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Negative Gating Modulation by (*R*)-*N*-(Benzimidazol-2-yl)-tetrahydro-1-naphtylamine (NS8593) Depends on Residues in the Inner Pore Vestibule: Pharmacological Evidence of Deep-Pore Gating of $K_{Ca}2$ Channels

David Paul Jenkins, Dorte Strøbæk, Charlotte Hougaard, Marianne L. Jensen, Rene Hummel, Ulrik S. Sørensen, Palle Christophersen, Heike Wulff

Department of Pharmacology, University of California, Davis, CA 95616, USA (D.P.J., H.W.); and NeuroSearch A/S, Pederstrupvej 93, DK2750 Ballerup, Denmark (C.H., M.L.J., R.H., U.S.S., P.C., D.S.)

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Running title:

Negative gating modulation by pore amino acids

Address correspondence to:

Heike Wulff, Department of Pharmacology, Genome and Biomedical Sciences Facility, Room 3502, 451 Health Sciences Drive, University of California, Davis, Davis, CA 95616; phone: 530-754-6136; email: hwulff@ucdavis.edu

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Abbreviations:

BMB, bicuculline methobromid; CaM, calmodulin; CaMBD, calmodulin binding domain; CyPPA, cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methylpyrimidin-4-yl]-amine; 1-EBIO, 1-ethyl-2-benzimidazolinone; CK2, casein kinase 2; GFP, Green Fluorescent Protein; GW542573X, 4-(2-methoxy-phenylcarbamoyloxymethyl)-piperidine-1-carboxylic acid *tert*-butyl ester; HEK293, human embryonic kidney 293; K_{Ca}, Ca^{2+}-activated K^+ channel; $K_{Ca}1.1$, big conductance Ca^{2+}-activated K^+ channel (BK channel); $K_{Ca}2$, small conductance Ca^{2+}-activated K^+ channel (SK channel); $K_{Ca}3.1$, intermediate conductance Ca^{2+}-activated K^+ channel (IK 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-naphtylamine; P_o, open state probability; TM, Transmembrane; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; WT, wild type.

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ABSTRACT

Acting as a negative gating modulator, NS8593 shifts the apparent Ca^{2+}-dependence of the small-conductance Ca^{2+}-activated K^+ channels $K_{Ca}2.1$-2.3 to higher Ca^{2+} concentrations. Similar to the positive K_{Ca} channel gating-modulators 1-EBIO and CyPPA the binding site for NS8593 has been assumed to be located in the C-terminal region, where these channels interact with their calcium sensor calmodulin. However, by employing a progressive chimeric approach, we were able to localize the site-of-action of NS8593 to the $K_{Ca}2$ pore. For example, when we transferred the C-terminus from the NS8593-insensitive intermediate-conductance $K_{Ca}3.1$ channel to $K_{Ca}2.3$ the chimeric channel remained as sensitive to NS8593 as WT-$K_{Ca}2.3$. In contrast, when we transferred the $K_{Ca}2.3$ pore to $K_{Ca}3.1$, the channel became sensitive to NS8593. Using site-directed mutagenesis we subsequently identified two specific residues in the inner vestibule of $K_{Ca}2.3$ (S507 and A532) that determined the effect of NS8593. Mutation of these residues to the corresponding residues in $K_{Ca}3.1$ (T250 and V275) made $K_{Ca}2.3$ insensitive to NS8593, while introduction of S and A into $K_{Ca}3.1$ was sufficient to render this channel highly sensitive to NS8593. Interestingly, the same two residue positions have previously been found to mediate sensitivity of $K_{Ca}3.1$ to clotrimazole and TRAM-34. The location of S507 in the pore-loop near the selectivity filter and A532 in an adjacent position in S6, are within the region predicted to contain the $K_{Ca}2$ 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.

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Introduction

Within the superfamily of Ca^{2+}-activated K^+ channels (K_{Ca}) the group of small (SK, K_{Ca}2.1-2.3) and intermediate-conductance (IK, K_{Ca}3.1) channels are closely related in both structure and function. In contrast to the big conductance K_{Ca}1.1 (BK) channel, which is activated by both voltage and Ca^{2+}, K_{Ca}2 and K_{Ca}3.1 channels are inward rectifying, voltage-independent and activated solely by intracellular Ca^{2+} (for reviews, see (Stocker, 2004) and (Wulff et al., 2007)). The opening of both K_{Ca}2 and K_{Ca}3.1 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 (Fanger et al., 1999; Xia et al., 1998). The energy of the ensuing conformational change is transferred 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 helixes, 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., 2007; Bruening-Wright et al., 2002; Garneau et al., 2009; Klein et al., 2007).

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 like charybdotoxin and maurotoxin, interact with extracellularly exposed amino acids in the outer pore vestibule (Castle et al., 2003; Ishii et al., 1997; Rauer et al., 2000) and are usually conceived to inhibit via a simple blocking mechanism (but see (Lamy et al., 2010), for an emergent different view on the apamin mode-of-action). The same applies to the positively charged small molecule K_{Ca}2 selective blockers like UCL1684

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(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 TRAM-34 (Wulff et al., 2001), is mediated via two amino acids located in the lower part of the pore loop (T250) and in the S6 segment (V275), 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 T250 and V275 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.

Recently, a new principle for selective $K_{Ca}2$ channel inhibition by small molecules has been described: Certain 2-(*N*-substituted)-aminobenzimidazoles, such as NS8593 (Fig. 1) and NS11757, inhibit all three $K_{Ca}2$ channels via negative gating modulation rather than via a simple pore blocking mechanism (Sørensen et al., 2008; Strøbæk et al., 2006). 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+}-dependency of inhibition. The physical binding site for the negative gating modulators is clearly distinct from the apamin binding site, since these compounds do not displace radiolabelled 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 CK2-mediated threonine phosphorylation of CaM (T80) attached to the $K_{Ca}2$ channel (Allen et al., 2007; Bildl et al., 2004). 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 to the effect of NS8593. Hence, we originally hypothesised that negative gating modulation by NS8593 is a phosphorylation-independent, direct down-stream effect on the physiological gating process. Further, since 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 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 paper, 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 S507 and A532 in $K_{Ca}2.3$, which are in equivalent positions to T250 and V275 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.

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Materials and Methods

Molecular biology

HEK293 cell lines stably expressing WT hK_{Ca}2.3 and hK_{Ca}3.1 channels were previously described (Hougaard et al., 2009). The N-terminal K_{Ca}3.1-K_{Ca}2.3 chimera was generated with overlapping PCR (Expand High Fidelity PCR System, Roche, Germany) using the oligonucleotides hIK-hSK3s GTCTCTGGCCGGCTGGGCActgatttttgggatgttt and hSK3-hIKas aaacatcccaaaaatcagTGCCCAGCCGGCCAGAGAC, 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 corporation (Piscataway, NJ) ACCGGTATCATGGGTGCAGGCTGCACTGCCCTTGTGGTGGCCGTGGTGGCCCGAAAG CTGGAGTTTAACAAGGCAGAGAAGCACGTGCACAACTTCATGATGGATATCCAGTA TACCAAAGAGATGAAGGAGTCCGCTGCCCGAGTGCTACAAGAAGCCTGGATGTTCT ACAAACATACTCGCAGGAAGGAGTCTCATGCTGCCCGCAGGCATCAGCGCAAGCTG CTGGCCGCCATCAACGCGTTCCGCCAGGTGCGGCTGAAACACCGGAAGCTCCGGGA ACAAGTGAACTCCATGGTGGACATCTCCAAGATGCACATGATCCTGTATGACCTGCA GCAGAATCTGAGCAGCTCACACCGGGCCCTGGAGAAACAGATTGACACGCTGGCGG GGAAGCTGGATGCCCTGACTGAGCTGCTTAGCACTGCCCTGGGGCCGAGGCAGCTT CCAGAACCCAGCCAGCAGTCCAAGTAGCTGGAGTGGCGGCCGC into hK_{Ca}2.3 with an AgeI-site 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 previously described (Wulff et al., 2001). Additional point mutations were generated using WT K_{Ca}2.3 and K_{Ca}3.1 plasmids uracilated via *E. coli* RZ1032 (Stratagene, La Jolla, CA, USA) as templates in mutagenesis reactions. Four oligonucleotides (MWG Biotec, Germany) were used to introduce the mutations:

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$hK_{Ca}2.3$ S507T: CTCCATCACATTCCTTaCaATTGGTTATGGGGACA

$hK_{Ca}2.3$ A532V: TCACTGGCATCATGGGTGtAGGCTGtACaGCCCTTGTGGTGGCCG

$hK_{Ca}3.1$ T250S: GATCCCCATCACATTCCTGtCaATtGGCTATGGTGACGTG

$hK_{Ca}3.1$ V275A: GCACTGGAGTCATGGGTGcaTGCTGCACAGCCCTGCT

The mutagenesis reactions were performed using T7 DNA polymerase and T4 DNA ligase (New England Biolabs, USA). *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 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 trypsination and plated on cover slips (3.5 mm Ø) on the day of the experiments, whereas cells for inside-out recordings were plated 1 day prior to the experiments to attach them more firmly. For recordings a cover slip was placed in a 15 µl recording chamber mounted on the cross-board of an inverted microscope (Olympus XI-70, equipped with fluorescence burner and filters) and cell selection was guided by fluorescence from the co-transfected green fluorescent protein. The extracellular solutions contained (in mM): 154 KCl, 10 HEPES (pH = 7.4), 2 or 0.1 $CaCl_2$, 1 or 3 $MgCl_2$ for inside-out/whole-cell experiments, respectively. Solutions on the intracellular side contained (in mM): 154 KCl, 10 HEPES (pH = 7.2), 10 EGTA or 1 EGTA plus 9 nitriloacetic acid (NTA), $CaCl_2$ and $MgCl_2$ to yield a calculated free Mg^{2+} concentration of 1 mM and calculated free Ca^{2+}-concentrations of

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0.01, 0.2, 0.3, 0.4, 0.5, 3, 10, and 30 μM. Solutions used for experiments with ATP were made with Na_2ATP (Sigma/Aldrich) and adjusted to yield 30 μM free Ca^{2+} and 1.6 mM MgATP. 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, Denmark) or soda lime glass (micro-hematocrit tubes, Kimble Chase, Rochester, NY) and had resistances of 2-3 MΩ when submerged in the bath solution. Positioning of the patch electrode was controlled with a Patchman micromanipulator (Eppendorf, USA). 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, Bellmore, NY and Lambrecht, Germany). Cells were clamped to a holding potential of at 0 mV and K_{Ca} currents were elicited by 200-ms voltage-ramps from -80 to +80 mV applied every 5 sec. Data analysis, fitting and plotting were performed with IGOR-Pro (WaveMetrics, Lake Oswego, OR) or Origin 7 (OriginLab, Northampton, MA).

Chemicals and reagents

(*R*)-*N*-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphtylamine (NS8593) and 6,7-dichloro-1*H*-indole-2,3-dione-3-oxime (NS309) were synthesized at NeuroSearch A/S as described (Strøbæk et al., 2006; Strøbæk et al., 2004). Bicuculline methobromid (BMB) and apamin were from Sigma/Aldrich and charybdotoxin from Bachem Bioscience (King of Prussia, PA). TRAM-34 was synthesized in the Wulff laboratory as previously described (Wulff et al., 2001). Standard laboratory chemicals were purchased from commercial dealers and were of the purest grade available.

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Quantification and statistics

IC_{50} values were calculated as previously described (Strøbæk et al., 2006). Summary values are given as means ± standard deviation.

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 [Ca^{2+}] of 400 nM, while the related K_{Ca}3.1 channel is insensitive to this negative gating modifier (Strøbæk et al., 2006). In order to delineate K_{Ca}2 channel regions important for the rightward shift in the Ca^{2+}-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 bicuculline methobromid (BMB) and to 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*).

Figures 2A and 2B show representative recordings from wild-type K_{Ca}2.3 and K_{Ca}3.1 currents elicited by 200 ms voltage-ramps from -80 mV to +80 mV applied every 5 seconds with 400 nM of free Ca^{2+} in the patch-pipette. As previously published for recordings with symmetrical K^+ concentrations in this expression system (Hougaard et al., 2007; Strøbæk et al., 2006), 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

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pharmacology. $K_{Ca}2.3$ was potently inhibited by NS8593 with an IC_{50} of 104 nM (Table 1) and almost fully blocked by 100 μM of BMB (IC_{50} 5 μM), which we routinely use to estimate leak when working with symmetrical K^+ solutions. $K_{Ca}3.1$ in contrast was completely insensitive to 10 μM of NS8593 and 100 μM BMB but could be blocked completely by TRAM-34 (Table 1). 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 (Strøbæk 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 $K_{Ca}3.1$. Since 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. 2C and 2D), 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 expressed too poorly to allow for evaluation of modulator sensitivity (*Supplemental Table 1*). However, an important chimera, which contained the transmembrane regions S1-S4 from $K_{Ca}3.1$ and the pore

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and C-terminus of $K_{Ca}2.3$ (Fig. 2E) expressed sufficiently well for pharmacological experiments. Interestingly, 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. In consequence, we decided to follow up this observation with point mutations in the inner pore region (see below). The latter observation could indicate that the presence of the S1-S4 region from $K_{Ca}3.1$ in the chimera might have disturbed the architecture of the outer $K_{Ca}2.3$ vestibule, where 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), where it apparently acts more as an allosteric modulator of the selectivity filter than as a direct blocker like 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 previously reported to confer TRAM-34 and clotrimazole sensitivity to $K_{Ca}3.1$ (Wulff et al., 2001). These amino acids, T250 and V275 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 Table 1 and Figure 3 mutations of each of the corresponding residues in $K_{Ca}2.3$ (S507 and A532) reduced the potency of NS8593 roughly 20-fold, while introduction of the double mutation produced a $K_{Ca}2.3$ channel that was completely insensitive to NS8593. The importance of S507 and A532 was verified by showing that the reverse $K_{Ca}3.1$ mutants (T250S and V275A) became

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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 Table 1). 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 480-590 nM and Hill coefficients of 3.8-4.7 (Fig. 4A). In whole-cell recordings both double mutants also showed the characteristic pharmacology of the WT channels. While the $K_{Ca}3.1_{T250S+V275A}$ mutant was sensitive to ChTX (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 Table 1). 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 (Table 1) 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). While 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. The inward-rectification of $K_{Ca}2$ channels has previously been shown 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 S359 in rat $K_{Ca}2.2$ (Soh and Park, 2001; Soh and Park, 2002), which corresponds to S507 in human $K_{Ca}2.3$. Mutation of this position to alanine or the larger threonine demonstrated that the

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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

Since the residues conferring NS8593 sensitivity to $K_{Ca}2.3$ and transferring it to $K_{Ca}3.1$, S507 in the pore-loop and A532 in the S6 segment, are located within in the region predicted to contain the gate of $K_{Ca}2$ and $K_{Ca}3.1$ channels, we wondered if their mutations would also affect the mechanism of action of NS8593. In order to address this issue we investigated two defining characteristics of negative gating modulation: 1) dependence on $[Ca^{2+}]_i$ and 2) reversibility in presence of the positive modulator NS309.

As previously described (Strøbæk 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 μM NS8593 onto $K_{Ca}2.3$ currents activated with 300 nM of $[Ca^{2+}]_i$ in inside-out patches blocked 74 ± 6% (n = 6) of the calcium-dependent inward current at –80 mV. However, when the same patch was subsequently exposed to 30 μM of $[Ca^{2+}]_i$ in order to maximally activate $K_{Ca}2.3$, 1 μM of NS8593 only blocked 21 ± 5% (n = 6) of the current. After washout and another control for absence of contaminating leak current by a switch to 10 nM of $[Ca^{2+}]_i$, $K_{Ca}2.3$ was then exposed to 0.5 μM of $[Ca^{2+}]_i$ and 1 μM of NS8593 was observed to exhibit an intermediate potency (58 ± 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

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calcium-dependent (Fig. 4B and Fig. 5). In a similar inside-out experiment, in which $[Ca^{2+}]_i$ was varied from 0.3 μM to 30 μM, NS8593 at 1 μM inhibited 87 ± 3% (n = 3) at 0.3 μM, 80 ± 4% (n = 3) at 0.5 μM, and 70 ± 6% (n = 5) of the current at 30 μM $[Ca^{2+}]_i$. 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 since inhibition remained less calcium-sensitive even when the NS8593 concentration was reduced 10-fold from 1 μM to 100 nM (Fig. 6).

The NS8593 inhibition of K_{Ca} channels at low $[Ca^{2+}]_i$ is essentially abolished upon addition of the positive modulator NS309 (Ji et al., 2009; Strøbæk et al., 2006). Fig. 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 panels) 1 μM of NS8593 was superfused causing nearly 100 % inhibition (red traces and middle graph after 150 sec). Increasing concentrations of NS309 were then co-superfused with NS8593 leading to a concentration dependent reversal and "overshoot" of the $K_{Ca}2.3$ current, while the $K_{Ca}3.1_{T250S+V275A}$ current remained largely inhibited even at an NS309 concentration of 1 μM (green traces and time course figure). This series of experiments has been quantified in the right panels where the fold decrease/increase relative to the control current is plotted for the NS8593 (1 μM) equilibrium inhibition and for the combined NS8593+NS309 (1 μM) 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

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$[Ca^{2+}]_i$ (30 μM) and MgATP (1.6 mM) or NS309 (1 μM). The rationale for using MgATP 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 MgATP. In our hands, MgATP 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 μM) caused current reductions that were not significantly different from the effects without MgATP or NS309 (53 ± 8%, n = 5 vs. 52 ± 7%, n = 3, and 51 ± 7%, n = 4, respectively). Likewise, the higher NS8593 concentration of 1 μM 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 dependency of inhibition, and by the preservation of its inhibitory potency in the presence of MgATP.

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Discussion

In comparison to the classical $K_{Ca}2$ channel blocker apamin, NS8593 acts by a different mechanism and via a different site since $K_{Ca}2.3$ channels mutated in the apamin binding site remain sensitive to its actions (Sørensen 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 (Sørensen et al., 2008; Strøbæk et al., 2006). The present paper attempted 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 chimaeric/mutagenesis approach to identify regions and specific amino acids important for the NS8593 effect. In short, we have shown that $K_{Ca}2.3$ looses sensitivity towards NS8593 inhibition by the point mutations S507T and A532V and conversely 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, where they define open channel properties like inward rectification/divalent cation block. Hence, due to the coupling between NS8593 inhibition and Ca^{2+}-dependent gating (Strøbæk et al., 2006), we assume that S507 and A532 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) towards triarylmethanes like 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 between the $K_{Ca}2$ and $K_{Ca}3$ families. However, our data also points towards significant functional differences between

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the two channel families. While NS8593 inhibition via this region in $K_{Ca}2.3$ exhibits strong negative gating modulation (abolishable by Ca^{2+} and co-application of positive modulators) Ca^{2+}-dependence and NS309 mediated reversion is barely detectable for its inhibition of the $K_{Ca}3.1_{T250S+V275A}$ 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), since combined experimental conditions tending towards a high P_o (high $[Ca^{2+}]_i$ + NS309 or MgATP), do not shift the potency significantly. It is noteworthy that the same is the case for the TRAM-34/clotrimazole inhibition of $K_{Ca}3.1$, where we have previously found that clotrimazole and TRAM-34 inhibited NS309 or SKA-31 activated $K_{Ca}3.1$ channels with essentially the same potency as non-activated channels (Sankaranarayanan et al., 2009; Strøbæk et al., 2004). Collectively, this series of results may warrant the consideration whether these residue positions are simply less intimately coupled to the gating process in $K_{Ca}3.1$ than the equivalent positions in $K_{Ca}2.3$.

Equivalent deep-pore amino acid positions allowing three orders of magnitude of inhibitory selectivity between $K_{Ca}2$ and $K_{Ca}3$ families provoke the question, whether interaction with these positions might potentially also cause selectivity between the $K_{Ca}2.1$, $K_{Ca}2.2$ and $K_{Ca}2.3$ subtypes, a matter of considerable pharmacological and potential clinical relevance: $K_{Ca}2.1$ and $K_{Ca}2.2$ are mostly expressed in the cortical/limbic structures of the brain, whereas $K_{Ca}2.3$ is preferentially expressed in the basal ganglia and in other subcortical regions (Sailer et al., 2004). K_{Ca} inhibitors have been considered for improvement of cognitive performance (primarily $K_{Ca}2.2$) (Hammond et al., 2006), whereas there is evidence for use of $K_{Ca}2.3$ inhibitors for mood disorders like depression (Jacobsen et al., 2008). Unfortunately, both S507 and A532 are conserved among the $K_{Ca}2$ subtypes (S330 and A355 in $K_{Ca}2.1$; S359 and S384 in

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$K_{Ca}2.2$), which implies that selective inhibition of the $K_{Ca}2$ channel members may be difficult to achieve. In support of this interpretation a detailed structure-activity-analysis on NS8593-analogues revealed no subtype selectivity between $K_{Ca}2.2$ and $K_{Ca}2.3$, despite the achievement of considerably increased potency for negative gating modulation (Sørensen et al., 2008). In a study of non-apamin displacing $K_{Ca}2$ inhibitors of a different chemotype (4-(aminomethylaryl)pyrrazolopyrimidines) 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 amongst the $K_{Ca}2$ subtypes based on the different Ca^{2+} concentrations in the microscopic environment surrounding the channel's calmodulin and its phosphorylation state (Allen et al., 2007; Bildl et al., 2004) in different types of neurons and brain regions.

An emerging picture of $K_{Ca}2$ channel gating pharmacology

Since 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 $K_{Ca}2$ 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 $K_{Ca}2.1$ (Pedarzani et al., 2001), a finding that has been confirmed by the $K_{Ca}2.3/K_{Ca}2.2$-selective CyPPA ($K_{Ca}2.3>K_{Ca}2.2>>K_{Ca}2.1=K_{Ca}3.1$) (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

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effect of CK2 mediated phosphorylation of T80 in the attached CaM (Allen et al., 2007; Bildl et al., 2004).

However, a number of findings significantly complicate this simple, unifying picture. First of all, 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). Additionally, recent findings show that NS309 - which is a classic positive modulator like 1-EBIO - strengthens the link between CaM-Ca^{2+} and channel opening, rather than increasing Ca^{2+} binding to CaM *per se* (Li et al., 2009). Furthermore, we have recently described a $K_{Ca}2.1$ selective activator ($K_{Ca}2.1$>$K_{Ca}2.2$= $K_{Ca}2.3$>>$K_{Ca}3.1$) 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 dependent on selective interaction with S293 (L476 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., 2007; Bruening-Wright et al., 2002; Garneau et al., 2009; Klein et al., 2007); a finding that has to be reconciled with the primary Ca^{2+}-binding event occurring on CaM at the cytoplasmic C-terminus. We think of $K_{Ca}2$ (and $K_{Ca}3$) channel gating as

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a series of events comprising Ca^{2+}-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, both in terms of site-of-actions, selectivity and mode-of-actions, most likely reflect the existence of several points for pharmacological intervention along the chain of molecular events leading from Ca^{2+}-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 appears to exist between 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 S507 and A532. Indeed, different sites of modulation coupled to different selectivities are not unprecedented for K^+ channel gating modulators. While 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 ICA-27243 was recently found 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: Dorte Strøbæk, David P. Jenkins, Palle Christophersen, Heike Wulff

Conducted experiments: David P. Jenkins, Dorte Strøbæk, Charlotte Hougaard

Contributed new reagents or analytic tools: Ulrik S. Sørensen (Chemistry); Marianne L. Jensen and Rene Hummel (Molecular Biology)

Performed data analysis: Dorte Strøbæk, David P. Jenkins, Heike Wulff

Wrote or contributed to the writing of the manuscript: Palle Christophersen, Heike Wulff, Dorte Strøbæk, David P. Jenkins

Other: Dorte Strøbæk made the pivotal observation leading to the NS8593 binding site identification.

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Footnote

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Legends for Figures

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

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), $K_{Ca}3.1$-$K_{Ca}2.3_{(287-731)}$ (D) and $K_{Ca}3.1$-$K_{Ca}2.3_{(435-731)}$ (E). Voltage-ramps were applied every 5 sec from a holding potential of 0 mV. Control traces (last sweep before compound application) are shown in black, compound traces (last sweep before compound wash-out) in red. Wash-out 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).

Fig. 3. S507 and A532 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 μM of 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 $hK_{Ca}3.1$ and $hK_{Ca}2.3$ in the P-loop and the S6 region with the position of the residues highlighted is shown on top. Experimental details and color-coding as stated in text to Figure 1.

Fig. 4. The $K_{Ca}2.3_{S507T+A532V}$ and the $K_{Ca}3.1_{T250S+V275A}$ double mutants exhibit similar biophysical and pharmacological properties as the WT channels. (A) Overlay of the calcium-response curves

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of $K_{Ca}2.3$ and the two double mutants measured from inside-out patches exposed to increasing Ca^{2+} concentrations. Data points are mean ± SD from 7 or 8 experiments per construct. The fit of the data to the Hill equation yielded the following results: $K_{Ca}2.3$ (EC_{50} 480 ± 50 nM, n_H = 4.8 ± 1.0); $K_{Ca}2.3_{S507T+A532A}$ (EC_{50} 590 ± 40 nM, n_H = 4.4 ± 0.5) and $K_{Ca}3.1_{T250S+V275A}$ (EC_{50} 520 ± 70 nM, n_H = 3.8 ± 0.9). (B) Whole-cell recording showing effects of the pore-blocking toxins apamin *(left)* and charybdotoxin *(right)* on the $K_{Ca}2.3_{S507T+A532V}$ and the $K_{Ca}3.1_{T250S+V275A}$ double mutants. Experimental details as stated in text to Figure 1. (C) Rectification of the different $K_{Ca}2.3$ and $K_{Ca}3.1$ constructs determined by the ratio of the current amplitude at -80 and +80 mV. Values are mean ± SD (n = 2-15 per data point).

Fig. 5. Inhibition of WT $K_{Ca}2.3$ (A) by NS8593 is more Ca^{2+}-dependent than inhibition of the $K_{Ca}3.1_{T250S+V275A}$ mutant (B). Inside-out patches were exposed to a $[Ca^{2+}]_i$ of 0.01, 0.3, 0.5 or 30 μM 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 as described in text to Figure 1.

Fig. 6. NS8593 inhibition of the $K_{Ca}3.1_{T250S+V275A}$ mutant shows reduced calcium-dependence at both 1 μM and 100 nM NS8593. Plot of the percentage of current inhibition by NS8593 versus $[Ca^{2+}]_i$ concentration for WT $K_{Ca}2.3$ (*right*) and the $K_{Ca}3.1_{T250S+V275A}$ mutant (*left*). Values are mean ± SD (n = 3-15).

Fig. 7. Whole-cell experiments (symmetric K^+, free pipette $[Ca^{2+}]$ buffered to 400 nM) showing that NS8593 inhibition of $K_{Ca}2.3$ is abolished by the positive modulator NS309 (A), whereas

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inhibition of $K_{Ca}3.1_{T250S+V275A}$ is not (B). *Right panels*: Ramp traces obtained before (black) and after (red) superfusion with 1 µM NS8593 as well as after co-superfusion with 1 µM NS309 (green). *Middle panels*: Time courses (tick separation 100 sec) of the currents recorded at -80 mV. *Right panels*: Diagram showing quantification of these effects relative to the control current (before NS8593 addition) defined as 1. The concentration for the co-application with NS309 is 1 µM.

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Table 1. Pharmacology of $K_{Ca}2.3$ and $K_{Ca}3.1$ chimeric and point mutated channels.

Chimeras	**NS8593 IC_{50} (μM)**	**% current inhibition by 100 μM BMB**	**Fold current increase by 30 nM NS309**
$K_{Ca}2.3$ 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)
$K_{Ca}3.1$-$K_{Ca}2.3_{(287-731)}$	0.115 ± 0.040 (6)	91 ± 3 (6)	1.1 ± 0.5 (6)
$K_{Ca}3.1$-$K_{Ca}2.3_{(435-731)}$	0.947 ± 0.031 (4)	0 ± 0 (3)	2.3 ± 0.4 (3)
Mutants	**NS8593 IC_{50} (μM)**	**% current inhibition by 100 μM BMB**	**TRAM-34 IC_{50} (μM)**
$K_{Ca}2.3$ wt	0.104 ± 0.034 (13)	83 ± 13 (10)	> 20*
$K_{Ca}2.3_{S507T}$	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*

*(Wulff et al., 2001)

For information on the pharmacological properties of additional $K_{Ca}2.3$/$K_{Ca}3.1$ chimeras please see *Supplemental Table 1* online.

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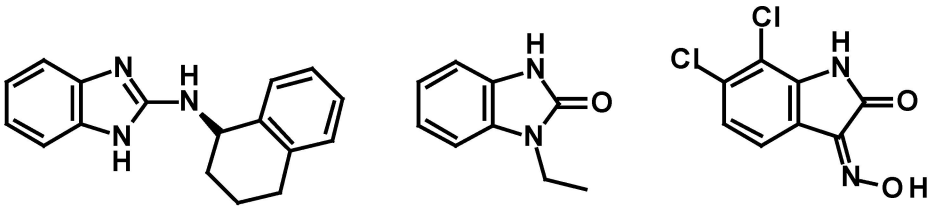

NS8593 1-EBIO NS309

Figure 1

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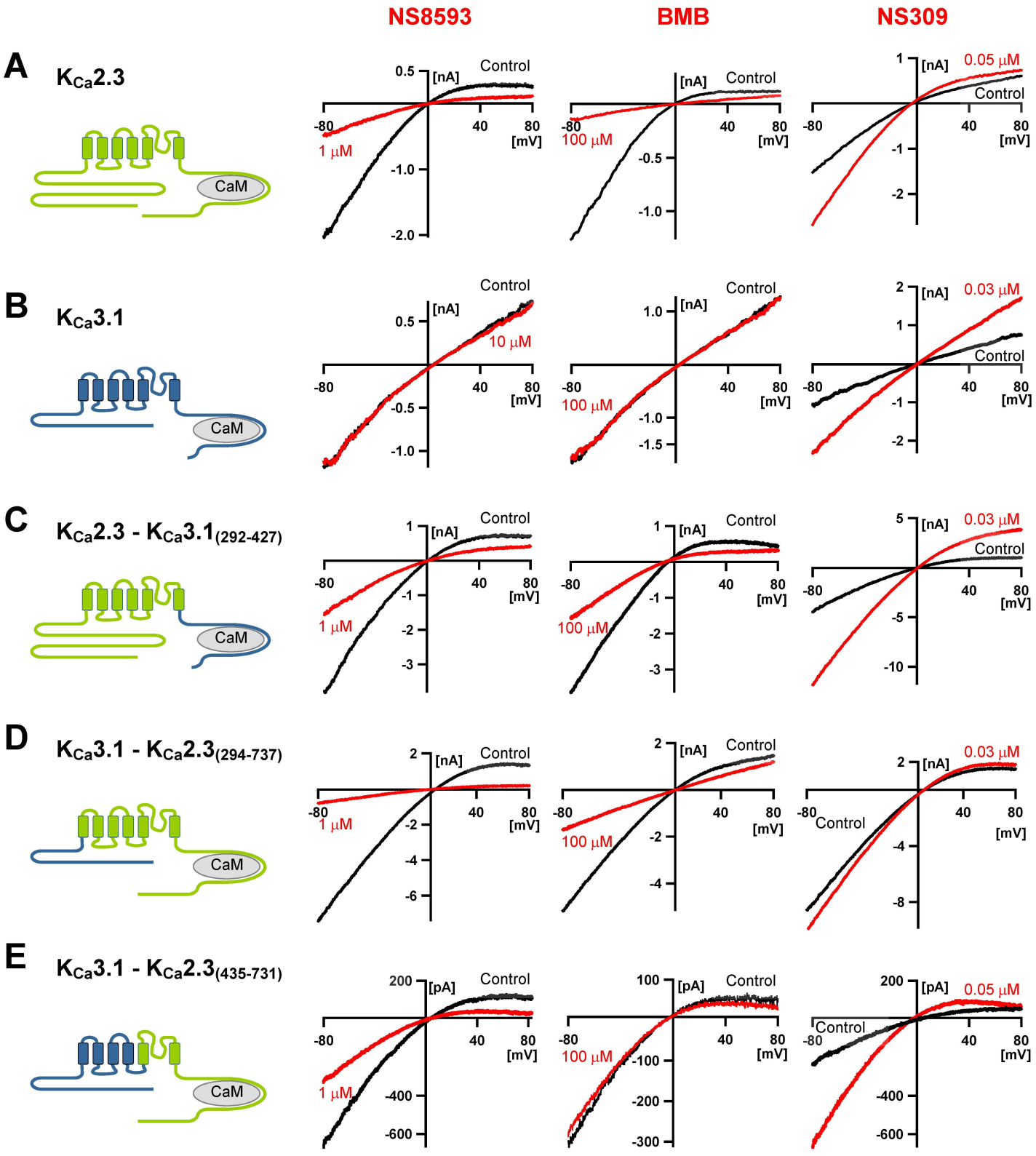

Figure 2

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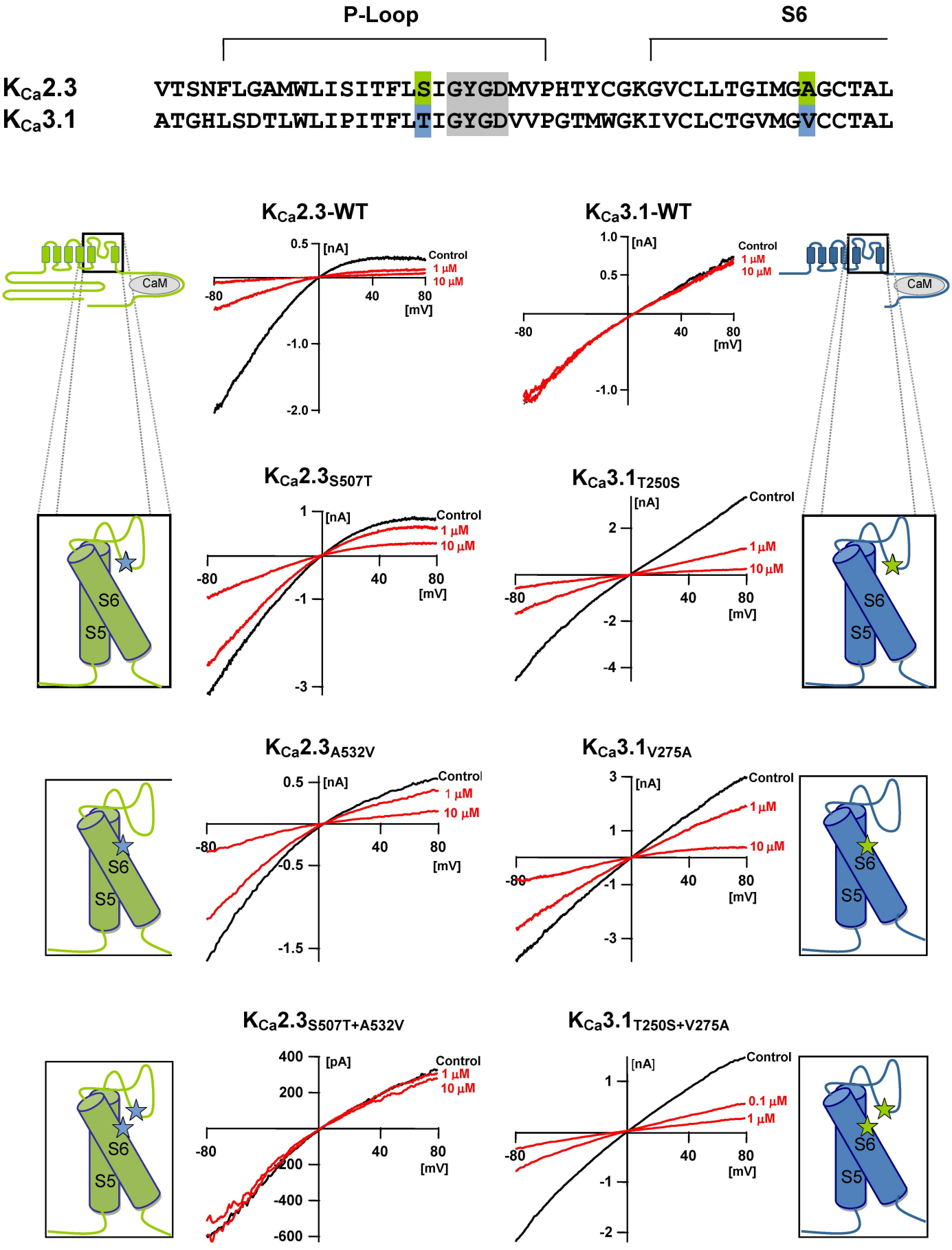

Figure 3

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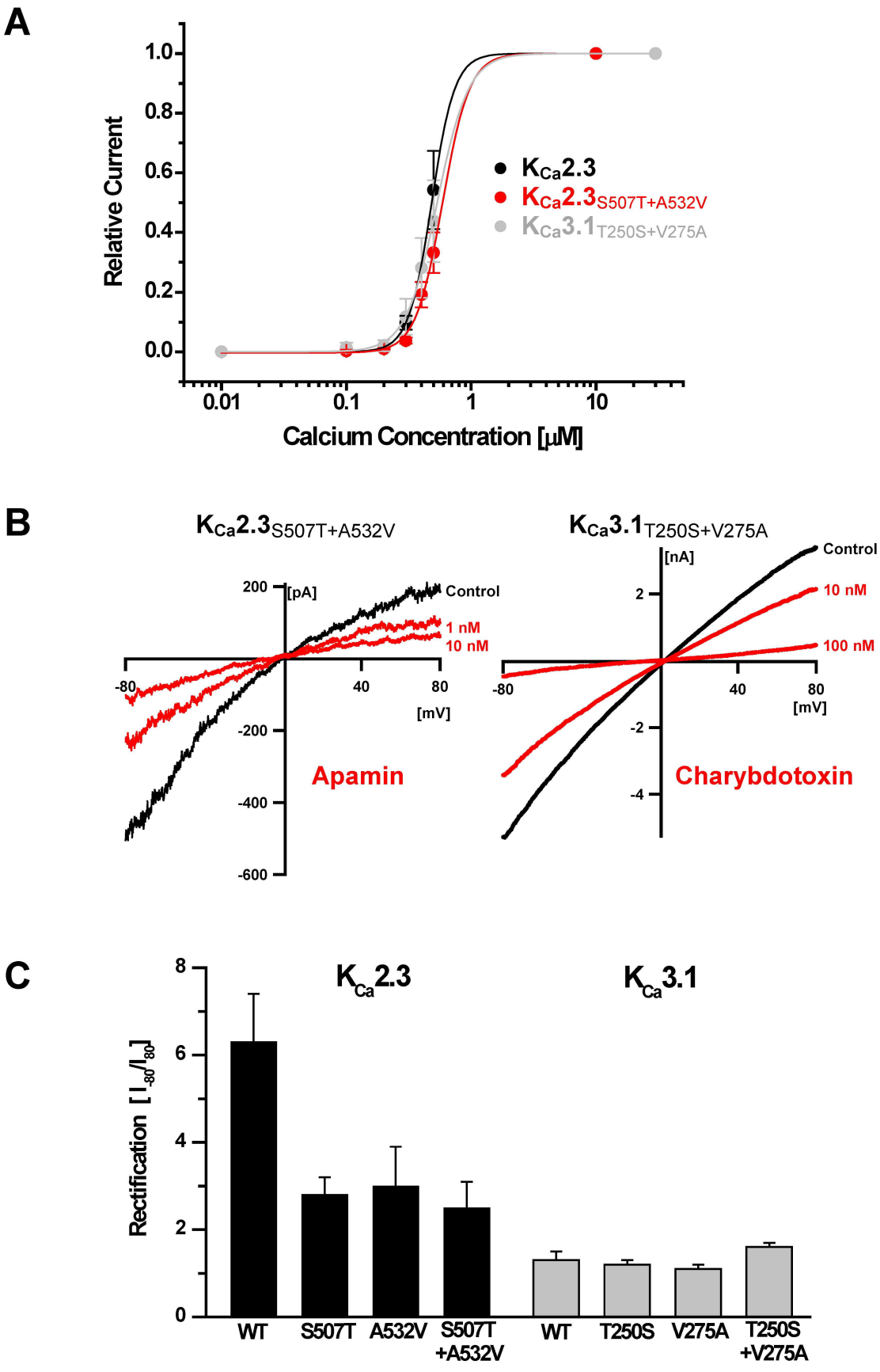

Figure 4

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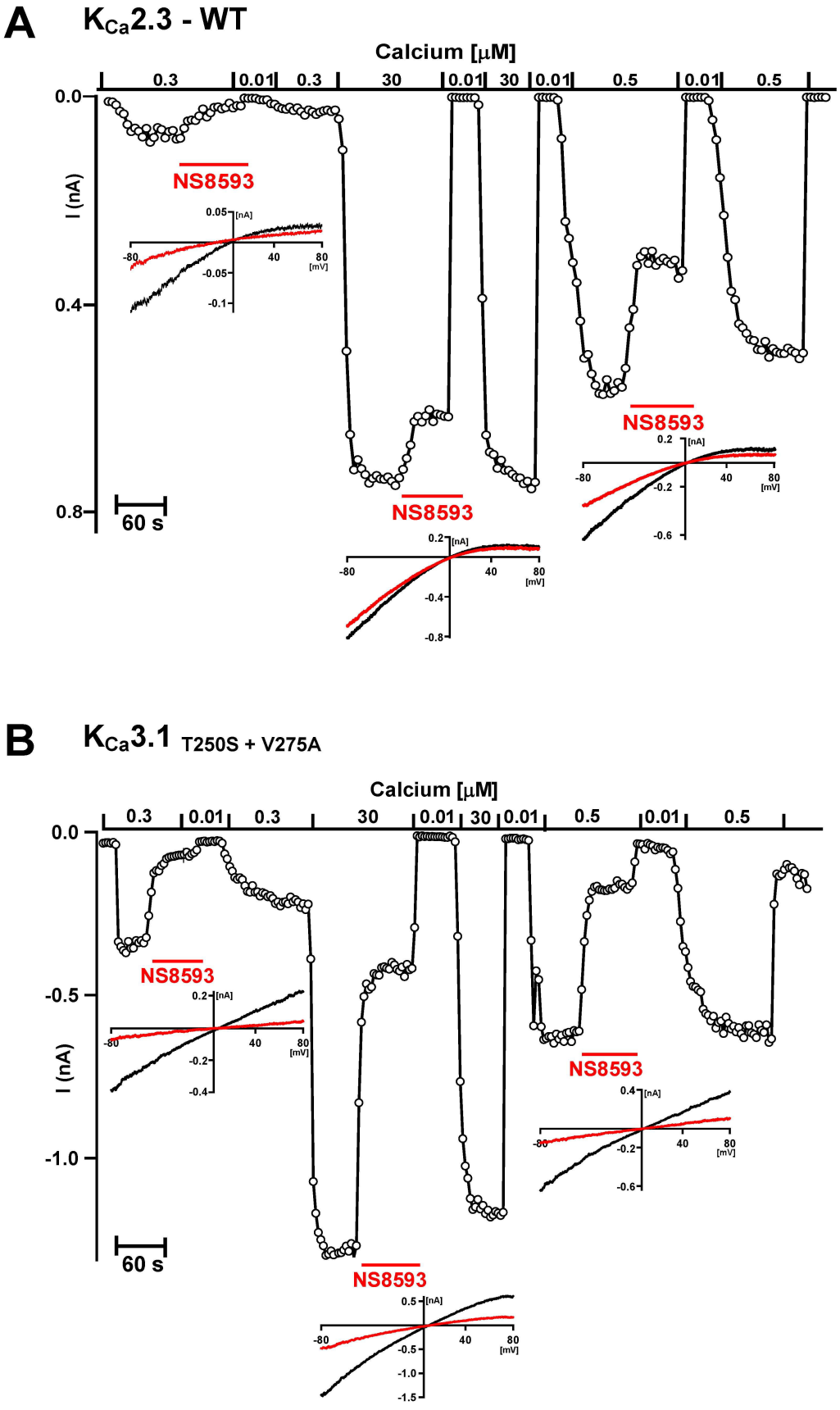

Figure 5

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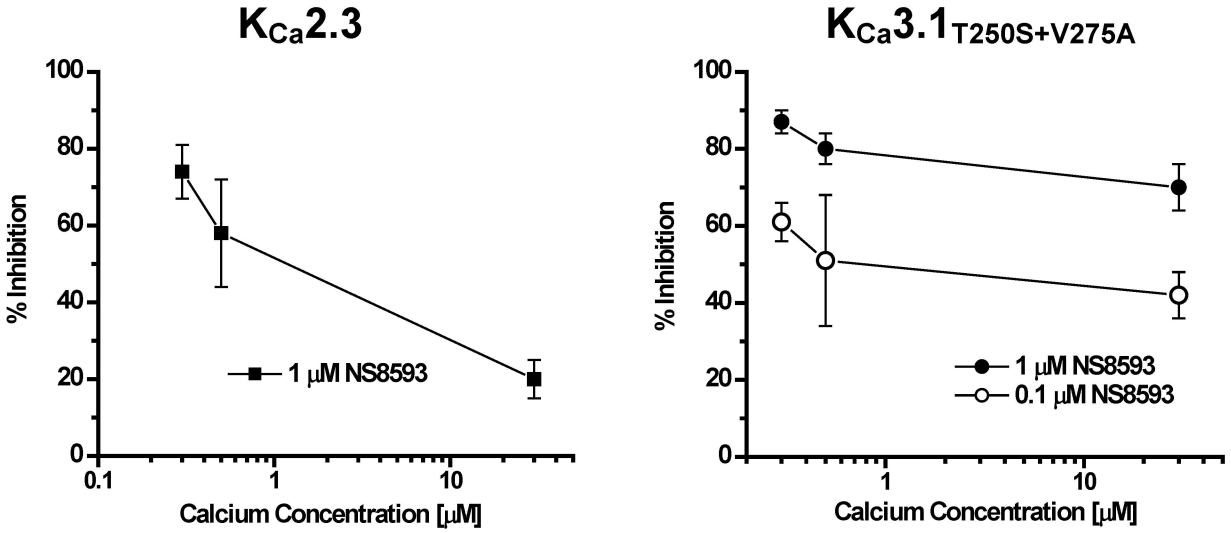

Figure 6

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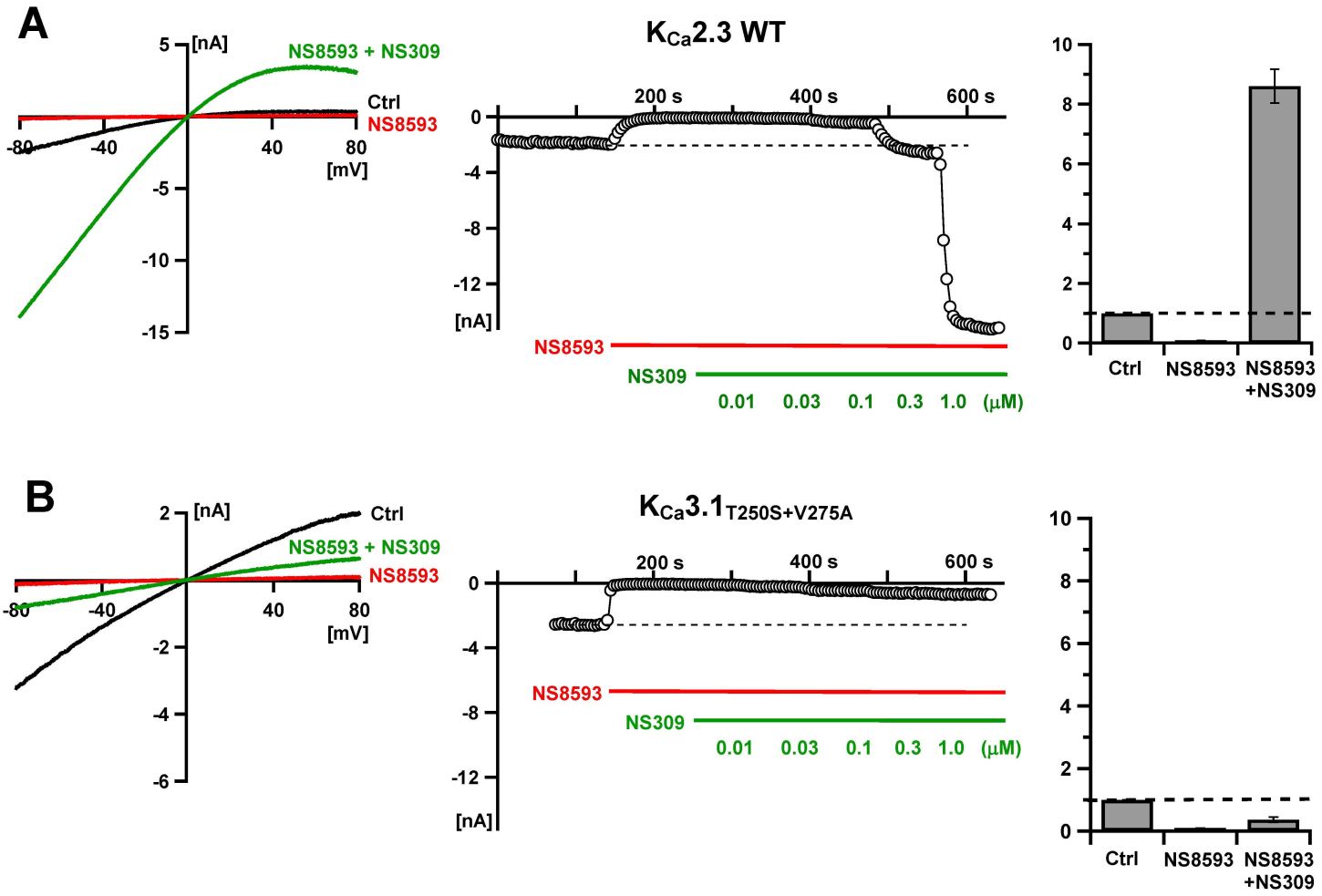

Figure 7