0026-895X/96/061672-08$3.00/0

MOLECULAR PHARMACOLOGY, 50:1672–1679 (1996).

ACCELERATED COMMUNICATION

Novel Nonpeptide Agents Potently Block the C-Type Inactivated Conformation of Kv1.3 and Suppress T Cell Activation

ANGELA NGUYEN, JOHN C. KATH, DOUGLAS C. HANSON, MICHAEL S. BIGGERS, PAUL C. CANNIFF, CAROL B. DONOVAN, ROBERT J. MATHER, MATHEW J. BRUNS, HEIKO RAUER, JAYASHREE AIYAR, ALBRECHT LEPPLE-WIENHUES, GEORGE A. GUTMAN, STEPHAN GRISSMER, MICHAEL D. CAHALAN, and K. GEORGE CHANDY

Departments of Physiology and Biophysics (A.N., J.A., A.L.-W., M.D.C., K.G.C.) and Microbiology and Molecular Genetics (G.A.G.), University of California, Irvine, California 92717, Pfizer Central Research, Groton, Connecticut 06340 (J.C.K., D.C.H., M.S.B., P.C.C., C.B.D., R.J.M., M.J.B.), and Department of Applied Physiology, University of Ulm, Ulm, Germany (H.R., S.G.)

Received July 25, 1996; Accepted September 14, 1996

SUMMARY

The nonpeptide agent CP-339,818 (1-benzyl-4-pentylimino-1,4-dihydroquinoline) and two analogs (CP-393,223 and CP-394,322) that differ only with respect to the type of substituent at the N1 position, potently blocked the Kv1.3 channel in T lymphocytes. A fourth compound (CP-393,224), which has a smaller and less-lipophilic group at N1, was 100–200-fold less potent, suggesting that a large lipophilic group at this position is necessary for drug activity. CP-339,818 blocked Kv1.3 from the outside with a IC_{50} value of ~200 nM and 1:1 stoichiometry and competitively inhibited ^{125}I-charybdotoxin from binding to the external vestibule of Kv1.3. This drug inhibited Kv1.3 in a use-dependent manner by preferentially blocking the C-type inactivated state of the channel. CP-339,818 was a significantly less potent blocker of Kv1.1, Kv1.2, Kv1.5, Kv1.6, Kv3.1-4, and Kv4.2; the only exception was Kv1.4, a cardiac and neuronal A-type K^+ channel. CP-339,818 had no effect on two other T cell channels (I_{CRAC} and intermediate-conductance K_{Ca}) implicated in T cell mitogenesis. This drug suppresses human T cell activation, suggesting that blockade of Kv1.3 alone is sufficient to inhibit this process.

Peripheral blood human T lymphocytes express a K^+ channel termed type *n* (1–3), which is encoded by the *Shaker*-related Kv1.3 gene (4–6). The functional channel, a homotetramer of Kv1.3 subunits, is voltage dependent ($V_{1/2}$ = −35 mV), shows cumulative C-type inactivation, has a single-channel conductance of 18 pS, and is potently blocked by several scorpion toxins (2, 4–8), including ChTX, MgTX, noxiustoxin, and KTX. Several nonpeptide antagonists (e.g., 4-aminopyridine, tetraethylammonium, quinine, verapamil, nifedipine) also block this channel but with significantly lower potency (1, 2, 9). Quiescent human T cells express ~400 Kv1.3 channels, and this number increases ~2-fold after activation (3).

A pharmacological approach has served to define the role of Kv1.3 in T lymphocytes. 4-Aminopyridine, tetraethylammonium, quinine, verapamil, nifedipine, and several other nonpeptide agents, although differing in structure, block Kv1.3 and in a parallel potency sequence inhibit T lymphocyte activation (1–3, 9). Results of studies with the more selective and potent peptide antagonists ChTX, MgTX, and KTX have corroborated these findings (10–12). Kv1.3 antagonists are thought to influence mitogenesis by regulating the membrane potential (3, 10–13). MgTX, for example, induces membrane depolarization, which in turn attenuates the calcium-signaling response (11, 12), presumably by reducing the electrical driving force for calcium influx through the I_{CRAC}

This research was supported by Grants AI24783 (K.G.C.), NS14609 (M.D.C.), and GM41514 (M.D.C.) from the National Institutes of Health and by Pfizer Central Research (Groton, CT) (K.G.C., M.D.C., G.A.G., J.A.).

ABBREVIATIONS: ChTX, charybdotoxin; MgTX, margatoxin; mKv1.3, mouse Kv1.3; KTX, kaliotoxin; PMA, phorbol-12-myristate-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA; 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; MNC, mononuclear cell; HBSS, Hanks' balanced saline solution; VV, vaccinia viruses; $[K^+]_o$, extracellular potassium; I_{CRAC}, calcium-release activated calcium.

current (3). Membrane depolarization also inhibits interleukin-2 production and ^{3}H-thymidine incorporation (14). MgTX also suppresses delayed-type hypersensitivity and allogeneic-antibody responses in miniswine (15). All these lines of evidence indicate that Kv1.3 channels play an important role in T cell activation, both *in vitro* and *in vivo*.

Due to its restricted tissue distribution (16) and important role in T lymphocyte activation (3), the Kv1.3 channel is an attractive target for the development of novel, therapeutically useful immunosuppressive agents. To this end, several groups have mounted high-throughput screens to identify potent and selective nonpeptide Kv1.3 blockers, and the compound WIN 17317–3 (1-benzyl-7-chloro-4-*n*-pentylimino-1,4-dihydroquinoline hydrochloride) was discovered through such an effort (17, 18). This drug potently blocks Kv1.3 (IC_{50} = ~200 nM), competitively inhibits ^{125}I-ChTX binding to the channel, and suppresses T cell activation (17, 18). In this report, we describe three novel analogs of WIN 17317–3 that are potent and selective inhibitors of Kv1.3. Detailed analysis of one of these compounds, CP-339,818, revealed that the drug blocks the channel by interacting with residues exposed during C-type inactivation and suppresses T cell activation at similar concentrations.

Materials and Methods

Synthesis of CP-339,818 and three related analogs. Four analogs of WIN 17317–3 (CP-339,818, CP-393,223, CP-393,224, and CP-394,322) were synthesized as described previously (17). Purification of these compounds was accomplished by recrystallization from ethanol. All compounds were fully characterized by proton NMR, carbon NMR, and mass spectroscopy.

Chemicals and solutions. Cells were bathed in mammalian Ringer's solution containing 160 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with NaOH. The internal pipette solution contained 134 mM KF, 2 mM $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, and 10 mM EGTA, adjusted to pH 7.2 with KOH (290–320 mOsM). A syringe-driven perfusion system was used to exchange the bathing solution. KTX was purchased from Peptides International (Louisville, KY), PMA was from Sigma Chemical (St. Louis, MO), ionomycin was from Calbiochem (San Diego, CA), anti-CD3 antibody (Leu-4) was from Becton Dickinson (San Jose, CA), ^{3}H-thymidine was from New England Nuclear Research Products (Cambridge, MA), and Ficoll-Paque was from Pharmacia (Piscataway, NY). For the experiments on the I_{CRAC} current, internal solutions contained 128 mM Cs^+ aspartate, 10 mM HEPES, pH 7.2, 12 mM BAPTA, 3-16 mM $MgCl_2$, and 0.9 mM $CaCl_2$; free [Ca^{2+}] was 10 nM. The external solution contained 120 mM Na, 20 mM Ca^{2+}, 2 mM Cl^-, 158 mM methanesulfonate, 10 mM HEPES, and 5 mM glucose, pH 7.4. I_{CRAC} was activated by passive store depletion. For the K_{Ca} channel studies, the pipette solution contained 150 mM K^+-aspartate, 2 mM $MgCl_2$, 5 mM HEPES, and 10 mM EGTA, 8.7 mM $CaCl_2$, pH 7.2 (290 mOsM). For the experiments on swelling activated Cl^- channels, the internal solution contained 145 mM Cs^+-glutamate, 10 mM Cs^+-EGTA, 4 mM Na-ATP, 2.3 mM $CaCl_2$, 2.3 mM $MgCl_2$, and 10 mM Cs^+-HEPES, pH 7.2.

Cell lines, cells, and activation assays. Rat basophilic leukemia cells, maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 1% L-glutamine, were plated onto glass coverslips 1 day before use in electrophysiological experiments.

Purification of MNCs. Human MNCs were separated on a Ficoll-Paque gradient and further purified by depleting non-T cells using the T Kwik monoclonal antibody cocktail and complement lysis (One Lambda, Canoga Park, CA). T cells were then resuspended in medium (RPMI-1640 containing 10% heat-inactivated fetal calf serum, 25 mM HEPES, 2 mM L-glutamine, 1 × nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin). Cell purity was determined by FACScan analysis. Cell lines stably expressing Kv1.1, Kv1.2, Kv1.3, Kv1.5, and Kv3.1 (7) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and G418 (1 mg/ml).

Activation of human T cells by anti-CD3 antibody and by ionomycin/PMA. Anti-CD3 antibody was added to each well (25 ng/well) of a 96-well plate (125 μl total volume) and incubated for 2 hr at 37°. Human MNCs (1.25×10^5 cells/well) were plated with or without the Kv1.3 antagonists, incubated at 37° for 24 hr, pulsed with ^{3}H-thymidine (1 μCi/ml, 25 μl/well) for an additional 18 hr, and then harvested with a TOMTEC Mach-2 harvester (Tomtech International, Orange, CT). Control wells contained MNCs without drug.

Human T cells were plated onto a 96-well plate (1.25×10^5 cells/well; 125 μl total volume) and incubated with the Kv1.3 antagonists for 15 min at room temperature. After the addition of ionomycin (15.6 ng/well) and PMA (0.625 ng/well), cells were incubated for 24 hr at 37°, pulsed with ^{3}H-thymidine (1 μCi/ml, 25 μl/well) for an additional 18 hr, and then harvested. Control wells contained T cells without drug.

Rubidium efflux assay. Human T cells (10^7 cells/ml) in medium were incubated with ^{86}RbCl (10 mCi/ml; New England Nuclear) for 18 hr at 37° in 5% CO_2. Cells were then washed in low-K^+ HBSS medium [4.5 mM K^+, prepared according to the GIBCO (Grand Island, NY) using ultrapure water] and resuspended at 5.5×10^6 cells/ml in low-K^+ HBSS. Fifty microliters of the cell suspension was incubated for 15 min at an ambient temperature with 10 μl of the test compound (in 4.5 mM K^+ HBSS) in a well of a Millipore Multiscreen 96-well 0.65-μm filtration plate (no. MADV-N6550). The mixture was then incubated for 20 min at the same temperature with 125 μl of 140 mM K^+ HBSS (NaCl replaced by KCl) added to initiate ^{86}Rb efflux via membrane depolarization (125 μl of 4.5 mM K^+ HBSS was added to control wells) and filtered with a TV-1 vacuum transfer manifold (Pall Trinity Micro, Cortland, NY) into standard 96-well microtiter plates (Costar, Cambridge, MA); 100 μl of the filtrate was removed and counted in a Beckman LSC (Beckman Instruments, Palo Alto, CA) for 1 min.

ChTX binding assay. HeLa cells were coinfected with two recombinant VV. VV:TF7-3 virus encodes bacteriophage T7 RNA polymerase, whereas VV:Kv1.3 contains the entire mKv1.3 coding sequence under control of the T7 promoter. In dually infected cells, T7 RNA polymerase is made, which in turns activates the T7 promoter and results in overexpression of Kv1.3 channels. These cells were incubated in normal-ionic-strength buffer (150 mM NaCl, 5 mM KCl, 10 mM Na-HEPES, 6 mM glucose, pH 7.4) with 25 pM ^{125}I-ChTX (custom iodinated by Amersham, Arlington Heights, IL) and increasing concentrations of either cold ChTX (0–100 nM; Peptides International) or the test compound in a total volume of ~800 μl in 12 × 75-mm glass tubes. Test compounds were added 15 min before the radioiodinated probe, and incubation was carried out on a rotary shaker for 1 hour at an ambient temperature. Binding was stopped with 4 ml of ice-cold quench buffer (200 mM NaCl, 20 mM HEPES, free acid, pH 7.4). Cells were collected using a Hoeffer filtration device onto Whatman GF/C filters that were presoaked in 0.3% polyethyleneimine in 18 MΩ of water for 15 min. Filters were counted for 1 min with a Beckman 5500 γ counter.

Generation and analysis of Kv1.3 mutants and related Kv expression constructs. All of the mKv1.3 mutants and the mKv1.4 expression construct used in this study, with the exception of mKv1.3 V406D, have been described previously (8, 19). All of these mutants were generated through a two-step polymerase chain reaction method (20). Cell lines stably expressing Kv1.1, Kv1.2, Kv1.3, Kv1.5, and Kv3.1 have been previously described (7). The other expression constructs used in this study were gifts from Dr. Olaf Pongs [Hamburg, Germany (Kv1.6, Kv3.4)], Dr. Mark Tanouye [University of

California, Berkeley, Berkeley, CA (Kv3.2)], and Dr. David MacKinnon [State University of New York at Stonybrook, Stonybrook, NY (Kv4.2)].

cRNA was transcribed *in vitro* (Stratagene, La Jolla, CA, or Boehringer-Mannheim Biochemica, Mannheim, Germany) and diluted in a 0.1–0.5% fluorescein-Dextran (M_r, 10,000; Molecular Probes, Eugene, OR) in 100 mM KCl. Rat basophilic leukemia cells were injected using prepulled injection capillaries (Femtotips) in combination with an Eppendorf microinjection system (micromanipulator 5171 and tranjector 5242, Madison, WI) as previously described (21). Rat basophilic leukemia cells were chosen because they lack endogenous voltage-gated channels. The inward rectifier K^+ channels present in these cells are blocked by G protein stimulation initiated by dialysis with F^- or by depletion of nucleotides (22). Four hours later, fluorescent cRNA-injected cells cells were evaluated electrophysiologically.

Electrophysiological analysis. Most experiments were carried out in the whole-cell configuration of the patch-clamp technique (2, 23). All membrane currents were recorded at room temperature (22–26°) with either a LIST EPC-7 or an EPC-9 amplifier (Heka Elektronik, Lambrecht, Germany). Series-resistance compensation (80%) was used if the current exceeded 2 nA. Capacitative and leak currents were subtracted using the P/8 procedure. The command input of the EPC-7 amplifier was controlled by a PDP 11/73 computer via a digital-to-analog converter. The EPC-9 amplifier was interfaced to a Macintosh computer running acquisition and analysis software (Pulse/Pulsefit). The holding potential in all experiments was −80 mV, unless indicated otherwise.

Results

CP-339,818 and analogs block Kv1.3. CP-339,818 (1-benzyl-4-pentylimino-1,4-dihydroquinoline) is shown in Fig. 1, along with three analogs that differ from the parent compound only in the type of substituent at the N1 position. Drug potency on the channel was measured using three independent assays: patch-clamp, competitive inhibition of ^{125}I-ChTX binding, and ^{86}Rb efflux. We found good correspondence between the IC_{50} values measured with the patch-clamp and ^{125}I-ChTX competitive-inhibition binding assays (Table 1 and Fig. 2). Surprisingly, the drugs were significantly less effective in the ^{86}Rb-efflux assay (Table 1). The reasons for this difference are discussed later.

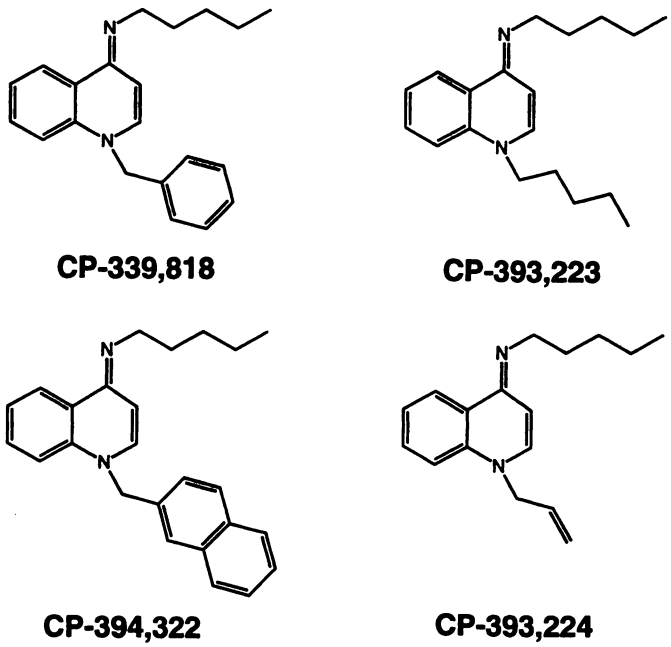

Fig. 1. Chemical structures of CP 339,818 and analogs.

The three compounds containing either benzyl (CP-339,818), pentyl (CP-393,223), or naphthyl (CP-394 322) groups at the N1 position were all equally potent blockers of Kv1.3 (Table 1). The first two compounds have approximately the same size and lipophilic character, whereas the third is larger and more lipophilic. Substitution with the smaller and less-lipophilic allyl group (CP-393,224) reduced potency ~10-200-fold in all three assays (Table 1). Collectively, these results suggest that a large lipophilic group at the N1 position is important for block of Kv1.3.

More detailed studies were performed on CP-339,818. Consistent with its ability to competitively inhibit ^{125}I-ChTX (Table 1) binding to its receptor within the outer vestibule (8), externally applied CP-339,818 blocked Kv1.3 currents in peripheral blood human T cells in a concentration-dependent manner (Fig. 2, A and B), with a IC_{50} value of 230 nM and a Hill coefficient close to unity. This block was almost completely reversed after extensive washing. The pK_a value of this compound is 11 (17), suggesting that CP-339,818 most likely blocks the channel in a charged form at pH 7.4

Blockade occurs in a use-dependent manner via drug binding to the inactivated state of the channel. CP-339,818 at a concentration (1 μM), which blocks almost all of the Kv1.3 current, required multiple openings of the channel to reach steady state block, a phenomenon termed "use-dependent inhibition." The time to reach steady state block depended on the duration of the depolarizing pulse and on the holding potential (Fig. 3). When repeated depolarizing pulses (200 msec at 30-sec intervals) were applied from a holding potential of −80 mV, steady state block was reached within ~600 sec. Lengthening the pulse duration (e.g., 2 sec) significantly shortened the time to reach steady state block, whereas a shorter pulse duration (e.g., 20 msec) had the reverse effect (Fig. 3A). This result suggests that CP-339,818 blocks a postactivation state, possibly the open and/or inactivated conformations of the channel.

To distinguish between these two possibilities, we examined the effect of changing the holding potential from −80 to −50 mV on the time required to reach steady state block while the pulse duration was kept constant at 200 msec. The more-depolarized holding potential would significantly increase the proportion of channels in the inactivated conformation by retarding recovery from inactivation without changing the open time duration (2, 23). As shown in Fig. 3B, CP-339,818 blocked Kv1.3 more rapidly at a holding potential of −50 mV than at −80 mV, suggesting that the drug preferentially blocks the inactivated state of the channel.

Sensitivity of the channel to block by CP-339,818 is dependent on its ability to undergo C-type inactivation. Inactivation in voltage-gated K^+ channels occurs via two distinct mechanisms. The first of these, N-type inactivation, involves a tethered ball in the amino terminus interacting with and blocking the internal mouth of the pore (for a review, see Ref. 16). C-type inactivation, on the other hand, involves a conformational change resulting in the closure of the external mouth of the channel (24–28). Kv1.3 exhibits only C-type inactivation (24, 25). If CP-339,818 binds to this inactivated conformation, then removal or slowing of inactivation should reduce the sensitivity of the channel to this

TABLE 1

Binding assay values

Values shown represent K_d values ± standard error; numbers in parentheses represent the number of experiments performed.

Compound	^{125}I-ChTX binding	Patch-clamp	^{86}Rb-efflux
		nM	
CP-339818-04	120 ± 10 (88)	150 ± 50 (7)	2,300 ± 400 (7)
CP-394322-01	90 ± 20 (3)	230 ± 150 (2)	2,100 ± 300 (3)
CP-393223-01	70 ± 30 (2)	N.D.	3,800 ± 100 (2)
CP-393224-01	3,600 ± 950 (3)	32,000 ± 10,000 (2)	24,000 ± 500 (2)
ChTX	0.81 ± 0.13 (11)	1.5 ± 0.12 (10)	1.3 ± 0.5 (4)
MgTX	0.24 ± 0.06 (2)	0.09 ± 0.02 (4)	0.09 ± 0.04 (3)

N.D., not determined.

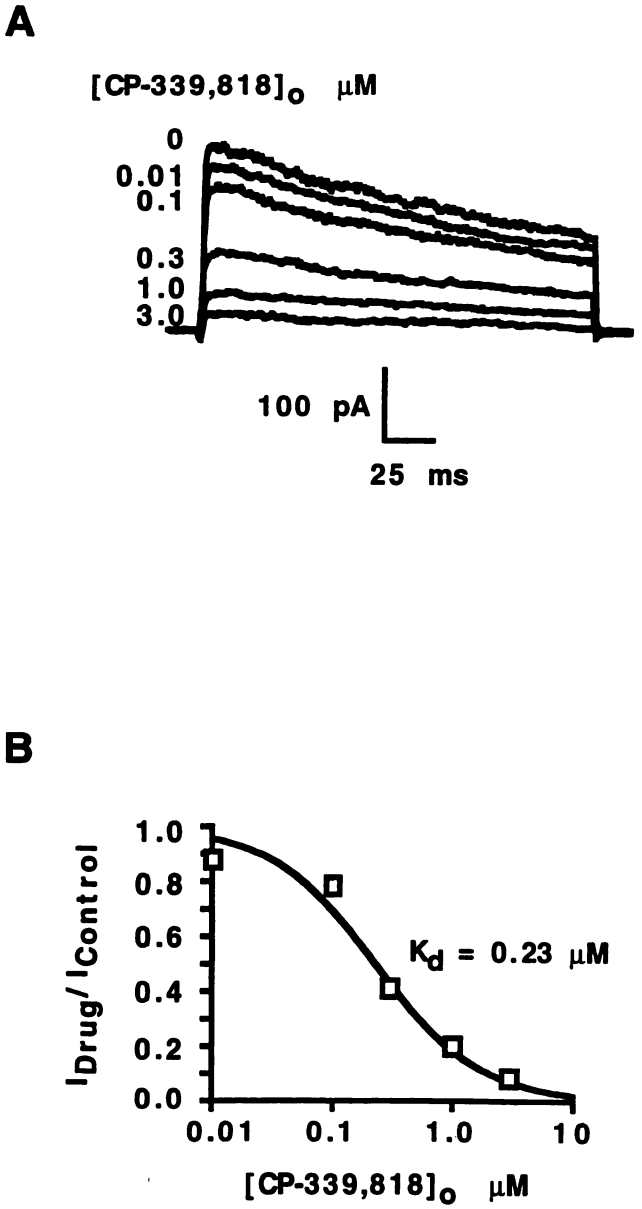

Fig. 2. A, CP-339,818 blocked Kv1.3 from the outside. Typical Kv1.3 currents in human peripheral blood T cells studied in the whole-cell configuration and blocked with drug added to the external bathing solution. B, Hill plot of data in A.

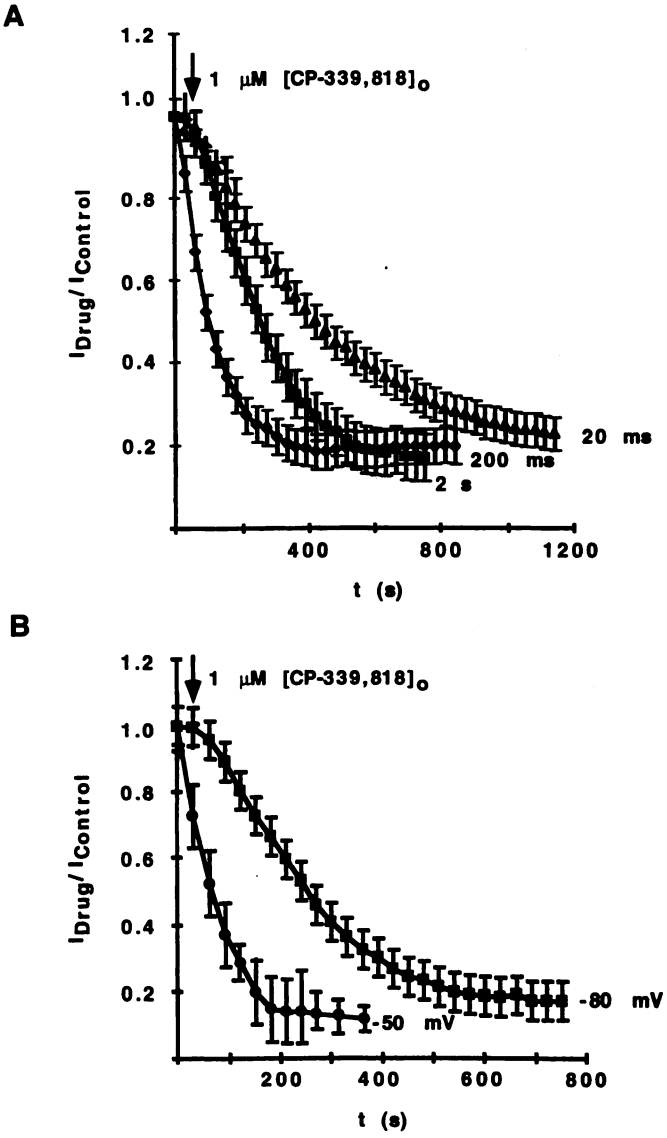

Fig. 3. Use-dependent block of Kv1.3 currents in human T cells by 1 µM CP-339,818. A, Effect of varying the pulse duration on time to reach steady state block. *Vertical axis*, ratio of current at different times after drug addition versus at time 0. All experiments were performed in the whole-cell mode. Holding potential, −80 mV. Depolarizing durations: 20 msec, 200 msec, and 2 sec. Interpulse interval, 60 sec. B, Effect of varying the holding potential (as indicated) on time to reach steady state block. Pulse duration, 200 msec. Interpulse interval, 60 sec.

drug by decreasing the substrate for drug-binding (i.e., the fraction of channels in the inactivated conformation). Alterations in C-type inactivation can be accomplished through site-specific mutagenesis of vestibular residues (25, 27, 28) or by increasing the external K^+ ion $[K^+]_o$ concentration (23, 27, 29).

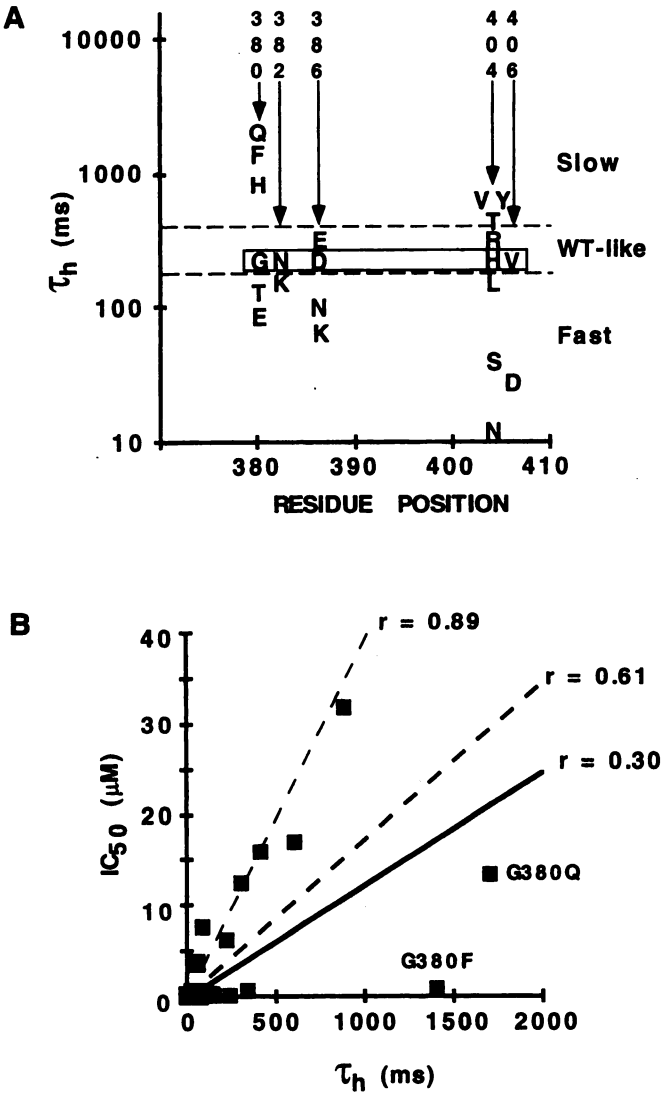

Fig. 4. A, τ_h of Kv1.3 vestibular mutants. *Horizontal axis*, residue positions in the P region and adjacent sequence. *Vertical axis*, τ_h. B, Direct relationship between τ_h for each Kv1.3 mutant and its sensitivity to block by CP-339,818 (IC_{50}). All experiments were performed in the whole-cell mode, with repeated 200-msec pulses to +40 mV at 30-sec intervals. *Solid regression line*, all data included; *heavy dashed regression line*, G380F omitted; *dashed regression line*, G380F and G380Q omitted; *WT*, wild-type.

External vestibular mutants alter τ_h. We analyzed the effects on C-type inactivation of various substitutions at five positions in the external vestibule of Kv1.3 (G380, N382, D386, H404, and V406). G380 lies at the outer margin of the vestibule; N382 and D386 are positioned at the periphery of its base; and H404 and V406 are located near the central axis, at the entrance to the pore (8). Mutations at all five positions alter τ_h (Fig. 4A and Table 2), suggesting that the entire vestibule might participate in the conformational change associated with C-type inactivation. The mutants fall into three groups: those that inactivate faster than WT Kv1.3 ("fast," or group 1; τ_h = <200 msec); those that remain unchanged ("wild-type-like," or group 2; τ_h = 200–400 msec); and those that inactivate more slowly ("slow," or group 3; τ_h = >400 msec). In general, mutants in group 1 have charged or polar substitutions, whereas those in group 3 have hydrophobic or aromatic residues in place of the native residues.

TABLE 2

Alterations in τ_h by mutations

Values are mean ± standard error; numbers in parentheses represent the number of experiments performed.

	Position	τ_h	IC_{50}
		msec	*μM*
Slow	H404T	410 ± 4.6 (9)	16 ± 2.8 (2)
	H404V	600 ± 220 (12)	17 ± 4.8 (4)
	H404Y	605 ± 130 (7)	17 ± 2.1 (2)
	G380H	880 ± 240 (7)	32 ± 1.4 (2)
	G380Q	1700 ± 340 (6)	14 ± 4.4 (5)
	G380F	1400 ± 300 (5)	1.02 ± 0.19 (3)
Wild-type-like	Wild-type	230 ± 92 (12)	0.23 ± 0.12 (4)
	H404R	340 ± 61 (6)	0.64 ± 0.04 (2)
	H404L	220 ± 16 (6)	6.3 ± 0.06 (2)
	D386E	300 ± 75 (9)	13 ± 5 (2)
Fast	V406D	29 ± 9 (5)	0.46 ± 0.15 (3)
	H404N	4 ± 0 (5)	0.25 ± 0.10 (2)
	H404S	41 ± 21 (8)	3.8 ± 2.0 (3)
	D386K	67 ± 16 (9)	0.19 ± 0.04 (2)
	D386N	100 ± 41 (7)	0.73 ± 0.29 (5)
	G380T	130 ± 59 (4)	0.28 ± 0.21 (4)
	G380E	86 ± 22 (10)	7.7 ± 0.92 (2)

Many of these mutants were then tested for their sensitivity to block by CP-339,818.

Vestibular mutations that slow τ_h also reduce sensitivity to CP-339,818. In general, mutants belonging to the slow group were least sensitive to CP-339,818, whereas those belonging to the rapid and wild-type-like groups were potently blocked by the drug (Table 2). A direct, although weak, linear relationship ($r = 0.30$; $p > 0.1$) was noted between τ_h and IC_{50} values for these mutants (Fig. 4B, *solid line*). This relationship becomes statistically significant ($r = 0.61$; $p = 0.01$) when the single G380F mutant that deviates most significantly from this relationship is omitted (*heavy dashed line*) and even more striking ($r = 0.89$; $p = 0.0001$) when both G380F and G380Q are omitted (*dashed line*). These results, in combination with those presented above (Fig. 3B), are consistent with idea that CP-339,818 binds to residues in the vestibule that are solvent exposed in the inactivated conformation and become less accessible in noninactivating or slowly inactivating mutants.

$[K^+]_o$ (160 mM) slows C-type inactivation and reduces sensitivity to CP-339,818. A second method to slow C-type inactivation is to increase $[K^+]_o$ (23, 27, 29). We therefore evaluated the effect of 160 mM $[K^+]_o$ on drug block of representative channels belonging to each of the three inactivation groups: fast (H404N), wild-type-like (wild-type Kv1.3, N382K) and slow (H404V). As shown in Fig. 5A, 160 mM $[K^+]_o$ significantly slowed inactivation of wild-type Kv1.3 and N382K channels. Replacement of H404 with the hydrophobic residue valine (H404V) or the polar residue asparagine (H404N) substantially reduced $[K^+]_o$-dependent slowing of C-type inactivation, suggesting that H404 might serve as one potential $[K^+]_o$-modulatory site (Fig. 5A). In keeping with our mutant data, the degree of slowing of τ_h induced by 160 mM $[K^+]_o$ (Fig. 5A) correlated well with the decrease in sensitivity of the channels to block by CP-339,818 (Fig. 5B). Collectively, these results provide further support for the idea that CP-339,818 preferentially binds to residues that become accessible in the inactivated conformation.

CP-339,818 is a selective blocker of Kv1.3 and Kv1.4. To determine the selectivity of the compound for Kv1.3, we

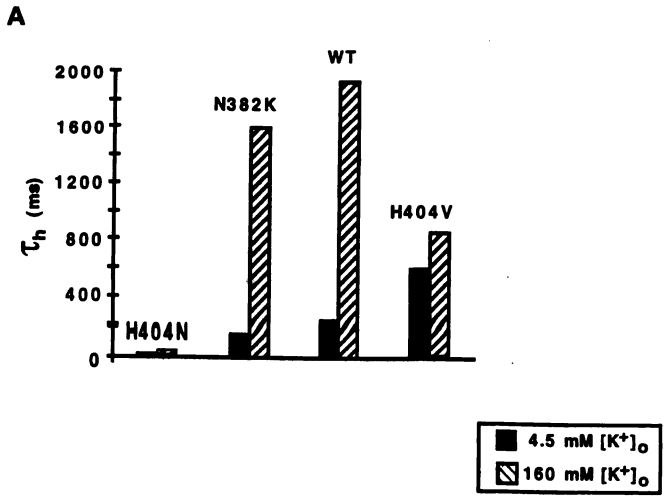

Fig. 5. Effect of $[K^+]_o$ concentration on τ_h and IC_{50} values. Increasing $[K^+]_o$ slowed C-type inactivation (A) and reduced sensitivity to block by CP-339,818 of representative channels (B) from each of the three classes: rapid (H404N), wild-type-like (WT Kv1.3, N382K), and slow (H404V).

TABLE 3

Sensitivities of channels to block

Values are mean ± standard error; numbers in parentheses represent the number of experiments performed.

Channel	CP-339,818
	μM
Cloned channels	
Kv1 family	
Kv1.1	62 ± 9.9 (2)
Kv1.2	14 ± 1.8 (2)
Kv1.3	0.23 ± 0.12 (4)
Kv1.4	0.3 ± 0.1 (3)
Kv1.5	19 ± 0.71 (2)
Kv1.6	20 ± 3 (2)
Kv3 family	
Kv3.1	17 ± 2.1 (2)
Kv3.2	10 ± 1 (3)
Kv3.4	36 ± 5.7 (2)
Kv4 family	
Kv4.2	>10
T cells	
K_{Ca}	> 500 (2)
I_{CRAC}	>100 (3)
SWAC[a]	>500 (2)

[a] Swelling activated Cl^- channel.

tested its potency against a panel of 10 related cloned K^+ channel targets. Nine of these channels were 1–3 orders of magnitude less sensitive to block by CP-339,818 (Table 3). The single exception, Kv1.4, is an A-type channel expressed in the heart and the brain (30–33). CP-339,818 blocked Kv1.4 with a IC_{50} value of ~300 nM (Table 3), making it the most potent antagonist of this channel.

As a further test of selectivity, we examined the effects of CP-339,818 on two other channels in human peripheral blood T cells that have been implicated in mitogenesis. The first of these, a ChTX-sensitive intermediate-conductance K_{Ca} channel, is found in low numbers in resting human T cells, with the number increasing 10–20-fold after activation (3). The second, I_{CRAC}, is a voltage-independent calcium influx current that plays an essential role in the signaling events during mitogenesis (3). Both these channels are 100–200-fold less sensitive to CP-339,818 than Kv1.3 (Table 3). Another channel that was not affected was the swelling activated chloride channel in human Jurkat T cells (Table 3). Because Kv1.4 is not expressed in T cells, CP-339,818 can be used as specific pharmacological blocker of Kv1.3 to determine the role of this channel in T cell activation.

CP-339,818 suppresses human peripheral blood T cell activation. There has been controversy regarding the suppressive effect of Kv1.3 blockers on human T cell activation. Using the Kv1.3-specific peptide blocker MgTX, Leonard *et al.* (11) reported that blockade of Kv1.3 alone inhibited T cell activation. More recent studies with other peptide toxins, KTX and ChTX, suggest that blockade of both Kv1.3 and the intermediate conductance K_{Ca} channel are required for inhibition of mitogenesis (13). To distinguish between these possibilities, we used CP-339,818 as a selective nonpeptide pharmacological probe of Kv1.3. This drug suppressed ^{3}H-thymidine incorporation by human peripheral blood T cells induced by anti-CD3 antibody (IC_{50} = 4.7 ± 2.1 μM, six experiments) or a combination of ionomycin and PMA (IC_{50} = 5.2 ± 0.6 μM, six experiments). These data indicate that blockade of Kv1.3 channels alone is sufficient to inhibit T cell activation and validate our results with less-specific nonpeptide antagonists (1, 9).

Discussion

We described three related 1,4-dihydroquinoline compounds that are potent and selective antagonists of the Kv1.3 channel in T cells. In an earlier report on this class of compounds, the length of the 4-alkylimino group was determined to be 5 or 6 carbons for optimal activity (17). In the current study, we demonstrate the requirement for a large lipophilic group at the N1 position for optimal function. One of these compounds, CP-339,818, was studied in detail.

Three independent lines of evidence suggest that the drug preferentially blocks the inactivated conformation of the channel. First, the sensitivity of the channel to CP-339,818 was enhanced as the fraction of inactivated channels increased by changing the holding potential from −80 to −50 mV (Fig. 3B). Second, significant reduction in the proportion of inactivated channels via exposure to 160 mM external K^+ greatly diminished drug potency (Fig. 5). Last, studies with a panel of external vestibular Kv1.3 mutants revealed a direct relationship between the inactivation time constant and IC_{50}. G380F and G380Q, the two mutants that deviate most strikingly from this relationship, may be capable of forming novel positive contacts with CP-339,818 that mask the overall selectivity of the drug for the inactivated state. Because CP-339,818 competitively inhibits ^{125}I-ChTX binding to res-

idues in the external vestibule of Kv1.3, this drug may interact with vestibular residues that become exposed to solvent in the inactivated state. However, the precise residues responsible for binding the drug remain to be identified.

Cysteine-scanning mutagenesis studies on the *Shaker* channel indicate that the side chains of residues corresponding to M403, H404, and P405 in Kv1.3 become more accessible to MTSET reagents in the C-type inactivated conformation compared with the closed or open states of the channel (26). These data suggest that conformational changes associated with inactivation enhance the exposure of external vestibular residues to the solvent phase (26). If a similar conformational change occurs in Kv1.3, the increased exposure of vestibular residues during inactivation might provide binding sites for CP-339,818. For example, CP-339,818 could bind to H404 via an amino-aromatic interaction, similar to the one reported between a histidine in the neurokinin-1 receptor and a benzhydryl in its antagonist, CP-96,345 (34). Once bound, the drug might "lock" the channel in the inactivated conformation. This notion is generally consistent with the effects of vestibular mutations on the inactivation rate of the channel and its sensitivity to block by CP-339,818. Mutants containing hydrophobic residues (e.g., valine or tyrosine) that might prefer to remain buried have slow inactivation rates and may be insensitive to CP-339,818 because the drug has lost critical binding sites on the channel. In contrast, scorpion toxins, which rely on multiple contacts in the vestibule to stabilize their interaction with the channel, block inactivating and noninactivating Kv1.3 mutants with almost equal potency (8).

CP-339,818 is significantly less potent in the ^{86}Rb-efflux assay compared with its activity as measured by patch-clamp or ^{125}I-ChTX binding (Table 1), possibly because the ^{86}Rb-efflux assay relies on the presence of a high external concentration of $[K^+]_o$ to initiate ionic flux, which also slows C-type inactivation and reduces the sensitivity of the channel to CP-339,818 (Fig. 5). In contrast, ChTX and MgTX, which block both inactivating and noninactivating channels with comparable potency (8), are equally effective in the ^{86}Rb-efflux, ^{125}I-ChTX-binding, and patch clamp assays (Table 1). Thus, ^{86}Rb-efflux assays may underestimate the potency of some blockers that preferentially interact with the inactivated conformation of the channel. Interestingly, of the three methods used to assess block of Kv1.3 by CP-339,818, the IC_{50} value obtained through the ^{86}Rb-efflux assay was closest to the IC_{50} value for block of T cell activation by this drug (Table 1). This result suggests that a significant proportion of the channels in the resting T cell may not reside in the inactivated conformation. Consistent with this idea, a 15-min preincubation of human T cells with CP-339,818 before 140 mM $[K^+]_o$ initiation of ^{86}Rb-efflux (during which the drug should have been able to interact with any inactivated channels in T cells) did not change drug potency. However, many other factors can contribute to potency shifts in functional assays, such as binding of drugs to serum proteins.

In addition to its effect on Kv1.3, CP-339,818 inhibits the *Shaker*-related A-type K^+ channel, Kv1.4, with a IC_{50} value of ~300 nM. This channel has phenotypic properties resembling those of the neuronal afterhyperpolarization-inducing channel (30) and the 4-aminopyridine-sensitive component of the cardiac I_{to} current (31–33). This channel exhibits only N-type inactivation (35). Because none of the known toxins block this channel (16), CP-339,818 seems to be the most potent known blocker of this channel. Because Kv1.3 is not expressed in the heart (16) and because CP-339,818 does not block other cardiac channels (e.g., Kv1.1, Kv1.2, Kv1.5, Kv4.2), this compound could be used as a selective Kv1.4 blocker to determine the contribution of this channel to the cardiac action potential and in particular to the I_{to} current.

The potential of Kv1.3 as a target for an immunosuppressive drug has long been recognized. Although CP-339,818 is a potent inhibitor of Kv1.3 and inhibits lymphocyte activation, it lacks the selectivity and potency required of a therapeutic drug. Nevertheless, it is a useful experimental reagent for studying the physiological roles of Kv1.3 in lymphocytes, and it might potentiate other therapeutic immunosuppressants such as cyclosporin and FK-506. In addition, it could serve as a template for the design of a new generation of channel blockers selective for either Kv1.3 or Kv1.4, which could have therapeutic roles in modifying the immune system or the cardiac action potential.

Acknowledgments

We thank Luette Forrest and Brent Dethlefs for technical assistance.

References

1. DeCoursey, T. E., K. G. Chandy, S. Gupta, and M. D. Cahalan. Voltage-gated potassium channels in human T lymphocytes: a role in mitogenesis? *Nature (Lond.)* **307**:465–467 (1984).
2. Cahalan, M. D., K. G. Chandy, T. E. DeCoursey, and S. Gupta. A voltage-gated K^+ channel in human T lymphocytes. *J. Physiol.* **358**:197–237 (1985).
3. Lewis, R. S., and M. D. Cahalan. Potassium and calcium channels in lymphocytes. *Annu. Rev. Immunol.* **13**:623–654 (1995).
4. Grissmer, S., B. Dethlefs, J. J. Wasmuth, A. L. Goldin, G. A. Gutman, M. D. Cahalan, and K. G. Chandy. Expression and chromosomal localization of a lymphocyte K^+ channel gene. *Proc. Natl. Acad. Sci. USA* **87**:9411–9415 (1990).
5. Douglass, J., P. B. Osborne, Y. C. Cai, M. Wilkinson, M. J. Christie, and J. P. Adelman. Characterization of RGK5, a genomic clone encoding a lymphocyte channel. *J. Immunol.* **144**:4841–4850 (1990).
6. Attali, B., G. Romey, E. Honore, A. Schmid-Alliana, M.-G. Mattei, F.Lesage, P. Ricard, J. Barhanin, and M. Ladzunski. Cloning, functional expression, and regulation of two K^+ channels in human T lymphocytes. *J. Biol. Chem.* **267**:8650–8657 (1992).
7. Grissmer, S., A. N. Nguyen, J. Aiyar, D. C. Hanson, R. J. Mather, G. A. Gutman, M. J. Karmilowicz, D. D. Auperin, and K. G. Chandy. Pharmacological characterization of five cloned voltage-gated K^+ channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol. Pharmacol.* **45**:1227–1234 (1994).
8. Aiyar, J., J. Withka, J. Rizzi, D. H. Singleton, G. C. Andrews, W. Lin, J. Boyd, D. C. Hanson, M. Simon, B. Dethlefs, C.-L. Lee, J. Hall, G. A. Gutman, and K. G. Chandy. Topology of the pore-region of a K^+ channel revealed by the NMR-derived structures of scorpion toxins. *Neuron* **15**:1169–1181 (1995).
9. Chandy, K. G., T. E. DeCoursey, M. D. Cahalan, C. McLaughlin, and S. Gupta. Voltage-gated potassium channels are required for human T cell activation. *J. Exp. Med.* **160**:369–385 (1984).
10. Price, M., S. C. Lee, and C. Deutsch. Charybdotoxin inhibits proliferation and interleukin 2 production in human peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* **86**:10171–10175 (1989).
11. Leonard, R. J., M. L. Garcia, R. S. Slaughter, and J. P. Reuben. Selective blockers of voltage-gated K^+ channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc. Natl. Acad. Sci. USA* **89**:10094–10098 (1992).
12. Lin, C. S., R. C. Boltz, J. T. Blake, M. Nguyen, A. Talento, P. A. Fischer, M. S. Springer, N. H. Sigal, R. S. Slaughter, M. L. Garcia, G. J. Kaczorowski, and G. C. Koo. Voltage-gated K^+ channels regulate calcium-dependent pathways involved in human T lymphocyte activation. *J. Exp. Med.* **177**:637–645 (1993).
13. Rader, R. K., L. E. Kahn, G. D. Anderson, C. L. Martin, K. S. Chinn, and S. A. Gregory. T-cell activation is regulated by voltage-dependent and calcium-activated potassium channels. *J. Immunol.* **156**:1425–1430 (1996).
14. Freedman, B. D., M. A. Price, and C. J. Deutsch. Evidence for voltage-modulation of IL2 production in mitogen-stimulated human peripheral blood lymphocytes. *J. Immunol.* **149**:3789–94 (1992).
15. Koo, G. C, J. T. Blake, A. Talento, M. Nguyen, S. Lin, A. Sirotina, K. Shah,

K. Mulvany, D. Hora, Jr., P. Cunninghan, R. Slaughter, R. Bugianesi, J. Felix, M. Garcia, J. Williamson, G. Kaczorowski, N. Sigal, M. Springer, and W. Feeney. Margatoxin, a blocker of Kv1.3 channels, inhibits immune responses. *FASEB J.* A1447, (1996)
16. Chandy, K. G., and G. A. Gutman. Voltage-gated K^+ channel genes, in *Handbook of Receptors and Channels: Ligand and Voltage-gated Ion Channels* (A. North, ed.). CRC Press, Boca Raton, FL, 1–71 (1995).
17. Michne, W. F., J. W. Guiles, A. M. Treasurywala, L. A. Castonguay, B. O'Connor, W. A. Volberg, A. M. Grant, C. C. Chadwick, D. S. Krafte, and R. Hill. Novel inhibitors of potassium ion channels on human T lymphocytes. *J. Med. Chem.* **38**:1877–83 (1995).
18. Hill, R. J., A. M. Grant, W. Volberg, L. Rapp, C. Faltynek, D. Miller, K. Pagani, E. Baizman, S. Wang, J. W. Guiles, and D. Krafte. WIN 17317-3: novel nonpeptide antagonist of voltage-activated channels in human T-lymphocytes. *Mol. Pharmacol.* **48**:98–104 (1995).
19. Wymore, R., J. R. Korenberg, C. Coyne, X.-N. Chen, C. Hustad, N. G. Copeland, G. A., Gutman, N. A. Jenkins, and K. G. Chandy. Genomic organization, nucleotide sequence, biophysical properties and localization of the voltage-gated K^+ channel gene, KCNA4/Kv1.4 to mouse chromosome 2/human 11p14, and mapping of KCNC1/Kv3.1 and KCNA1/Kv1.1 to mouse 7/human 11p14.3-15.2 and human 12p13, respectively. *Genomics* **20**:191–202 (1993).
20. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pearse. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**: 51–59 (1989).
21. Ikeda, S. R., F. Soler, R. D. Zuhlke, and D. L. Lewis. Heterologous expression of the human potassium channel, Kv2.1, in clonal mammalian cells by direct cell injection. *Pflueg. Arch. Eur. J. Physiol* **422**:201–203 (1992)
22. McCloskey, M., and M. D. Cahalan. G protein control of potassium channel activity in a mast cell line. *J. Gen. Physiol.* **95**:208–222 (1990)
23. Levy, D. I., and C. Deutsch. Recovery from C-type inactivation is modulated by extracellular potassium. *Biophys. J.* **70**:798–805 (1996)
24. Grissmer, S., and M. D. Cahalan. TEA prevents inactivation while blocking open K^+ channels in human T-lymphocytes. *Biophys. J.* **55**:203–6 (1989)
25. Panyi, G., Z. Sheng, and C. Deutsch. C-type inactivation of a voltage-gated K^+ channel occurs by a cooperative mechanism. *Biophys. J.* **69**:896–903 (1995).
26. Liu, Y., M. E. Jurman, and G. Yellen. Dynamic rearrangement of the outer mouth of a K^+ channel during gating. *Neuron* **16**:859–867 (1996).
27. Lopez-Barneo, J., T. Hoshi, S. H. Heinemann, and R. W. Aldrich. Effects of external cations and mutations in the pore region on C-type inactivation of *Shaker* potassium channels. *Recept. Channels* **1**:61–71 (1993)
28. Ogielska, E. M., W. N. Zagotta, T. Hoshi, S. H. Heinemann, and R. W. Aldrich. Cooperative subunit interaction in C-type inactivation of K^+ channels. *Biophys. J.* **69**:2449–2457 (1995)
29. Grissmer S., and M. D. Cahalan. Divalent ion trapping inside potassium channels of human T lymphocytes. *J. Gen. Physiol.* **93**:609–630 (1989)
30. Ruppersberg, J. P., R. Frank, O. Pongs, and M. Stocker. Cloned neuronal I_k (A) channels reopen during recovery from inactivation. *Nature (Lond.)* **17**:657–660 (1991).
31. Po, S., D. J. Snyders, M. M. Tamkun, and P. B. Bennett. Functional expression of an inactivating potassium channel cloned from human heart. *Circ. Res.* **71**:732–736 (1992)
32. Yatani, Y., M. Wakamori, G. Mikala, and A. Bahinski. Blocking of transient outward-type cloned cardiac K^+ channel currents by quinidine. *Circ. Res.* **73**:351–359 (1993).
33. Murray, K. T., S. A. Fahrig, K. K. Deal, S. S. Po, N. N. Hu, D. J. Snyders, M. M. Tamkun, and P. B. Bennet. Modulation of an inactivating human cardiac K^+ channel by protein kinase C. *Circ. Res.* **75**:999–1004 (1994).
34. Fong, T. M., M. A. Cascieri, H. Yu, A. Bansal, C. Swain, and C. D. Strader. Amino-aromatic interaction between histidine 197 of the neurokinin-1 receptor and CP-96345. *Nature (Lond.)* **362**:350–353 (1993).
35. Tseng, G. N., and J. Tseng-Crank. Differential effects of elevating $[K]_o$ on three transient outward potassium channels. Dependence on channel inactivation mechanisms. *Circ. Res.* **71**:657–672 (1992).

Send reprint requests to: Dr. K. George Chandy, Department of Physiology and Biophysics, Joan Irvine Smith Hall, University of California Irvine, Irvine, CA 92697. E-mail: gchandy@uci.edu