Design of a potent and selective inhibitor of the intermediate-conductance Ca^{2+}-activated K^+ channel, *IKCa1*: A potential immunosuppressant

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The antimycotic clotrimazole, a potent inhibitor of the intermediate-conductance calcium-activated K^+ channel, *IKCa1*, is in clinical trials for the treatment of sickle cell disease and diarrhea and is effective in ameliorating the symptoms of rheumatoid arthritis. However, inhibition of cytochrome P450 enzymes by clotrimazole limits its therapeutic value. We have used a rational design strategy to develop a clotrimazole analog that selectively inhibits *IKCa1* without blocking cytochrome P450 enzymes. A screen of 83 triarylmethanes revealed the pharmacophore for channel block to be different from that required for cytochrome P450 inhibition. The "*IKCa1*-pharmacophore" consists of a (2-halogenophenyl)diphenylmethane moiety substituted by an unsubstituted polar π-electron-rich heterocycle (pyrazole or tetrazole) or a $-C\equiv N$ group, whereas cytochrome P450 inhibition absolutely requires the imidazole ring. A series of pyrazoles, acetonitriles, and tetrazoles were synthesized and found to selectively block *IKCa1*. TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole) inhibits the cloned and the native *IKCa1* channel in human T lymphocytes with a K_d of 20–25 nM and is 200- to 1,500-fold selective over other ion channels. Using TRAM-34, we show that blocking *IKCa1* in human lymphocytes, in the absence of P450-inhibition, results in suppression of mitogen-stimulated [^{3}H]thymidine incorporation of preactivated lymphocytes with EC_{50}-values of 100 nM-1 μM depending on the donor. Combinations of TRAM-34 and cyclosporin A are more effective in suppressing lymphocyte mitogenesis than either compound alone. Our studies suggest that TRAM-34 and related compounds may hold therapeutic promise as immunosuppressants.

Clotrimazole, a topically used antimycotic, exerts its fungicidal effect by inhibiting fungal P450-dependent enzymes (1). Clotrimazole has also been reported to inhibit mammalian P450 enzymes (2–4), as well as directly block the intermediate-conductance Ca^{2+}-activated potassium (IK_{Ca}) channel, a product of the *IKCa1* gene (5–7), in human erythrocytes, colonic epithelium, and human T lymphocytes at nanomolar concentrations (8–13). This lack of specificity clouds the use of clotrimazole as a pharmacological tool, creating a need for a truly selective *IKCa1* inhibitor. Because of its potent channel-blocking activity, clotrimazole is being clinically evaluated for the treatment of erythrocyte dehydration in sickle cell disease and secretory diarrheas (9, 14). Recent studies have also raised the possibility of using clotrimazole as an immunosuppressant (12, 13). Clotrimazole was previously reported to be effective in rheumatoid arthritis (15). However, the gastrointestinal and urinary disturbances caused by clotrimazole, coupled with elevation of hepatic enzymes (9, 16) and changes in plasma cortisol levels (15) caused by its acute inhibition (2–4) and chronic induction of human P450-dependent enzymes (3, 17), may limit its systemic use (18).

Resting human T lymphocytes possess ≈400 *Kv1.3* channels and roughly 2–20 functional *IKCa1* channels. The membrane potential of resting T cells is maintained by *Kv1.3* channels rather than by *IKCa1*, and selective inhibitors of *Kv1.3* suppress the activation response (19). In contrast, mitogen-activated human T lymphocytes exhibit 300–800 functional *IKCa1* channels (20) along with 400–500 *Kv1.3* channels. Because expression of *IKCa1* channels is dramatically enhanced in activated T cells (20), in parallel with enhanced $[Ca^{2+}]_i$ signaling (21, 22), a strategy targeting *IKCa1* channels could be especially effective in suppressing chronically activated T cells and could perhaps lead to therapy for autoimmune disorders.

By identifying and exploiting differences in the pharmacophores required for channel block and cytochrome P450 inhibition, we have designed a triarylmethane (TRAM-34) that selectively blocks the *IKCa1* channel. TRAM-34 may have a therapeutic profile similar to clotrimazole but may lack its toxic side effects.

Materials and Methods

Compounds. Clotrimazole (**1a**), econazole, and ketoconazole were purchased from Sigma. Clotrimazole was subjected to the same physical analysis as the synthesized triarylmethanes (see supplementary Table 2, www.pnas.org) to ensure its purity. Nifedipine, nimodipine, and nitrendipine were obtained from Research Biochemicals. Triarylmethanes were synthesized according to the route described for clotrimazole (23) with modifications according to ref. 24 and of our own. Compounds were characterized by melting point, IR, ^{1}H-NMR, mass spectrometry, and combustion analysis. Briefly, triarylmethanols (**2a-p**) were prepared from benzophenones and aryl bromides by a Grignard reaction and then converted into triaryl chlorides with freshly distilled thionyl chloride in petroleum ether, which then were further reacted with an excess of the required amine in anhydrous acetonitrile to give compounds **1b-f, 3h-l, 4a-q, 6a-m**, and **7a**. Bivalent and trivalent compounds **8a-f** were synthesized according to ref. 25. Compounds **3a-d** were prepared from the triaryl chlorides in a mixture of diethyl ether and 25% aqueous ammonia solution (26). Compounds **3e-g** were prepared by reacting **3a, 3b,** and **3d** with freshly distilled acetic anhydride. Compounds **3h-k** were synthesized from triaryl chlorides and urea according to the method given for **8a-f**. Compounds **5a-f** were synthesized by heating triaryl chlorides with copper cyanide without solvent (27).

Clones, Cells, and Cell Lines. The cloning of human *IKCa1* and transient transfection into COS-7 cells have been previously

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Abbreviations: CRAC, calcium release-activated Ca^{2+}; K_{Ca}, Ca^{2+}-activated K^+; IK_{Ca}, intermediate-conductance K_{Ca}; PBMCs, peripheral blood mononuclear cells; PMA, phorbol-12-myristate 13-acetate; [^{3}H]TdR, tritiated thymidine.

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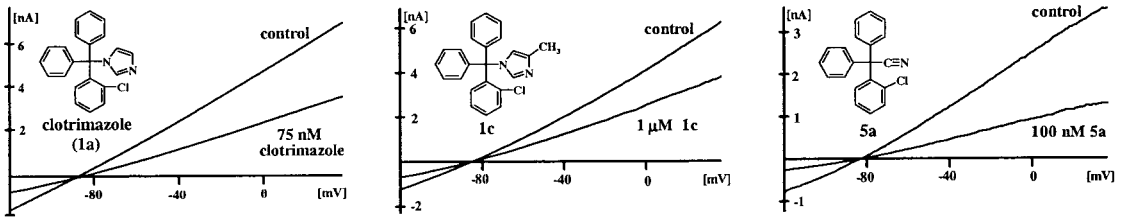


Fig. 1. Inhibitory effects of compounds **1a** (clotrimazole), **1c**, and **5a** on *IKCa1* currents expressed in COS-7 cells. Voltage ramps were applied from −120 mV to 40 mV.

reported (11). Cell lines stably expressing *mKv1.1*, *rKv1.2*, *mKv1.3*, *mKv3.1*, and *hKv1.5* have been previously described (28). Human *SKCa2* (expressed sequence tag: GenBank accession no. AI810558) and human *SKCa3* (AJ251016), were cloned in-frame downstream to green fluorescent protein in the pEGFP-C1 expression vector (CLONTECH). Rat *SKCa2* (U69882) was previously described (29). These clones were transiently expressed in COS-7 cells. LTK cells expressing *hKv1.4* and *rKv4.2* were obtained from M. Tamkun (University of Colorado, Boulder, CO), HEK-293 cells expressing the skeletal muscle sodium channel *hSkM1* (SCN4A) from F. Lehmann-Horn (University of Ulm, Ulm, Germany), and HEK-293 cells expressing *hSlo*α (30) from A. Tinker (Centre for Clinical Pharmacology, University College London). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples of healthy volunteers by using a lymphocyte separation medium (Accuspin System-Histopaque-1077, Sigma) and maintained in RPMI-1640 supplemented with 10% FCS/2 mM L-glutamine/1 mM Na^+ pyruvate/1% nonessential amino acids/100 units/ml penicillin/100 μg/ml streptomycin. Cells were rested for 24 h after isolation and then activated with 10 nM phorbol-12-myristate 13-acetate (PMA) or 5 ng/ml anti-CD3 Ab (Biomedia, Foster City, CA). T cells were isolated by nylon-wool purification immediately before electrophysiological experiments, typically yielding >90% $CD3^+$ T cells.

Electrophysiology. Cells were studied in the whole-cell configuration of the patch-clamp technique. The holding potential in all experiments was −80 mV. For measurement of IK_{Ca}, SK_{Ca}, and BK_{Ca} currents, we used an internal pipette solution containing (in mM): 145 K^+ aspartate, 2 $MgCl_2$, 10 Hepes, 10 K_2 EGTA, and 8.5 $CaCl_2$ (1 μM free Ca^{2+}), pH 7.2, 290–310 mOsm. To reduce currents from the native chloride channels in COS-7, T84, and T cells, Na^+ aspartate Ringer was used as an external solution (in mM): 160 Na^+ aspartate/4.5 KCl/2 $CaCl_2$/1 $MgCl_2$/5 Hepes, pH 7.4/290–310 mOsm. IK_{Ca} currents in COS-7 and T84 cells were elicited by 200-ms voltage ramps from −120 mV to 40 mV applied every 10 s and the reduction of slope conductance at −80 mV by drug taken as a measure of channel block. For activated T lymphocytes, the same voltage ramp was applied every 30 s to avoid inactivation of *Kv1.3* channels. BK_{Ca} currents were elicited by 200-ms voltage ramps from −80 to 80 mV applied every 30 s and channel block measured at 35 mV. The inward rectifier (*rKir2.1*) in RBL cells was studied in Na^+ aspartate Ringer with a K^+ aspartate-based pipette solution containing 50 nM free Ca^{2+}. Recordings from Jurkat SK_{Ca} channels were made in K^+ aspartate Ringer. For both SK_{Ca} and inward rectifier currents, the reduction of slope conductance at −110 mV was taken as measure of channel block. For all currents elicited by voltage ramps, series resistance was not used. Recordings of Kv- (28), monovalent currents through Jurkat calcium release-activated Ca^{2+} (CRAC) channels (31), and swelling-activated chloride currents (32) were made as previously described.

Inhibition Studies of CYP3A4. Inhibition of the catalytic activity of purified recombinant human cytochrome P450 3A4 in microsomes (Gentest Corporation, Woburn, MA) was assayed on the turnover of 7-benzyloxy-4-trifluoromethyl-coumarin by the detection of its fluorescent metabolite 7-hydroxy-4-trifluoromethylcoumarin as described (33). All experiments were done in duplicate, and results are reported as percent inhibition. Positive controls (5 μM ketoconazole and 100 nM clotrimazole) were run on the same plate producing 99% inhibition.

[^{3}H]Thymidine Incorporation Assay. Resting or 2-day-activated (10 nM PMA or 5 ng/ml anti-CD3 Ab) PBMCs were seeded at 2×10^5 cells per well in culture medium in flat-bottom 96-well plates (final volume 200 μl). Cells preincubated with drug (60 min) were stimulated with mitogen (10 nM PMA + 175 nM ionomycin or 5 ng/ml anti-CD3 Ab) for 48 h. Triated thymidine ([^{3}H]TdR) (1 μCi per well) was added for the last 6 h. Cells were harvested onto glass fiber filters and radioactivity measured in a scintillation counter.

Flow Cytometric Measurement of Cell Viability. Cells were seeded at 5×10^5 cells/ml (Jurkat E6–1, MEL cells, human T lymphocytes) or 10^5 cells/ml (C_2F_3 myoblasts, CHO, COS-7, L929, NGP and NLF neuroblastoma, RBL-2H3) in 12-well plates. Drug (5 μM) was added in a final DMSO concentration of 0.1% which was found not to affect cell viability. After 48 h cells were harvested by suction (suspension cells) or by trypsinization (adherent cell lines), centrifuged, resuspended in 0.5 ml PBS containing 1 μg/ml propidium iodide (PI), and red fluorescence measured on a FACScan flow cytometer (Becton Dickinson). The percentage of dead cells was determined by their PI uptake, 10^4 cells of every sample being analyzed.

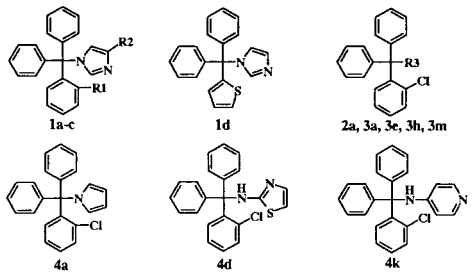
Acute *in Vivo* Toxicity Determinations. Five CF-1BR mice (17–19 g) were injected intravenously with a single 1.0-ml dose of 0.5 mg/kg TRAM-34 (in mammalian Ringer solution with 1% ethanol and 2.5% BSA). Five control mice were injected with an equal volume of the vehicle. Mice were observed for adverse effects immediately after dosing, at 4 h after injection and daily for 7 days.

Results

Defining the Triarylmethane Oharmacophore for *IKCa1* Block. Fig. 1 shows currents from *IKCa1*-transfected COS-7 cells elicited by voltage ramps with 1 μM free calcium in the pipette solution. Clotrimazole (compound **1a**) potently blocks the *IKCa1* channel with a K_d of 70 nM. In contrast, two related antimycotic agents, ketoconazole (K_d = 30 μM) and econazole (K_d = 12 μM), as well as the dihydropyridines nifedipine (K_d = 4 μM), nimodipine (K_d = 1 μM), and nitrendipine (K_d = 0.9 μM), are significantly less potent.

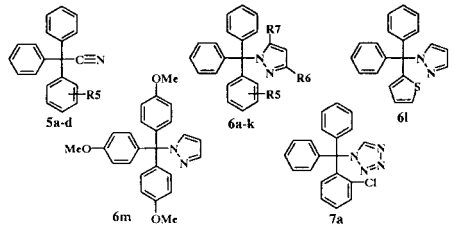
We synthesized 83 triarylmethanes and tested them by whole-cell patch clamp against *IKCa1* channels, the compounds being added externally in every case. The structures and channel-

A



	R1	R2	R3	K_d for *IKCa1* [nM]	CYP3A4 % Inhibition
1a	Cl	H		70 (± 10)	99
1b	H	H		1500 (± 500)	
1c	Cl	CH_3		1200 (± 100)	
1d				1000 (± 200)	
2a			OH	520 (± 30)	No inhibition
3a			NH_2	1000	
3e			$NHCOCH_3$	1200	No inhibition
3h			$NHCONH_2$	5000	
3m			$CH(COOC_2H_5)_2$	400	No inhibition
4a				7000	
4d				15 000	87
4k				30 000	74

B



	R1	R2	R3	K_d for *IKCa1* [nM]	CYP3A4 % Inhibition
5a	o-Cl			60 (± 5)	10
5b	o-Fl			70 (± 10)	9
5c	p-Cl			750 (± 90)	
5d	p-Fl			800 (± 100)	
6a	H	H	H	2500 (± 5400)	
6b	o-Cl	H	H	20 (± 53)	No inhibition
6c	o-Fl	H	H	40 (± 5)	No inhibition
6d	p-Cl	H	H	90 (± 10)	
6e	p-Fl	H	H	200 (± 40)	
6f	o-CF_3	H	H	3800 (± 500)	
6g	p-CF_3	H	H	1500 (± 300)	No inhibition
6h	o-Cl	CH_3	H	1100 (± 300)	
6i	o-Cl	CH_3	CH_3	12 000 (± 1000)	
6j	o-Cl	CF_3	H	2000 (± 500)	
6k	o-CF_3	CF_3	H	25 000 (± 3000)	
6l				1100 (± 100)	
6m				> 20 μM	
7a				45 (± 5)	

C

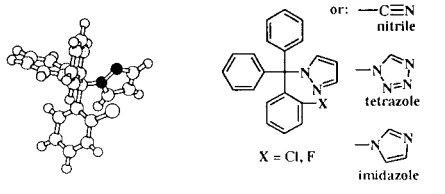


Fig. 2. (*A*) Structures of triarylmethanes and blocking potencies of *IKCa1* and CYP3A4. Clotrimazole and compound **1a** were tested at five concentrations (n = 3). K_d and Hill coefficient (Hill coefficient = 1.2) were determined by fitting the Hill equation to the reduction of slope conductance at −80 mV. The remaining compounds were screened at 100 nM, 1 μM, and 10 μM and their K_d values determined by fitting the data with the same Hill coefficient as clotrimazole. Inhibition of CYP3A4 was tested at 10 μM except for clotrimazole, which was tested at 100 nM. (*B*) Structures of triarylmethyl-acetonitriles, -pyrazoles, and -tetrazoles, and blocking potencies of IKCa1 and CYP3A4. Compound **6b** was tested at five concentrations (15 cells); the other compounds were tested at three concentrations (9 cells). Hill coefficient = 1.0 to 1.2. (*C*) Pharmacophore for triphenylmethane IKCa1 blockers. (*Left*) AM1-optimized molecular structure of TRAM-34 (color code: white, hydrogen and chlorine; light gray, carbon; black, nitrogen). (*Right*) General structure of the pharmacophore for channel block.

blocking potencies of 30 exemplary compounds that highlight our design strategy are described in Fig. 2, and their physical data are listed in supplementary Table 3 (www.pnas.org). The structures and channel affinities of the remaining 53 compounds are provided in supplementary Table 3 and their physical data in supplementary Table 4. The hydrolytic stability of TRAM-34 is shown in supplementary Fig. 5.

To test whether the imidazole ring is necessary for channel blocking activity, we generated several analogs where this moiety is replaced by a hydroxyl- (**2a-p**), an amino- (**3a-d**), an acetamido- (**3e-g**), an ureido- (**3h-k**), a malono- (**3 l**), an aromatic pyrrole- (**4a**), an aminothiazol- (**4d**), or an aminopyridine- (**4k**) group. All these analogs are significantly less potent than clotrimazole (Fig. 2*A*), indicating the need of the imidazole moiety for channel block. Five bivalent compounds and one trivalent compound (supplementary Table 3, **8a-f**) are inert. The triphenylmethyl moiety of the molecule is equally important for channel block, because replacement of one or more of the phenyl rings by thiophene (**1c, 2 m-p**) or pyrimidine (supplementary Table 3) reduces activity 10- to 20-fold (Fig. 2*A*). Our analysis also reveals the requirement of the *o*-halogen on the triphenylmethane, because imidazole analogs lacking an *o*-chlorine substituent (**1b**) are 20-fold less potent than clotrimazole (**1a**), whereas compounds containing more than one chlorine (**2d-e**, supplementary Table 3) are inert. Collectively, our data indicate that low nanomolar block of *IKCa1* requires the presence of both the (2-halogenophenyl)diphenylmethane and the imidazole moieties.

Comparison of the Pharmacophores for Channel Block and Cytochrome P450 Inhibition. Extensive structure-activity studies of azole antimycotics have shown that the imidazole ring is absolutely required for block of cytochrome P450 enzymes. These compounds exert their inhibitory effect by coordinately binding to the heme iron of P450-dependent enzymes with the N_3 nitrogen of the imidazole ring (1). Replacement of the imidazole ring by other heterocyles lacking this crucial nitrogen atom abolishes inhibition and induction of cytochrome P450 enzyme activity (17, 23). To determine whether such substituents might retain potency against *IKCa1*, we generated a new series of analogs (Fig. 2*B*) where the imidazole moiety was replaced with functional groups of similar size, lipophilicity, and π-electron density, such as −C≡N (**5a-d**), pyrazole (**6a-m**), and tetrazole (**7a**). Two acetonitriles (**5a**, **5b**), four pyrazoles (**6b-e**), and the tetrazole (**7a**) analog are potent inhibitors of *IKCa1*, four of these having higher affinities than clotrimazole (Fig. 2*B*). However, any substitution on these small heterocycles (**6h-k**) dramatically diminishes affinity (K_d 1–25 μM), an effect we also witnessed in the corresponding imidazole compounds (Fig. 1, Table 1, **1c**, **1e,** and **1f**, and supplementary Table 3, www.pnas.org). As with the imidazole series, the *o*-halogen is required for optimal activity, because **6a** lacking this group is 100-fold less effective. Ten compounds, including representative acetonitriles (**5a**, **5b**) and pyrazoles (**6b**, **6k**), were tested at a single high concentration of 10 μM for inhibition of the catalytic activity of recombinant human cytochrome P450 3A4, the major xenobiotic metabolizing

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Table 1. Selectivity

	Channel	Clotrimazole, nM	TRAM-34, nM
IK_{Ca}	*IKCal*	70 ± 10	20 ± 3
	lymphocyte IK	100	25 ± 5
	T84 *IK*	90 ± 15	22 ± 10
K^+	*Kv1.1*	10,000 ± 850	9,500 ± 1,000
	Kv1.2	5,000 ± 730	4,500 ± 520
	Kv1.3	6,000 ± 440	5,000 ± 350
	Kv1.4	6,000 ± 520	7,500 ± 410
	Kv1.5	8,000 ± 890	7,000 ± 620
	Kv3.1	33,000 ± 4,000	30,000 ± 5,000
	Kv4.2	8,000 ± 950	6,000 ± 870
	Jurkat-SK	22,000 ± 1,200	23,000 ± 3,000
	hSKCa2	21,000 ± 2,000	18,000 ± 3,000
	rSKCa2	>100 μM (29)	20,000 ± 3,000
	hSKCa3	28,000 ± 5,000	25,000 ± 3,000
	Slo	24,000 ± 2,000	25,000 ± 1,800
	Kir2.1	>20 μM	>20 μM
Na^+	*SKM1*	7,000 ± 550	8,000 ± 600
Ca^{2+}	Jurkat-CRAC	>20 μM	>20 μM
Cl^-	T cell swelling-activated	not done	10,000 ± 3,000
	COS-7	>20 μM	>20 μM

enzyme in human liver. These compounds do not inhibit CYP3A4 activity at 10 μM, whereas clotrimazole, for which reported EC_{50} values vary from 250 pM (34) to 30 nM (4), completely inhibits CYP3A4 at 100 nM (Fig. 2). Thus, we have successfully separated the *IKCa1*-blocking activities from cytochrome P450 inhibition.

Our results suggest that optimal potency against the *IKCa1* channel is achieved with a (2-halogenophenyl)diphenylmethane moiety substituted by a small unsubstituted polar π-electron-rich heterocyle (pyrazole or tetrazole) or a −C≡N group (Fig. 2*C*). Molecular modeling studies (AM1) render a propeller-shaped structure for the pharmacophore. The three phenyl rings are almost perpendicular to the central C—N bond axis between the triphenylmethane moiety and the imidazole or pyrazole ring. This modeled structure is in agreement with the crystal structure for clotrimazole (35).

TRAM-34 Is a Highly Selective Inhibitor of Cloned *IKCa1* and Native IK_{Ca} Currents. A pyrazole 1-[(2-chlorophenyl)diphenylmethyl]-1*H*. pyrazole (**6b**, TRAM-34) was characterized further. This highly lipophilic compound (logP value of 4.0) is readily membrane permeable. Fig. 3*A* shows the effect of externally applied TRAM-34 on *IKCa1* currents in COS-7 cells. The dose-response curve (Fig. 3*B*) reveals a K_d of 20 ± 3 nM and a Hill coefficient of 1.2 with 1 μM calcium in the pipette. Because the *IKCa1* channel is activated by cytoplasmic calcium (half activation: ≈300 nM) via a calmodulin-dependent mechanism and is not voltage dependent (7, 11, 20), we examined whether the channel's sensitivity to block by TRAM-34 depends on the intracellular calcium concentration. The K_ds measured at lower internal calcium concentrations (500 nM Ca^{2+} K_d = 24 ± 8 nM; 250 nM Ca^{2+} K_d = 28 ± 6 nM) suggest that block by TRAM-34 is not calcium dependent. The block by all triarylmethanes is voltage independent and slow in onset, taking 3–6 minutes to reach equilibrium.

Activation of human T lymphocytes via the receptor signaling complex by anti-CD3 Ab or the PKC-dependent cascade by PMA results in a 20- to 50-fold increase in *IKCa1* conductance after 48 h (Fig. 3*C*). Currents at potentials more negative than −40 mV are through the *IKCa1* channel, whereas at more depolarized potentials, K^+ currents are carried by a combination of *IKCa1* and the voltage-gated K^+ channel, *Kv1.3*. As shown in Fig. 3*D*, TRAM-34 selectively blocks the *IKCa1* current (K_d = 25 nM), whereas the residual *Kv1.3* current is blocked by the selective peptide inhibitor, ShK-Dap^{22} (22, 36). TRAM-34 also blocks *IKCa1* currents in human T84 colonic epithelial cells with equivalent potency (K_d = 22 nM).

To test the selectivity of the compound, we screened it against a panel of 15 other channels (Table 1). TRAM-34 is 200- to 1,500-fold less effective against several related mammalian potassium channels: *Kv1.1-Kv1.5*, *Kv3.1*, *Kv4.2*, *Kir2.1*, BK_{Ca}, including the closely related cloned SK_{Ca} channels (*rSKCa2, hSKCa2, hSKCa3)* as well as the native SK_{Ca} in Jurkat T cells. The CRAC calcium channel, the human *SKMI*-sodium channel, the swelling-activated chloride channel in activated human T lymphocytes, and the native chloride channel in COS-7 cells are also insensitive to TRAM-34.

TRAM-34 Suppresses Human T Lymphocyte Activation. Jensen *et al.* (13) recently showed that 10 μM clotrimazole suppresses anti-

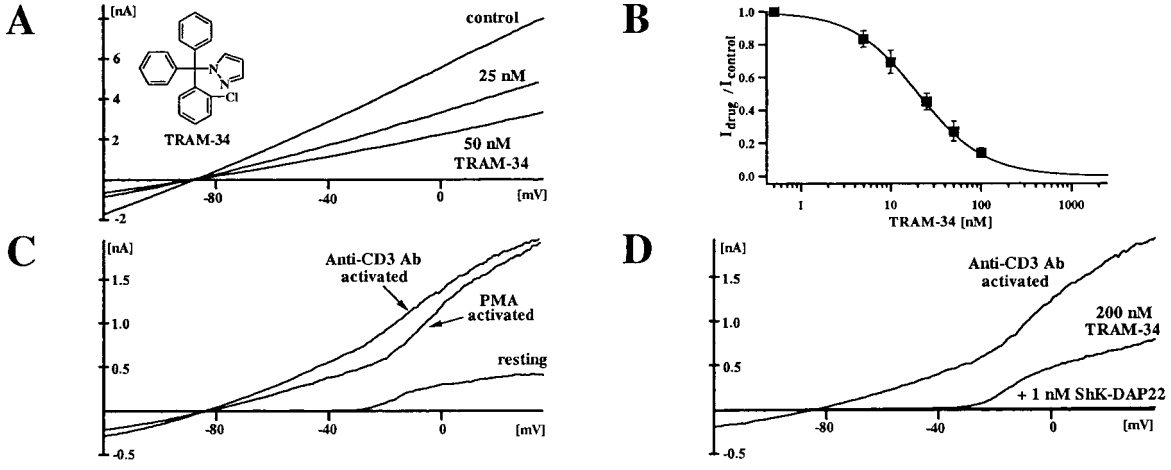

Fig. 3. (*A*) *IKCa1* currents in COS-7 cells blocked by TRAM-34. (*B*) Dose response for IKCa1 channel block by TRAM-34. The Hill equation was fitted to the reduction of slope conductance at −80 mV (15 cells). (*C*) IK_{Ca} currents in resting human T lymphocytes and in T lymphocytes activated for 2 days with PMA or anti-CD3 Ab. Mean IK_{Ca} conductance in resting cells = 0.098 (±0.17) ns (n = 24), PMA activated (10 nM) = 3.45 (±2.21) ns (n = 21), anti-CD3 Ab-activated (5 ng/ml) = 5.59 (±3.91) ns (n = 20). (*D*) Effect of TRAM-34 and ShK-Dap^{22} on K^+ currents in activated T lymphocytes.

gen- and mitogen-induced proliferation of resting human lymphocytes. Since this concentration is ≈100 times the channel-blocking dose, suppression is probably due to a nonspecific mechanism. Studies done at the same time by Khanna *et al.* (12) showed that 250 nM clotrimazole (a concentration closer to the channel-blocking dose) suppresses the activation of phytohemagglutinin-preactivated T cells more effectively than the activation of quiescent cells. However, because clotrimazole blocks both *IKCa1* and cytochrome P450 enzymes, the mechanism underlying this suppression remains unclear.

We have used TRAM-34 to evaluate the role of *IKCa1* in resting and activated lymphocytes. Quiescent cells were activated for 48 h through the T-cell-receptor signaling pathway with anti-CD3 Ab or with a combination of the PKC-activator PMA and calcium-ionophore ionomycin, in the presence or absence of TRAM-34, and the incorporation of [^{3}H]TdR measured. In parallel, cells were preactivated with either anti-CD3 Ab or PMA for 2 days to up-regulate *IKCa1* channels and then restimulated with the mitogenic combinations used on quiescent cells. Up-regulated *IKCa1* expression, to a level of several hundred channels in T cells preactivated by either stimulus, was confirmed in four of the six donors by whole-cell recording (n = 20/donor). In keeping with our expectations, TRAM-34 suppresses reactivation of lymphocytes by both mitogenic stimuli (Fig. 4 *A* and *B*, closed symbols). Sensitivity varies with the different stimuli and from donor to donor. In anti-CD3 Ab-stimulated T cells, the mean EC_{50} value among sensitive donors is 295 (±130) nM and 910 (±70) nM for less sensitive donors. In PMA + ionomycin-activated cells, including both T and B lymphocytes, the EC_{50} values are 85 (±30) nM for sensitive and 830 (±300) nM for less sensitive donors. In contrast, TRAM-34 has little effect at nanomolar concentrations on the activation of resting human lymphocytes and requires a dose 250–500 times the channel-blocking dose (5–10 μM) to inhibit [^{3}H]TdR incorporation (Fig. 4 *A* and *B*, open symbols), which may be caused by nonspecific effects. Thus, our results with TRAM-34 demonstrate that selective blockade of *IKCa1* channels preferentially suppresses mitogenesis in preactivated lymphocytes, in response to either PMA + ionomycin or to specific T-cell stimulation via CD3.

TRAM-34 Combined with Cyclosporin A. Cyclosporin A inhibits T-cell proliferation by acting on the calcineurin-dependent step in the activation cascade (19), whereas TRAM-34 acts on an earlier event, namely the modulation of calcium entry. A combination of the two compounds might therefore suppress mitogenesis more substantially than either compound alone. To test this idea, preactivated T cells were stimulated with PMA and ionomycin in the presence or absence of cyclosporin A and increasing doses of TRAM-34. The dose response for cyclosporin A-mediated suppression of [^{3}H]TdR incorporation was shifted by TRAM-34 to more sensitive values by a factor of 2- to 10-fold for donor 1 (Fig. 4*C*). Similar results were obtained with donors 2 and 6 (data not shown).

TRAM-34 Is Nontoxic in an *in Vitro* Assay and in a Limited Short-Term Acute *in Vivo* Toxicity Test. TRAM-34 (5 μM) does not reduce cell viability of human T lymphocytes or several cell lines incubated for 48 h with the compound (supplementary Table 5). Mice (n = 5) injected intravenously with a single dose of TRAM-34 (0.5 mg/kg; 29 μM) appeared clinically normal during the 7-day study. The body-weight data of the TRAM-34-treated group (day 1:17.8 g; day 7: 27.0 g) were similar to control mice injected with the vehicle (day 1: 17.4 g; day 7: 23.4 g). Collectively, data from these limited toxicity studies suggest that TRAM-34 is not acutely toxic at ≈500–1,000 times the channel-blocking dose.

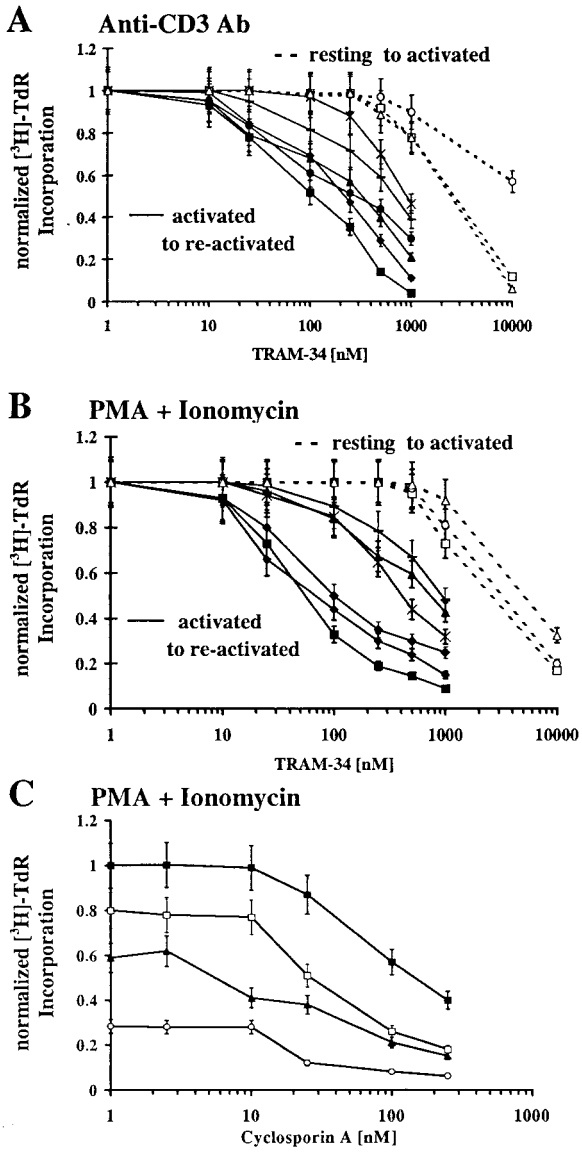

Fig. 4. Effect of TRAM-34 on anti-CD3 Ab- (*A*) or PMA + ionomycin- (*B*) stimulated [(3H)-TdR incorporation by resting and preactivated lymphocytes. PBMCs from different donors were activated with anti-CD3 Ab (5 ng/ml) or a combination of PMA (10 nM) + ionomycin (175 nM) for 48 h. [^{3}H]TdR was added to the culture for the last 6 h. In parallel, PBMCs were preactivated with either anti-CD3 Ab (5 ng/ml) or 10 nM PMA for 48 h (to up-regulate *IKCa1* channels) and then restimulated for a further 48 h with anti-CD3 Ab or PMA + ionomycin. Donor 1 resting (□), donor 2 resting (○), donor 3 resting (Δ), donor 1 preactivated (■), donor 2 preactivated (●), donor 3 preactivated (▲), donor 4 preactivated (♦), donor 5 preactivated [Xρ, donor 6 preactivated (−)]. (*C*) Effects of TRAM-34 on cyclosporin A-mediated inhibition of [(3H)-TdR incorporation. PBMCs from donor 1 were preactivated with 10 nM PMA for 48 h and then restimulated for a further 48 h with PMA + ionomycin in the presence or absence of cyclosporin A and TRAM-34. [^{3}H]TdR was added to the culture for the last 6 h. Cyclosporin (CsA) alone (■), CsA + 250 nM TRAM-34 (□), CsA + 500 nM TRAM-34 (▲), CsA + 1 μM TRAM-34 (○).

Discussion

Starting with clotrimazole, an azole antimycotic that blocks both the *IKCa1* channel and mammalian cytochrome P450 enzymes at nanomolar concentrations, we have developed compounds that selectively target *IKCa1*. The pharmacophore for channel block consists of a triphenyl moiety with an orthohalogen on one of the phenyl rings and substituted by a small unsubstituted polar

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π-electron-rich heterocyle (pyrazole or tetrazole) or a nitrile group (Fig. 2*C*). The molecular dimensions of this pharmacophore are ≈9.5 Å by 9.5Å by 8.6 Å, giving a molecular volume of 308 Å^3. Smaller molecules that keep the perfect propeller shape of the molecule retain potency (**5a** and **5b**), whereas the introduction of even small substituents such as a methyl group (**6h**, **6i**) on the heterocycle lower potency by increasing size. Replacing the heterocycle with nonaromatic substituents (e.g., **2a, 3a, 3e, 3h**) greatly reduces activity, the only exception being the nitrile group (**5a, 5b**) that has a π-electron density similar to imidazole (Figs. 1 and 2*B*). Other substitutions that alter the π-electron density in the heterocycle (**6j**) and/or distort the molecular shape (**6k**) also reduce potency. Affinity of these compounds for the channel does not correlate with their lipophilicity (supplementary Fig. 6, www.pnas.org). From these structure-activity relationships, we postulate that triphenylmethanes bind to a size-restricted pocket in the *IKCa1* channel, possibly via π–π electron interactions involving the three phenyl rings and the pyrazole, tetrazole, or imidazole moiety. Another possibility is that the benzphenone phenyl groups do not participate in binding but instead serve as a scaffold, holding the π-bonded nitrogen, quaternary carbon, and ortho-halogen in place (Fig. 2*C*).

Clotrimazole and the related triarylmethanes, although applied externally in our studies, should readily cross the cell membrane because of their lipophilicity (clotrimazole: logP: 3.5; TRAM-34 logP: 4.0) and may interact with a site on the inner surface of the channel, possibly accounting for the slow onset of block. Consistent with this idea, an earlier study with a membrane-impermeant quaternary derivative of clotrimazole revealed an internal binding site on the *IKCa1* channel (37). A molecular model of the *IKCa1* inner vestibule (38) based on the KcsA crystal structure (39) contains a putative binding pocket that is lined by residues from the cytoplasmic ends of S5 and S6 and with dimensions to match the estimated size of the triphenylmethane pharmacophore.

The most potent channel inhibitor, TRAM-34 (K_d = 20 nM), exhibits a 200- to 1,500-fold selectivity for *IKCa1* over K_v, BK_{Ca}, SK_{Ca}, Na, CRAC, and chloride channels, and unlike clotrimazole does not inhibit the major mammalian cytochrome P450 enzyme, CYP3A4. TRAM-34 also does not exhibit toxicity in an *in vitro* assay or cause obvious deleterious changes in a limited short-term acute toxicity study in rodents. [^{3}H]thymidine incorporation assays using TRAM-34 as a selective inhibitor of *IKCa1* demonstrate that the channel plays an important role in the reactivation process of human lymphocytes. *IKCa1* blockers might therefore have use for the treatment of diverse autoimmune disorders in which reactivation of T lymphocytes contributes to the pathogenesis of the disease. Because TRAM-34 and cyclosporin A suppress T-cell mitogenesis more potently than either compound alone, *IKCa1* blockers may be useful for combination therapy to reduce cyclosporin A toxicity. These encouraging results suggest that TRAM-34 should be further evaluated for possible therapeutic applications. TRAM-34 also has immediate value as a pharmacological tool to define the role of *IKCa1* channels in human tissues.

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