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Delineation of the Clotrimazole/TRAM-34 Binding Site on the Intermediate Conductance Calcium-activated Potassium Channel, IKCa1*

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Heike Wulff‡, George A. Gutman‡§, Michael D. Cahalan‡, and K. George Chandy‡¶

From the ‡Departments of Physiology and Biophysics and §Microbiology and Molecular Genetics, University of California, Irvine, California 92697

Selective and potent triarylmethane blockers of the intermediate conductance calcium-activated potassium channel, IKCa1, have therapeutic use in sickle cell disease and secretory diarrhea and as immunosuppressants. Clotrimazole, a membrane-permeant triarylmethane, blocked IKCa1 with equal affinity when applied externally or internally, whereas a membrane-impermeant derivative TRAM-30 blocked the channel only when applied to the cytoplasmic side, indicating an internal drug-binding site. Introduction of the S5-P-S6 region of the triarylmethane-insensitive small conductance calcium-activated potassium channel SKCa3 into IKCa1 rendered the channel resistant to triarylmethanes. Replacement of Thr^{250} or Val^{275} in IKCa1 with the corresponding SKCa3 residues selectively abolished triarylmethane sensitivity without affecting the affinity of the channel for tetraethylammonium, charybdotoxin, and nifedipine. Introduction of these two residues into SKCa3 rendered the channel sensitive to triarylmethanes. In a molecular model of IKCa1, Thr^{250} and Val^{275} line a water-filled cavity just below the selectivity filter. Structure-activity studies suggest that the side chain methyl groups of Thr^{250} and Val^{275} may lock the triarylmethanes in place via hydrophobic interactions with the π-electron clouds of the phenyl rings. The heterocyclic moiety may project into the selectivity filter and obstruct the ion-conducting pathway from the inside.

The intermediate conductance calcium-activated potassium channel IKCa1[1] (also known as IK1, KCa4, SK4, KCNN4, and the "Gardos channel") plays an important role in the physiology of lymphocytes, erythrocytes, and intestinal and airway epithelial cells (1–4). IKCa1 channels are voltage-independent, and their activation is steeply sensitive to rises in intracellular Ca^{2+}, calcium sensitivity being conferred by calmodulin tightly complexed to the C terminus of the channel (5). Pharmacologically, IKCa1 channels are distinguished from SK_{Ca} (*SKCa1–3*) channels by their sensitivity to clotrimazole and charybdotoxin and by their insensitivity to apamin.

In mitogen-activated human lymphocytes, IKCa1 regulates membrane potential and calcium signaling, and selective inhibitors of IKCa1 suppress lymphocyte proliferation and cytokine secretion by attenuating Ca^{2+} influx (4, 6–9). In red blood cells, IKCa1 participates in volume control. Erythrocyte dehydration in sickle cell disease can be attributed partly to excessive K^+ loss through IKCa1 channels that are activated by a rise in intracellular Ca^{2+} during sickling (10). Clotrimazole and ICA-15451, two potent triarylmethane (TRAM) blockers of this channel, are in clinical trials for the treatment of sickle cell disease (11, 12). In intestinal and airway epithelium cells, basolateral expression of the IKCa1 channel modulates apical water and Cl^- secretion (13, 14), and blockade of this channel by clotrimazole has been reported to ameliorate secretory diarrhea (15). However, the therapeutic usefulness of clotrimazole is seriously limited by its nanomolar affinity for cytochrome P450-dependent enzymes, especially CYP3A4 (the major xenobiotic metabolizing enzyme in human liver), which leads to a high incidence of side effects (11, 16).

Starting with clotrimazole as a template, we recently designed and synthesized TRAM analogs that block IKCa1 potently and specifically without inhibiting P450-dependent enzymes (8). The most potent of these, TRAM-34, blocks IKCa1 with a K_d of 20 nM and exhibits exquisite selectivity for the channel. Related TRAMs containing tetrazole (TRAM-84) or acetonitrile (TRAM-39) in place of the pyrazole are also selective and potent inhibitors of IKCa1. Although applied externally, the TRAMs are readily membrane-permeant because of their high lipophilicity (logP value 3.5–5.0) and may interact with a site on the inner face of the channel. Furthermore, a membrane-impermeant clotrimazole analog has been reported previously to block the native IK_{Ca} channel in erythrocytes only when applied to the cytoplasmic surface, suggesting an internal binding site (17). In the present study, we have used molecular chimeras and mutagenesis to identify the channel residues that interact with the TRAM inhibitors.

EXPERIMENTAL PROCEDURES

Reagents—Clotrimazole, econazole, ketoconazole, nifedipine, and TEA were purchased from Sigma. Charybdotoxin (ChTX) was from Bachem (King of Prussia, PA). TRAM-34 ([1-(2-chlorophenyl)diphenyl) methyl]-1*H*-pyrazole), TRAM-3 ((2-chlorophenyl)diphenylmethanol), and TRAM-39 (2-(2-chlorophenyl)-2,2-diphenylacetonitrile) were synthesized as described previously (8). TRAM-30 was prepared by refluxing clotrimazole (2.00 g, 5.81 mmol) with an excess of methyl iodide in butanone for 36 h. The reaction mixture was evaporated to dryness. On addition of petroleum ether to the resulting oily residue and intensive cooling and scratching, the material began to solidify and was then washed thoroughly with acetone and petroleum ether (mp: 139 °C; 1H

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¶ To whom correspondence should be addressed: Room 291, Joan Irvine Smith Hall, Medical School, University of California Irvine, Irvine, CA 92697. Tel.: 949-824-2133; Fax: 949-824-3143; E-mail: gchandy@uci.edu.

[1] The abbreviations used are: K_{Ca}, calcium activated K^+ channel; IKCa, intermediate K_{Ca}; SKCa, small conductance K_{Ca}; TRAM, triarylmethane; TEA, tetraethylammonium; ChTX, charybdotoxin; GFP, green fluorescent protein.

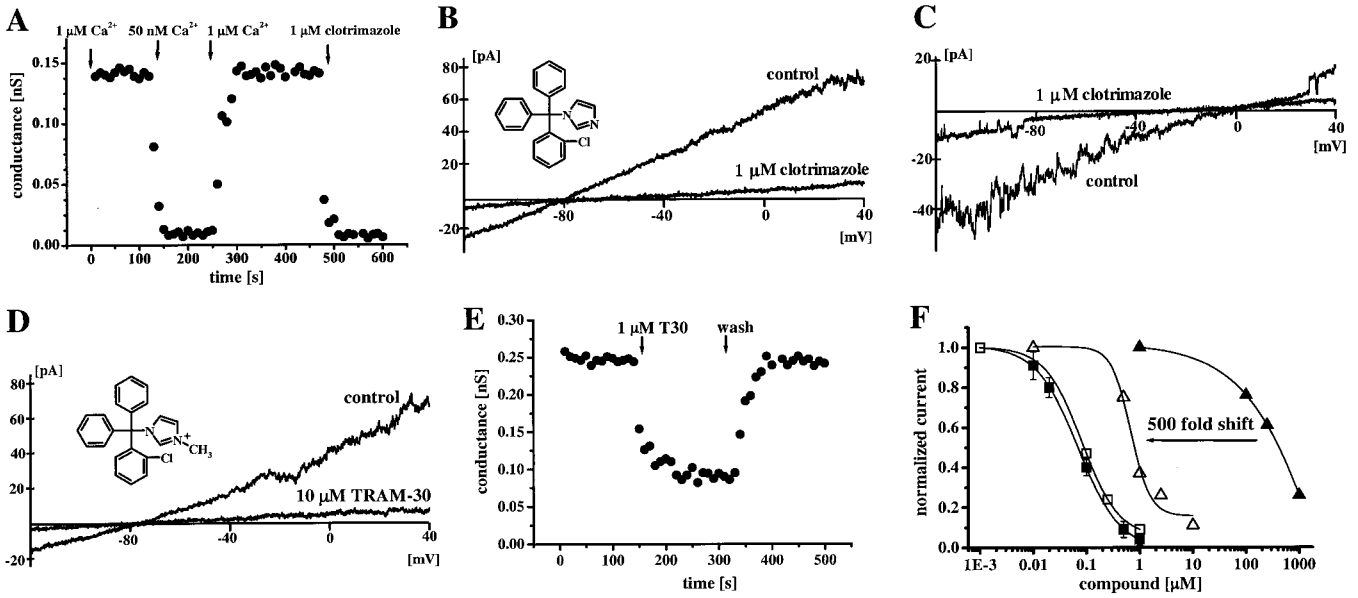

FIG. 1. **Effect of clotrimazole and TRAM-30 on IKCa1 currents in inside-out patches.** *A*, IKCa1 conductance with 50 nM or 1 μM free Ca^{2+} in the presence or absence of 1 μM clotrimazole. *B* and *C*, effect of 1 μM clotrimazole on IKCa1 current in a patch with either Na^+ aspartate (*B*) or K^+ aspartate (*C*) in the pipette and a K^+ aspartate bath solution containing 1 μM free Ca^{2+}. *D*, TRAM-30 (10 μM) inhibits IKCa1 current. *E*, IKCa1 conductance during the application of 1 μM TRAM-30 and after wash-out of drug. *F*, dose-response curves for clotrimazole (■, whole cell, K_d = 70 ± 10 nM; □, inside-out, K_d = 85 nM) and TRAM-30 (▲, whole cell, K_d = 350 μM; Δ, inside-out, K_d = 700 nM) when applied to the external *versus* the internal side of the cell membrane.

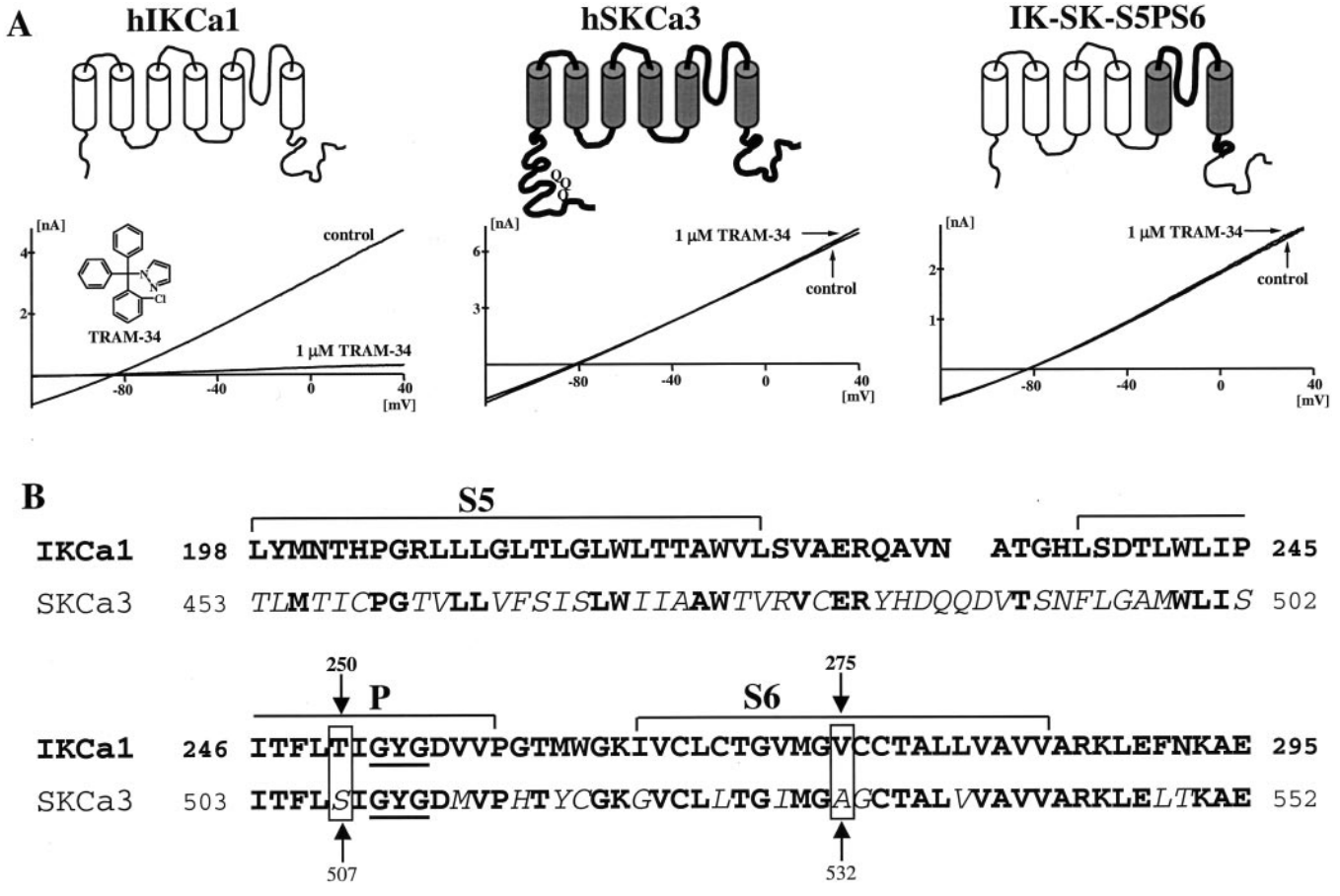

FIG. 2. *A*, effect of 1 μM TRAM-34 on currents through IKCa1 (*left*), SKCa3 (*middle*), and an IK chimera containing the S5-P-S6 segment from SKCa3 (*right*). *B*, alignment of the S5-P-S6 region of human *IKCa1* and *SKCa3*. The *arrows* indicate where the two critical mutations Thr^{250} and Val^{275} are located.

NMR (300 MHz, Me_2SO): δ 9.13 (s, 1H, -(2-H), 7.72 (s, 1H), 7.48–7.41 (m, 9H), 7.27–7.21 (m, 5H), 6.96 (s, 1H), 4.26 (s, 3H, N^+-CH_3); MS (70eV) *m/z* 277 (52), 254 (13), 241 (42), 226 (8), 199 (14), 165 (100), 127 (11), 119 (30), 106 (10), 82 (47), 63 (10), 54 (20).

$C_{23}H_{20}CClN_2$

Calculated:	C 56.75%	H 4.14%	N 5.75%
Found:	C 56.56%	H 4.12%	N 5.75%

TABLE I
K_d values of TRAM-34 for wild-type (WT) and mutant IKCa1 channels

	K_d for TRAM-34
WT-*IKCa1*	20 nM
Thr^{250}	
P245S + T250S	>20 μM
T250S	>20 μM
D239K + P245S	20 nM
Val^{275}	
V272I + V275A + C276G + L281V	not functional
D239K + P245S + L281V	28 nM
V272I	not functional
V275A + C276G	>20 μM
V275A	>20 μM
Others	
Y199L + N201T + T202I + H203C + R206T + L207V	25 nM
G259H + M261Y + W262C	120 nM
I265G	30 nM
C269L	20 nM
F291L + N292T	20 nM

Thr^{250}: Asp^{239} and Pro^{245} are not part of the TRAM-binding site, because mutations at these positions do not alter TRAM sensitivity. Mutations involving Thr^{250} abolish TRAM binding. Val^{275}: Leu^{281} is not part of the binding site, because a mutation involving this residue did not alter TRAM sensitivity. Mutations involving Val^{272} could not be studied, because they were nonfunctional. Mutations involving Val^{275} completely abolished TRAM sensitivity. The C276G point mutant was not generated, because Cys^{276} faces away from the pore in a published model of IKCa1 (20). Others: these mutants did not alter TRAM sensitivity significantly with the exception of the G259H + 261Y + W262C mutant, which reduced TRAM sensitivity 6-fold. These residues are buried in the IKCa1 model, and the small change in TRAM sensitivity resulting from their substitution with SKCa3 residues may be caused by an indirect effect via alteration of the shape of the protein.

Clones and Mutants—The cloning of human *IKCa1* (GenBank™ accession number AF033021) and human *SKCa3* containing 19 polyglutamines in the N terminus (GenBank™ accession numbers AF031815 and AJ251016) have been reported previously (2, 18, 19). Both channel genes were subcloned into the pEGFP-C1 vector (CLONTECH) in frame with GFP. Mutant *IKCa1* channels were generated by either one- or two-step polymerase chain reaction. Polymerase chain reaction products were digested with *Hin*dIII/*Bbr*PI and cloned into *Hin*dIII/*Bbr*PI-cut GFP-*IKCa1*. For *SKCa3* mutations, *Kpn*I and a C-terminal-introduced *Bam*HI site were used. All clones were verified by sequencing. The DNA for transfection was prepared with the Qiagen (Valencia, CA) mini-prep kit.

Electrophysiological Analysis—COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 1 mM Na^+ pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were transiently transfected with GFP-tagged wild-type or mutant *IKCa1* and *SKCa3* cDNAs using FuGene™ 6 (Roche) according to manufacturer protocol. Cells were trypsinized 16–72 h after transfection, plated onto polylysine-coated coverslips, and used within the next 4 h. Whole-cell patch clamp recordings (EPC-9, HEKA Elektronik) were made at a holding potential of −80 mV with a pipette solution containing 145 mM K^+ aspartate, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM K_2EGTA, and 8.5 mM $CaCl_2$ (1 μM free $[Ca^{2+}]$), pH 7.2, 290–310 mosM. To reduce currents from the native chloride channels in COS-7, Na^+ aspartate Ringer was used as an external solution containing 160 mM Na^+ aspartate, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4, 290–310 mosM. Currents were elicited by 200-ms voltage ramps from −120 to 40 mV applied every 10 s, and the reduction of slope conductance at −80 mV by drug was taken as a measure of channel block. The recordings in Fig. 1 were done on excised inside-out patches with the external solution described above as pipette solution. K^+ aspartate solutions containing 1 μM and 50 nM free $[Ca^{2+}]$ were applied to the cytoplasmic side of the patch.

RESULTS

The Binding Site for TRAMs Is Situated on the Cytoplasmic Surface of IKCa1—IKCa1 channels are potently blocked by clotrimazole and related TRAMs with a Hill coefficient of unity. These highly lipophilic compounds readily cross the plasma membrane and block the channel with a slow onset. An earlier study (17) that used a membrane-impermeant clotrimazole analog on the native IK_{Ca} channel in erythrocytes reported a drug-binding site on the cytoplasmic surface of the channel. To confirm this prediction on the cloned channel we generated a membrane-impermeant quaternary TRAM (TRAM-30) and evaluated its ability to block IKCa1 when added from the outside (in the whole-cell configuration) or the intracellular side in inside-outside patches. As a control we studied clotrimazole (logP = 3.5), which is freely membrane-permeant.

Currents (10–200 pA) in inside-out patches from IKCa1-expressing COS-7 cells excised into 1 μM Ca^{2+} disappeared when the Ca^{2+} concentration was reduced to 50 nM and were restored after the re-addition of 1 μM Ca^{2+} (Fig. 1*A*). This Ca^{2+}-activated current was completely and rapidly blocked by 1 μM clotrimazole (Fig. 1, *A* and *B*), and its K^+ selectivity was confirmed by the shift in reversal potential when the Na^+ aspartate pipette solution was replaced by K^+ aspartate (Fig. 1*C*). These IKCa1 currents were also reversibly blocked by the membrane-impermeant quaternary compound, TRAM-30 (Fig. 1, *D* and *E*) with a K_d of 700 nM (Fig. 1*F*, Δ), which was almost ineffective (K_d = 350 μM, ▲) when applied to the external surface of the channel in the whole-cell configuration. In contrast, the membrane-permeant clotrimazole blocked the channel with equal potency when applied to either side of the membrane (Fig. 1*F*, ■ and □). The 10-fold lower potency of TRAM-30 compared with clotrimazole is caused by the bulk of the additional methyl group on the imidazole ring, consistent with the published pharmacophore of the TRAMs (8). These results suggest that TRAMs interact with a site on the intracellular surface of the channel.

The TRAM-binding Site Is Located in the S5-P-S6 Region—To localize the region on the channel to which the TRAMs bind, we generated a chimera between IKCa1 and the phylogenetically related TRAM-insensitive small conductance SKCa3 channel (Fig. 2*A*). In whole-cell recordings, IKCa1 currents were potently blocked by externally applied TRAM-34 (Fig. 2*A*, *left*), whereas SKCa3 was insensitive (Fig. 2*A*, *middle*). A chimera containing the SKCa3 S5-P-S6 region in the IKCa1 body was insensitive to 1 μM TRAM-34 (Fig. 2*A*, *right*). Together with the data obtained in inside-out experiments (see above), these results suggest that the TRAM-binding site is situated on the cytoplasmic side of the S5-P-S6 region of IKCa1.

An alignment of the IKCa1 and SKCa3 amino acid sequences through this region reveals 45 differences (Fig. 2*B*). To define the residues involved we replaced IKCa1 residues with the corresponding ones from SKCa3. Mutations were made in the cytoplasmic half of S5, the intracellular portion of the P-loop and the entire S6 segment, and as a control we also mutated residue 239 that had been shown previously to be involved in ChTX binding (20). Mutations involving Thr^{250} (P245S + T250S; or T250S) or Val^{275} (V275A +C276G; or V275A) abolished sensitivity to TRAM-34 and clotrimazole, whereas all other mutants showed unchanged TRAM-34 sensitivity (Fig. 2*B*, Table I). The V272I mutant could not be investigated because it was nonfunctional. The T250S and V275A mutants were studied in more detail.

Mutations of Thr^{250} and Val^{275} Selectively Abolish TRAM Sensitivity—The T250S mutant produced a K^+-selective current equally sensitive to block by ChTX (K_d = 5 ± 1 nM), TEA (K_d = 20 ± 4 mM), and nifedipine (K_d = 5 ± 1 μM) as the wild-type IKCa1 channel (4, 8, 20), whereas it was completely insensitive to TRAM-34 (Fig. 3, *A–D*). Similar results were obtained with the V275A mutant (Fig. 3, *E–H*). Thus, these two point mutations selectively abolished TRAM-34 sensitivity

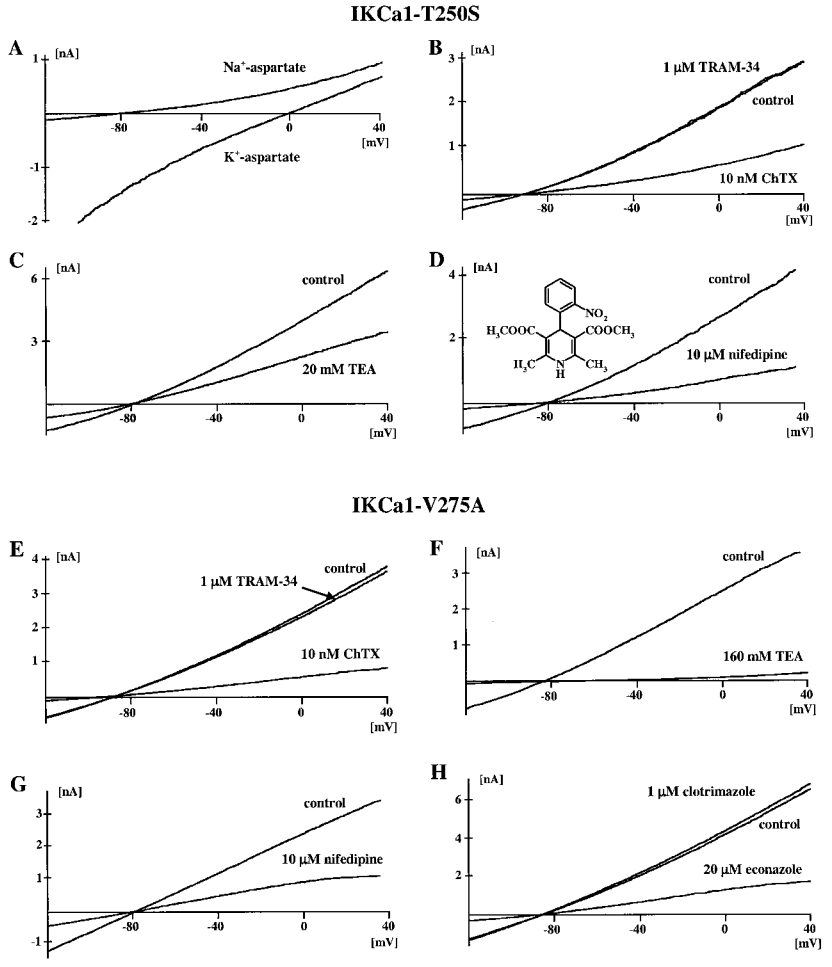

FIG. 3. *A–D*, currents from the IKCa1-T250S mutant channel: K^+ selectivity (*A*), no block by 1 μM TRAM-34 and block by 10 nM ChTX (*B*), 20 mM TEA (*C*), and 10 μM nifedipine (*D*). *E–H*, currents from the IKCa1-V275A mutant channel showing no block by 1 μM TRAM-34 and block by 10 nM ChTX (*E*, K_d = 6 ± 1.5 nM), 160 mM TEA (*F*, K_d = 22 ± 3 mM), and 10 μM nifedipine (*G*, K_d = 7 ± 1 μM). Current is blocked by 20 μM econazole (K_d = 10 ± 2 μM) but not by 1 μM clotrimazole (*H*). In all cases K_d values were determined by testing three concentrations of drug (n = 3) and fitting with a Hill coefficient of unity.

	K_d-WT	K_d-T250S	K_d-V275A
TRAM-34	20 nM	> 20 μM	> 20 μM
TRAM-39	60 nM	> 20 μM	> 20 μM
TRAM-3	520 nM	> 50 μM	> 50 μM
clotrimazole	70 nM	> 20 μM	> 20 μM
econazole	10 μM	12 μM	10 μM
ketoconazole	35 μM	33 μM	35 μM

FIG. 4. **Blocking potencies of TRAM-34, TRAM-39, TRAM-3, clotrimazole, econazole, and ketoconazole on the wild-type (*WT*) IKCa1 channel and the T250S and V275A mutants (n = 3).**

without altering other pharmacological properties of the channel or its K^+ selectivity, indicating that the overall conformation of these mutant channels had not been grossly perturbed. It also shows that the TRAM-binding site is distinct from those of ChTX, TEA, and nifedipine.

The TRAM pharmacophore consists of two parts, a triphenyl moiety and either a small heterocycle or another π-electron-rich substituent such as an acetonitrile or hydroxyl group. To identify the part of the molecule that interacts with the channel, we first examined four TRAMs that contain the triphenyl moiety but different substituents, TRAM-34 (pyrazole), TRAM-39 (acetonitrile), TRAM-3 (hydroxyl), and clotrimazole (imidazole). All four compounds were inactive on the T250S and V275A mutant channels (Fig. 4). In contrast, two azole inhibitors of IKCa1 (econazole and ketoconazole) containing an imidazole moiety such as clotrimazole but lacking the triphenyl group blocked both mutant channels as effectively as the wild-type channel (Figs. 3*H* and 4). Collectively, these results suggest that the triphenyl moiety interacts with Thr^{250} and Val^{275}.

Introducing TRAM Sensitivity into SKCa3—If Thr^{250} and Val^{275} constitute the TRAM-binding site, introduction of these residues into the SKCa3 channel should confer sensitivity to the TRAMs. The SKCa3-S507T+A532V mutant produced a K^+-selective channel (Fig. 5*A*) that was blocked by apamin with a potency roughly equal to the wild-type SKCa3 channel (Fig. 5*B*). Unlike SKCa3, which was relatively insensitive to TRAM-34 (K_d = 28 μM), the S507T+A532V-SKCa3 mutant was substantially blocked by 1 and 5 μM TRAM-34 (K_d = 680 ± 70 nM, Fig. 5, *C* and *D*).

A Heuristic Model of the TRAM-binding Site—The positions of Thr^{250} and Val^{274} are highlighted in a published model of the

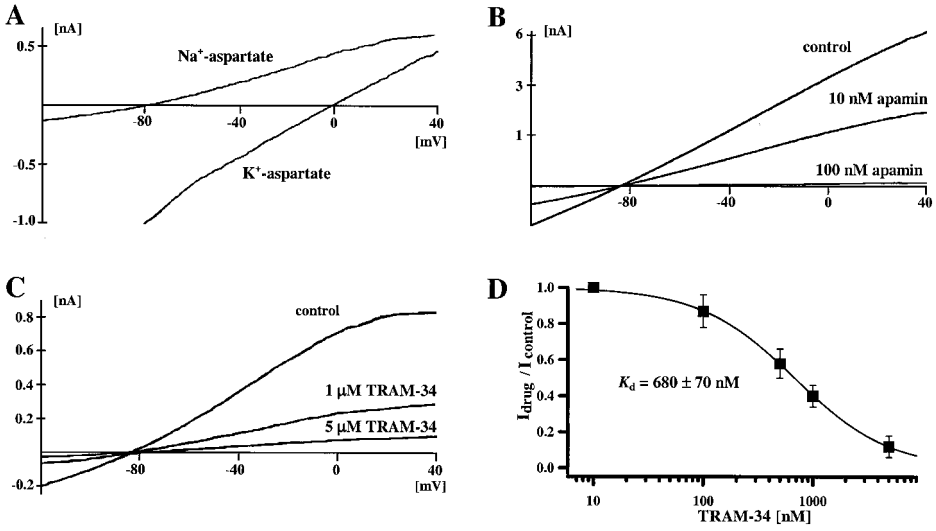

FIG. 5. Currents through the SKCa3-T250S+A532V mutant channel: K^+ selectivity (*A*), block by 10 and 100 nM apamin (*B*), block by 1 and 5 μM TRAM-34 (*C*), and dose-response curve for TRAM-34 (K_d = 680 ± 70 nM, n_H = 1.01, n = 3).

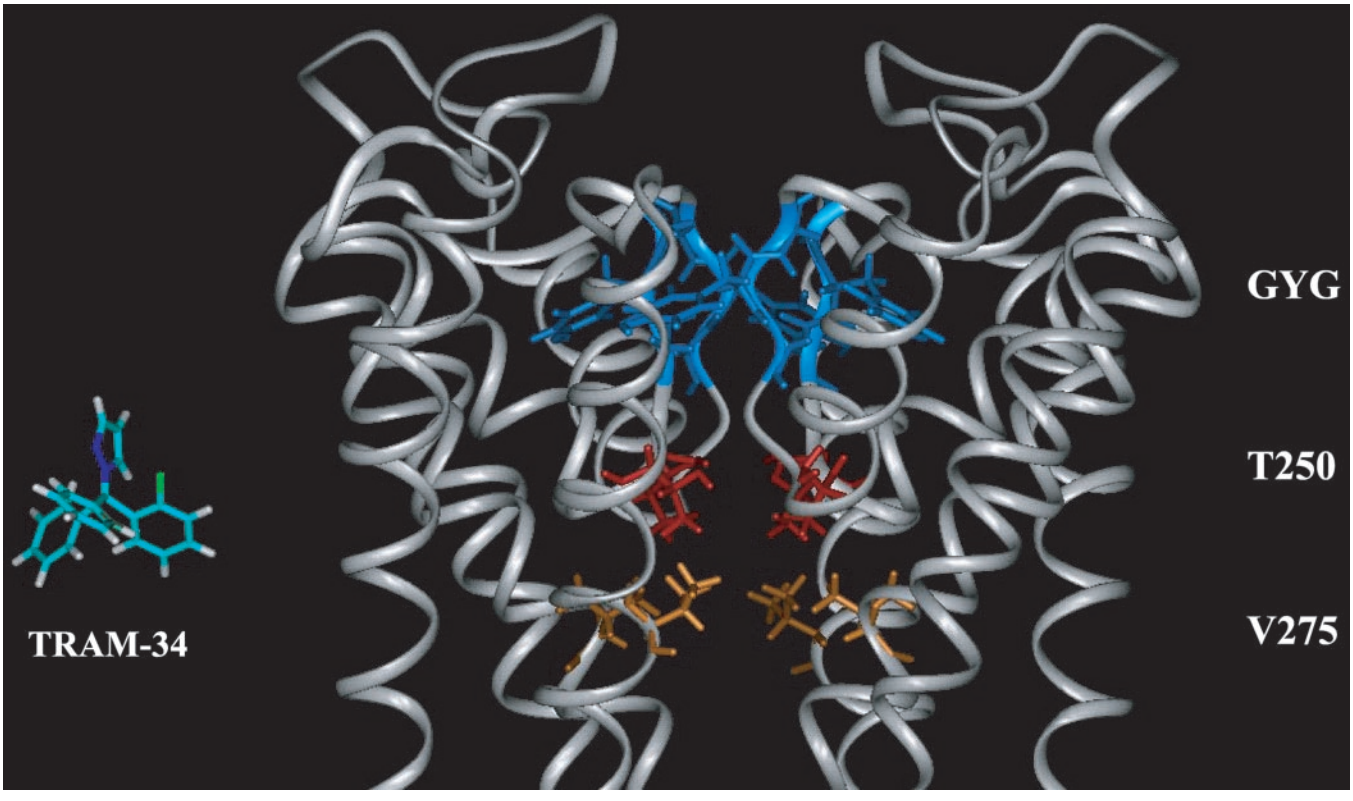

FIG. 6. **Model of IKCa1 based on the KcsA structure (20).** Only the upper part of the pore region of the channel is shown. The GYG residues that constitute the K^+ selectivity filter are *highlighted in light blue*, Thr^{250} in *red*, and Val^{275} in *gold*. One of the three subunits is removed to make the internal cavity more visible. An AM-1-optimized structure of TRAM-34 is shown on scale (*cyan*, carbon; *white*, hydrogen; *dark blue*, nitrogen; *green*, chlorine).

IKCa1 pore (20) based on the KcsA crystal structure (Fig. 6). Both residues line a large water-filled cavity that lies just below the narrow selectivity filter of the channel, which is formed by the conserved GYG motif. If this cavity in IKCa1 is as wide as the 10-Å cavity in KcsA, it should easily accommodate clotrimazole and TRAM-34, both of which have dimensions of 9.5 × 9.5 × 8.6 Å. An AM-1-optimized structure of TRAM-34 drawn to scale is shown in Fig. 6 next to the channel model. A highly lipophilic membrane-permeant compound like TRAM-34 (logP = 4.0) could easily reach the internal cavity either by traversing the ion conduction pathway from the inside or through crevices and tunnels in the protein similar to those recently described in sodium channels (21).

DISCUSSION

Using a combination of electrophysiology and mutagenesis we have defined the TRAM-binding site on IKCa1. Our data suggest that the binding site is located on the cytoplasmic side of the S5-P-S6 region of the channel. First, a membrane-impermeant analog blocks the channel with 500-fold greater potency when added from the inside *versus* the outside, and second, introduction of the S5-P-S6 region of the TRAM-resistant SKCa3 into IKCa1 renders the channel insensitive to these compounds. Single and multiple replacements of IKCa1 residues in the S5-P-S6 region with the corresponding residues in SKCa3 identified Thr^{250} in the P-region and Val^{275} in S6 as being critical for TRAM binding. Based on our IKCa1 model

(20), both these residues lie in close proximity to each other in the water-filled cavity below the ion selectivity filter. The introduction of these two residues into SKCa3 made this channel TRAM-sensitive. Given the tetrameric nature of K^+ channels, the TRAM-binding site could involve Thr^{250} and Val^{275} from more than one subunit.

The exquisite selectivity of IKCa1 for the TRAMs is most likely caused by the tight interactions between the propeller-shaped TRAMs and the Thr^{250} and Val^{275} of multiple IKCa1 subunits. The three SK_{Ca} channels contain serine and alanine at positions equivalent to Thr^{250} and Val^{275}, and these residues contribute to their TRAM insensitivity. Consistent with this idea, the introduction of Thr^{250} and Val^{275} into SKCa3 (SK3-S507T+A532V) produces an ~40-fold enhancement in TRAM-34 sensitivity compared with the wild-type SKCa3 channel (Fig. 5). Other TRAM-resistant K_v (Kv1.1–1.5, 3.1, 4.2), K_{ir} (Kir2.1), and BK_{Ca} (Slo) channels contain threonine and valine at positions homologous to Thr^{250} and Val^{275}, although they share little sequence similarity with IKCa1 in the residues neighboring the threonine and valine (22). The threonine and valine residues in these channels may be oriented differently from Thr^{250} and Val^{275} in IKCa1, resulting in a less than optimal interaction with the phenyl rings of TRAM-34. Differences in the shape of the internal cavities of these channels compared with IKCa1 may also contribute to TRAM insensitivity. Bulky residues in the vicinity of the threonine or valine in these channels may also sterically interfere with their ability to interact with TRAMs.

In an earlier study, we proposed that the TRAM-binding site had dimensions of roughly $9.5 \times 9.5 \times 8.6$ Å based on structure-activity relationships of the TRAMs; introduction of bulky substituents on the triphenyl moiety or on the heterocycle abolished affinity (8). Consistent with this earlier report, our heuristic model suggests that TRAM-34 could fit snugly into the internal cavity just below the selectivity filter with each of the phenyl rings locked in place by interactions with Thr^{250} and Val^{275} from three of the four IKCa1 subunits. We propose that the π-electron cloud of the phenyl rings of TRAM-34 form hydrophobic interactions with the positively polarized hydrogen atoms of the methyl group of Thr^{250}. The conservative substitution of serine in place of Thr^{250} abolishes TRAM sensitivity, possibly because serine lacks the side chain methyl group of threonine. Val^{275} could also form a hydrophobic interaction with the phenyl rings. This interaction must be size-dependent, because the introduction of the smaller alanine (88 $Å^3$) in place of the valine (140 $Å^3$) abolishes TRAM sensitivity. Although each hydrophobic interaction in itself is weak, taken together they may well account for the 20 nM K_d value of TRAM-34. Our model also predicts that the heterocyclic moiety could project upwards into the selectivity filter and occlude the ion conduction pathway.

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