Molecular Localization of the Inhibitory Arachidonic Acid Binding Site to the Pore of hIK1*

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We previously demonstrated that the endogenously expressed human intermediate conductance, Ca2+-activated K+ channel (hIK1) was inhibited by arachidonic acid (AA) (Devor, D. C., and Frizzell, R. A. (1998) Am. J. Physiol. 274, C138–C148). Here we demonstrate, using the excised, inside-out patch-clamp technique, that hIK1, heterologously expressed in HEK293 cells, is inhibited 82 ± 2% (n = 16) with 3 μM AA, being half-maximally inhibited (IC50) at 1.4 ± 0.7 μM. In contrast, AA does not inhibit the Ca2+-dependent, small conductance K+ channel, rSK2, another member of the KCNN gene family. Therefore, we utilized chimeric hIK1/rSK2 channels to define the AA binding domain on hIK1 to the SS-Pore-S6 region of the channel. Subsequent site-directed mutagenesis revealed that mutation of Thr250 to Ser (T250S) resulted in a channel with limited sensitivity to block by AA (8 ± 2%, n = 8), demonstrating that Thr250 is a key molecular determinant for the inhibitory activity of hIK1 by AA. Likewise, when Val275 in S6 was mutated to Ala (V275A) AA inhibited only 43 ± 11% (n = 9) of current flow. The double mutation T250S/V275A eliminated the AA sensitivity of hIK1. Introducing the complimentary single amino acid substitutions into rSK2 (S359T and A384V) conferred partial AA sensitivity to rSK2, 21 ± 3% and 31 ± 3%, respectively. Further, introducing the double mutation S359T/A384V into rSK2 resulted in a 63 ± 8% (n = 9) inhibition by AA, thereby demonstrating the ability to introduce this inhibitory AA binding site into another member of the KCNN gene family. These results demonstrate that AA interacts with the pore-lining amino acids, Thr250 and Val275 in hIK1, conferring inhibition of hIK1 by AA and that AA and clotrimazole share similar, if not identical, molecular sites of interaction.

Intermediate conductance, Ca2+-activated K+ channels play crucial roles in a wide array of physiological processes, including agonist-mediated transepithelial Cl– secretion across airway and intestinal epithelia (1–6). Indeed, Dharmasathaphorn and Pandol (7) initially proposed that Ca2+-mediated intestinal Cl– secretion was dependent upon the activation of a basolateral membrane K+ conductance in the absence of a change in apical membrane Cl– conductance. As a consequence of the increased K+ conductance there would be a hyperpolarization of the membrane potential, thereby increasing the electrochemical driving force for Cl– exit across the apical membrane via constitutively active Cl– channels (7, 8). An increase in intracellular Ca2+ alone can stimulate Cl– secretion across the colonic epithelial cell line, T84 (5, 9). Yet, there is a disparity between the magnitude and time course of the rise in intracellular Ca2+ and the subsequent Cl– secretory response (1, 4) such that the Cl– secretory response is transient in nature. These data suggest that second messengers other than Ca2+ may down-regulate the secretory response. Various inhibitory second messengers have been proposed, including protein kinase C (5, 10, 11), inositol tetrakisphosphate (12, 13), and arachidonic acid (AA) (2, 14, 15). It is proposed that these second messengers may inhibit the K+ conductance, thus modulating the Cl– secretory response even in the presence of elevated intracellular Ca2+ levels.

Ca2+-mediated agonists are known to increase AA levels in a wide range of tissues where hIK1 is expressed, including colon and lung (14, 16–18). This can occur in several ways (19), including: 1) Ca2+ directly activating PLA2, 2) either diacylglycerol itself or protein kinase C activating PLA2, or 3) diacylglycerol lipase directly generating AA from diacylglycerol. Thus, the generation of AA by Ca2+-mediated agonists would be expected to lag behind the rise in intracellular Ca2+. Given this, an effect of AA on hIK1 would be temporally appropriate to explain the dissociation between changes in intracellular Ca2+ and the resultant Cl– secretory response. Likewise, AA is released during inflammatory responses such as asthma (20) and irritable bowel disease (21) such that AA may play an important role in modulating ion channels in these diseases.

Arachidonic acid has been shown to modulate a wide variety of ion channels, including K+, Na+, Ca2+, and Cl– channels (22–25). Indeed, we previously demonstrated that inhibition of cytosolic PLA2 resulted in a potentiated Cl– secretory response to the Ca2+-mediated agonist, carbachol in T84 cells and that AA was a potent negative modulator of the intermediate conductance, Ca2+-dependent K+ channel in these cells (2). Recently, we (26) and others (3) have confirmed the molecular identity of this colonic epithelial K+ channel as being the recently cloned hIK1/hSK4 (27, 28). hIK1 is a member of the KCNN gene family, exhibiting significant homology with the SK channels (SK1–3), having 40% identity at the amino acid level (27, 28).

In the present study, we demonstrate that AA directly inhibits heterologously expressed hIK1, whereas rSK2 is insensitive to AA inhibition. This inhibitory activity of AA may be due to the availability of the Ca2+ channel.

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to AA. Using a series of hIK1/rSK2 chimeras and point mutations we demonstrate that AA inhibits hIK1 via an interaction with two pore-lining amino acids, Thr250 and Val275. Importantly, substitution of these amino acids into rSK2 induces sensitivity to AA, confirming the critical nature of these amino acids in defining the molecular binding site for AA on hIK1.

**MATERIALS AND METHODS**

**Cell Culture**—Human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified 5% CO2/95% O2 incubator at 37 °C. Cells were transfected using LipofectAMINE 2000 (Invitrogen) following the manufacturer’s instructions. Stable cell lines were generated for all constructs by subjecting cells to antibiotic selection (1 mg/ml G418) 48 h post-transfection. Selection was typically complete within 14 days post-transfection. The chimeras were subcloned into the pcDNA 3.1 vector using EcoRI and XhoI restriction sites. Point mutations were generated using Pfu polymerase (Invitrogen). The fidelity of all constructs utilized in this study was characterized the AA sensitivity of heterologously expressed hIK1 in T84 cells with high affinity (2). Therefore, we initially characterized the AA sensitivity of heterologously expressed hIK1 in HEK293 cells as excised, inside-out patches. As shown in a representative experiment in Fig. 1B, following the establishment of a stable current, the addition of AA (3 μM) induced a significant reduction in current. The average current prior to AA

**Electrophysiology—Inside/Out Patch-Clamp Experiments**—The effects of AA on hIK1, rSK2, chimeric, and point-mutation channels were assessed with excised, inside-out patch-clamp experiments as a functional assay. Currents were recorded using a List EPC-7 amplifier (Medical Systems, Greenvile, NY) and stored on videotape for later analysis. Electrodes were fabricated from thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL), pulled on a vertical puller (Narishige, Long Island, NY), fire-polished, and had a resistance of 1–4 MΩ. During patch-clamp experiments, the bath solution was (in mM) potassium glutonate 145, KCl 5, MgCl2 1, HEPES 10, CaCl2 10, pH 7.2 (adjusted with KOH). To obtain a Ca2+-free bath solution EGTA (1 mM) was added without CaCl2 (estimated free Ca2+ <10 nM). All bath solutions contained 100 μM 1-ethyl-2-benzimidazolinoine to maximally activate channels and 300 μM ATP to prevent channel rundown (31). The pipette solution was (in mM) potassium glutonate 140, KCl 5, MgCl2 1, HEPES 10, CaCl2 1, pH 7.2 (adjusted with KOH). The chimeras tested were strictly Ca2+-sensitive as a Ca2+-free bath solution eliminated all channel activity. Generally, a 3-μM concentration of AA was used (except for the concentration-response experiments). Constructs that were not sensitive to block by AA were always tested in parallel with hIK1 or a chimera known to be sensitive to AA as a positive control. All experiments were performed at room temperature. All patches were held at a holding potential of −100 mV. The voltage is referenced to the extracellular compartment, as is the standard method for membrane potentials. Inward currents are defined as the movement of positive charge from the extracellular compartment to the intracellular compartment and are presented as downward deflections from the baseline in all recordings. Channel data were digitized with the Fetchex application within the pCLAMP suite of programs (version 6.04, Axon Instruments, Foster City, CA) using a PC computer. Single channel analysis was performed on records after low pass filtering at 200 Hz and sampling at 500 Hz (Digidata 1200, Axon Instruments). Total channel current was determined using Biopatch software (version 3.3, Bio-Logic).

**Chemicals**—1-Ethyl-2-benzimidazolinoine, AA, 5,8,11,14-eicosatetraynoic (ETYA), clotrimazole, and all general chemicals were obtained from Sigma, unless otherwise stated. ATP was purchased from Roche Applied Science and added directly to a Ringer’s solution as a dry powder. 1-Ethyl-2-benzimidazolinoine, AA, and clotrimazole were made as 10,000-fold stock solutions in Me2SO.

**Statistics**—All data are presented as means ± S.E., where n indicates the number of experiments. Statistical analysis was performed using a Student’s t test (paired or unpaired) or analysis of variance with Student-Newman-Keuls multiple posttest. A value of p < 0.05 is considered statistically significant and is reported.

**RESULTS**

**Arachidonic Acid Inhibits Heterologously Expressed hIK1**—We previously demonstrated that AA inhibits endogenously expressed hIK1 in T84 cells with high affinity (2). Therefore, we initially characterized the AA sensitivity of heterologously expressed hIK1 (Fig. 1A) in HEK293 cells in excised, inside-out patches. As shown in a representative experiment in Fig. 1B, following the establishment of a stable current, the addition of AA (3 μM) induced a significant reduction in current. The average current prior to AA...
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FIG. 2. Effect of arachidonic acid on rSK2. A, schematic of rSK2. B, representative current (pA) trace of the effect of arachidonic acid (3 μM) on rSK2. rSK2 was heterologously expressed in HEK cells and recorded in excised, inside-out patches at a holding potential of −100 mV. 0 Ca²⁺ steps occurred at the beginning and the end of the experiment to illustrate the Ca²⁺ dependence of rSK2.

was 468 ± 60 pA, which was reduced by 82 ± 2% to 81 ± 18 pA in the presence of AA (p < 0.001, n = 16). Arachidonic acid inhibited hIK1 in a concentration-dependent manner, being half-maximal (IC₅₀) at 1.4 ± 0.7 μM (n = 5, Fig. 1C), a value similar to what we previously reported for endogenously expressed hIK1 (IC₅₀ = 0.42 μM) in T84 cells (2).

Direct Effect of Arachidonic Acid on hIK1—Arachidonic acid can elicit its effect by interacting directly with ion channels or indirectly by metabolites produced via cyclo-oxygenase (COX), lipooxygenase (LOX), and/or cytochrome P450 pathways (22, 23). We previously demonstrated that inhibitors of COX, LOX, and cytochrome P-450 did not affect the AA-dependent inhibition of endogenously expressed hIK1 in T84 cells (2). We confirmed these results in the present study by examining the effects of AA in the presence of ETYA (10 μM), a blocker of COX, LOX, and P450 pathways (32, 33) using excised, inside-out patches. As shown in Fig. 1D, ETYA had no effect on hIK1 current. Under control conditions channel current was 622 ± 83 pA, and ETYA did not significantly reduce this current (560 ± 94 pA). However, subsequent addition of AA (3 μM) reduced channel current by 77% (117 ± 26 pA, p < 0.01, n = 3) in the continued presence of ETYA. These data suggest that oxidative metabolites of AA are not involved in the inhibition of hIK1 observed.

rSK2 Is Insensitive to Inhibition by Arachidonic Acid—Since hIK1 shares ~40% sequence homology with rSK2 (27, 28) we examined whether rSK2 could be inhibited by AA in excised, inside-out patches. A representative experiment is shown in Fig. 2B in which AA did not reduce channel current in HEK cells heterologously expressing rSK2. The average channel current was 491 ± 169 pA in the absence of AA and 438 ± 136 pA in the presence of AA (n = 4), representing an 8 ± 3% inhibition of current flow. The Ca²⁺ dependence of rSK2 was subsequently verified by demonstrating that our 0 Ca²⁺ solution reduced channel current to zero (Fig. 2B). These data demonstrate that even though hIK1 and rSK2 share ~40% sequence homology AA sensitivity is specific to hIK1.

Arachidonic Acid Sensitivity of hIK1 Is Localized to the S5-Pore-S6 Region—Based on our observation that AA inhibits hIK1, while having no effect on rSK2, we used a chimeric hIK1/rSK2 strategy to identify the region of the channel that confers AA sensitivity to hIK1. Wang and co-workers (34) have demonstrated that the NH₂ terminus (particularly Ser⁵) of ROMK1 plays a key role in the determination of the AA effect on that channel. hIK1 has a serine (Ser⁵) just prior to S1. Thus, we initially generated the chimera 26IK-SK (Met⁴-Ala⁶ of hIK1 with Asp¹⁸⁷-Ser⁵⁰⁸ of rSK2; schematic diagram in Fig. 3A) in which only the cytoplasmic NH₂ terminus is derived from hIK1. As shown for one experiment in Fig. 3A (right panel), after a sustained current was established, AA had little effect (7 ± 3% inhibition, Fig. 3E) on this construct; however, 0 bath Ca²⁺ eliminated channel current. In five experiments, the channel current prior to the addition of AA was 296 ± 16 pA and 277 ± 17 pA in the presence of AA. The absence of inhibition of the 26IK-SK channel suggests that the cytoplasmic NH₂ terminus of hIK1 is insufficient to confer AA sensitivity to rSK2.

FIG. 3. S5-pore-S6 region of hIK1 confers arachidonic acid sensitivity. Schematics of the 26IK-SK (A), SK-287IK (B), 200IK-SK (C), and SK-200IK (D) chimeras in which open circles represent amino acids from hIK1 and solid circles represent amino acids from rSK2. Single amino acid nomenclature and arrows indicate the hIK1 amino acid that defines the chimeric junction. Also shown are representative current traces of the 26IK-SK to 3 μM AA. E, percentage inhibition summary data for all experiments for the chimeric channels are given (number of experiments shown above the bars). Data for hIK1 and rSK2 are also presented for comparison. Chimeras were heterologously expressed in HEK cells and recorded in excised inside-out patches at a holding potential of −100 mV. 0 Ca²⁺ steps occurred at the beginning and the end of the experiment to illustrate the Ca²⁺ dependence of the chimeric channels.
Fig. 4. Amino acid sequence of hIK1 and rSK2 for the S5 linker-pore region. The amino acid sequences for hIK1 (Gln295-Cys307) and rSK2 (Asp355-Cys367) are provided in single-letter code. The selectivity filter (GYGD) is indicated by a dashed box while the pore-helix is indicated by an open box. The S5 and S6 transmembrane domains are prior to Gln295 and subsequent to Cys367, respectively. Amino acid residues that are not similar for hIK1 and rSK2 are in bold type. The underlined residues comprise the mutated hIK1 constructs in which the appropriate aligned residue of rSK2 was substituted into hIK1. These mutations include: GHL/SNF, SDTL/LGAM, VGMW/MNYC, and T250S.

Kim et al. (35) reported that the COOH terminus is important in the AA sensitivity of TREK-2, a two-pore, four-transmembrane domain K+ channel. To elucidate the role of the COOH terminus in the AA sensitivity of hIK1, we generated the chimeric channel SK-287IK (Met1—Met200 of hIK1 with Arg287—Lys427 of hIK1; see Fig. 3B for schematic) in which only the cytoplasmic COOH terminus is derived from hIK1. As shown in Fig. 3B (right panel), AA did not alter the channel current of the SK-287IK channel. In six excised, inside-out patch-clamp experiments, the average current was 467 ± 169 pA in the absence of AA and 472 ± 176 pA in the presence of AA. The failure of AA to reduce the activity of the SK-287IK channel suggests that the cytoplasmic COOH terminus of hIK1 is not sufficient to confer AA sensitivity to rSK2.

To determine whether the S1-S4 or pore region of hIK1 was important in the AA sensitivity, we generated two additional channel constructs that altered the first half of hIK1 and rSK2. The first construct, 200IK-SK (Met1—Met200 of hIK1 with Thr250—Ser359 of rSK2; see Fig. 3C for schematic) is composed of the entire NH2 terminus to the beginning of S5 of hIK1, while prior to Gln229 and subsequent to Cys267, respectively. Amino acid residues comprise the mutated hIK1 constructs in which the appropriate aligned residue of rSK2 was substituted into hIK1. These mutations include: GHL/SNF, SDTL/LGAM, VGMW/MNYC, and T250S.

Multiple Amino Acid Mutations of the S5 Linker-Pore Region of hIK1—To identify the specific amino acid residue(s) responsible for the inhibition of hIK1 by AA, we made selected amino acid mutations in hIK1 with their rSK2 amino acid counterparts. The amino acid alignment of the S5 linker-pore region is shown in Fig. 4. The amino acids that are different between hIK1 and rSK2 are shown in bold type. Initially, three multiple amino acid mutations of hIK1 were generated. These mutations are shown in Fig. 4 (underlined) and include: GHL (G235S/H236N/L237P), SDTL (S238L/D239G/T240A/L241M), and VGMW (V256M/G258N/M261Y/W262C). Fig. 5 illustrates representative current traces for GHL/SNF (5A), SDTL/LGAM (5B) and VGMW/MNYC (5C). For each of these channels, AA significantly (p < 0.05) inhibited current flow (GHL/SNF, 623 ± 199 pA to 133 ± 49 pA, n = 5, Fig. 5A; SDTL/LGAM 494 ± 115 pA to 83 ± 19 pA, n = 6, Fig. 5B; VGMW/MNYC 246 ± 85 pA to 53 ± 19 pA, n = 5, Fig. 5C). These data are summarized in the bar graph shown in Fig. 5D and suggest that these amino acids are not important in conferring the AA sensitivity of hIK1.

Mutations of Thr250 and Val275 Selectively Abolish AA Sensitivity of hIK1—We next mutated the threonine (Thr250) just prior to the K+ selectivity filter GYG (Gly-Tyr-Gly) motif of hIK1 to serine (T250S-hIK1), which is present in rSK2 (see Fig. 4). A representative excised patch-clamp experiment of this construct is shown in Fig. 5D. The T250S-hIK1 channel possessed significantly reduced (p < 0.05) AA sensitivity compared with hIK1 (Fig. 5B). Arachidonic acid reduced the channel current of this construct by only 8 ± 2% from 304 ± 59 pA to 280 ± 62 pA (Fig. 5F, n = 8). The effect of AA on the T250S-hIK1 channel was not different than that for rSK2 (8 ± 3% inhibition, Fig. 2). These data suggest that Thr250 is the critical amino acid responsible for conferring the AA sensitivity to hIK1.

It is interesting to note that Chandy and co-workers (36) previously reported that Thr250, in combination with Val275 (within S6), of hIK1 are crucial in conferring the clozapine sensitivity of hIK1. Based on the crystal structure of the KCa3.1 channel (37), Chandy and co-workers postulated that Thr250 and Val275 line the water-filled pocket that lies just below the narrow K+ selectivity filter of hIK1. In light of the data from our T250S-hIK1 construct, it was of interest to determine whether Val275 played a similar role in the AA sensitivity of hIK1. Therefore, we generated separate hIK1 constructs with V275A or the double mutation T250S/V275A to assess the effect of these amino acids on the AA sensitivity of hIK1. A representative experiment for the V275A-hIK1 construct is shown in Fig. 5E. Although AA significantly inhibited 43 ± 11% (315 ± 81 pA and 165 ± 37 pA in the absence and presence of AA, respectively, n = 9; p < 0.05) of the V275A current (Fig. 5F) this inhibition is significantly less than the 82 ± 2% inhibition observed for wild type hIK1 (p < 0.05). These data suggest that Val275 is also contributing to the inhibition of hIK1 by AA.

Lastly, we predicted that the double mutated T250S/V275A-hIK1 channel would be relatively insensitive to AA. Similar to the T250S channel construct, the double-mutated channel was not sensitive to AA, with current averaging 191 ± 39 pA and 192 ± 47 pA (n = 4) in the absence and presence of AA, respectively (Fig. 5F). Clearly, these data suggest that the amino acids Thr250 and Val275 are critical in conferring the AA sensitivity of hIK1.

Can the Thr250 and Val275 Substitutions Confer AA Sensitivity to rSK2?—If Thr250 and Val275 are crucial for AA inhibition of hIK1, then we hypothesized that introducing these amino acids at the appropriate amino acid alignment positions in rSK2 would cause rSK2 to become sensitive to AA inhibition. Thus, we made separate rSK2 constructs in which Ser359 was mutated to threonine (S359T-rSK2), Ala384 was mutated to valine (A384V-rSK2), or the double mutation S359T/A384V-rSK2. A representative experiment of the S359T-rSK2 channel is shown in Fig. 6A. The average channel current prior to the addition of AA was 277 ± 27 pA, which was reduced (p < 0.001) to 223 ± 30 pA in the presence of AA, indicative of a 21 ± 3% inhibition of channel current (Fig. 6D, n = 4). This inhibition is significantly greater than the 8 ± 3% inhibition observed for wild-type rSK2 (Fig. 2), indicating that the S359T mutation confers a partial AA binding site to rSK2. Similarly, the A384V-
Arachidonic Acid and Clotrimazole Share Overlapping Inhibitory Sites—As noted above, our results suggest that AA and clotrimazole share similar molecular sites of interaction, resulting in block of hIK1. That is, Chandy and co-workers (36) previously demonstrated that T250 and V275 in hIK1 were critical for inhibition by clotrimazole. Similarly, we demonstrate that these amino acids play a crucial role in the inhibition of hIK1 by AA. Indeed, substituting these amino acids into their corresponding positions in rSK2 (S359T/A384V) results in the generation of an AA-sensitive rSK2 channel. To confirm the extent of molecular overlap between the AA and clotrimazole inhibitory sites we similarly evaluated the sensitivity of hIK1, rSK2, and the double mutations hIK1-T250S/V275A and rSK2-S359T/A384V to 3 μM clotrimazole. As shown for one representative experiment in Fig. 7A, clotrimazole dramatically inhibited hIK1, similar to what has been previously reported (27, 36, 38, 39). In five experiments, clotrimazole inhibited hIK1 an average of 94 ± 3%, from 379 ± 171 pA to 17 ± 7 pA (p < 0.01, Fig. 7E). In contrast, in patches containing rSK2 the current averaged 571 ± 279 pA, and this was not inhibited by clotrimazole (540 ± 262 pA; 3 ± 2%, n = 6; Fig. 7, B and E), as previously reported (36). Similar to what was reported by Chandy and co-workers (36), we demonstrate that the effect of clotrimazole on hIK1-T250S/V275A is significantly reduced compared with wild-type hIK1 (p < 0.01, Fig. 7C). That is, on average clotrimazole reduced (p < 0.01) the current an average of only 31 ± 8%, from 64 ± 30 pA to 45 ± 25 pA (n = 5, Fig. 7E).
Finally, as shown in Fig. 7, D and E, we demonstrate that mutating Ser\(^{359}\) and Ala\(^{384}\) in rSK2 to their corresponding amino acids in hIK1 (rSK2-S359T/A384V) confers clotrimazole sensitivity onto rSK2. In six patches, clotrimazole inhibited 92/11006 of current flow across patches expressing rSK2-S359T/A384V from an average of 80/11006.0 pA to 3/11006.1 pA (p < 0.01).

These results confirm previous studies (36) and further demonstrate that identical pore lining amino acids are required to confer both AA- and clotrimazole-dependent inhibition of hIK1.

DISCUSSION

Arachidonic acid is an ubiquitous second messenger released in response to both Ca\(^{2+}\)-and cAMP-mediated agonists, including acetylcholine (40, 41), UTP (42–45), bile acids (17), adenosine (16), and vasoactive intestinal polypeptide (14). Inflammatory responses are also characterized by significant increases in AA production, both via direct release from inflammatory cells (20) as well as through Ca\(^{2+}\)-dependent, kinin-mediated generation (18). Given the crucial role of ion channels in generating the physiological as well as patho-physiological responses to these agonists it is not surprising that K\(^{+}\), Na\(^{+}\), Ca\(^{2+}\), and Cl\(^{-}\) channels have all been demonstrated to be modulated by either AA directly or one of its oxygenated metabolites (22, 23).

In this regard, we previously demonstrated that hIK1, endogenously expressed in the colonic cell line T84, was inhibited with high affinity (IC\(_{50}\) = 0.42 \(\mu\)M) by AA (2). This inhibitory action appeared to be a direct effect on hIK1, as inhibitors of COX, LOX, or cytochrome P-450 did not alter the inhibitory effect of AA on hIK1 (Fig. 1D), further supporting our conclusion that the effects of AA on hIK1 are direct. We further demonstrate that AA does not inhibit the activity of rSK2, another member of the KCNN gene family. As we previously demonstrated that hIK1 is activated by ATP/PKA while SK1–3 are not (31), the present results further highlight the unique regulatory properties of hIK1 relative to the SK channels.

Given the high degree of homology between IK and SK channels throughout their core regions (S1-S6) we anticipated that the inhibitory action observed would be directed against either the cytoplasmic NH\(_{2}\) or COOH terminus. Indeed, three different NH\(_{2}\)-terminal splice variants of ROMK exist, only one of which is sensitive to AA (34). Interestingly, AA induces the phosphorylation of ROMK at a non-consensus NH\(_{2}\)-terminal phosphorylation site (34). In contrast to these results, we demonstrate that the cytoplasmic NH\(_{2}\)-terminus of hIK1 does not play a role in the AA-dependent inhibition observed (Fig. 3A).

In the present study, we confirm that AA inhibits hIK1, heterologously expressed in HEK293 cells, with high affinity (IC\(_{50}\) = 1.4 ± 0.7 \(\mu\)M; Fig. 1C), a value very similar to what we previously reported for endogenously expressed hIK1 (2). Consistent with our previous studies, we demonstrate that ETYA, a blocker of COX, LOX, and P450 pathways (32, 33) did not alter the inhibitory effect of AA on hIK1 (Fig. 1D), further supporting our conclusion that the effects of AA on hIK1 are direct. We further demonstrate that AA does not inhibit the activity of rSK2, another member of the KCNN gene family. As we previously demonstrated that hIK1 is activated by ATP/PKA while SK1–3 are not (31), the present results further highlight the unique regulatory properties of hIK1 relative to the SK channels.

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![Fig. 6. Thr\(^{250}\) and Val\(^{275}\) substitutions into rSK2 (S359T and A384V) confer AA sensitivity. Representative current (pA) traces of the mutated rSK2 channels with S359T (A), A384V (B), or the double mutation S359T/A384V (C) in response to 3 \(\mu\)M AA. D, percentage inhibition summary data for all experiments for the mutated channels are given (number of experiments shown above the bar). Data for rSK2 are also presented for comparison. Mutated rSK2 channels were heterologously expressed in HEK cells and recorded in excised inside-out patches at a holding potential of −100 mV. 0 Ca\(^{2+}\) steps occurred at the end of the experiments to illustrate the Ca\(^{2+}\) dependence of the mutated channels.](http://www.jbc.org/)

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Mutational analysis demonstrates that AA interacts with the pore of hIK1. Indeed, while the T250S mutation completely abrogated the effect of AA, we further demonstrate that Val\(^{275}\), an amino acid in S6 predicted to line the pore, also plays a crucial role in the AA-dependent inhibition of hIK1. As the side
chains of Thr\textsuperscript{250} and Val\textsuperscript{275} are predicted to extend into the water-filled pore of hIK1 (36). Our results would most parsimoniously be interpreted as demonstrating a direct pore block of hIK1 by AA. Somewhat surprisingly, these exact amino acids were previously demonstrated to be required for the inhibition of hIK1 by clotrimazole (36). Indeed, our own studies have confirmed the role of Thr\textsuperscript{250} and Val\textsuperscript{275} in the inhibition of hIK1 by clotrimazole. Thus, our results indicate that clotrimazole and its analogues have been synthesized to take advantage of an already pre-existing second-messenger binding site (AA), which similarly blocks current flow through the pore of hIK1. Clearly, the most convincing argument in favor of Thr\textsuperscript{250} and Val\textsuperscript{275} defining the binding site for AA is our demonstrated ability to recapitulate this binding site in a closely related but AA-insensitive channel, rSK2 (Fig. 6). Indeed, while mutation of Thr\textsuperscript{250} alone in hIK1 was sufficient to completely eliminate the inhibition by AA, introducing this single point mutation into rSK2 (S359T) conferred only partial inhibition by AA (Fig. 6A). Similarly, the A384V mutation resulted in only a partial block of rSK2 in response to AA (Fig. 6B). However, mutation of both amino acids resulted in a rSK2 channel that was nearly as sensitive to AA as hIK1 (Fig. 6C). In total, our results suggest that the molecular motif required for both AA and clotrimazole inhibition of hIK1 share a high degree of overlap and may indeed be identical.

It is interesting to note that both clotrimazole- (36) and AA-dependent inhibition of hIK1 is highly dependent upon a threonine in the pore, as this threonine is conserved in virtu-
ally all K+ channels, including voltage-gated (e.g. Kv1–4), inward rectifying (e.g. ROMK, KATP, GIRK), and two pore-domain (e.g. TWIK, TREK, TRAAK) K+ channels. Indeed, AA is known to both inhibit and activate a wide array of K+ channels, all of which share this pore threonine. Thus, the specificity of actions is likely conferred by additional amino acids, including Val275 in SK2. Indeed, we demonstrate that a single S/T mutation in SK2 confers only modest sensitivity to inhibition (21%) by AA (Fig. 6). While we have not evaluated the contribution of all of the pore-lining amino acids on AA sensitivity, other amino acids may also modulate AA affinity, perhaps explaining the wide range of affinities reported for AA-dependent modulation of K+ channels (22, 23). Based on our studies, it would be predicted that channels sharing both a threonine corresponding to Thr250 in hIK1 and valine corresponding to Val275 in hIK1 would be sensitive to AA. This is indeed the case in many instances, including Kv1.1 (46), Kv1.3 (47), Kv1.5 (48), and Kv4.2 (49), although the K+ channel-interacting protein KCbH1 modifies this effect on Kv4.2 (32). Future studies will be required to determine whether the conserved, pore-lining threonine and valine are responsible for the inhibition of these channels by arachidonic acid. However, in other channels sharing both this threonine and valine, including Kir2.1 and the Maxi (BKCa) K+ channel, AA has been shown to have either no effect (Kir2.1, Ref. 33) or is stimulatory (BKCa, Refs. 50–52). Importantly, Chandry and co-workers (53) previously demonstrated that Kv1.1, Kv1.3, Kv1.5, Kv4.2, Kir2.1, and BKCa were also insensitive to clotrimazole despite the conservation of the threonine and valine. As previously suggested (53), this may indicate that the orientation of the threonine and valine side chains are altered by the surrounding amino acids resulting in widely disparate sensitivities to both clotrimazole and AA despite an apparently conserved binding site.

In conclusion, we demonstrate that the inhibition of hIK1 by AA is dependent upon two critical pore lining amino acids, Thr250 and Val275. As the pore of the related channels, SK1 and AA despite an apparently conserved binding site.

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Molecular Localization of the Inhibitory Arachidonic Acid Binding Site to the Pore of hIK1
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