

Negative Gating Modulation by (*R*)-*N*-(Benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine (NS8593) Depends on Residues in the Inner Pore Vestibule: Pharmacological Evidence of Deep-Pore Gating of K_{Ca2} Channels[§]

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ABSTRACT

Acting as a negative gating modulator, (*R*)-*N*-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine (NS8593) shifts the apparent Ca^{2+} -dependence of the small-conductance Ca^{2+} -activated K^+ channels $K_{Ca2.1-2.3}$ to higher Ca^{2+} concentrations. Similar to the positive K_{Ca} channel-gating modulators 1-ethyl-2-benzimidazolinone (1-EBIO) and cyclohexyl-[2-(3,5-dimethylpyrazol-1-yl)-6-methylpyrimidin-4-yl]-amine (CyPPA), the binding site for NS8593 has been assumed to be located in the C-terminal region, in which these channels interact with their Ca^{2+} sensor calmodulin. However, by using a progressive chimeric approach, we were able to localize the site-of-action of NS8593 to the K_{Ca2} pore. For example, when we transferred the C terminus from the NS8593-insensitive intermediate-conductance $K_{Ca3.1}$ channel to $K_{Ca2.3}$, the chimeric channel remained as sensitive to NS8593 as wild-type $K_{Ca2.3}$. In contrast, when we transferred the $K_{Ca2.3}$ pore to $K_{Ca3.1}$, the channel

became sensitive to NS8593. Using site-directed mutagenesis, we subsequently identified two specific residues in the inner vestibule of $K_{Ca2.3}$ (Ser507 and Ala532) that determined the effect of NS8593. Mutation of these residues to the corresponding residues in $K_{Ca3.1}$ (Thr250 and Val275) made $K_{Ca2.3}$ insensitive to NS8593, whereas introduction of serine and alanine into $K_{Ca3.1}$ was sufficient to render this channel highly sensitive to NS8593. It is noteworthy that the same two residue positions have been found previously to mediate sensitivity of $K_{Ca3.1}$ to clotrimazole and 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34). The location of Ser507 in the pore-loop near the selectivity filter and Ala532 in an adjacent position in S6 are within the region predicted to contain the K_{Ca2} channel gate. Hence, we propose that NS8593-mediated gating modulation occurs via interaction with gating structures at a position deep within the inner pore vestibule.

Introduction

Within the superfamily of Ca^{2+} -activated K^+ channels (K_{Ca}) the group of small ($K_{Ca2.1-2.3}$) and intermediate-con-

ductance ($K_{Ca3.1}$) channels are closely related in both structure and function. In contrast to the big conductance $K_{Ca1.1}$ channel, which is activated by both voltage and Ca^{2+} , K_{Ca2} and $K_{Ca3.1}$ channels are inward-rectifying, voltage-independent, and activated solely by intracellular Ca^{2+} (Stocker, 2004; Wulff et al., 2007). The opening of both K_{Ca2} and $K_{Ca3.1}$ channels is initiated via Ca^{2+} -binding to the N-lopes of calmodulin (CaM) constitutively attached to a calmodulin binding domain (CaMBD) located in the proximal intracellular C terminus (Xia et al., 1998; Fanger et al., 1999). The energy of the ensuing conformational change is transferred

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ABBREVIATIONS: K_{Ca} , Ca^{2+} -activated K^+ channel; CaM, calmodulin; BMB, bicuculline methobromid; CaMBD, calmodulin binding domain; 1-EBIO, 1-ethyl-2-benzimidazolinone; GW542573X, 4-(2-methoxy-phenylcarbamoyloxymethyl)-piperidine-1-carboxylic acid *tert*-butyl ester; TM, transmembrane; HEK, human embryonic kidney; $K_{Ca1.1}$, big conductance Ca^{2+} -activated K^+ channel; K_{Ca2} , small conductance Ca^{2+} -activated K^+ channel; $K_{Ca3.1}$, intermediate conductance Ca^{2+} -activated K^+ channel; NS309, 6,7-dichloro-1*H*-indole-2,3-dione 3-oxime; NS8593, (*R*)-*N*-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; WT, wild type; SKA-31, naphtho[1,2-*d*]thiazol-2-ylamine; UCL1684, 6,10-diaza-3(1,3)8(1,4)-dibenzena-1,5(1,4)-diquinolinacyclodecaphane; NS11757, (1*H*-benzimidazol-2-yl)-(6,7-dichloro-1,2,3,4-tetrahydronaphthalen-1-yl)amine; ICA-27243, *N*-(6-chloro-pyridin-3-yl)-3,4-difluoro-benzamide.

to the transmembrane (TM) regions to open the gate. Unlike K_v channels, which are gated by a rotational constriction of the intracellular aperture formed by the lower part of the four S6 TM helices, the physical gates of $K_{Ca}2$ and $K_{Ca}3.1$ channels seem to be deeply buried in the inner pore vestibule, close to or even overlapping with the K^+ selectivity filter (Bruening-Wright et al., 2002, 2007; Klein et al., 2007; Garneau et al., 2009).

Despite their structural and functional similarity, $K_{Ca}2$ and $K_{Ca}3.1$ channels have a very different pharmacology, the details of which are increasingly becoming better defined at the molecular level. Selective peptide inhibitors of $K_{Ca}2$ channels, such as apamin or scyllatoxin, and $K_{Ca}3.1$ channel-inhibiting peptides, such as charybdotoxin and maurotoxin, interact with extracellularly exposed amino acids in the outer pore vestibule (Ishii et al., 1997; Rauer et al., 2000; Castle et al., 2003) and are usually conceived to inhibit via a simple blocking mechanism (Lamy et al., 2010 describes an emergent different view on the apamin mode-of-action). The same applies to the positively charged small-molecule $K_{Ca}2$ -selective blockers such as 6,10-diaza-3(1,3)8(1,4)-dibenzena-1,5(1,4)-diquinolincyclodecaphane (UCL1684) (Campos Rosa et al., 2000), which were designed to mimic the charged part of the apamin molecule, as well as to bicuculline methiodide (Johnson and Seutin, 1997), a lower-affinity blocker.

In contrast, the inhibition by the established small-molecule blockers of $K_{Ca}3.1$, the triarylmethanes, exemplified by clotrimazole and the more selective 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34) (Wulff et al., 2001), is mediated via two amino acids located in the lower part of the pore loop (Thr250) and in the S6 segment (Val275), respectively (Wulff et al., 2001). These amino acids form part of a ring of hydrophobic residues, which line and thus isolate the upper part of the watery inner pore vestibule. Their position close to the selectivity filter suggested a model in which TRAM-34 coordinates via its aryl groups to Thr250 and Val275 and interacts with the lower part of the selectivity filter via its pyrazole moiety, thereby blocking the $K_{Ca}3.1$ pore from the inside.

A new principle for selective $K_{Ca}2$ channel inhibition by small molecules has been described. Certain 2-(*N*-substituted)-aminobenzimidazoles, such as (*R*)-*N*-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine (NS8593) (Fig. 1) and (1*H*-benzimidazol-2-yl)-(6,7-dichloro-1,2,3,4-tetrahydronaphthalen-1-yl)amine (NS11757), inhibit all three $K_{Ca}2$ channels via negative gating modulation rather than via a simple pore-blocking mechanism (Strøbaek et al., 2006; Sørensen et al., 2008). The hallmark of this mode-of-action is a right-shifted and less steep $K_{Ca}2$ channel Ca^{2+} -response curve, leading to a strong Ca^{2+} -dependence of inhibition. The physical binding site for the negative gating modulators is clearly distinct from the apamin binding site, because these com-

pounds do not displace radiolabeled apamin. Furthermore, $K_{Ca}2$ channels that have been point-mutated to apamin insensitivity are still sensitive to NS8593 (Sørensen et al., 2008). Physiological gating modulation of $K_{Ca}2$ channels occurs via casein kinase 2-mediated threonine phosphorylation of CaM (Thr80) attached to the $K_{Ca}2$ channel (Bildl et al., 2004; Allen et al., 2007). This phosphorylation reduces the apparent Ca^{2+} affinity and the resulting right-shift and reduced steepness of the $K_{Ca}2$ channel Ca^{2+} response curve is essentially identical with the effect of NS8593. Hence, we originally hypothesized that negative gating modulation by NS8593 is a phosphorylation-independent, direct downstream effect on the physiological gating process. Furthermore, because the C-terminal CaM/CaMBD is the site-of-action for the classic positive $K_{Ca}2$ channel gating modulator 1-EBIO (Pedarzani et al., 2001), we assumed, because of the structural similarity between NS8593 and the positive gating modulators 1-EBIO and 6,7-dichloro-1*H*-indole-2,3-dione-3-oxime (NS309) (Fig. 1), that negative modulation by NS8593 was also mediated via interaction with the CaM/CaMBD. All three compounds contain a benzimidazole or related indole-dione scaffold, and it seemed reasonable to hypothesize that they might be binding to the same site with the larger and more lipophilic NS8593 reducing gating. In the present article, we adopted a $K_{Ca}2/K_{Ca}3.1$ channel chimeric/mutational approach to delineate the amino acids important for negative pharmacological $K_{Ca}2$ channel modulation. Surprisingly, NS8593 sensitivity was found to reside at residues Ser507 and Ala532 in $K_{Ca}2.3$, which are in equivalent positions to Thr250 and Val275 in $K_{Ca}3.1$, the amino acids conferring TRAM-34 sensitivity. We therefore conclude that NS8593 inhibition involves deep-pore amino acids and suggest that the compound's negative gating modulation may result from a direct interaction with the physical gate of $K_{Ca}2$ channels.

Materials and Methods

Molecular Biology. HEK293 cell lines stably expressing WT $hK_{Ca}2.3$ and $hK_{Ca}3.1$ channels were described previously (Hougaard et al., 2009). The N-terminal $K_{Ca}3.1$ - $K_{Ca}2.3$ chimera was generated with overlapping polymerase chain reaction (Expand High Fidelity PCR System; Roche Diagnostics, Mannheim, Germany) using the oligonucleotides hIK-hSK3s GTCTCTGGCCGGCTGGGCACTgatttttgggatgttt and hSK3-hIKas aaacatcccaaaaatcagTGCCAGCCGGCCAGAGAC, a T7 primer, and an antisense primer in the 3'-polylinker end. The C-terminal $K_{Ca}2.3$ - $K_{Ca}3.1$ chimera was generated by ligation of the following DNA fragment purchased from GenScript (Piscataway, NJ): ACCGGTATCATGGGTGCAGGCTGCACTGCCCTGTGGTGGCCGTGGTGGCCCGAAAGCTGGAGTTTAAACAAGGCAGAGAAGCACGTGCACTTTCATGATGGATATCCAGTATACCAAAGAGATGAAGGAGTCCGCTGCCCGAGTGCTACAAGAAGCCCTGGATGTTCTACAAACATACTCGCAGGAAGGAGTCTCATGCTGCCCGCAGGCATCAGCGCAAGCTGCTGGCCGCATCAACCGCTCCGCCAGGTGGCCGCTGAAACACCGGAAGCTCCGGGAACAAGTGAACCTCCATGGTGGACATCTCCAAGATGCACATGATCCTGTATGACCTGCAGCAGAATCTGAGCAGCTCACACCGGGCCCTGGAGAAACAGATTGACACGCTGGCCGGGGAAGCTGGATGCCCTGACTGAGCTGCTTAGCACTGCCCTGGGGCCGAGGCAGCTTCCAGAACCAGCCAGCAGTCCAAGTAGCTGGAGTGGCGGCCG into $hK_{Ca}2.3$ with an AgeI site as described in Hougaard et al., (2009). The $K_{Ca}3.1$ mutants T250S and V275A and the $K_{Ca}2.3$ mutants S507T and S507T + A532V were described previously (Wulff et al., 2001). Additional point mutations were generated using WT $K_{Ca}2.3$ and $K_{Ca}3.1$ plasmids uracilated via *Escherichia coli* RZ1032 (Stratagene, La Jolla, CA)

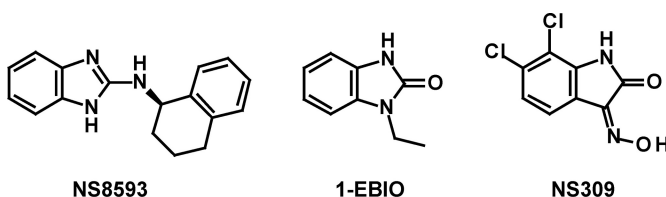


Fig. 1. Chemical structures of the negative $K_{Ca}2$ channel modulator NS8593 and the positive modulators 1-EBIO and NS309.

as templates in mutagenesis reactions. Four oligonucleotides (MWG-Biotech AG, Ebersberg, Germany) were used to introduce the mutations: hK_{Ca}2.3 S507T, CTCCATCACATTTCCTTaCaATTGGTTATGGGGACA; hK_{Ca}2.3 A532V, TCACTGGCATCATGGGTGtAGGCTGtAcAGCCCTTGTGGTGGCCG; hK_{Ca}3.1 T250S, GATCCCCATCACATTCTGTtCaATtGGCTATGGTGACGTG; and hK_{Ca}3.1 V275A, GCACTGGAGTCATGGGTGcaTGCTGCACAGCCCTGCT. The mutagenesis reactions were performed using T7 DNA polymerase and T4 DNA ligase (New England Biolabs, Ipswich, MA). *E. coli* XL1-Blue (Stratagene) was transformed with an aliquot of the reaction, and the resulting plasmid DNA was purified using standard methods. All constructs were verified by sequencing.

Electrophysiology. All experiments were performed on transiently transfected HEK 293 cells in either the inside-out or the whole-cell configuration of the patch-clamp technique. Lipofectamine and standard transfection methods were used, and recordings were conducted 2 days after transfection. Cells for whole-cell experiments were detached by trypsinization and plated on coverslips (3.5 mm Ø) on the day of the experiments, whereas cells for inside-out recordings were plated 1 day before the experiments to attach them more firmly. For recordings, a coverslip was placed in a 15- μ l recording chamber mounted on the cross-board of an inverted microscope (Olympus XI-70 equipped with fluorescence burner and filters; Olympus, Tokyo, Japan), and cell selection was guided by fluorescence from the cotransfected green fluorescent protein. The extracellular solutions contained 154 mM KCl, 10 mM HEPES, pH 7.4, 2 or 0.1 mM CaCl₂, and 1 or 3 mM MgCl₂ for inside-out/whole-cell experiments, respectively. Solutions on the intracellular side contained 154 mM KCl, 10 mM HEPES, pH 7.2, 10 mM EGTA or 1 mM EGTA plus 9 mM nitriloacetic acid, CaCl₂, and MgCl₂ to yield a calculated free Mg²⁺ concentration of 1 mM and calculated free Ca²⁺ concentrations of 0.01, 0.2, 0.3, 0.4, 0.5, 3, 10, and 30 μ M. Solutions used for experiments with ATP were made with Na²⁺-ATP (Sigma-Aldrich, St. Louis, MO) and adjusted to yield 30 μ M free Ca²⁺ and 1.6 mM Mg²⁺-ATP. Cells or membrane patches were perfused at 1 ml/min by gravity from a 10-position solution exchanger. Patch pipettes were pulled from borosilicate (Vitrex Medical A/S, Herlev, Denmark) or soda lime glass (micro-hematocrit tubes; Kimble Chase, Rochester, NY) and had resistances of 2 to 3 M Ω when submerged in the bath solution. Positioning of the patch electrode was controlled with a Patchman micromanipulator (Eppendorf/North America, New York, NY). Any initial voltage difference between the patch electrode and the integrated and grounded bath electrode was eliminated before the pipette was attached to the cell. Experiments were controlled with a HEKA EPC-9 or EPC-10 amplifier and Pulse software (HEKA, Lambrecht/Pfalz, Germany). Cells were clamped to a holding potential of 0 mV, and K_{Ca} currents were elicited by 200-ms voltage ramps from -80 to +80 mV applied every 5 s. Data analysis, fitting, and plotting were performed with IGOR-Pro (Wavemetrics, Lake Oswego, OR) or Origin 7 (OriginLab Corporation, Northampton, MA).

Chemicals and Reagents. NS8593 and NS309 were synthesized at NeuroSearch A/S as described previously (Strøbaek et al., 2004, 2006). Bicuculline methobromide (BMB) and apamin were from Sigma-Aldrich, and charybdotoxin was from Bachem Biosciences (King of Prussia, PA). TRAM-34 was synthesized in the Wulff laboratory as described previously (Wulff et al., 2001). Standard laboratory chemicals were purchased from commercial dealers and were of the purest grade available.

Quantification and Statistics. IC₅₀ values were calculated as described previously (Strøbaek et al., 2006). Summary values are given as means \pm S.D.

Results

The Inhibition of K_{Ca}2.3 by NS8593 Is Not Dependent on N- and C-Terminal Regions. In whole-cell experiments, NS8593 inhibits K_{Ca}2.3 channels with a potency of 90 nM at a pipette [Ca²⁺] of 400 nM, whereas the related K_{Ca}3.1

channel is insensitive to this negative gating modifier (Strøbaek et al., 2006). To delineate K_{Ca}2 channel regions important for the rightward shift in the Ca²⁺ response curve induced by NS8593, we constructed a number of chimeras between the hK_{Ca}2.3 channel and the hK_{Ca}3.1 channel. The responses of these chimeras to NS8593, the K_{Ca}2 channel blocker BMB and NS309, a positive modulator of K_{Ca}2 and K_{Ca}3.1 channels, were tested in whole-cell patch-clamp experiments (Fig. 2 and Supplemental Table 1).

Figure 2, A and B, show representative recordings from wild-type K_{Ca}2.3 and K_{Ca}3.1 currents elicited by 200-ms voltage ramps from -80 to +80 mV applied every 5 s with 400 nM free Ca²⁺ in the patch pipette. As published previously for recordings with symmetrical K⁺ concentrations in this expression system (Strøbaek et al., 2006; Hougaard et al., 2007), K_{Ca}2.3 exhibited a much more pronounced inward rectification than K_{Ca}3.1, which showed a nearly linear IV relationship. Both channels exhibited their characteristic pharmacology. K_{Ca}2.3 was potently inhibited by NS8593 with an IC₅₀ of 104 nM (Tables 1 and 2) and almost fully blocked by 100 μ M BMB (IC₅₀ 5 μ M), which we routinely use to estimate leak when working with symmetrical K⁺ solutions. K_{Ca}3.1, in contrast, was completely insensitive to 10 μ M NS8593 and 100 μ M BMB but could be blocked completely by TRAM-34 (Tables 1 and 2). NS309 increased both K_{Ca}2.3 and K_{Ca}3.1 currents but was distinctly more effective on K_{Ca}3.1 in keeping with previous reports (Strøbaek et al., 2004).

We next tested the effect of NS8593 on K_{Ca}2.3 chimeras that contained either the N- or the C-terminal regions of K_{Ca}3.1. Because both chimeras contained the pore region of K_{Ca}2.3, they exhibited the characteristic inward rectification of K_{Ca}2.3. Surprisingly, NS8593 inhibited both chimeras as potently as the WT K_{Ca}2.3 channel (Fig. 2, C and D), suggesting that the NS8593-induced negative gating modulation is not mediated via the N- or the C-terminal regions. Exchanging the C terminus between K_{Ca}2.3 and K_{Ca}3.1 also transferred the higher NS309 sensitivity of K_{Ca}3.1 to the resulting chimeric channel (Fig. 2C, right). This observation is reminiscent of the increase in 1-EBIO sensitivity that was reported for a K_{Ca}2.2 chimeric channel containing the C terminus of K_{Ca}3.1 (Pedarzani et al., 2001) and suggests that the binding site of the positive gating modulator NS309 is also located in this region.

Amino Acids in the Pore Region Confer Sensitivity to NS8593. After having obtained the above results, we next focused our attention on the TM regions. Unfortunately, some K_{Ca}2.3/K_{Ca}3.1 chimeras in which various parts of TM5 and/or TM6 were swapped between the two channels did not seem to form functional channels or were expressed too poorly to allow for evaluation of modulator sensitivity (Supplemental Table 1). However, an important chimera that contained the transmembrane regions S1 to S4 from K_{Ca}3.1 and the pore and C terminus of K_{Ca}2.3 (Fig. 2E) expressed sufficiently well for pharmacological experiments. It is noteworthy that this chimera was as sensitive to NS8593 as the wild-type K_{Ca}2.3 channel (Fig. 2E, left) and was further found to be insensitive to the K_{Ca}2.3 pore blocker BMB. Both observations were surprising, the first suggesting that NS8593 might be exerting its effect by interacting with residues in the pore. As a consequence, we decided to follow-up this observation with point mutations in the inner pore re-

gion (see below). The latter observation could indicate that the presence of the S1 to S4 region from $K_{Ca}3.1$ in the chimera might have disturbed the architecture of the outer $K_{Ca}2.3$ vestibule, in which BMB, which exhibits voltage-

dependent block (Grunnet et al., 2001) and displaces apamin (Finlayson et al., 2001), presumably binds. In this context, it should be mentioned that apamin was very recently reported to bind to the outer pore of $K_{Ca}2$ channels (Lamy et al., 2010),

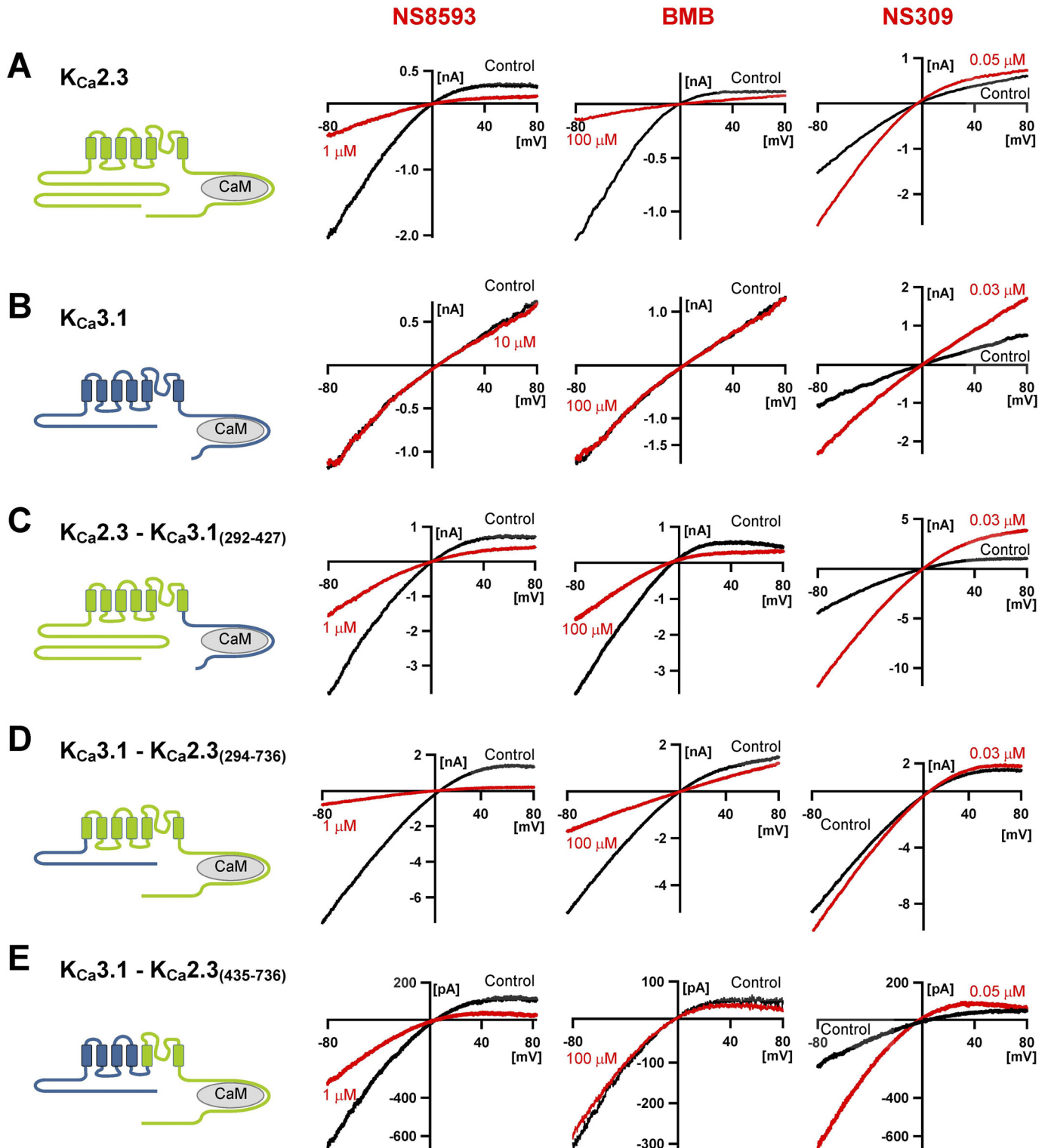


Fig. 2. The inhibitory effect of NS8593 is not dependent on N- and C-terminal regions of $K_{Ca}2.3$ but on pore regions. Effect of NS8593, BMB, and NS309 on whole-cell currents from HEK293 cells expressing WT $K_{Ca}2.3$ (A), WT $K_{Ca}3.1$ (B), and the chimeric constructs $K_{Ca}2.3$ - $K_{Ca}3.1(292-427)$ (C), $K_{Ca}3.1$ - $K_{Ca}2.3(287-736)$ (D), and $K_{Ca}3.1$ - $K_{Ca}2.3(435-736)$ (E). Voltage ramps were applied every 5 s from a holding potential of 0 mV. Control traces (last sweep before compound application) are shown in black, and compound traces (last sweep before compound washout) are shown in red. Washout traces have been omitted for clarity. Symmetric K^+ distributions with free $[Ca^{2+}]$ in the pipette solution buffered to 400 nM (see *Materials and Methods* for specifications).

where it apparently acts more as an allosteric modulator of the selectivity filter than as a direct blocker such as tetraethyl ammonium or the larger scorpion toxins tamapin and scyllatoxin, which are able to span the $K_{Ca}2$ channel pore (Weatherall et al., 2010).

Two Amino Acids in the Inner Pore Region Are Required and Sufficient for Inhibition by NS8593. Two amino acids in the inner pore vestibule were reported previously to confer TRAM-34 and clotrimazole sensitivity to $K_{Ca}3.1$ (Wulff et al., 2001). These amino acids, Thr250 and Val275 in the $K_{Ca}3.1$ numbering, are located on either side of the selectivity filter, and we therefore speculated whether the effect of NS8593 might also be dependent on these amino acids. As shown in Tables 1 and 2 and Fig. 3, mutations of each of the corresponding residues in $K_{Ca}2.3$ (Ser507 and Ala532) reduced the potency of NS8593 roughly 20-fold, whereas introduction of the double mutation produced a $K_{Ca}2.3$ channel that was completely insensitive to NS8593. The importance of Ser507 and Ala532 was verified by showing that the reverse $K_{Ca}3.1$ mutants (T250S and V275A) became sensitive to NS8593 and that the $K_{Ca}3.1$ double mutant was at least as sensitive to NS8593 as the wild-type $K_{Ca}2.3$ channel (Fig. 3 and Tables 1 and 2). As a control of whether the point mutations disturbed the overall architecture and gating function of the channels we determined the calcium sensitivity and the pharmacology of the two double mutants. In inside-out patches, the $K_{Ca}2.3_{S507T+A532V}$ and the $K_{Ca}3.1_{T250S+V275A}$ mutants exhibited Ca^{2+} response curves that were very similar to the WT channels with EC_{50} values of 480 to 590 nM and Hill coefficients of 3.8 to 4.7 (Fig. 4A). In whole-cell recordings, both double mutants also showed the characteristic pharmacology of the WT channels. Although the $K_{Ca}3.1_{T250S+V275A}$ mutant was sensitive to charybdotoxin (IC_{50} 17 ± 5.4 nM, $n = 4$) and insensitive to BMB and apamin, the $K_{Ca}2.3_{S507T+A532V}$ mutant was inhibited by apamin (IC_{50} 1.4 ± 0.2 nM, $n = 6$) and BMB (Fig. 4B

and Tables 1 and 2). These results suggest that the double point mutations did not significantly change the overall conformation of the K_{Ca} channels. However, as expected, the single and the double mutations changed TRAM-34 sensitivity (Tables 1 and 2), in keeping with our previous observations (Wulff et al., 2001).

Another interesting feature of the inner pore mutations was that introduction of the respective $K_{Ca}3.1$ residues into $K_{Ca}2.3$ significantly reduced this channel's strong inward rectification. This is illustrated by comparing the ratio of the current amplitude at -80 and $+80$ mV between the different constructs (Fig. 4C). Although both $K_{Ca}2.3$ single mutants and the double mutant showed a pronounced reduction in rectification, the reverse substitutions in $K_{Ca}3.1$ did not increase the generally weak rectification of this channel. Part of the inward-rectification of $K_{Ca}2$ channels has been shown previously to be the result of voltage-dependent block by intracellular divalent cations, such as Ca^{2+} and Mg^{2+} , at a site located below the selectivity filter involving Ser359 in rat $K_{Ca}2.2$ (Soh and Park, 2001, 2002), which corresponds to Ser507 in human $K_{Ca}2.3$. Mutation of this position to alanine or the larger threonine demonstrated that the hydroxyl group of this serine residue in $K_{Ca}2.2$ is critical for the binding of divalent cations to this site and thus for inward-rectification in their presence. Our results here confirm these observations in $K_{Ca}2.3$ and strongly underscore these amino acids as functionally important pore residues.

Gating Modulation of the $K_{Ca}3.1_{T250S+V275A}$ Mutant by NS8593 Is Much Less Ca^{2+} - and NS309-Dependent than Gating Modulation of the WT $K_{Ca}2.3$ Channel. Because the residues conferring NS8593 sensitivity to $K_{Ca}2.3$ and transferring it to $K_{Ca}3.1$ are located within in the region predicted to contain the gate of $K_{Ca}2$ and $K_{Ca}3.1$ channels, we wondered whether their mutations would also affect the mechanism of action of NS8593. To address this issue, we investigated two defining characteristics of negative gating

TABLE 1

Pharmacology of $K_{Ca}2.3$ and $K_{Ca}3.1$ chimeric channelsNumbers in a parentheses indicate the number of independent experiments. For information on the pharmacological properties of additional $K_{Ca}2.3/K_{Ca}3.1$ chimeras, see Supplemental Table 1.

Chimeras	NS8593 IC_{50}	Current Inhibition by 100 μ M BMB		Current Increase by 30 nM NS309
	μ M	%		fold
$K_{Ca}2.3$ WT	0.104 ± 0.034 (13)	83 ± 13 (10)		2.1 ± 0.5 (6)
$K_{Ca}3.1$ WT	>10 (11)	0 ± 0 (5)		5.1 ± 2.1 (6)
$K_{Ca}2.3$ - $K_{Ca}3.1_{(292-427)}$	0.117 ± 0.013 (3)	57 ± 24 (3)		2.9 ± 0.6 (3)
$K_{Ca}3.1$ - $K_{Ca}2.3_{(287-736)}$	0.115 ± 0.040 (6)	91 ± 3 (6)		1.1 ± 0.5 (6)
$K_{Ca}3.1$ - $K_{Ca}2.3_{(435-736)}$	0.947 ± 0.031 (4)	0 ± 0 (3)		2.3 ± 0.4 (3)

TABLE 2

Pharmacology of $K_{Ca}2.3$ and $K_{Ca}3.1$ point-mutated channelsNumbers in a parentheses indicate the number of independent experiments. For information on the pharmacological properties of additional $K_{Ca}2.3/K_{Ca}3.1$ chimeras, see Supplemental Table 1.

Mutants	NS8593 IC_{50}	Current Inhibition by 100 μ M BMB		TRAM-34 IC_{50}
	μ M	%		μ M
$K_{Ca}2.3$ WT	0.104 ± 0.034 (13)	83 ± 13 (10)		$>20^a$
$K_{Ca}2.3_{S507T}$	2.5 ± 0.78 (9)	42 ± 18 (5)		$>>1$ (3)
$K_{Ca}2.3_{A532V}$	3.1 ± 1.3 (4)	67 ± 12 (2)		2.7 (1)
$K_{Ca}2.3_{S507T+A532V}$	>10 (3)	65 ± 15 (3)		0.064 ± 0.068 (2)
$K_{Ca}3.1$ WT	>10 (11)	0 ± 0 (5)		0.004 ± 0.002 (4)
$K_{Ca}3.1_{T250S}$	0.503 ± 0.209 (11)	0 ± 0 (5)		22 ± 14 (2)
$K_{Ca}3.1_{V275A}$	3.5 ± 1.6 (14)	0 ± 0 (7)		21 ± 4.3 (3)
$K_{Ca}3.1_{T250S+V275A}$	0.056 ± 0.024 (3)	1 ± 1 (2)		$>20^a$

^a Wulff et al. (2001).

P-Loop

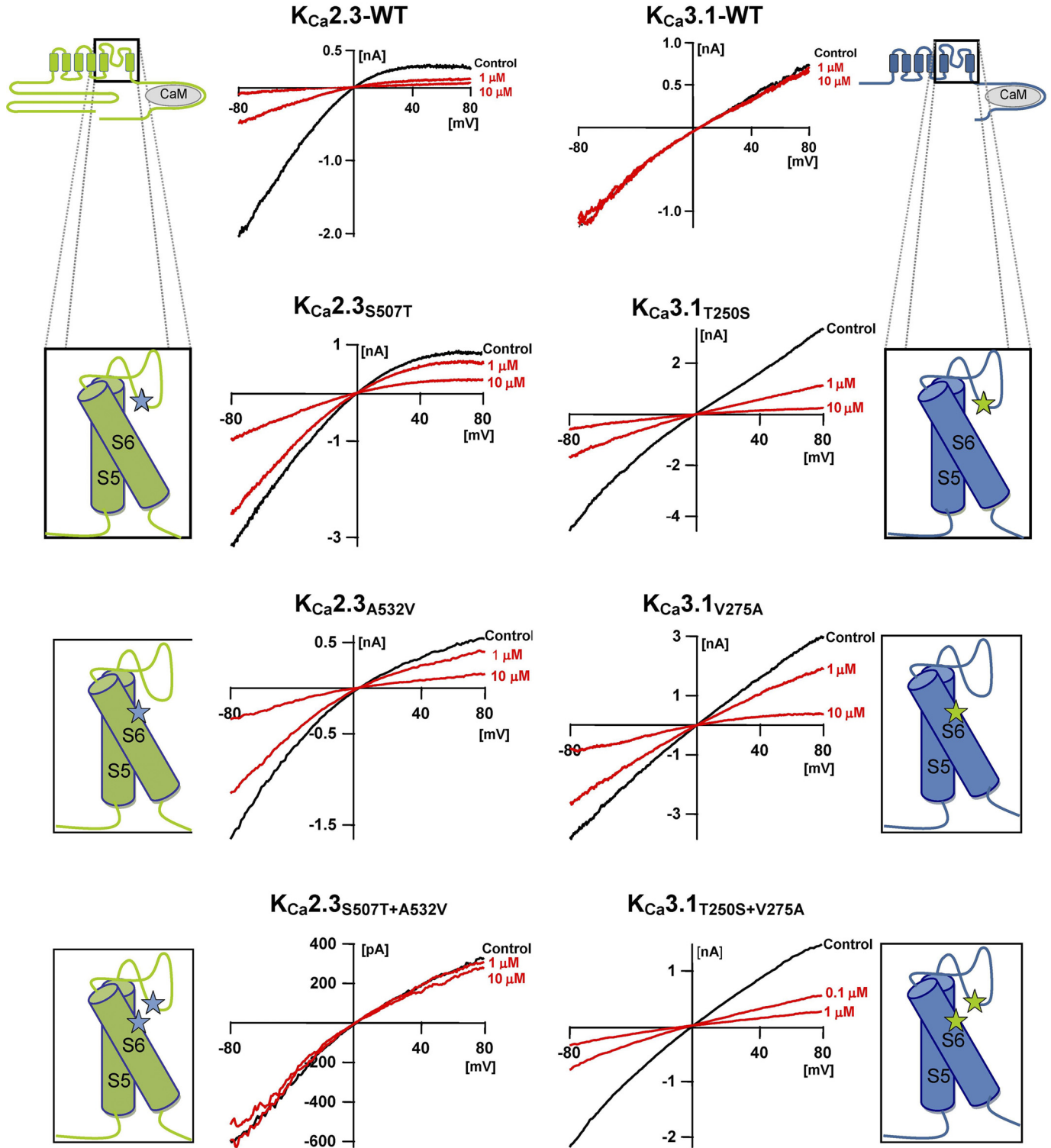
S6

K_{Ca}2.3

K_{Ca}3.1

VTSNFLGAMWLI SITFLS IGYGDMVPHTYCGKGVCLLTGIMGAGCTAL

ATGHLSDTLWLI PITFLT IGYGDVVPGMTWVGKIVCLCTGVMGVCCTAL



modulation: 1) dependence on $[Ca^{2+}]_i$; and 2) reversibility in presence of the positive modulator NS309.

As described previously (Strøbaek et al., 2006), current reduction by the negative gating modulator NS8593 is strongly dependent on the $[Ca^{2+}]_i$, with the compound's potency decreasing with increasing $[Ca^{2+}]_i$. As shown in Fig. 5A, perfusion of 1 μ M NS8593 onto $K_{Ca}2.3$ currents activated with 300 nM $[Ca^{2+}]_i$ in inside-out patches blocked $74 \pm 6\%$ ($n = 6$) of the calcium-dependent inward current at -80 mV. However, when the same patch was subsequently exposed to 30 μ M $[Ca^{2+}]_i$ to maximally activate $K_{Ca}2.3$, 1 μ M NS8593 only blocked $21 \pm 5\%$ ($n = 6$) of the current. After washout and another control for absence of contaminating leak current by a switch to 10 nM $[Ca^{2+}]_i$, $K_{Ca}2.3$ was then exposed to 0.5 μ M $[Ca^{2+}]_i$, and 1 μ M NS8593 was observed to exhibit an intermediate potency ($58 \pm 14\%$ blockade, $n = 5$). In contrast, inhibition of the $K_{Ca}3.1$ double mutant ($K_{Ca}3.1_{T250S+V275A}$) by NS8593 was much less calcium-dependent (Figs. 5B and 6). In a similar inside-out experiment, in which $[Ca^{2+}]_i$ varied from 0.3 to 30 μ M, NS8593 at 1 μ M inhibited $87 \pm 3\%$ ($n = 3$) at 0.3 μ M, $80 \pm 4\%$ ($n = 3$) at 0.5 μ M, and $70 \pm 6\%$ ($n = 5$) of the current at 30 μ M $[Ca^{2+}]_i$. However, the reduced calcium-dependence of the inhibitory effect of NS8593 on the $K_{Ca}3.1$ double mutant was not simply the result of the slightly higher affinity of NS8593 to the mutant channel because inhibition remained less calcium-sensitive even when the NS8593 concentration was reduced 10-fold from 1 μ M to 100 nM (Fig. 6).

The NS8593 inhibition of K_{Ca} channels at low $[Ca^{2+}]_i$ is essentially abolished upon the addition of the positive modulator NS309 (Strøbaek et al., 2006; Ji et al., 2009). Figure 7 compares the ability of NS309 to reverse the inhibitory effect of NS8593 on $K_{Ca}2.3$ (Fig. 7A) and the $K_{Ca}3.1_{T250S+V275A}$ mutant (Fig. 7B). In both cases, upon stabilization of the whole-cell current (black traces, left) 1 μ M NS8593 was superfused causing nearly 100% inhibition (red traces, middle, after 150 s). Increasing concentrations of NS309 were then cosuperfused with NS8593 leading to a concentration-dependent reversal and "overshoot" of the $K_{Ca}2.3$ current, whereas the $K_{Ca}3.1_{T250S+V275A}$ current remained largely inhibited even at an NS309 concentration of 1 μ M (green traces, middle, after 250 s). This series of experiments has been quantified (Fig. 7, right), in which the fold decrease/increase relative to the control current is plotted for the NS8593 (1 μ M) equilibrium inhibition and for the combined NS8593 + NS309 (1 μ M) effect. The overall conclusion from these experiments is that NS8593-mediated inhibition of the $K_{Ca}3.1_{T250S+V275A}$ construct is not reversible with a positive modulator and in this respect has lost its negative modulation character and rather resembles a blocker.

Finally, in a last attempt to demonstrate negative gating modulation characteristics on the $K_{Ca}3.1_{T250S+V275A}$ construct, we performed a series of inside-out experiments with combined high $[Ca^{2+}]_i$ (30 μ M) and Mg^{2+} -ATP (1.6 mM) or NS309 (1 μ M). The rationale for using Mg^{2+} -ATP quote back to the observation that the Ca^{2+} -dependent open channel probability $P_o(\max)$ value of $K_{Ca}3.1$ is significantly lower

than the corresponding value for $K_{Ca}2$ in some studies (Gerlach et al., 2001; Jones et al., 2007), and that this value can be increased significantly in the presence of Mg^{2+} -ATP. In our hands, Mg^{2+} -ATP and NS309 both induced slight increases (28%, $n = 2$, and 23%, $n = 3$, respectively), in the maximal current level of $K_{Ca}3.1_{T250S+V275A}$ (results not shown), and the ensuing addition of NS8593 (0.3 μ M) caused current reductions that were not significantly different from the effects without Mg^{2+} -ATP or NS309 ($53 \pm 8\%$, $n = 5$ versus $52 \pm 7\%$, $n = 3$, and $51 \pm 7\%$, $n = 4$, respectively). Likewise, the higher NS8593 concentration of 1 μ M blocked the $K_{Ca}3.1_{T250S+V275A}$ mutant channel by 70% in both the absence ($n = 5$) and the presence of 1.6 mM ATP ($n = 2$).

Taken together, these data suggest that although the $K_{Ca}3.1_{T250S+V275A}$ mutation perfectly transfers the interaction site for NS8593 in terms of potency, the characteristic coupling to the gate is less pronounced in this construct, as shown by diminished Ca^{2+} - and NS309-dependence of inhibition and by the preservation of its inhibitory potency in the presence of Mg^{2+} -ATP.

Discussion

In comparison with the classic $K_{Ca}2$ channel blocker apamin, NS8593 acts by a different mechanism and via a different site because $K_{Ca}2.3$ channels mutated in the apamin binding site remain sensitive to its actions (Sørensen et al., 2008). NS8593 produces a rightward-shift in the $K_{Ca}2$ channel Ca^{2+} activation curve and has therefore been termed a negative gating modulator rather than a pore blocker (Strøbaek et al., 2006; Sørensen et al., 2008). This article attempts to define the site-of-action for NS8593.

The selectivity of NS8593 for $K_{Ca}2$ channels over the structurally related $K_{Ca}3.1$ channel allowed us to use a chimeric/mutagenesis approach to identify regions and specific amino acids important for the NS8593 effect. In short, we have shown that $K_{Ca}2.3$ loses sensitivity toward NS8593 inhibition by the point mutations S507T and A532V and, on the other hand, that $K_{Ca}3.1$ gains sensitivity to NS8593 by the equivalently positioned mutations T250S and V275A. These results have at least two noteworthy implications. First, according to the generally accepted gross architecture of 6-TM K^+ -channels, both of these amino acids are located in the inner pore vestibule (inner pore helix of S5 and S6, respectively) close to the inside of the selectivity filter, in which they define open channel properties such as inward rectification/divalent cation block. Hence, because of the coupling between NS8593 inhibition and Ca^{2+} -dependent gating (Strøbaek et al., 2006), we assume that Ser507 and Ala532 are positioned close to the physical gate of the channel. Second, these positions are the same two positions that define $K_{Ca}3.1$ sensitivity (and $K_{Ca}2$ insensitivity) toward triaryl-methanes such as TRAM-34 and clotrimazole (Wulff et al., 2001) and to arachidonic acid (Hamilton et al., 2003). This coincidence of equivalent sites for selective inhibitors of the two channels underscores the close structural resemblance

Fig. 3. Ser507 and Ala532 confer NS8593 sensitivity to $K_{Ca}2.3$, and their transfer into $K_{Ca}3.1$ renders $K_{Ca}3.1$ sensitive to NS8593. Effects of 1 and 10 μ M NS8593 on $K_{Ca}2.3$ (left) and $K_{Ca}3.1$ (right) single and double mutants, in which inner pore residues were mutated to the equivalent amino acids of the other channel. A partial sequence alignment of h $K_{Ca}3.1$ and h $K_{Ca}2.3$ in the P-loop and the S6 region with the position of the residues highlighted is shown on top. Experimental details and color-coding are as stated in Fig. 1.

between the K_{Ca2} and K_{Ca3} families. However, our data also point toward significant functional differences between the two channel families. Although NS8593 inhibition via this region in $K_{Ca2.3}$ exhibits strong negative gating modulation (abolishable by Ca^{2+} and coapplication of positive modulators), Ca^{2+} -dependence and NS309-mediated reversion are barely detectable for its inhibition of the $K_{Ca3.1}^{T250S+V275A}$ construct. The present results seem not to support the interpretation that the increased potency and abolished gating dependence is due solely to a low $P_o(max)$ value (favoring a possible closed state binding of NS8593), because combined experimental conditions tending toward a high P_o (high $[Ca^{2+}]_i$ + NS309 or Mg^{2+} -ATP), do not shift the potency significantly. It is noteworthy that the same is the case for the TRAM-34/clotrimazole inhibition of $K_{Ca3.1}$, in which we have previously found that clotrimazole and TRAM-34 inhib-

ited NS309 or naphtho[1,2-*d*]thiazol-2-ylamine (SKA-31) activated $K_{Ca3.1}$ channels with essentially the same potency as nonactivated channels (Strøbaek et al., 2004; Sankaranarayanan et al., 2009). Together, this series of results may warrant the consideration of whether these residue positions are simply less intimately coupled to the gating process in $K_{Ca3.1}$ than the equivalent positions in $K_{Ca2.3}$.

Equivalent deep-pore amino acid positions allowing 3 orders of magnitude of inhibitory selectivity between K_{Ca2} and K_{Ca3} families provoke the question of whether interaction with these positions might potentially also cause selectivity between the $K_{Ca2.1}$, $K_{Ca2.2}$, and $K_{Ca2.3}$ subtypes, a matter of considerable pharmacological and potential clinical relevance: $K_{Ca2.1}$ and $K_{Ca2.2}$ are mostly expressed in the cortical/limbic structures of the brain, whereas $K_{Ca2.3}$ is preferentially expressed in the basal ganglia and in other subcortical regions (Sailer et al., 2004). K_{Ca} inhibitors have

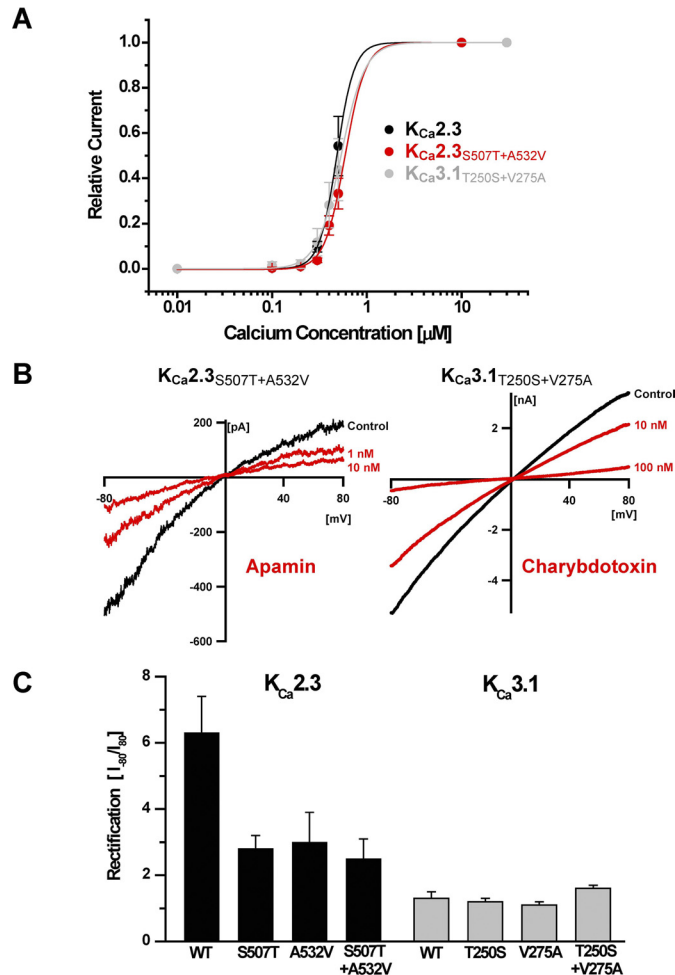


Fig. 4. The $K_{Ca2.3}^{S507T+A532V}$ and the $K_{Ca3.1}^{T250S+V275A}$ double mutants exhibit similar biophysical and pharmacological properties as the WT channels. **A**, overlay of the calcium-response curves of $K_{Ca2.3}$ and the two double mutants measured from inside-out patches exposed to increasing Ca^{2+} concentrations. Data points are mean \pm S.D. from seven or eight experiments per construct. The fit of the data to the Hill equation yielded the following results: $K_{Ca2.3}$ (EC_{50} 480 ± 50 nM, $n_H = 4.8 \pm 1.0$); $K_{Ca2.3}^{S507T+A532V}$ (EC_{50} 590 ± 40 nM, $n_H = 4.4 \pm 0.5$), and $K_{Ca3.1}^{T250S+V275A}$ (EC_{50} 520 ± 70 nM, $n_H = 3.8 \pm 0.9$). **B**, whole-cell recording showing effects of the pore-blocking toxins apamin (left) and charybdotoxin (right) on the $K_{Ca2.3}^{S507T+A532V}$ and the $K_{Ca3.1}^{T250S+V275A}$ double mutants. Experimental details as stated in text to Fig. 1. **C**, Rectification of the different $K_{Ca2.3}$ and $K_{Ca3.1}$ constructs determined by the ratio of the current amplitude at -80 and $+80$ mV. Values are mean \pm S.D. ($n = 2-15$ per data point).

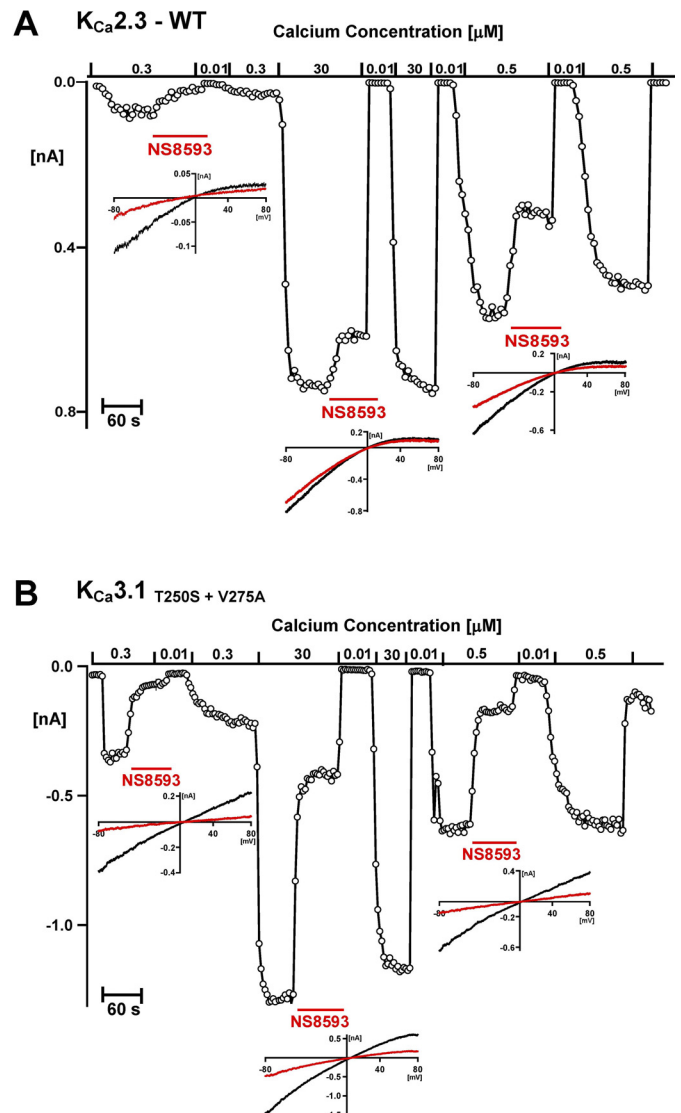


Fig. 5. Inhibition of WT $K_{Ca2.3}$ (**A**) by NS8593 is more Ca^{2+} -dependent than inhibition of the $K_{Ca3.1}^{T250S+V275A}$ mutant (**B**). Inside-out patches were exposed to a $[Ca^{2+}]_i$ of 0.01, 0.3, 0.5, or 30 μ M as indicated in the presence or absence of NS8593. The main figures show a continuous plot of the currents recorded at -75 mV. The inserts show control and NS8593 current traces. Experimental details and color-coding are as described in Fig. 1.

been considered for improvement of cognitive performance (primarily $K_{Ca2.2}$) (Hammond et al., 2006), whereas there is evidence for the use of $K_{Ca2.3}$ inhibitors for mood disorders such as depression (Jacobsen et al., 2008). Unfortunately, both Ser507 and Ala532 are conserved among the K_{Ca2} subtypes (Ser330 and Ala355 in $K_{Ca2.1}$; Ser359 and Ala384 in $K_{Ca2.2}$), which implies that selective inhibition of the K_{Ca2} channel members may be difficult to achieve. In support of this interpretation, a detailed structure-activity analysis on NS8593 analogs revealed no subtype-selectivity between $K_{Ca2.2}$ and $K_{Ca2.3}$, despite the achievement of considerably increased potency for negative gating modulation (Sørensen et al., 2008). In a study of non-apamin-displacing K_{Ca2} inhibitors of a different chemotype [4-(aminomethylaryl)pyrazolopyrimidine], subtype selectivity was also not observed (Gentles et al., 2008), but whether these compounds are interacting with the same site as NS8593 is currently not known. However, it might of course be possible to achieve some functional selectivity among the K_{Ca2} subtypes based on the different Ca^{2+} concentrations in the microscopic environment surrounding the channel's calmodulin and its phosphorylation state (Bildl et al., 2004; Allen et al., 2007) in different types of neurons and brain regions.

An Emerging Picture of K_{Ca2} Channel-Gating Pharmacology. Because the predominant effects of the prototypical positive and negative gating modulators are left-shifting/

right-shifting of the $[Ca^{2+}]_i$ -response curve, respectively, their phenomenological mode-of-actions were originally attributed to selective increases/decreases in the “apparent Ca^{2+} affinity” of K_{Ca2} channels, not excluding a priori an interference with the genuine CaM binding affinity for Ca^{2+} . The demonstration that the C terminus is the site of 1-EBIO-mediated positive modulation of $K_{Ca2.1}$ (Pedarzani et al., 2001), a finding that has been confirmed by the $K_{Ca2.3}/K_{Ca2.2}$ -selective cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methylpyrimidin-4-yl]-amine ($K_{Ca2.3} > K_{Ca2.2} \gg K_{Ca2.1} = K_{Ca3.1}$) (Hougaard et al., 2008), further strengthened the view of positive modulation occurring by a comparatively simple “local” C-terminal mechanism. We initially imagined a similar C-terminally “delimited” action for negative gating modulation, in particular with reference to the quite similar negative gating effect of casein kinase 2-mediated phosphorylation of Thr80 in the attached CaM (Bildl et al., 2004; Allen et al., 2007).

However, a number of findings significantly complicate this simple, unifying picture. First, localization of the site-of-action for NS8593 to deep-pore amino acids immediately excludes the possibility that positive and negative modulators simply share the same binding site and just differ in their (positive or negative) coupling to the gating process in analogy to the action of benzodiazepines on the GABA_A receptor (Sieghart, 1994). In addition, recent findings show

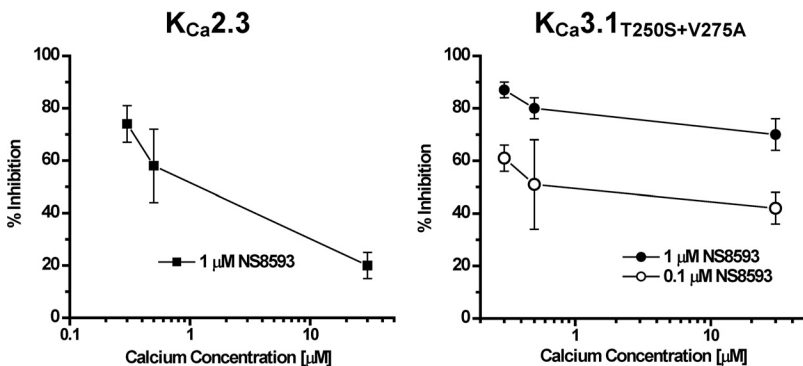


Fig. 6. NS8593 inhibition of the $K_{Ca3.1T250S+V275A}$ mutant shows reduced calcium-dependence at both 1 μM and 0.1 μM NS8593. Plot of the percentage of current inhibition by NS8593 versus $[Ca^{2+}]_i$ concentration for WT $K_{Ca2.3}$ (right) and the $K_{Ca3.1T250S+V275A}$ mutant (left). Values are mean \pm S.D. ($n = 3-15$).

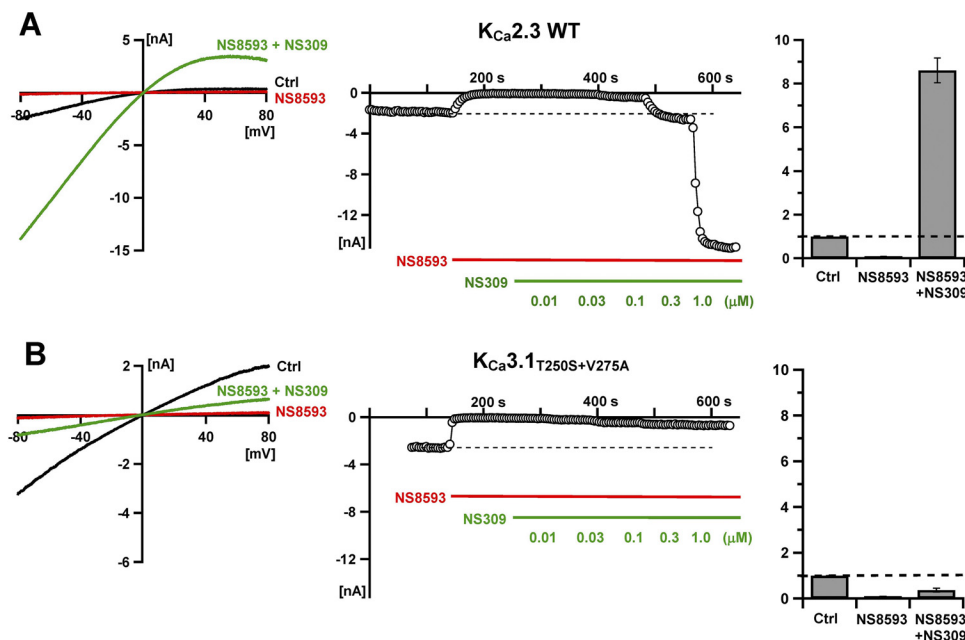


Fig. 7. Whole-cell experiments (symmetric K^+ , free pipette $[Ca^{2+}]_i$ buffered to 400 nM) showing that NS8593 inhibition of $K_{Ca2.3}$ is abolished by the positive modulator NS309 (A), whereas inhibition of $K_{Ca3.1T250S+V275A}$ is not (B). Right, ramp traces obtained before (black) and after (red) superfusion with 1 μM NS8593 and after cosuperfusion with 1 μM NS309 (green). Middle, time courses (tick separation, 100 s) of the currents recorded at -80 mV. Right, diagram showing quantification of these effects relative to the control current (before NS8593 addition) defined as 1. The concentration for the coapplication with NS309 is 1 μM .

that NS309, which is a classic positive modulator like 1-EBIO, strengthens the link between CaM-Ca²⁺ and channel opening rather than increasing Ca²⁺ binding to CaM per se (Li et al., 2009). Furthermore, we have described recently a K_{Ca}2.1-selective activator (K_{Ca}2.1 > K_{Ca}2.2 = K_{Ca}2.3 ≫ K_{Ca}3.1) 4-(2-methoxy-phenylcarbamoyloxymethyl)-piperidine-1-carboxylic acid *tert*-butyl ester (GW542573X), which exhibits a complex, partial agonist-like mode-of-action (Hougaard et al., 2009) that is also independent of the CaMBD/C-terminal region but is dependent on selective interaction with Ser293 (Leu476 in K_{Ca}2.3) in the S5 segment. In line with the present analysis of the interaction site for negative gating modulation by NS8593, the GW542573X results were interpreted as evidence for K_{Ca}2.1 activation via “deep-pore” gating structures. At the present time we therefore favor the hypothesis, that the diverse positive/negative modulator pharmacology directly reflects the complexity and extended participation of even remotely positioned parts of K_{Ca}2 (and accessory proteins) in the gating process. Cysteine scanning experiments have clearly shown that the gate of both K_{Ca}2 and K_{Ca}3 channels is positioned very close to or even encompasses the quite outwardly displaced K⁺ selectivity filter (Bruening-Wright et al., 2002, 2007; Klein et al., 2007; Garneau et al., 2009), a finding that has to be reconciled with the primary Ca²⁺-binding event occurring on CaM at the cytoplasmic C terminus. We think of K_{Ca}2 (and K_{Ca}3) channel gating as a series of events comprising Ca²⁺-binding, CaM/CaMBD/C-terminal conformational change, leading to a transduction via S6 (possibly involving S5 stabilization) to deep-pore gating structures and eventual opening of the channel. The emerging complexity of the gating modulators, in terms of site-of-actions, selectivity, and mode-of-actions, most likely reflects the existence of several points for pharmacological intervention along the chain of molecular events leading from Ca²⁺ binding to the eventual channel opening. Both negative and positive allosteric-like gating modulation can be achieved, as well as partial agonism-like activation. However, no simple unifying relation seems to exist between the position of interaction sites and mode-of-actions. Despite the present results obtained with NS8593, we do therefore not exclude that future negative gating modulators of K_{Ca}2 channels might act on different sites than Ser507 and Ala532. Indeed, different sites of modulation coupled to different selectivities are not unprecedented for K⁺ channel gating modulators. Although the “classic” K_v7.2-K_v7.5 channel activator retigabine interacts with a hydrophobic pocket formed upon channel opening between the cytoplasmic parts of S5 and S6 (Wuttke et al., 2005), the more K_v7.2/7.3-selective activator *N*-(6-chloro-pyridin-3-yl)-3,4-difluoro-benzamide (ICA-27243) was found recently to act through a voltage-sensor domain site located in transmembrane segments S1–S3 (Padilla et al., 2009). In analogy, alternative gating inhibitor sites on K_{Ca}2 channels may exist, possibly providing a better opportunity for the achievement of subtype-selectivity.

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Authorship Contributions

Participated in research design: Jenkins, Strøbæk, Christophersen, and Wulff.

Conducted experiments: Jenkins, Strøbæk, and Hougaard.

Contributed new reagents or analytic tools: Jensen, Hummel (molecular biology), and Sørensen (chemistry).

Performed data analysis: Jenkins, Strøbæk, and Wulff.

Wrote or contributed to the writing of the manuscript: Jenkins, Strøbæk, Christophersen, and Wulff.

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