Rapid report

Activation of human IK and SK Ca²⁺-activated K⁺ channels by NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime)

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Abstract

We have identified and characterized the compound NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) as a potent activator of human Ca²⁺-activated K⁺ channels of SK and IK types, whereas it is devoid of effect on BK type channels. IK- and SK-channels have previously been reported to be activated by the benzimidazolinone, 1-EBIO and more potently by its dichloronated-analogue, DC-EBIO. NS309 is at least 1000 times more potent than 1-EBIO and at least 30 times more potent than DC-EBIO when the compounds are compared on the same cell.
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1. Results and discussion

Many physiological processes are regulated by the free concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and calcium-activated K⁺ channels (K_{Ca}) transmit changes in $[Ca^{2+}]_i$ into changes in membrane potentials or cell excitability. K_{Ca} channels of small (SK1–3) and intermediate (IK) conductance are activated by $[Ca^{2+}]_i$ via constitutively bound calmodulin [1,2], which acts as an accessory subunit that is also essential for cell surface expression of SK channels [3]. Open K⁺ channels draw the membrane potential towards the equilibrium potential for K⁺, which in neurons, certain gland and muscle cells will serve as a brake on excitability. In non-excitable cells, such as endothelial, epithelial and blood cells, the K⁺ channels are responsible for a hyperpolarization that increases the driving force for influx of Ca^{2+} via voltage-independent Ca^{2+} channels and thereby prolongs and strengthens the activating Ca^{2+} signal. SK channels are predominantly expressed in excitable cells, whereas IK channels mostly are found in non-excitable cells [4,5]. This implies that the physiological roles of SK and IK channels may be quite different despite the similarity in mode of activation and function. Some positive gating modifiers of SK and IK channels have been identified: The benzimidazolinone, 1-EBIO, was found to stimulate secretion across colon epithelium via activation of basolateral Ca²⁺-activated K⁺ channels with intermediate conductance [6] and later to activate both the cloned hIK channel [5] as well as cloned rSK2 and hSK3 channels [7,8]. The close analogue DC-EBIO [9] and other small heterocyclic compounds, such as the drugs riluzole [10,11], chlorzoxazone [7,11] and theophylline [12] also act as positive modulators. These compounds exhibit relatively low potency (range 10^{-6}–10^{-3} M) and are thus poor pharmacological tools. Here we present a new compound, 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309), which is a selective and potent activator of IK/SK-channels.

All experiments were performed at room temperature applying the whole-cell version of the patch clamp

technique. For each experiment, a cover slip (Ø 3.5 mm) containing the cells was positioned in a perfusion chamber (volume ≈ 15 μl) and superfused at 1 ml/min with an extracellular solution, as detailed in the legend of Fig. 1. The electrodes were allowed to stabilize 1–2 min before equalization and giga-sealing and after break-through to the whole-cell configuration the pipette/cytoplasm were allowed to equilibrate for 5–8 min in order to attain a stable (Ca^{2+}-dependent) baseline current before addition of compounds. The series resistance (R_S, 80% compensation) and cell capacitance were monitored before each stimulus and only experiments with stable read-outs and initial R_S values below 5 MΩ were used.

The structure of NS309 as well as Ca^{2+}-dependent IV-ramps for human IK and SK1-3 recorded in the presence and absence of the compound is shown in Fig. 1. In accordance with the high expression level in the transfected cell lines and the high K^+/Na^+-selectivity of these channels, reversal potentials close to the K^+-equilibrium potentials were consistently recorded. Non-linear IV-characteristics were obtained for all channel subtypes: Around E_K the conductance was lower for inward than for the corresponding outward currents. At positive membrane potentials, the SK channels (especially hSK2 and hSK3) exhibited a pronounced conductance decrease. Application of NS309 at a concentration of 30 nM augmented the SK-currents to the same extend (a factor

of 1.5–2) as 10 nM did on the IK currents, without any influence on ion selectivity or IV characteristics. The concentration differences used in these experiments reflect that the hSK channels are two to four times less sensitive to NS309 than hIK. In experiments with buffered Ca^{2+}-free pipette solutions (10 mM EGTA with no added Ca^{2+}), NS309 was not able to activate the IK/SK channels (results not shown) as previously demonstrated for 1-EBIO [13]. NS309 also activated the rat SK2 and SK3 subtypes with similar potencies as the human orthologs (data not shown).

The time course of an experiment on hIK channels is shown in Fig 2A. After 5 min of equilibration, the intracellular Ca^{2+} concentration stabilized at the new level (influenced by the buffered 300 nM pipette concentration) and NS309 at a concentration of 3 nM was applied. A higher current level was reached within approximately 1 min and upon wash the current returned to the baseline with approximately the same time characteristic. Application of NS309 at higher concentrations clearly demonstrated the concentration-dependency as well as the reversible nature of this compound on hIK channels. Fig. 2B shows the concentration–response relation for NS309 compiled from several experiments with hIK expressing cells. An EC_{50} value of 10 nM was inferred from these experiments. We have previously determined an EC_{50} value for 1-EBIO of 74 μM [5] using the same hIK cell line.
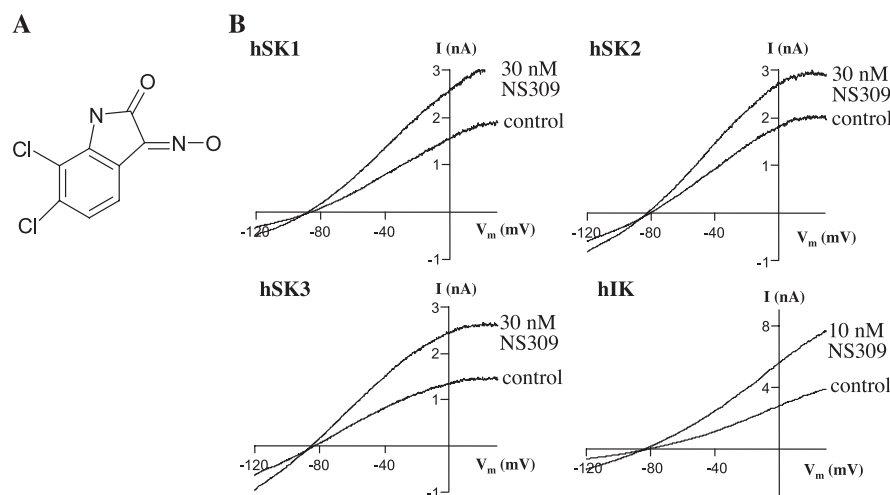

Fig. 1. (A) Structure of NS309. Implicit hydrogens are not shown. The compound was synthesized according to Ref. [17] (shown as compound 1g in the patent). All starting materials were from Aldrich. (B) The effect of NS309 on whole cell currents obtained by voltage ramps applied to HEK293 cells expressing hSK1, hSK2, hSK3 and hIK, respectively. The holding potential was -90 mV and voltage ramps going from -120 to $+30$ mV during 200 ms were applied every 5 s. Each panel shows the control current as well as the NS309-modulated current from a representative experiment on the indicated subtype. The extracellular solution had the following composition (mM): 140 NaCl, 4 KCl, 0.1 $CaCl_2$, 3 $MgCl_2$ and 10 HEPES (pH=7.4). The patch pipette contained (mM): 144 KCl, 10 EGTA, 7.625 $CaCl_2$, 1.205 $MgCl_2$ and 10 mM HEPES (pH=7.2). The free Ca^{2+} and Mg^{2+} concentration was calculated as 300 nM and 1 mM, respectively. All salts and buffer substances were of analytical grade or higher and obtained from commercial dealers. The generation of cell lines stably expressing hIK and hSK1 has been described previously [5,14]. For the present study, pcDNA3_hSK2 and pNS3n_hSK3 were stably transfected into HEK293 cells by lipofectamine (Invitrogen) using standard-methods. pNS3n is a customized vector derived from pcDNA3, which contains an internal ribosomal entry site element and a neomycin resistance open reading frame downstream of the polylinker. hSK3 was cloned from total human skeletal muscle RNA (Stratagene) using RobusT RT-PCR kit (Finnzymes) with -20S (5′-GAATACAGCCAGGCCCCAAG) and 2228AS primer (5′-GGAGTGGGGAGATTTAT). After the initial PCR, the coding region of hSK3 was amplified with 1S primer containing a Kozak sequence (5′-GCCGCCACCATGGACACTTCTGGG-CACTTCCATG) and 2211 as (5′-TTAGCAACTGCTTGAACTTGTGTAC) using Expand High Fidelity (Roche). 5% DMSO was included to the reaction.
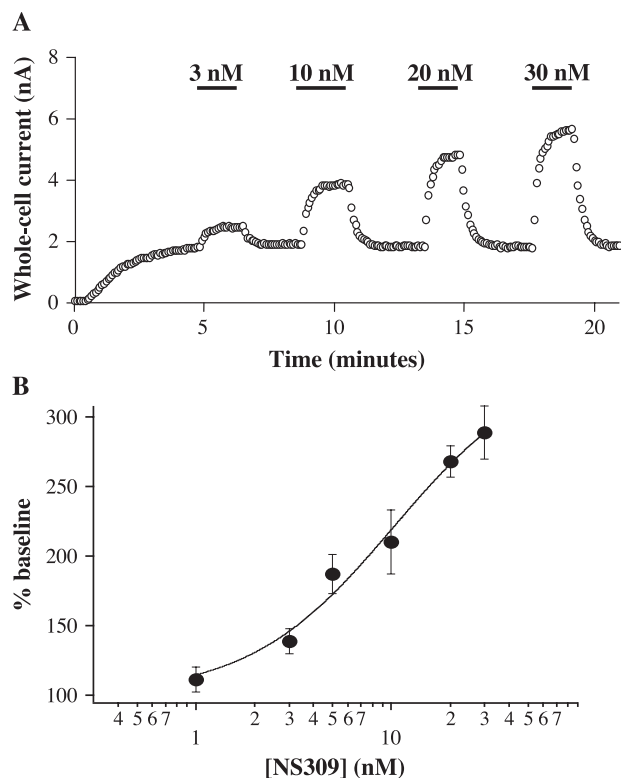
Fig. 2. (A) Concentration- and time-dependency of NS309-induced increase in hIK current. The current was measured at 0 mV from the voltage ramps described in Fig. 1 and plotted as a function of time (5 s between each data point). NS309 (3, 10, 20, and 30 nM) was present in the bath solution during the periods indicated by the solid bars. (B) Concentration–response relation for NS309 on hIK. 100% denotes the baseline current level at 300 nM $[Ca^{2+}]_i$. The points represent means±S.E. (four to nine individual experiments). The line represents the best fit to a standard Hill equation with the following parameters: EC_{50}=10 nM, n=1.18, I_{max}=342%.

Fig. 3 shows a head-to-head comparison of NS309, 1-EBIO, and DC-EBIO on hIK channels. After the initial equilibration period, the compounds were added sequentially at concentrations (10 nM, 10 μM, and 300 nM, respectively) selected to give approximately identical responses. Defining the current shortly before the individual applications as 100%, the current was increased to 213% by NS309, to 294% by 1-EBIO, to 240% by DC-EBIO and finally to 208% by a second addition of NS309.

Despite the large difference in the concentrations applied it is evident that all the three compounds act fast (steady-state within 30 s) and are fully reversible upon washout of the compounds.

Specific blockers such as clotrimazole (hIK) and apamin (hSK) were tested on the NS309-modulated channels as shown with hSK1 and hIK in Fig. 4. After the initial equilibration with the pipette solution, the current was further increased by application of NS309 (30 nM on hSK1 and 10 nM on hIK). After a current plateau was reached increasing concentrations of apamin (hSK1) and clotrimazole (hIK) was co-administered with NS309. Both blockers were able to block the modulated currents completely with

potencies consistent with the K_i values previously published for the unmodulated channels (K_i=2.5 nM for apamin on hSK1 [14]; K_i=125 nM for clotrimazole on hIK [5]). Also the characteristic washout kinetics of the blockers were preserved (i.e. extremely slow for clotrimazole and fast for apamin). Thus, potency and kinetics of inhibition were not notably influenced by NS309, indicating that the binding site for activation is functionally (and most likely also physically) separated from the blocker sites.

In contrast to the potent modulation of hSK/hIK channels demonstrated here, the human BK channel (for cell line information, see Ref. [15]) was insensitive to NS309 at concentrations up to 10 μM as were human KCNQ4 channels (for cell line information, see Ref. [16]), TTX-sensitive Na^+ channels, high-threshold voltage-dependent Ca^{2+} channels and delayed rectifier K^+ channels from primary cultures of chick DRG cells. However, hERG channels expressed in HEK293 cells, were blocked by NS309 with a K_i value of 1.3 μM. Furthermore, in a screening program for activity on 60 different receptor binding and enzyme assays, NS309 (10 μM) only showed up in a binding assay for adenosine A_{2A}, an effect which could not be reproduced with a functional assay (concentrations up to 30 μM) (results from selectivity screening not shown).

In conclusion, a new positive modulator of hSK and hIK channels has been presented, which is orders of magnitudes more potent than the standard reference compound 1-EBIO. Except for the potency difference, NS309 shares many qualitative properties with 1-EBIO, such as slight selectivity for IK over SK, no effect on BK channels, and an absolute requirement for a minimum concentration of intracellular Ca^{2+}. NS309 is probably a good alternative to 1-EBIO as a pharmacological tool in many situations, where the functional impact of increasing the IK/SK activity is studied. At this point, it is not known if NS309 exactly parallels the documented effects of 1-EBIO in complex tissue prepara-
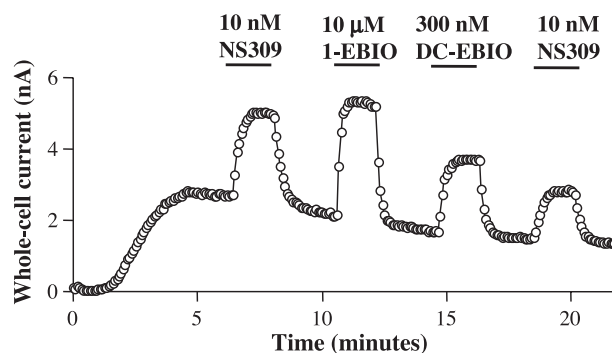

Fig. 3. Concentration- and time-dependency of NS309 (10 nM), 1-EBIO (10 μM), and DC-EBIO (300 nM) for activation of hIK channels. Experimental conditions as outlined in Figs. 1 and 2. 1-EBIO was purchased from Sigma. DC-EBIO (5,6-dichloro-1-ethyl-2-benzimidazolinone) was synthesized at NeuroSearch A/S.
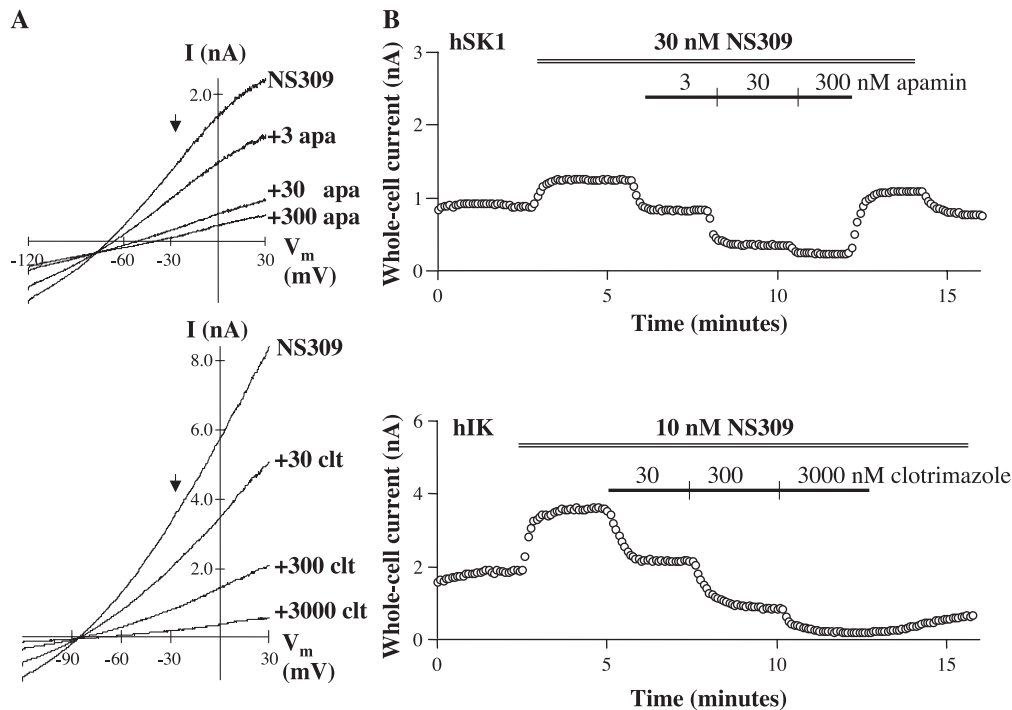
Fig. 4. Block of NS309 modulated hIK and hSK channels. (A) Upper panel: NS309-modulated (30 nM NS309) hSK1 channels co-applied with 3, 30, and 300 nM apamin (apa). Lower panel: NS309-modulated (10 nM NS309) hIK channels co-applied with 30, 300, and 3000 nM clotrimazole (clt). (B) Concentration- and time-dependency of apamin and clotrimazole inhibition of NS309-modified hSK1 (upper panel) and hIK (lower panel) channels, respectively. A K_i value of 4.4 ± 1.4 nM ($n=3$) was obtained for apamin on hSK1, and clotrimazole inhibited hIK with a K_i value of 43 ± 17 nM ($n=3$). Apamin and clotrimazole were purchased from Sigma.

tions, such as epithelial secretion (where 1-EBIO affects luminal Cl^- conductance as well) and neuronal m- and sAHP (where 1-EBIO augments the apamin-sensitive component). Furthermore, due to its high potency, NS309 may well be a valuable ligand for the future definition of the physical binding site for positive modulators of IK/SK channels.

Acknowledgement

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