab Corp).

The averaged results are presented as means $\pm$ S.E.M., with n representing the number of cells from which the data were obtained. The paired or unpaired t test and one-way analysis of variance with the least significance difference method for multiple comparisons were used for statistical evaluation of the differences among the mean values. Statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, IL). Differences between values were considered significant when $P < 0.05$.

Drugs and Solutions. 3,7-Dimethyl-1-propargylxanthine, NS1643 ($C_{15}H_{10}F_6N_2O_3$) (Fig. 1), and tetrodotoxin were obtained from Sigma Chemical (St. Louis, MO), and paxilline was from Alomone Labs (Jerusalem, Israel). Azimilide was a generous gift from Procter and Gamble Pharmaceuticals (Cincinnati, OH). The stock solution of NS1643 (50 mM) was made in dimethyl sulfoxide and diluted to final concentration (0.3–30 μM) in the bath solution. All other chemicals were commercially available and of reagent grade. The water used in this study was deionized using a Milli-Q water purification system (Millipore, Taipei, Taiwan).

The composition of normal Tyrode's solution was 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5.5 mM HEPES-NaOH buffer, pH 7.4. To record K^+ currents or membrane potential, a patch pipette was filled with a solution consisting of 140 mM KCl, 1 mM $MgCl_2$, 3 mM Na_2ATP, 0.1 mM Na_2GTP, 0.1 mM EGTA, and 5 mM HEPES-KOH buffer, pH 7.2. For single-channel recordings, high K^+ bathing solution contained 145 mM KCl, 0.53 mM $MgCl_2$, and 5 mM HEPES-KOH, pH 7.4, and pipette solution contained 145 mM KCl, 2 mM $MgCl_2$, and 5 mM HEPES-KOH, pH 7.2.

Results

Effect of NS1643 on $I_{K(Ca)}$ in Pituitary GH_3 Cells. In the initial set of experiments, a whole-cell configuration was used to investigate electrical properties of macroscopic $I_{K(Ca)}$ in these cells. Cells were bathed in normal Tyrode's solution,
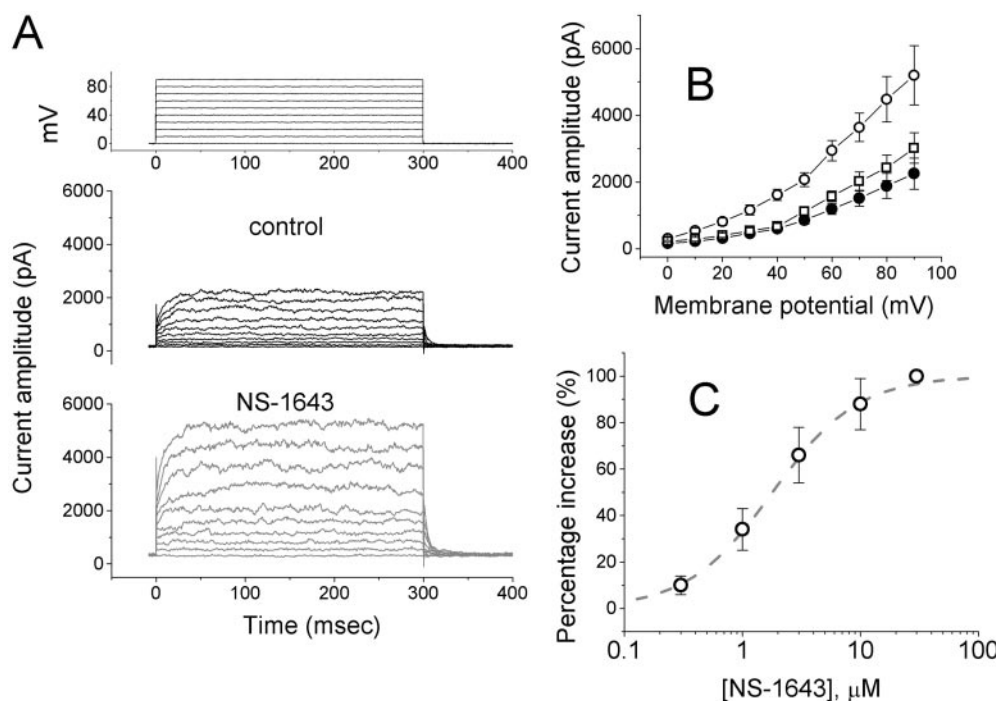
Fig. 2. Stimulatory effect of NS1643 on $I_{K(Ca)}$ in pituitary tumor (GH_3) cells. A, superimposed current traces obtained before (top) and during cell exposure (bottom) to 3 μM NS1643. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The cell was held at 0 mV, and different voltage steps ranging from 0 to +90 mV in 10-mV increments were applied. The uppermost side in A indicates the voltage protocol used. B, averaged I-V relationships of $I_{K(Ca)}$ measured at the end of each voltage pulse in control (●), during exposure to 3 μM NS1643 (○), and washout of NS1643 (□). Each point represents the mean $\pm$ S.E.M. (n = 6–11). C, concentration-response relationship for NS1643-induced stimulation of $I_{K(Ca)}$. The smooth line represents the best fit to eq. 2, as described under *Materials and Methods*. The values for EC_{50}, maximal percentage increase of $I_{K(Ca)}$, and Hill coefficient were calculated to be 1.8 μM, 100%, and 1.2, respectively. Each point represents the mean $\pm$ S.E.M. (n = 5–9).

which contained 1.8 mM CaCl$_2$. When each cell was held at 0 mV and different voltage pulses ranging from 0 to +90 mV with 10-mV increments were applied, a family of large, noisy outward currents with an outward rectification was elicited. Current amplitudes were increased with greater depolarization and reduced by removal of extracellular Ca^{2+} (Wu et al., 1999). However, when the cells were exposed to NS1643 (3 μM), the amplitude of the outward current was greatly increased throughout the entire voltage-clamp step (Fig. 2A). For example, when cells were depolarized from 0 to +80 mV, NS1643 (3 μM) significantly increased I$_{K(Ca)}$ amplitude from 1884 $\pm$ 378 to 4483 $\pm$ 684 pA ($n = 7, P < 0.05$). After washout of the compound, the current amplitude returned to 2431 $\pm$ 379 pA ($n = 5$). The averaged I-V relationships for current amplitudes in the absence and presence of NS1643 are illustrated in Fig. 2B. In addition, a further application of paxilline (1 μM) can reverse the increased amplitude of I$_{K(Ca)}$ induced by NS1643 (3 μM); however, azimilide (30 μM), a blocker of I$_{K(erg)}$ (Liu and Wu, 2003), was not found to have any effects on NS1643-induced increase of I$_{K(Ca)}$.

The relationship between the concentration of NS1643 and the percentage increase of I$_{K(Ca)}$ was constructed (Fig. 1C). The presence of NS1643 was found to increase the amplitude of I$_{K(Ca)}$ in a concentration-dependent manner. The value of half-maximal concentration (i.e., EC$_{50}$) required for the effect of NS1643 on I$_{K(Ca)}$ was 1.8 μM, and 30 μM NS1643 fully increased the current amplitude. The results reflect that NS1643 can have a stimulatory effect on I$_{K(Ca)}$ in pituitary GH$_3$ cells.

Stimulatory Effect of NS1643 on the Activity of BK$_{Ca}$ Channels in Pituitary GH$_3$ Cells. Paxilline, a selective inhibitor of BK$_{Ca}$ channels, could reverse NS1643-stimulated I$_{K(Ca)}$. The increased macroscopic I$_{K(Ca)}$ could be due to the increased open probability, an increase in the number of active channels, or both. For these reasons, the effects of NS1643 on BK$_{Ca}$-channel activity in these cells were further investigated. For these experiments, single-channel recordings with an inside-out configuration were performed in symmetrical K$^+$ solution (145 mM) (Fig. 3). It is interesting that when NS1643 (3 μM) was applied to the intracellular leaflet of the excised patch, channel activity that was calculated by eq. 1, was significantly increased to 0.665 $\pm$ 0.091 ($n = 6$) from a control of 0.0232 $\pm$ 0.0075 ($n = 6, P < 0.01$). However, no significant modification in single-channel amplitude of these channels could be demonstrated during the exposure to 3 μM NS1643 (Fig. 3C).

Lack of Effect of NS1643 on Single-Channel Conductance of BK$_{Ca}$ Channels. We next examined whether exposure to NS1643 might modify single-channel conductance. To construct the plots of current amplitude as a function of membrane potential, the voltage ramp pulses from +10 to +90 mV with a duration of 1 s were applied at a rate of 0.05 Hz. Figure 4A illustrates an ohmic I-V relationship of BK$_{Ca}$ channels obtained in the absence and presence of 3 μM NS1643. The single-channel conductance in the control was calculated to be 175 $\pm$ 12 pS ($n = 11$) with a reversal potential of 0 $\pm$ 3 mV ($n = 9$). The value for these channels did not differ significantly from that obtained in the presence of 3 μM NS1643 (177 $\pm$ 11 pS; $n = 10, P > 0.05$). Thus, application of this compound did not result in any modification in the single-channel conductance of BK$_{Ca}$ channels, although it was able to increase the channel open probability. Thus,

NS1643-induced increase of channel activity can result primarily from an increase in open state probability rather than an increase in the number of active channels.

Effect of NS1643 on the Activation Curve of BK$_{Ca}$ Channels. The voltage-dependence of BK$_{Ca}$ channels was also evaluated in the presence of this compound. In these experiments, the activation curves were obtained by use of voltage-ramp protocol. The ramp pulses were delivered from 0 to +90 mV with a duration of 1 s. Figure 4B shows the activation curve of BK$_{Ca}$ channels with or without application of NS1643 (3 μM). The plots of relative open probability as a function of membrane potential were fitted with eq. 3, as described under *Materials and Methods*. In control, $V_{1/2} = 48.1 \pm 1.9$ mV and $q = 2.6 \pm 0.8$ e ($n = 6$), whereas $V_{1/2} = 33.7 \pm 1.8$ mV and $q = 2.7 \pm 0.7$ e ($n = 6$). Thus, exposure to
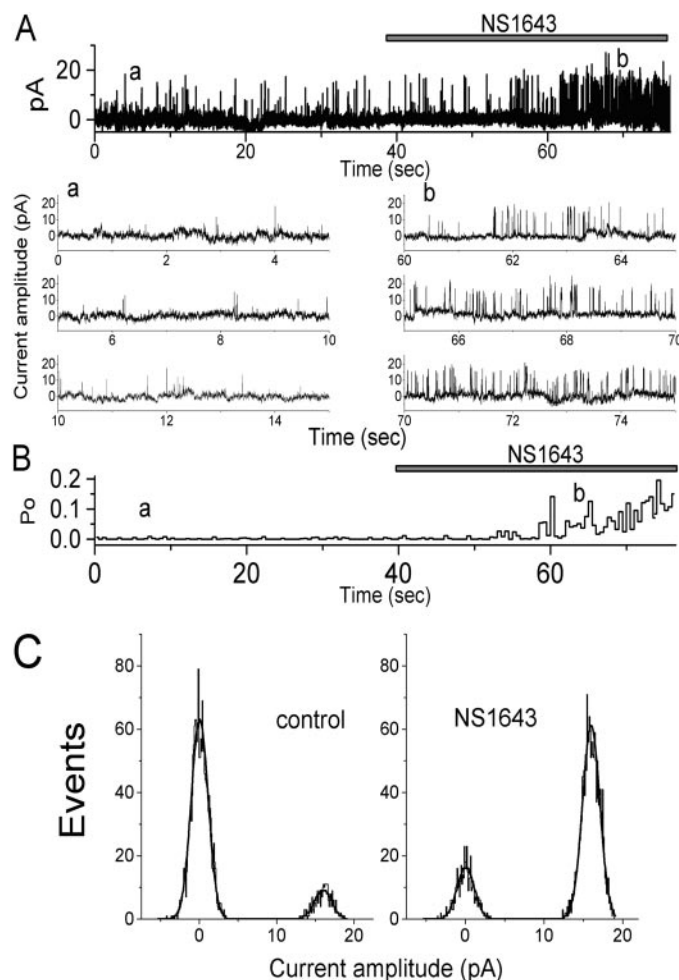

Fig. 3. Stimulatory effect of NS1643 on the activity of BK$_{Ca}$ channels recorded from pituitary GH$_3$ cells. The potential was held at +60 mV. A, original current traces recorded from a detached patch of GH$_3$ cells. The horizontal bar shown above indicates bath application of NS1643 (3 μM). The bottom of A shows the current traces displayed at an expanded time scale. Original current traces (a and b) shown at the top correspond to those labeled a and b in the bottom. An upward deflection of the current record indicates the opening events of the channel. B, time course of changes in channel open probability from A. It is noteworthy that there was a progressive increase in channel open probability when the detached patch was exposed to NS1643 (3 μM). C, amplitude histograms measured in the control (left) and in the presence of 3 μM NS1643 (right). All points shown in these histograms were fitted with simple Gaussian functions. The closed state corresponds to the peak at 0 pA. Note that no discernible change in single-channel amplitude can be demonstrated in the presence of NS1643.

NS1643 caused an approximately 15-mV leftward shift in voltage-dependent activation of BK_{Ca} channels, despite its ability to increase the probability of channel openings. However, there was no significant change in the gating charge (i.e., q) of BK_{Ca} channels in pituitary GH₃ cells. The results indicate that in addition to the increased open probability, this compound applied to the intracellular leaflet of the excised patch can modify the voltage dependence of these channels.

Effect of Internal Ca^{2+} Concentration on NS1643-Stimulated Activity of BK_{Ca} Channels. We further investigated whether NS1643 can alter Ca^{2+}-dependence of BK_{Ca} channels in GH₃ cells. In this set of experiments, when a detached patch was formed, different concentrations (i.e., 0.01, 0.1, and 10 μM) of Ca^{2+} with or without application of NS1643 (3 μM) were present in the bath. As shown in Fig. 4C, at a given concentration of NS1643 (3 μM), the magnitude of NS1643-induced increase in channel activity was increased as internal Ca^{2+} was elevated. In addition, NS1643 enhanced channel activity to a greater extent when internal Ca^{2+} was increased. Therefore, the results suggest that the magnitude of NS1643-stimulated channel activity seems to depend on the level of internal Ca^{2+}, although its stimulatory action on BK_{Ca} channels may not require internal Ca^{2+}.

Effect of NS1643 on Kinetic Behavior of BK_{Ca} Channels in Pituitary GH₃ Cells. Because NS1643 was found to enhance channel activity with no significant changes in single-channel conductance, we evaluated whether it could modify the gating of these channels. The effects of NS1643 on mean open and closed time of BK_{Ca} channels in these cells were examined and analyzed during recordings from the patches in which only one active channel was found. As shown in Fig. 5, in a detached patch under control conditions, the closed-time distributions obtained at the level of +60 mV were fitted with a two-exponential function. The time constants for fast and slow components of mean closed time were 8 ± 3 and 37 ± 7 ms, respectively ($n = 7$). NS1643 at a concentration of 3 μM added to the bath significantly decreased the mean lifetime of the closed state to 4 ± 2 and 27 ± 5 ms, respectively ($n = 7$, $P < 0.05$). On the other hand, no significant change in mean lifetime of opening events was observed during exposure to 3 μM NS1643 (1.87 ± 0.06 versus 1.89 ± 0.05 ms; $n = 7$, $P > 0.05$). These data suggest that the stimulation by this compound of BK_{Ca} channels in these cells is largely attributed to an increase in channel open probability caused by the decreased closed time. Therefore, the binding of this compound to the membrane in GH₃
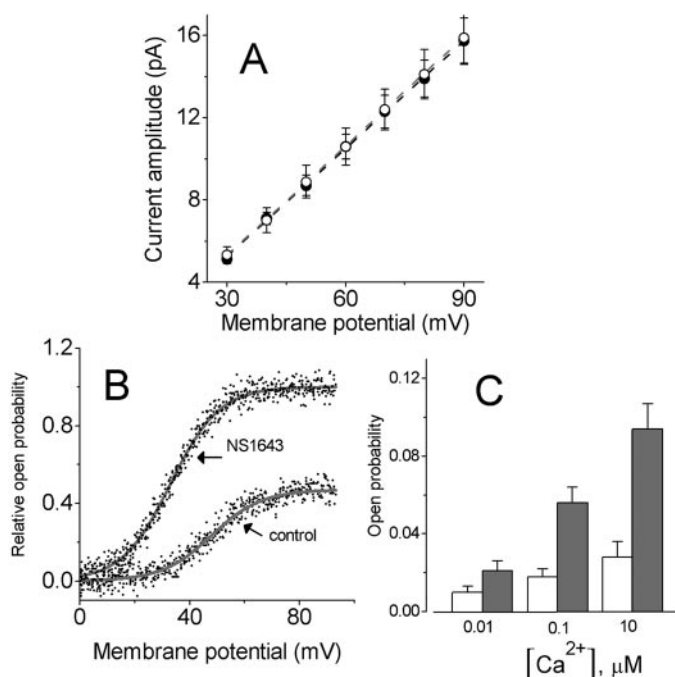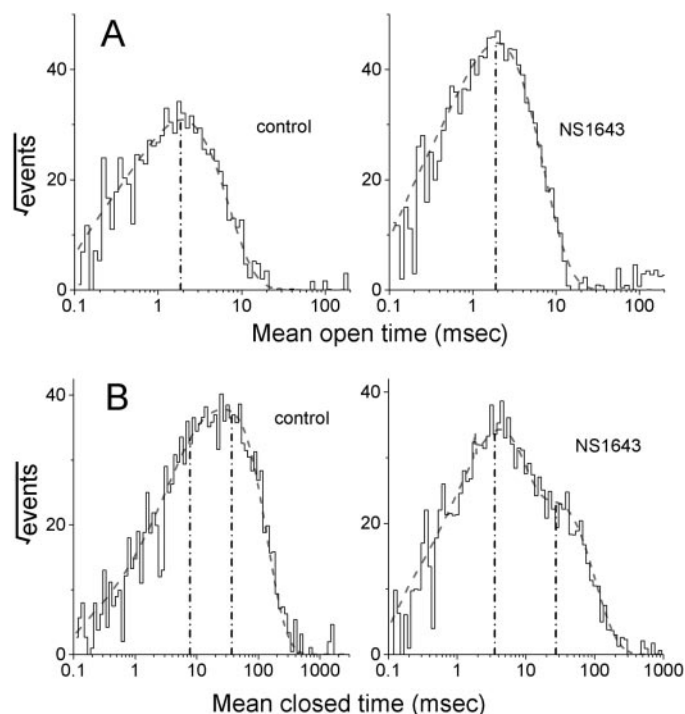

Fig. 4. Voltage- and Ca^{2+}-dependent effect of NS1643 on the activity of BK_{Ca} channels in GH₃ cells. The inside-out recordings were conducted with a symmetrical K⁺ solution (145 mM). A, lack of effect of NS1643 on single-channel conductance of BK_{Ca} channels. The broken lines represent the I-V relationships of BK_{Ca} channels obtained in the absence (●) and presence (○) of 3 μM NS1643. Each point represents the mean ± S.E.M. ($n = 9$–12). B, effect of NS1643 on the activation curve of BK_{Ca} channels. The activation curves were obtained when the ramp pulses were applied from 0 to +90 mV with a duration of 1 s. The smooth curves obtained in the absence and presence of 3 μM NS1643 represent the best fit to eq. 3, as described under *Materials and Methods*. C, the effect of internal Ca^{2+} concentration on NS1643-stimulated activity of BK_{Ca} channels. The potential was held at +60 mV, and different concentrations (0.1, 1.0, and 10 μM) of Ca^{2+} were present in the bath. Open bars are control and filled bars were obtained in the presence of 3 μM NS1643. Each point represents the mean ± S.E.M. ($n = 4$–7).

Fig. 5. Effect of NS1643 on mean open and closed time of BK_{Ca} channels in GH₃ cells. Inside-out configuration was performed in these experiments, and the potential was held at +60 mV. Cells were bathed in symmetrical K⁺ solution (145 mM), and the bath medium contained 0.1 μM Ca^{2+}. The open- and closed-time histograms obtained in the control (left) and during exposure (right) to 3 μM NS1643 are illustrated in the top (A) and bottom (B), respectively. Control data were obtained from measuring 266 channel openings with a total recording time of 1 min, whereas those obtained in the presence of NS1643 (3 μM) were measured from 561 channel openings with a total recording time of 30 s. The vertical broken lines shown in each lifetime distribution are placed at the value of time constant in the open or closed state of the channel. It is noteworthy that dwell-time data were plotted with a logarithmic time axis along the abscissa and a square-root ordinate, which represents the number of events in each bin. The broken smooth curves were well fitted by a one- or two-exponential function.

cells tends to cause the BK$_{Ca}$ channel to spend less time in the closed state.

Effect of NS1643 on I$_{K(erg)}$ in Pituitary GH$_3$ Cells. Previous studies have demonstrated that NS1643 is able to activate HERG channels in *X. laevis* oocytes and in HEK293 cells (Casis et al., 2006; Diness et al., 2006; Hansen et al., 2006). One would assume that the observed amplitude of I$_{K(Ca)}$ stimulated by NS1643 could be associated with its activation of I$_{K(erg)}$ present in GH$_3$ cells. We further explored whether this compound might have any effect on I$_{K(erg)}$ (Wu et al., 2000; Liu and Wu, 2003). For these experiments, cells were bathed in a high-K$^+$, Ca^{2+}-free solution. A family of large inward deactivating currents in response to long-lasting membrane hyperpolarizations could be seen in these cells. These tail currents elicited by membrane hyperpolarizations were sensitive to inhibition by azimilide (30 μM) or risperidone (30 μM), and correspond to those reported previously as I$_{K(erg)}$ (Wu et al., 2000; Liu and Wu, 2003). As shown in Fig. 6, NS1643 at a concentration of 3 μM had no effect on the peak amplitude of deactivating I$_{K(erg)}$ seen in GH$_3$ cells. As the time course for deactivating I$_{K(erg)}$ became slower, the current amplitude measured at the end of membrane hyperpolarization was significantly increased. However, a further application of paxilline (1 μM) could reverse the NS1643-induced increase in the time constant of deactivating I$_{K(erg)}$ (Fig. 6). The results showed that NS1643 had little or no effect on I$_{K(erg)}$ present in pituitary GH$_3$ cells.

Effect of NS1643 on the Firing of Spontaneous APs in GH$_3$ Cells. In the following experiments, we investigated the effect of NS1643 on repetitive firing of APs in these cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM CaCl$_2$, and current-clamp recordings were made. As shown in Fig. 7, when cells were exposed to NS1643, the resting potential was hyperpolarized, and the firing of APs

was progressively reduced. For example, NS1643 (10 μM) significantly decreased firing frequency from 0.45 $\pm$ 0.04 to 0.14 $\pm$ 0.02 Hz ($n = 6$, $P < 0.05$). A further application of paxilline (1 μM) returned the frequency to 0.41 $\pm$ 0.03 Hz ($n = 5$). Therefore, these results clearly showed that NS1643 was effective in altering the firing of APs in GH$_3$ cells. The inhibitory action of NS1643 on the firing of APs can be explained primarily by its activation of BK$_{Ca}$ channels. Therefore, the decrease in the firing of APs caused by this compound may attenuate the stimulus-secretion coupling in these cells.

Stimulatory Effect of NS1643 on the α-hSlo Channel Expressed in HEK293T Cells. In a final set of experiments, we tested the hypothesis that NS1643 had effects on the activity of BK$_{Ca}$ channels in HEK293T cells transfected with α-hSlo. Under our experimental conditions, the transfection of α-hSlo in HEK293T cells could result in the appearance of BK$_{Ca}$ channels. The functional properties of BK$_{Ca}$ channels obtained after transfection of α-hSlo into HEK293T cells were found to correspond to those measured from pituitary GH$_3$ cells. A single-channel current through α-hSlo was identified by adding paxilline (1 μM) to bath solution. In addition, in inside-out configuration, when NS1643 was applied to the intracellular side of excised patch, the activity of BK$_{Ca}$ channels in α-hSlo-expressing HEK293T cells was dramatically enhanced (Fig. 8). The time constants for fast and slow components of mean closed time were significantly reduced to 5 $\pm$ 1 and 22 $\pm$ 4 ms from a control value of 14 $\pm$ 4 and 39 $\pm$ 7 ms, respectively ($n = 7$, $P < 0.05$). However, the mean open time between the absence and presence of NS1643 did not differ significantly (2.33 $\pm$ 0.07 versus 2.36 $\pm$ 0.08 ms; $n = 7$, $P > 0.05$). No modification in single-channel conductance could be detected in the presence of 3 μM NS1643 (158 $\pm$ 12 versus 159 $\pm$ 11 pS; $n = 7$, $P >$
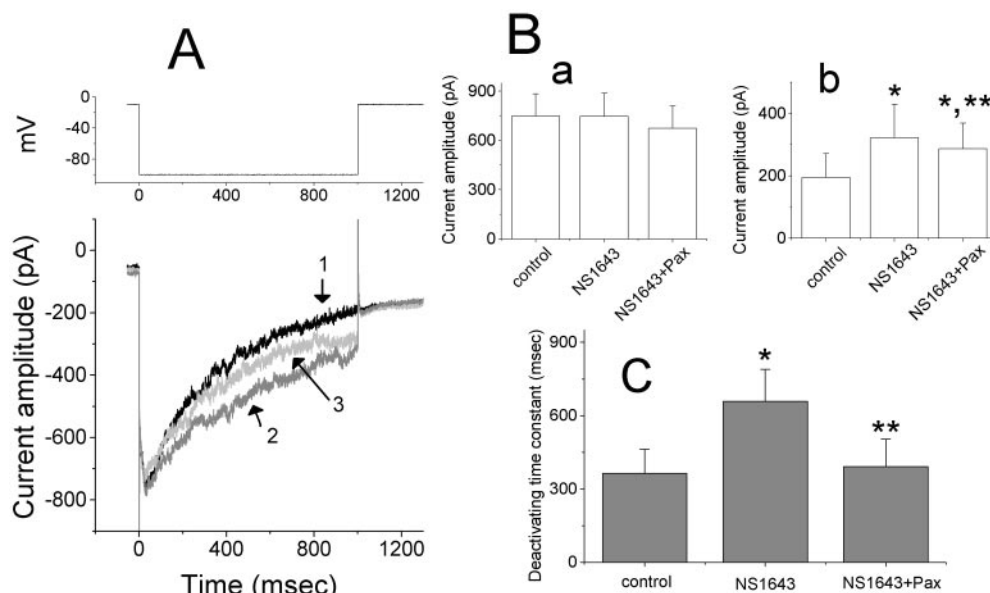
Fig. 6. Effect of NS1643 on I$_{K(erg)}$ in GH$_3$ cells. Cells were bathed in a high K$^+$, Ca^{2+}-free solution containing tetrodotoxin (1 μM) and CdCl$_2$ (0.5 mM). A, current traces obtained when the cell was held at -10 mV and hyperpolarizing pulses to -100 mV with a duration of 1 s were applied. 1, control; 2, NS1643 (3 μM); 3, NS1643 plus paxilline (1 μM). In the experiments of NS1643 plus paxilline, paxilline was applied in the continued presence of NS1643. The top of A indicates the voltage protocol used. B, bar graphs showing the effects of NS1643 (3 μM) and NS1643 plus paxilline (1 μM) on current amplitude measured at the beginning (a) and end (b) of the hyperpolarizing pulses (mean $\pm$ S.E.M.; $n = 5$–8 for each bar). Pax, paxilline (1 μM). ∗, significantly different from control. ∗∗, significantly different from the NS1643 (3 μM) alone group. C, summary of data depicting the effects of NS1643 and NS1643 plus paxilline on the time constant of I$_{K(erg)}$ deactivation in GH$_3$ cells (mean $\pm$ S.E.M.; $n = 5$–8 for each bar). ∗, significantly different from control. ∗∗, significantly different from the NS1643 (3 μM) alone group.
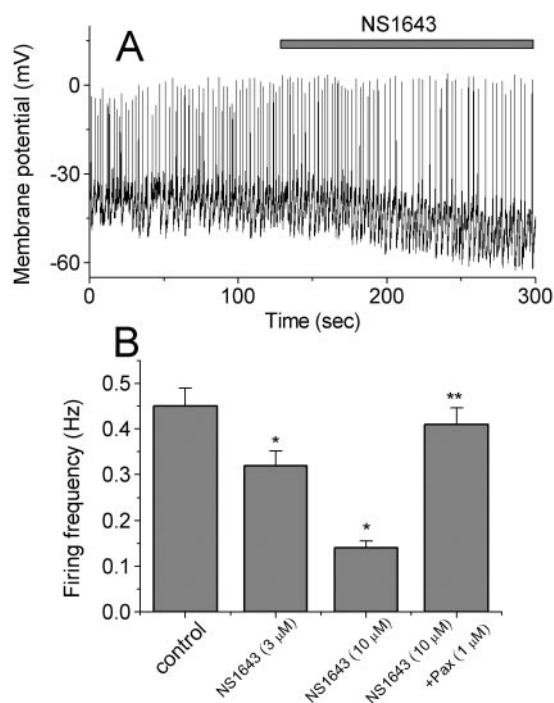
Fig. 7. Effect of NS1643 on the firing of APs in GH₃ cells. Cells were bathed in normal Tyrode's solution. Patch pipettes were filled with a K⁺-containing solution. Changes in membrane potential were measured under current-clamp configuration. A, continuous potential trace obtained before and after application of NS1643. The horizontal bar shown above indicates the application of NS1643 (3 μM) to the bath. B, summary of data showing inhibitory effect of NS1643 on the firing frequency of APs. Pax, paxilline (1 μM). ∗, significantly different from control (i.e., in the absence of NS1643). ∗∗, significantly different from NS1643 (10 μM) alone group. Each point represents the mean ± S.E.M. (n = 5–7).

0.05). A further application of paxilline (1 μM) reversed the NS1643-induced increase in channel open probability. The results showed that, similar to the results obtained in pituitary GH₃ cells, NS1643 can increase the probability of channel openings in HEK293T cells expressing α-hSlo.

Discussion

The major findings of this study are as follows. First, in differentiated GH₃ cells, NS1643, an activator of $I_{K(erg)}$, was effective in increasing the amplitude of $I_{K(Ca)}$. Second, NS1643 enhanced the activity of BK$_{Ca}$ channels from the cytoplasmic side in inside-out patch configuration, with no modification in single-channel conductance. The increased channel activity was accompanied by a shift of the steady-state activation toward a less depolarized range. Third, this compound had minimal or no effect on the open time of the channel, but reduced the mean closed time, thereby leading to an increase in channel open probability. Fourth, NS1643 decreased the repetitive firing of APs in GH₃ cells. Fifth, NS1643 enhanced the activity of BK$_{Ca}$ channels expressed in HEK293T cells transfected with α-hSlo. The stimulatory effect of NS1643 on BK$_{Ca}$ channels could be an important mechanism underlying NS1643-induced action in endocrine or neuroendocrine cells.

Our studies demonstrated that NS1643 applied to the cytoplasmic side of excised patch had no effect on single-channel conductance of BK$_{Ca}$ channels, although it increased the probability of channel openings significantly. The amplitude of macroscopic $I_{K(Ca)}$ enhanced by NS1643 was believed to be due to an increase in channel open probability accompanied by decreased mean closed time. Because it did not affect mean open time of the channel, unlike NS1608 or NS11021
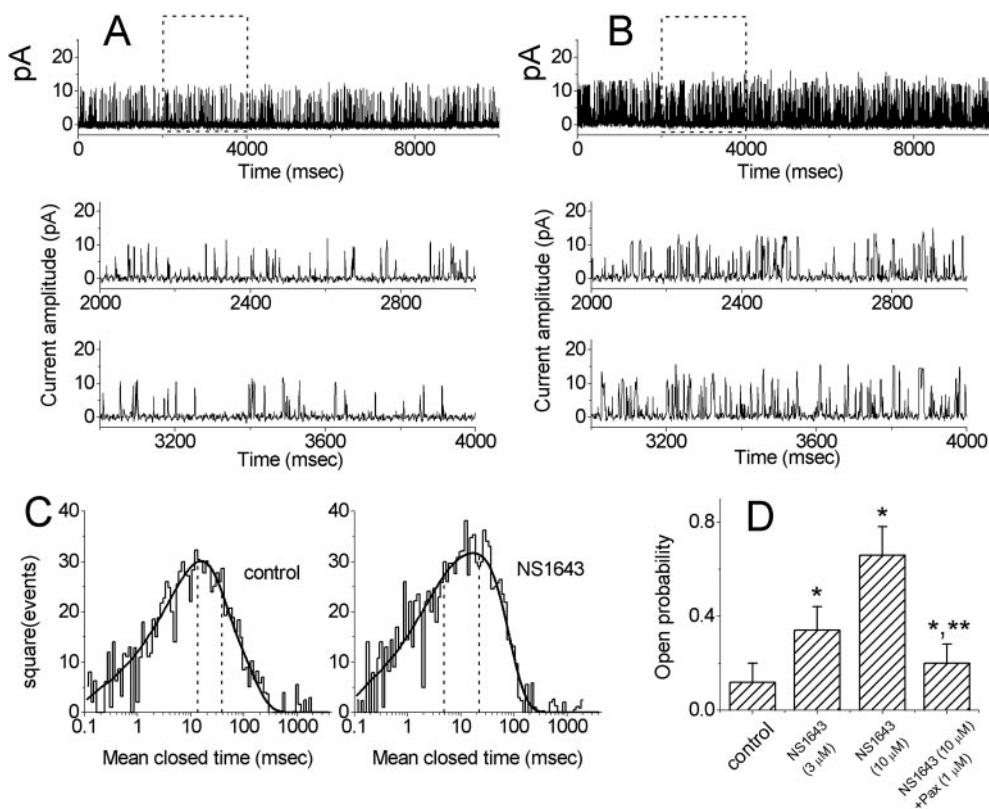

Fig. 8. Stimulatory effect of NS1643 on BK$_{Ca}$-channel activity recorded from α-hSlo-expressing HEK293T cells. Original current traces showing the activity of BK$_{Ca}$ channels before (A) and after the addition (B) of 3 μM NS1643. Inside-out recordings were conducted with symmetrical K⁺ concentration (145 mM). The potential was held at +60 mV, and the bath medium contained 0.1 μM. Ca²⁺. The bottom traces in A and B show the expanded records as indicated by the dashed box in the uppermost traces. C, closed-time histograms obtained in the control (left) and during exposure (right) to 3 μM NS1643. The vertical dashed lines indicate the value of the time constant in the closed state of the channel. The smooth curves were fitted by a two-exponential function. D, summary of data showing stimulatory effect of this compound on BK$_{Ca}$-channel activity present in α-hSlo-expressing HEK293T cells (mean ± S.E.M.; n = 7–9 for each bar). Pax, paxilline (1 μM). ∗, significantly different from control. ∗∗, significantly different from NS1643 (10 μM) alone group.

(Strøbaek et al., 1996; Bentzen et al., 2007), NS1643 might increase channel open probability mainly by its ability to bind the channel in closed conformation. It is also reasonable to assume that this compound may decrease the energy barrier for channel openings because of a destabilization of the closed state.

The inability of NS1643 to modify single-channel conductance observed in this study indicates that the increased responsiveness of the channel to this compound is unlikely to be located at the central ion-conducting pore of the channel. The lack of any change in the slope factor of the activation curve of BK$_{Ca}$ channels during exposure to this compound also prompted us to propose that the stimulatory effect of NS1643 on the BK$_{Ca}$ channel is not mediated by a direct effect on the voltage sensor per se, and that its binding site may lie outside of the transmembrane field. NS1643 stimulated the activity of BK$_{Ca}$ channels to a greater magnitude when intracellular Ca^{2+} was elevated, although in the absence of intracellular Ca^{2+}, it was able to activate channel activity. The results suggest that its activation could be dependent on the level of intracellular Ca^{2+}. The decreased closed time caused by NS1643 is likely to be associated with enhanced Ca^{2+} sensitivity of the channel. It remains to be determined whether this compound simply enhances the sensitivity of the channel to cytoplasmic Ca^{2+}, or instead it destabilizes the closed state by a direct effect on channel gating.

Previous studies showed that increased availability of intracellular Ca^{2+} resulting from the increase of Ca^{2+} influx through L-type Ca^{2+} channels is able to increase the amplitude of I$_{K(Ca)}$ in endocrine cells (Wu et al., 2003; Marcantoni et al., 2007; Tsaneva-Atanasova et al., 2007). However, the inability of NS1643 to increase L-type Ca^{2+} current in GH$_3$ cells (Supplementary Fig. 1) and as described previously in cardiac myocytes (Hansen et al., 2006) may exclude this possibility. No detectable change in intracellular Ca^{2+} in GH$_3$ cells was observed during the exposure to NS1643 (data not shown), and the results showing that NS1643 activated channel activity in HEK293T cells expressing *α-hSlo* strengthen this notion. In addition, the inability of 3,7-dimethyl-1-propargylxanthine, a blocker of adenosine A$_2$ receptors, to reverse the NS1643-induced increase of I$_{K(Ca)}$ in GH$_3$ cells was found in our study (data not shown). Therefore, its stimulatory action on I$_{K(Ca)}$ seen in these cells is unlikely to be mediated by the binding to adenosine receptors (Giorgi et al., 2008).

The concentration-dependent increase in the amplitude of I$_{K(Ca)}$ with an EC$_{50}$ value of 1.8 μM was observed in this study. NS1643 at a concentration of 3 μM was found to have little or no effect on the peak amplitude of deactivating I$_{K(erg)}$ in GH$_3$ cells, although the deactivating time course seemed to be slower in the presence of NS1643. Therefore, the experiments described in this study imply that even though NS1643 has been used to study the HERG channel function (Mewe et al., 2008), this compound at lower concentrations is also able to activate BK$_{Ca}$ channels directly. Because of its high potency, the data presented herein may be important in interpreting the in vivo mechanism of actions of this compound.

The reason why NS1643 has no effect on I$_{K(erg)}$ in GH$_3$ cells is unclear. These results may be explained as follows. First, the mechanism of stimulatory action of this compound is primarily due to altered inactivation of I$_{K(erg)}$ (Casis et al., 2006; Hansen et al., 2006; Xu et al., 2008). In GH$_3$ cells, I$_{K(erg)}$ elicited by membrane depolarization is rather difficult to be detected, because it consistently overlaps with other types of outward K$^+$ currents over a range of depolarizing voltages (Wu et al., 2000). Second, this cell line was reported to contain many different isoforms of endogenous *erg* channels (Schledermann et al., 2001), because the *erg*1 channel is more sensitive to stimulation by NS1643 (Casis et al., 2006; Diness et al., 2006; Hansen et al., 2006).

Activation of BK$_{Ca}$ channels induced by NS1643 in GH$_3$ cells shares similar characteristics in HEK293, in which *α-hSlo* channels are functionally expressed. These results are of importance because they suggest that NS1643 may bind to a site located in the cytoplasmic side of the α-subunit. It is thus tempting to speculate that in addition to the HERG channel, the BK$_{Ca}$ channel α-subunit may be an important target for the action of this compound. However, it still remains to be further investigated to what extent NS1643 affects other variants of BK$_{Ca}$ channels in different types of cells. In addition, niflumic acid, an activator of BK$_{Ca}$ channels, was shown to activate *erg* K$^+$ currents (Ottolia and Toro, 1994; Wu et al., 2001; Fernandez et al., 2008). Therefore, these two types of ion channels seem to share unique motifs with which some small compounds can interact.

The present findings are similar to those reported with its parent compound, NS1619 (Gribkoff et al., 1996), and the newer one, NS11021 (Bentzen et al., 2007). NS11021 is even more potent to activate BK$_{Ca}$ channels than NS1643 (Bentzen et al., 2007). Therefore, results from this study showed another diphenylurea that presents neither clear advantage nor distinction over other tens of activators that act similarly on BK$_{Ca}$ channels (Nardi and Olesen, 2008). However, our study also demonstrated that NS1643 could hyperpolarize the membrane and reduce the firing of APs in clonal pituitary GH$_3$ cells. Although the detailed mechanism of stimulatory actions of NS1643 has not yet been unraveled, our results provide the first evidence showing that this compound is able to interact with the BK$_{Ca}$ channel to increase the amplitude of I$_{K(Ca)}$. Assuming that similar results observed in this study are found in neuroendocrine or endocrine cells in vivo, the increase in K$^+$ efflux through NS1643-induced stimulation of BK$_{Ca}$ channels may underlie the mechanisms through which this compound influences cellular function.

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