Characterizing the Role of Thr352 in the Inhibition of the Large Conductance Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channels by 1-[1-Hexyl-6-(methyloxy)-1\textit{H}-indazol-3-yl]-2-methyl-1-propanone

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ABSTRACT

Large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) channels are known to be regulated by both intracellular Ca\textsuperscript{2+} and voltage. Although BK channel modulators have been identified, there is a paucity of information regarding the molecular entities of this channel that govern interaction with blockers and activators. Using both whole-cell and single-channel electrophysiological studies we have characterized the possible role that a threonine residue in the pore region of the channel has on function and interaction with BK channel modulators. A threonine-to-serine substitution at position 352 (T352S) resulted in a 59-mV leftward shift in the voltage-dependent activation curve. Single-channel conductance was 236 pS for the wild-type channel and 100 pS for the T352S mutant, measured over the range −80 mV to +80 mV. In addition, there was an almost 10-fold reduction in the potency of the BK channel inhibitor 1-[1-hexyl-6-(methyloxy)-1\textit{H}-indazol-3-yl]-2-methyl-1-propanone (HMIMP), the IC\textsubscript{50} values being 4.3 ± 0.3 and 38.2 ± 3.3 nM for wild-type and mutant channel, respectively. There was no significant difference between wild type and the mutant channel in response to inhibition by iberiotoxin. The IC\textsubscript{50} was 8.1 ± 0.3 nM for the wild type and 7.7 ± 0.3 nM for the mutant channel. Here, we have identified a residue in the pore region of the BK channel that alters voltage sensitivity and reduces the potency of the blocker HMIMP.

Large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) channels are known to be regulated by both intracellular Ca\textsuperscript{2+} and voltage (Toro et al., 1998). These channels are ubiquitously expressed and have important functions in the physiology of various organs and tissue groups. These include, but are not limited to, the brain (Piwonska et al., 2008), kidney (Grunnet et al., 2005), urinary bladder (Imaizumi et al., 1998; Ohi et al., 2001), and neurons (Vergara et al., 1998). The diverse role BK channels play in physiology (Ghatta et al., 2006) and the possible link to some pathophysiological states make them tractable targets for therapeutic intervention.

Functional BK channels are formed from the coassembly of four identical α-subunits, and it is also known that the channel complex has β-subunits that are tissue-specific in their distribution (Brenner et al., 2000). BK channels are believed to play a key role in hyperpolarization in excitable cells and changes in the expression pattern of BK (Bloch et al., 2007), and mutations in the channel are associated with some disease states. In particular, a missense mutation, D434G, resulting in a gain of function, is associated with epilepsy and paroxysmal dyskinesia in a large family cohort (Du et al., 2005; Díez-Sampedro et al., 2006). Likewise, a mutation in the β\textsubscript{3}-subunit resulting in loss of function has been identified in some African-American asthmatics (Seibold et al., 2008), and carriers are believed to be at a greater risk of asthma morbidity. In the present study we limit our analysis to channels that are composed of only the α-subunit.

Amino acid residues that play critical roles in Ca\textsuperscript{2+} (Xia et al., 2002) and voltage sensing (Ma et al., 2006) have been identified, but a lot less is known about residues that interact and form binding pockets for new chemical entities. Understanding how specific residues might influence channel modulation by small molecules will therefore provide a starting point for rational drug design if this channel is to be targeted. The BK channel is a member of the class of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, which are classified based on their conductance as large, intermediate, or small. It was previously reported that a threonine-to-serine substitution mutation at position 250 in human intermediate conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel (hIK) resulted in the elimination of arachidonic acid block (Hamilton et al., 2003), and sequence alignment anal-

ABBREVIATIONS: BK, large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+}; hBK, human BK; CHO, Chinese hamster ovary; HMIMP, 1-[1-hexyl-6-(methyloxy)-1\textit{H}-indazol-3-yl]-2-methyl-1-propanone; IbTx, iberiotoxin; WT, wild type; IK, intermediate conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+}; hIK, human IK; G-V, conductance-voltage; MOE, Molecular Operating Environment.
ysis identified threonine 352 as the corresponding residue in the human BK channel (Fig. 1A).

In the current study, we investigated the effect of the T352S substitution mutation on BK channel function. We also investigated how the substitution mutation at position 352 influenced the ability of the investigational BK channel blocker 1-[1-hexyl-6-(methyloxy)-1H-indazol-3-yl]-2-methyl-1-propanone (HMIMP) (Zeng et al., 2008) to modify channel activity. Using homology modeling we identified additional residues that might contribute to the binding and hence the activity of the compound. Using site-directed mutagenesis and whole-cell electrophysiology we were able to test the relative contribution of the identified residues to a putative binding region for HMIMP.

Materials and Methods

Mutagenesis. The T352S, F380A, A381S, and V384I BKα constructs were made by doing site-directed mutagenesis using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA). T352S BKα was subcloned into pFBMNA (GlaxoSmithKline, King of Prussia, PA), which was then sequenced to confirm the presence of the desired mutation and ensure that no extra unwanted mutations were present. The plasmid was then transformed into DH10bac cells, and the BacMam virus was generated by using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA).

Cell Culture. Chinese hamster ovary (CHO) cells were transduced with the BacMam virus to express the human BKα subunit. Cells were either transduced with WT or the T352S mutant BacMam. In experiments in which function of the channel mutants for F380A, A381S, or V384I were assessed, CHO cells were transfected with the construct for WT BKα that might have occurred because of the time between experiments, and the BacMam virus was generated by using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA).

Electrophysiology. Whole-cell currents were analyzed by using pCLAMP 8 (Molecular Devices). Single-channel currents were analyzed by using Clustalw (http://www.ebi.ac.uk/Tools/clustalw2). The putative S5 transmembrane domain and the pore region of hBK and hIK were aligned with Clustalw (http://www.ebi.ac.uk/Tools/clustalw2). The putative S5 transmembrane domain is shaded in blue, and the putative pore region is shaded in red. The conserved threonine residue shown in green was mutated to serine. B, topology diagram of a single α-subunit of the BK channel. The red circle shows the location of the T352S mutation.
The se-quence alignments for the putative S5 transmembrane domain and pore region of human BK and IK channels are shown in Fig. 1A. To determine whether Thr352 had any effect on the function or the pharmacology of BK channels, site-directed mutagenesis was used to make a serine substitution; the position of the mutation is shown in Fig. 1B. To first examine the effect of the point mutation on channel function, CHO cells expressing WT BK channel and the T352S construct were examined in the whole-cell patch clamp configuration. In brief, cells were stepped from a holding potential of −60 mV to voltages in the range of −120 to +200 mV, and tail currents were then recorded at −60 mV (Fig. 2, A and B). The rate of channel deactivation was slower for mutant channels compared with their wild-type counterparts. To quantify the deactivation time constant, the decay of the tail current at −60 mV after a +140-mV depolarization step was fitted with a single exponential function. The τ of deactivation in the WT channel was 1.1 ± 0.1 ms, compared with 7.7 ± 1.7 ms for the T352S channel mutant. The significantly slower rate of deactivation of the T352S mutant implies that Thr352 might play an important role in modulating the gating kinetics and specifically the deactivation of BK channels.

Results

Threonine 352 and BK Channel Function. The sequence alignments for the putative S5 transmembrane domain and pore region of human BK and IK channels are shown in Fig. 1A. To determine whether Thr352 had any effect on the function or the pharmacology of BK channels, site-directed mutagenesis was used to make a serine substitution; the position of the mutation is shown in Fig. 1B. To first examine the effect of the point mutation on channel function, CHO cells expressing WT BK channel and the T352S construct were examined in the whole-cell patch clamp configuration. In brief, cells were stepped from a holding potential of −60 mV to voltages in the range of −120 to +200 mV, and tail currents were then recorded at −60 mV (Fig. 2, A and B). The rate of channel deactivation was slower for mutant channels compared with their wild-type counterparts. To quantify the deactivation time constant, the decay of the tail current at −60 mV after a +140-mV depolarization step was fitted with a single exponential function. The τ of deactivation in the WT channel was 1.1 ± 0.1 ms, compared with 7.7 ± 1.7 ms for the T352S channel mutant. The significantly slower rate of deactivation of the T352S mutant implies that Thr352 might play an important role in modulating the gating kinetics and specifically the deactivation of BK channels.

Threonine to Serine Substitution at Position 352 also Alters Single-Channel Conductance. We then assessed the functional implication of the threonine to serine substitution at position 352 by measuring single-channel activity in the inside-out patch configuration. In Fig. 3A, we show sample single-channel recordings from WT (left) and T352S (right) BKα channels heterologously expressed in CHO cells at −80, +20, −20, and −80 mV. Single-channel current-voltage relationship in the range of −80 to +80 mV is summarized in Fig. 3B. Consistent with the observation in whole-cell recordings, the mutant channel was activated at more negative voltages than WT. Single-channel current amplitudes were measured at negative membrane potentials (−80, −60, −40, and −20 mV) for the T352S mutant but not for WT channels because of a lack of channel opening at these voltages. The single-channel conductance determined from the slope of the fits in Fig. 3B was 236 and 100 pS for the WT and the T352S mutant channels, respectively.

Threonine to Serine Substitution at Position 352 Impairs Inhibition by HMIMP. We previously reported that HMIMP was a potent and selective inhibitor of BK channels (Zeng et al., 2008), eliciting block by increasing the close dwell time of the channels. However, we did not identify a putative binding region. The contribution of T352S to the activity of HMIMP was therefore investigated by using whole-cell recording. Currents elicited in WT BKα channels were completely blocked after cells were perfused with 100 nM HMIMP (Fig. 4A). In comparison, mutant channels were
inhibited approximately 80% at this concentration, and complete channel block was observed in the presence of 1 μM HMIMP (Fig. 4B). It should be noted that different voltage protocols (Fig. 4, A and B, insets) were used because the mutant channels were activated in a voltage range that was more negative than that in the WT channels. The concentration-response curve (Fig. 4C) revealed that there was an almost 10-fold rightward shift in the IC50 for the mutant channels (38.2 ± 3.3 nM; n = 3) compared with WT channels (4.3 ± 0.3 nM; n = 3). This observation implies that the

**Fig. 3.** Threonine to serine substitution at position 352 alters voltage sensitivity and single-channel conductance. A, single-channel current traces from WT (left) and T352S (right) recorded in symmetrical potassium solutions. Acquisitions done at +80, +20, −20, and −80 mV are shown. Arrows indicate the closed state. B, the mean current-voltage plot for single channel currents from WT (■) and T352S (□) (n = 4–6 patches).

**Fig. 4.** Threonine to serine substitution at position 352 impairs inhibition by HMIMP. A, exemplar traces of currents elicited from WT channels, using the protocol shown (inset), for control (left), after bath perfusion with 10 nM HMIMP (center), and 100 nM HMIMP (right). B, exemplar traces of currents elicited from mutant (T352S) channels, using the protocol shown (inset), for control (left), after bath perfusion with 100 nM HMIMP (center), and 1 μM HMIMP (right). Currents from cells expressing the WT channels were elicited by pulses from +40 to +140 mV in 20-mV increments from a holding potential of −80 mV. In cells expressing the T352S mutant currents were elicited by pulses from −60 to +40 mV in 20-mV increments from a holding potential of −80 mV. The time between each sweep was 2 s. C, concentration-response curves for WT (■) and T352S (□) BKα channels expressed as current inhibition at +140 mV for WT and +40 mV for the mutant in the presence of increasing concentrations of HMIMP (n = 3).
Threonine residue at position 352 may form part of the binding pocket for HMIMP, but it is also possible that the effects may be caused by allosteric modification of the BKα channel. Furthermore, we can exclude the possibility that this effect is voltage-dependent because we had previously reported that the inhibition of BK by HMIMP was not affected by voltage (Zeng et al., 2008).

**Threonine to Serine Substitution at Position 352 Has No Effect on Iberiotoxin Activity.** Next, we investigated the effect that the residue at position 352 might have on the activity of the known BK channel blocker IbTx (Galvez et al., 1990; Giangiacomo et al., 1992). Assessing channel activity in the whole-cell patch clamp configuration we found that there was no significant difference in the inhibition of the WT and mutant channels, and there was more than 90% inhibition after bath perfusion with 300 nM IbTx in both cases (Fig. 5, A and B). As for the experiments with HMIMP, inhibition of WT channel activity was assessed at +140 mV and for the T352S mutant channel inhibition was assessed at +40 mV. The inability of IbTx to differentiate between the WT and T352S channels is probable evidence that the site of action for IbTx is not localized to the selectivity region of the pore where the threonine residue is thought to reside. The IC_{50} values for blockade by IbTx were found to be 8.1 ± 0.3 (n = 3) and 7.7 ± 0.3 nM (n = 3) for the WT and mutant channels, respectively (Fig. 5C).

**Homology Modeling of HMIMP Docking.** A homology model of the pore domain of the human BKα channel was created by using the crystal structure of MTHK as the template. The resulting model was used to investigate possible binding modes of the small-molecule inhibitor HMIMP. The modeled pore domain binding pocket is formed by the four Thr352 residues from each of the constituent helical bundles creating a polar bottom surface. The remainder of the pore pocket is lined with hydrophobic residues Phe380, Ala381, and Val384. The ligand is characterized by having a more polar lower portion, formed primarily by the carbonyl and methoxy oxygen atoms that can function as hydrogen-bond acceptors and a hydrophobic upper portion formed by the pendant alkyl chain. This is shown by the Connolly surface color-coded according to hydrogen bonding (magenta), polar (blue), and hydrophobic (green) domains in Fig. 6B. Consequently, the ligand was oriented in the pocket of the pore domain model with the polar oxygen-rich domain directed toward the threonine residues that could potentially act as hydrogen-bond donors. The protein–ligand complex was subsequently optimized with the MOE implementation of the MMFFx force field, where the anticipated interactions were indeed observed (Fig. 6C). The ligand forms hydrogen bonds with two of the Thr352 residues in the pocket, one via the carbonyl oxygen and the other by means of the methoxy oxygen. The interactions are shown in Fig. 6D, left, where the alkyl moieties are oriented toward the hydrophobic protein residues and the solvent front. This orientation of the ligand is an excellent match to the desired complementary interactions sought by the protein, as calculated by the MOE contact preference algorithm. Not surprisingly, the protein seeks a binding partner that is polar in nature around the threonine residues (Fig. 6D, right, magenta contour) and hydrophobic in the upper domains of the pore pocket (Fig. 6D, right, green contours).

**Role of Phe380, Ala381, and Val384 in the Activity of HMIMP.** Based on homology modeling, Phe380, Ala381, and Val384 were the amino acid residues that were shown to form part of the binding pocket for HMIMP. To deter-
mine the role played by these residues, three individual point mutants were designed and tested for channel activity and to ascertain the effect of HMIMP.

Site-directed mutagenesis was used to generate F380A, A381S, and V384S. Mutation of two of these residues, A381S and V384I, resulted in functional channels; a third mutant, F380A, was functionally silent. A381S mutant channels had similar voltage-dependent activation compared with T352S mutant, and the current was recorded in the range of $-60$ to $+40$ mV, whereas V384I mutant channels resembled WT in voltage-dependent activation and the current was recorded in the range of $+40$ to $+140$ mV. For both A381S and V384I channel mutants, the HMIMP potency was affected only slightly compared with the WT BK oxidations.
channels (Fig. 7). The IC\textsubscript{50} was 19.3 ± 2.9 nM (n = 5) for A381S and 8.9 ± 1.7 nM (n = 5) for V384I versus 13.1 ± 1.9 nM (n = 3) for WT.

Discussion

Substitution of a threonine residue at position 352 in BK\(\alpha\) channels reveals that this position in the selectivity region of the channel may play an important role in voltage sensitivity and the blocking activity of the compound HMIMP (Zeng et al., 2008). The corresponding threonine residue in IK channels was previously identified to be a molecular determinant of blockade of this channel by arachidonic acid (Hamilton et al., 2003). We therefore wanted to determine whether this threonine residue might play any role in the function and pharmacology of BK channels. Our findings indicated that this was indeed the case. The possible involvement of Thr352 in the gating mechanism of the channel is highlighted by the fact that a substitution mutation of threonine to serine revealed a channel that had slower deactivation kinetics. In fact, the deactivation time constant was 7-fold greater for the mutant compared with the wild-type channel.

The threonine to serine mutation also seemingly altered the voltage sensitivity of the channel compared with the WT. We observed a leftward shift of 59 mV for the half-maximal activation voltage when WT and mutant channels were compared under the same conditions. This shift in voltage sensitivity was confirmed at the single-channel levels where mutant channel openings were recorded at a voltage as low as −80 mV. In addition, the single-channel conductance of the mutant was approximately half that of the WT, 100 and 236 pS, respectively, giving rise to a highly voltage-sensitive and low conductance channel.

In assessing the ability of HMIMP, a potent blocker of BK channels whose mechanism of action we previously characterized (Zeng et al., 2008), to inhibit the mutant channel we found that there was an almost 10-fold shift in the IC\textsubscript{50} indicative of the role played by this region of the channel on compound activity. Threonine and serine are related amino acids, and because such a marked change was observed for the IC\textsubscript{50} values it can be implied that the binding of HMIMP is highly specific. In fact, differences in the overall structural topology between the wild-type and serine 352 mutant homology models are minimal and do not provide a rationale for the observed change in binding affinity of HMIMP. However, there are differences in the rotamer preferences between the threonine and serine side chains that may provide a plausible explanation (http://www.chemcomp.com/journal/rotxexpl.htm). In the binding model, HMIMP interacts with two threonine residues, forming hydrogen bonds through the hydroxyl proton to either the carbonyl or methoxy oxygen atoms. The Thr352 residue interacting with the carbonyl oxygen is the low-energy rotamer, whereas the methoxy-interacting threonine rotamer is 1.5 kcal/mol higher than the low-energy conformation. The Ser352 mutant model requires a much higher energy cost for the side-chain hydroxyls to adopt the binding interaction conformations. The Ser352 interacting with the carbonyl and methoxy oxygen atoms are 3.94 and 6.92 kcal/mol higher than their low-energy rotamers, respectively. Thus, the difference in binding affinity of HMIMP between wild-type and Ser352 mutant may be a function of the energy cost of the amino acid side chains adopting the required ligand interaction conformation. This specificity is further highlighted by the fact that IbTx inhibition of the channel was not affected by this mutation, and the binding region for IbTx was previously identified and is apparently distinct from that of HMIMP (Gianniacomo et al., 1992).

At this point we want to point out that, of the other residues identified to be part of the binding pocket for HMIMP, Ala381 and Val384 seemed to have only minor effect on the inhibitory actions of the compound compared with Thr352. We were unable to characterize the possible role of Phe380 because the generated mutant did not form a functional channel.

Here, we have reported the characterization of the possible role of a threonine residue at position 352 in the pore region of the BK channel. Our findings reveal that this residue might play an important role in the activation and the deactivation mechanism of the channel and its pharmacology. These findings could be the basis for further studies that are geared toward a clear understanding of the structure activity interactions that might be useful for rationale drug design if the BK channel is to be targeted.

References


