Potent Activation of Large-Conductance Ca\(^{2+}\)-Activated K\(^+\) Channels by the Diphenylurea 1,3-Bis-[2-hydroxy-5-(trifluoromethyl)phenyl]urea (NS1643) in Pituitary Tumor (GH\(_3\)) Cells

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Received May 23, 2008; accepted September 22, 2008

ABSTRACT

1,3-Bis-[2-hydroxy-5-(trifluoromethyl)phenyl]urea (NS1643) is reported to be an activator of human ether-\(\alpha\)-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 Ca\(^{2+}\)-activated K\(^+\) current (\(I_{\text{KCa}}\)) in a concentration-dependent manner with an EC\(_{50}\) value of 1.8 \(\mu\)M in pituitary tumor (GH\(_3\)) cells. In inside-out recordings, this compound applied to the intracellular side of the detached channels stimulated large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK\(_{\text{Ca}}\)) channels with no change in single-channel conductance. It shifted the activation curve of BK\(_{\text{Ca}}\) channels to less depolarized voltages without altering the gating charge of the channels. NS1643-stimulated channel activity depended on intracellular Ca\(^{2+}\), and mean closed time during exposure to NS1643 was reduced. NS1643 (3 \(\mu\)M) had little or no effect on peak amplitude of ether-\(\alpha\)-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 \(\mu\)M) had no effect on L-type Ca\(^{2+}\) 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\(_{\text{Ca}}\)-channel activity in human embryonic kidney 293T cells expressing \(\alpha\)-hSlo. In summary, we clearly show that NS1643 interacts directly with the BK\(_{\text{Ca}}\) channel to increase the amplitude of \(I_{\text{KCa}}\) in pituitary tumor (GH\(_3\)) cells. The \(\alpha\)-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 (Casais 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\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) channel, which is the product of a nearly ubiquitous, alternatively spliced gene (KC\(\alpha\)1.1, Slo1, or KC\(\text{NMA}1\)), 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\(_{\text{Ca}}\), large-conductance Ca\(^{2+}\)-activated K\(^+\); erg, ether-\(\alpha\)-go-go related gene; HEK, human embryonic kidney; HERG, human ether-\(\alpha\)-go-go-related gene; hSlo, human slowpoke; \(I_{\text{KCa}}\), Ca\(^{2+}\)-activated K\(^+\) current; \(I_{\text{Kslop}}\), ether-\(\alpha\)-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 Ca²⁺ concentrations. Organic compounds with varying structures capable of activating BKCa channels have been reported previously (Calderone, 2002; Wu et al., 2006; Nardi and Olesen, 2008). Because of their unique high conductance (≈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 BKCa channels represent a potential therapeutic approach to a wide variety of diseases (Nardi and Olesen, 2008).

In this study, we found that NS1643 can be effective in activating BKCa channels in pituitary tumor (GH₃) cells and in HEK293T cells expressing α-hSlo. The presence of this compound caused a shift in the voltage-dependence of BKCa 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 BKCa channel activator.

Materials and Methods

Cell Culture. GH₃, 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% CO₂ atmosphere. For transfection of HEK293T cells, cells numbering 2 to 2 × 10⁴ 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 Ca²⁺-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 BKCa 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 centrifugated 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, GH₃ 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 CaCl₂. Patch pipettes were made from Kmax-511 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., I₆K(Ca)ᵢ]⁻¹.

Single BKCa-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 NPo, which is estimated using the following equation:

\[ N_{Po} = \frac{(A_1 + 2A_2 + 3A_3 + \ldots + nA_n)}{(A_1 + A_2 + A_3 + \ldots + A_n)} \]

Fig. 1. Chemical structures of NS1619 (A), NS1643 (B), and NS11021 (C).
during exposure to NS1643 (3 mM CaCl2). 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 GH3 cells was determined by the Hill equation using a nonlinear regression analysis, that is:

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

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

To determine the effect of NS1643 on the activation curve of $B_{K_{Ca}}$, 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. 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.

The averaged results are presented as means ± 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 (C15H10F6N2O3) (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 CaCl2, 0.53 mM MgCl2, 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, 3 mM MgCl2, 3 mM Na2ATP, 0.1 mM Na2GTP, 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 MgCl2, and 5 mM HEPES-KOH, pH 7.4, and pipette solution contained 145 mM KCl, 2 mM MgCl2, and 5 mM HEPES-KOH, pH 7.2.

### Results

#### Effect of NS1643 on $I_{K(Ca)}$ in Pituitary GH3 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, 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 ± 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 $P < 0.05$.

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### Results

#### Effect of NS1643 on $I_{K(Ca)}$ in Pituitary GH3 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,
which contained 1.8 mM CaCl₂. 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 Ca²⁺ (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 $I_{\text{K(Ca)}}$ 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 paxilin (1 μM) can reverse the increased amplitude of $I_{\text{K(Ca)}}$ induced by NS1643 (3 μM); however, azimilide (30 μM), a blocker of $I_{\text{K(erg)}}$ (Liu and Wu, 2003), was not found to have any effects on NS1643-induced increase of $I_{\text{K(Ca)}}$.

The relationship between the concentration of NS1643 and the percentage increase of $I_{\text{K(Ca)}}$ was constructed (Fig. 1C). The presence of NS1643 was found to increase the amplitude of $I_{\text{K(Ca)}}$, in a concentration-dependent manner. The value of half-maximal concentration (i.e., $EC_{50}$) required for the effect of NS1643 on $I_{\text{K(Ca)}}$ was 1.8 μM, and 30 μM NS1643 fully increased the current amplitude. The results reflect that NS1643 can have a stimulatory effect on $I_{\text{K(Ca)}}$ in pituitary GH₃ cells.

**Stimulatory Effect of NS1643 on the Activity of $B\text{K}_{\text{Ca}}$ Channels in Pituitary GH₃ Cells.** Paxilin, a selective inhibitor of $B\text{K}_{\text{Ca}}$ channels, could reverse NS1643-stimulated $I_{\text{K(Ca)}}$. The increased macroscopic $I_{\text{K(Ca)}}$ 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 $B\text{K}_{\text{Ca}}$-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 $B\text{K}_{\text{Ca}}$ 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 $B\text{K}_{\text{Ca}}$ 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 ± 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 $B\text{K}_{\text{Ca}}$ 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 Activity Curve of $B\text{K}_{\text{Ca}}$ Channels.** The voltage-dependence of $B\text{K}_{\text{Ca}}$ 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 $B\text{K}_{\text{Ca}}$ 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 $B\text{K}_{\text{Ca}}$ channels recorded from pituitary GH₃ cells. The potential was held at +60 mV. A, original current traces recorded from a detached patch of GH₃ cells. The horizontal bar shown above indicates bath application of NS1643 (3 μM). B, 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. 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.](image)
NS1643 caused an approximately 15-mV leftward shift in voltage-dependent activation of BK$_{Ca}$ channels, despite its inability 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 GH$_3$ 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 GH$_3$ cells. In this set of experiments, when a detached patch was formed, different concentrations (i.e., 0.01, 0.1, and 10 $\mu$M) of Ca$^{2+}$ with or without application of NS1643 (3 $\mu$M) were present in the bath. As shown in Fig. 4C, at a given concentration of NS1643 (3 $\mu$M), 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$^{2+}$.

![Fig. 4. Voltage- and Ca$^{2+}$-dependent effect of NS1643 on the activity of BK$_{Ca}$ channels in GH$_3$ cells.](image)

![Fig. 5. Effect of NS1643 on mean open and closed time of BK$_{Ca}$ channels in GH$_3$ cells.](image)
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 (Casas et al., 2006; Diness et al., 2006; Hansen et al., 2006). One would assume that the observed amplitude of $I_{K_{erg}}$ 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 $I_{K_{erg}}$ 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 ± 0.04 to 0.14 ± 0.02 Hz ($n = 6$, $P < 0.05$). A further application of paxilline (1 µM) returned the frequency to 0.41 ± 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 ± 1 and 22 ± 4 ms from a control value of 14 ± 4 and 39 ± 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 ± 0.07 versus 2.36 ± 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 ± 12 versus 159 ± 11 pS; $n = 7$, $P > 0.05$).

![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 ± 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 ± S.E.M.; $n = 5–8$ for each bar). *, significantly different from control. **, significantly different from the NS1643 (3 µM) alone group.\]
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 IK(erg), was effective in increasing the amplitude of IK(Ca). Second, NS1643 enhanced the activity of BKCa 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 BKCa channels expressed in HEK293T cells transfected with α-hSlo. The stimulatory effect of NS1643 on BKCa 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 BKCa channels, although it increased the probability of channel openings significantly. The amplitude of macroscopic IK(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. 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).

**Fig. 8.** Stimulatory effect of NS1643 on BKCa-channel activity recorded from α-hSlo-expressing HEK293T cells. Original current traces showing the activity of BKCa 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 BKCa-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.
Effect of NS1643 on BKCa Channels in Pituitary GH3 Cells

Sence of intracellular Ca²⁺ is observed during the exposure to NS1643 (data also able to activate BKCa channels directly. Because of its selectivity, NS1643 has been used to study the HERG channel function. 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 BKCa channels than NS1643 (Bentzen et al., 2007). Therefore, results from this study showed that activation of BKCa channels induced by NS1643 in GH3 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 BKCa channel α-subunit may be an important target for the action of this compound. However, it still remains to be further investigated in what extent NS1643 affects other variants of BKCa channels in different types of cells. In addition, niflumic acid, an activator of BKCa 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 concentration-dependent increase in the amplitude of IKCa in GH3 cells was found in our study (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 A2 receptors, to reverse the NS1643-induced increase of IKCa in GH3 cells was found in our study (data not shown). Therefore, its stimulatory action on IKCa 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 IKCa with an EC50 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 IKCa in GH3 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 BKCa 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 IKCa in GH3 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 IKca (Casis et al., 2006; Hansen et al., 2006; Xu et al., 2008). In GH3 cells, IKca 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 erg1 channel is more sensitive to stimulation by NS1643 (Casis et al., 2006; Diness et al., 2006; Hansen et al., 2006).

Acknowledgments

We are grateful to Paul Steed for reading and editing the manuscript. We appreciate the support for this work from Professor Ruy J. Sung at the Cardiac Electrophysiology and Systems Biology Center, National Cheng Kung University Medical Center, Tainan, Taiwan.

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