Identification and Biochemical Characterization of a Novel Nortriterpene Inhibitor of the Human Lymphocyte Voltage-Gated Potassium Channel, Kv1.3

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ABSTRACT: A novel nortriterpene, termed correolide, purified from the tree *Spachea correae*, inhibits Kv1.3, a *Shaker*-type delayed rectifier potassium channel present in human T lymphocytes. Correolide inhibits $^{86}Rb^+$ efflux through Kv1.3 channels expressed in CHO cells (IC_{50} 86 nM; Hill coefficient 1) and displays a defined structure−activity relationship. Potency in this assay increases with preincubation time and with time after channel opening. Correolide displays marked selectivity against numerous receptors and voltage- and ligand-gated ion channels. Although correolide is most potent as a Kv1.3 inhibitor, it blocks all other members of the Kv1 family with 4−14-fold lower potency. C20−29-[^{3}H]dihydrocorreolide (diTC) was prepared and shown to bind in a specific, saturable, and reversible fashion (K_d = 11 nM) to a single class of sites in membranes prepared from CHO/Kv1.3 cells. The molecular pharmacology and stoichiometry of this binding reaction suggest that one diTC site is present per Kv1.3 channel tetramer. This site is allosterically coupled to peptide and potassium binding sites in the pore of the channel. DiTC binding to human brain synaptic membranes identifies channels composed of other Kv1 family members. Correolide depolarizes human T cells to the same extent as peptidyl inhibitors of Kv1.3, suggesting that it is a candidate for development as an immunosuppressant. Correolide is the first potent, small molecule inhibitor of Kv1 series channels to be identified from a natural product source and will be useful as a probe for studying potassium channel structure and the physiological role of such channels in target tissues of interest.

The voltage-gated delayed rectifier potassium channel, Kv1.3,[1] found in human T lymphocytes is a potential target for development of an immunosuppressant (*1*, *2*). Kv1.3 sets the resting potential in these cells (*2*), and peptidyl blockers of Kv1.3 such as margatoxin (MgTX) cause membrane depolarization. Depolarization is thought to reduce the electrochemical driving force for Ca^{2+} entry and limit the opening of calcium-release activated Ca^{2+} (CRAC) channels (*3*). Block of Kv1.3 also prevents K^+ counterion movement that facilitates Ca^{2+} entry (*4*). These processes lead to attenuation of the rise in Ca^{2+} observed after activation of the T-cell receptor, and as a consequence, to diminution in lymphokine release and synthesis (*1*), and to inhibition of delayed-type hypersensitivity in a porcine model (*5*). Since peptides are not ideal as systemic drugs, several groups have searched for small molecular weight inhibitors of Kv1.3 that might exhibit immunosuppressant effects similar to those of MgTX. Investigators at both Sterling−Winthrop (*6*) and Pfizer (*7*) have identified synthetic small molecules that inhibit Kv1.3. However, no compound yet reported appears to fulfill the requirements for developing a drug with this mechanism of action.

Kv1.3 is one of seven known members of the Kv1 (*Shaker*) family, among nine described families of six transmembrane voltage-gated potassium channels identified through molecular cloning techniques (*8*). Channels may consist of either homo- or heterotetramers of pore forming (α) subunits from within a particular family. Kv1 channels in human T cells are homotetramers of Kv1.3 (*9*), while in brain they exist as heterotetramers consisting mainly of Kv1.1 and Kv1.2 (*10*−*13*). In addition, some channel complexes contain accessory β subunits (*8*, *10*, *14*) which can affect both the biophysical and pharmacological properties of the pore-forming subunits. High-affinity peptidyl inhibitors have provided significant information about these channels (*15*). However, potent, small-molecule channel modulators that bind to distinct sites from the pore-blocking peptides would extend the scope of such studies.

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[1] Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Kv, voltage-gated potassium channel; Kv1, Kv2, Kv3, and Kv4 series, voltage-gated potassium channels of the *Shaker*, *Shab*, *Shaw*, and *Shal* families, respectively; maxi-K channel, high-conductance, calcium-activated potassium channel; diHC, dihydrocorreolide; diTC, ditritiocorreolide; MgTX, margatoxin; [^{125}I]MgTX, monoiodinated margatoxin; [$^{125}I_2$]MgTX, diiodinated margatoxin; ChTX, charybdotoxin; AgTX-1, agitoxin-1; AgTX-2, agitoxin-2; α-DaTX, α-dendrotoxin; IbTX, iberiotoxin; BSA, bovine serum albumin; *myc* tag, amino acid sequence EQKLISEEDL; WIN 17317-3, 1-benzyl-4-pentylimino, 7-chloro-1,4-dihydroquinoline.

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Goetz et al. have recently described the purification and structural elucidation of a nortriterpene inhibitor of Kv1.3 that was isolated from the Costa Rican tree, *Spachea correae* (*16*). The main active component, correolide, is a pentacyclic triterpenoid. Correolide is heavily oxygenated, displaying five acetoxy moieties and an epoxide group. Despite its complexity, correolide, along with analogues and derivatives, has been the focus of a series of studies to identify a compound for the development of a novel immunosuppressant.

Herein, we present the initial biochemical characterization of correolide and several other structurally related molecules as inhibitors of Kv1.3. This molecule is highly selective as an inhibitor of the Kv1 family and is devoid of activity against many other receptor and ion channel targets. We also report on a radiolabeled correolide derivative, [^{3}H]C20−29 dihydrocorreolide (diTC), which is useful as a ligand for the study of Kv1 family channels. We show that one molecule of diTC binds per Kv1.3 channel tetramer and that this binding site is allosterically coupled to other sites on the channel. DiTC also labels Kv1 channels in brain and can be used to determine the subunit composition of these channels. Correolide depolarizes human T cells to the same extent as do peptidyl inhibitors of Kv1.3, supporting development of this structural class as a novel immunosuppressant agent. Correolide is an important new tool for characterizing the structure of Kv1 series channels and for studying their function in target tissues of interest.

EXPERIMENTAL PROCEDURES

Materials

Correolide and naturally occurring correolide derivatives were isolated as described by Goetz et al. (*16*). Analogues and derivatives of correolide, including C20−29 dihydrocorreolide (diHC) were prepared according to the methods of Baker et al. (*17*−*19*). [^{3}H]C20−29 dihydrocorreolide was prepared by the Radiochemical Synthesis group, Merck and Co., at a specific activity of 30.6 Ci/mmol by catalytic hydrogenation. MgTX, agitoxin 1 (AgTX-1), agitoxin 2 (AgTX-2), and kaliotoxin (KTX) were prepared as part of a fusion protein in *E. coli*, cleaved, and purified as described (*20*). Charybdotoxin (ChTX) and iberiotoxin (IbTX) were purchased from Peninsula Laboratories and α-dendrotoxin (α-DaTX) from Sigma Chemicals (St Louis, MO). MgTX was radiolabeled with Na^{125}I as described (*9*). Na^{125}I, ^{86}RbCl, and [^{3}H]tetraphenylphosphonium ions were purchased from NEN Life Science Products. All tissue culture media were from Life Technologies. All other reagents were obtained from commercial sources and were of the highest purity commercially available.

Methods

Cell Lines. CHO cells stably transfected with Kv1.3 (CHO/Kv1.3) were prepared as described (*21*). HEK 293 cells stably transfected with either Kv1.1, 1.4, 1.5, or 1.6 were obtained from Professor Olaf Pongs (Zentrum für Molekulare Neurobiologie, Hamburg, Germany), whereas the cell line stably transfected with Kv1.2 was prepared as described for CHO/Kv1.3 (*21*).

Plasma Membrane Preparations. Plasma membranes derived from either CHO/Kv1.3 cells (*9*), HEK/Kv1.3 cells (*12*), or brain (*13*) were prepared as previously described. Human brain synaptic membranes, prepared as described for rat brain (*13*), were obtained from tissue provided by Drs. H.-G. Knaus and H. Glossmann, University of Innsbruck.

^{86}Rb$^+$ Efflux Assay. ^{86}Rb$^+$ efflux from CHO/Kv1.3 cells was performed essentially as described (*9*). Test compound in low-potassium buffer (in millimolar, 4.6 KCl, 126.9 NaCl, 1 CaCl$_2$, 2 MgCl$_2$, and 10 HEPES, pH 7.2, adjusted with NaOH) was added, and the mixture was preincubated for 10 min or other periods of time as indicated. ^{86}Rb$^+$ efflux was initiated by depolarization of the cells with high-potassium buffer (final concentration, in millimolar, 63.25 KCl, 69.2 NaCl, 1 CaCl$_2$, 2 MgCl$_2$, and 10 HEPES, adjusted to pH 7.2 with NaOH) in the presence of test compound (normal, 15 min). Activity was determined by the percent inhibition of efflux that is inhibitable by 50 nM MgTX. ^{86}Rb$^+$ efflux from HEK-293 cells containing either Kv1.1, 1.2, 1.5, or 1.6 channels was performed under similar conditions except that the concentration of potassium in the depolarization buffer was raised to 100 mM, with a concomitant reduction in sodium.

*[^{3}H]C20−29 Dihydrocorreolide (diTC) Binding Assay*s. The interaction of diTC with membranes derived from either CHO/Kv1.3 or human brain synaptosomes was measured in a medium consisting of (in millimolar): 4.6 KCl, 135 NaCl, 20 HEPES, pH 7.4 with NaOH, and 0.02% BSA. Nonspecific binding was determined in the presence of 10 μM unlabeled ligand (diHC) or correolide. Incubations were carried out at room temperature for the indicated periods of time, in a total volume of 0.2 mL. At the end of the incubation period, samples were diluted with 4 mL of ice-cold binding solution, membranes were collected on 25 mm Whatman GF/C glass fiber filters and then washed twice with ice-cold binding solution. Radioactivity associated with the filter was determined by liquid scintillation techniques. For dissociation experiments, membranes were preincubated with diTC until equilibrium was established. Measurement of dissociation kinetics was initiated by addition of 10 μM diHC, and samples were incubated at room temperature for different periods of time. DiTC binding to CHO/Kv1.3 cells was determined under the same conditions as those described for [^{125}I$_2$]MgTX binding (see below). Other details are given in the figure legends.

[^{125}I$_2$]MgTX Binding Assay. Diiodomargatoxin ([^{125}I$_2$]-MgTX) was used in binding studies with either CHO/Kv1.3 cells or membranes prepared from this source. [^{125}I$_2$]MgTX is more potent than the monoiodinated form ([^{125}I]MgTX) under the high ionic strength conditions of the binding assay, and exhibits a better signal-to-noise ratio than [^{125}I]MgTX when these studies are carried out in near physiological salt concentrations that favor cell stability over the extended period necessary to achieve equilibrium (*9*). Briefly, incubations were carried out in a medium consisting of (in millimolar) 4.6 KCl, 106 NaCl, 20 HEPES, pH 7.4 with NaOH, and 0.2% BSA, in a total volume of 0.2 mL. Nonspecific binding was determined in the presence of 10 nM MgTX. After a 1 h incubation at 37 °C (cells) or room temperature (membranes), samples were diluted with 4 mL of ice-cold quench buffer (in millimolar, 200 NaCl and 20 Tris, pH 7.4 with HCl) and cells or membranes were collected on 25 mm GF/C filters that had been presoaked in

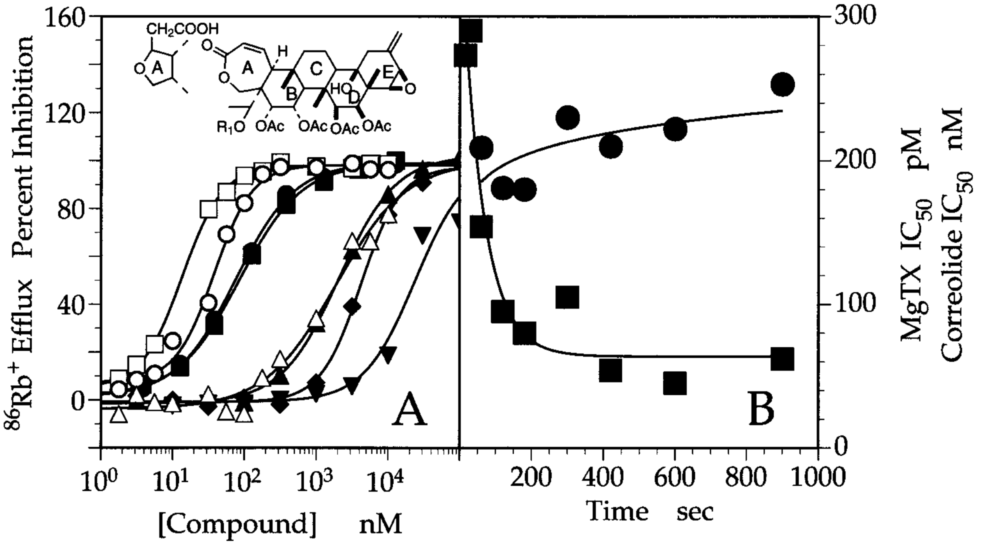

FIGURE 1: Correolide analogues and inhibition of $^{86}Rb^+$ efflux from CHO/Kv1.3 cells. Four nortriterpenes were isolated from *Spachea correa*: R_1 = Ac, correolide and C11−C12 dehydrocorreolide; R_1 = H, C4 desacetyl correolide and C4 desacetyl, C11−C12 dehydrocorreolide. The hydrogenation product of correolide (R_1 = Ac), C20−C29 dihydrocorreolide (diHC or diTC), and a weak analogue of correolide, C4 desacetyl correolide, THF C3 acid were prepared by synthetic procedures. (A) Cells loaded with $^{86}Rb^+$ were washed in low potassium buffer in the absence or presence of correolide (■, □), C11−12 dehydrocorreolide (●, ○), C4 desacetyl correolide (▲, △), C4 desacetyl, C11−12 dehydrocorreolide (◆) and C4 desacetyl correolide, THF C3 acid (▼) for either 10 min (filled symbols) or 4 h (open symbols). Efflux was initiated by addition of high potassium buffer in the presence of test compounds, and cells were incubated at room temperature for 15 min. Inhibition of $^{86}Rb^+$ efflux was assessed relative to untreated controls. (B) $^{86}Rb^+$ preloaded CHO/Kv1.3 cells were preincubated for 10 min with increasing concentrations of either correolide (■) or MgTX (●) in low potassium buffer. Efflux was initiated by addition of high potassium buffer to cells treated with either correolide or MgTX, or to an untreated control, and cells were incubated at room temperature for different time periods. IC_{50} values were based upon the untreated control at any particular time point.

1.0% polyethylenimine. Filters were washed twice in ice-cold quench buffer. The amount of ligand present on the filter was determined by γ radiation detection techniques.

Immunoprecipitation. Polyclonal antibodies raised against Kv1.1−1.6 were produced as previously described (*20*). Human brain synaptic membranes were incubated overnight with 80 nM diTC at ambient temperature and solubilized in 20 mM Tris-HCl, pH 7.4, 400 mM KCl, and 2.0% digitonin. Solubilized material was separated by centrifugation, diluted 4.5-fold with 20 mM Tris-HCl, pH 7.4, and 0.1% digitonin, and incubated with respective anti-Kv1 antibodies that had been bound to protein A-sepharose. All other experimental conditions have been previously described (*22*).

Membrane Potential Measurements. The distribution of the lipophilic cation [^{3}H]tetraphenylphosphonium in human peripheral blood T lymphocytes was measured as previously described (*2*).

Protein Determination. Membrane protein was determined by the amido blue-black spot method of Schaffner and Weissmann (*23*), as modified by Newman et al. (*24*).

Data Analysis. Equilibrium binding models and curve fitting of the experimental data have been previously described (*9*). For association experiments, kinetic parameters were determined by nonlinear least-squares fitting to Bound = Beq(1 − exp[−ln(2) × time/Thalf)], where Beq is the amount bound at equilibrium and Thalf is the time at which Bound = 0.5Beq. The time course of dissociation was fitted to the equation Bound = Bnd0 × exp[−ln(2) × time/Thalf], where Bnd0 is the amount of ligand bound at time = 0. Modulation of diTC binding by KCl was fit to arbitrary parameters, not based on any mathematically derived model.

RESULTS

Identification and Selectivity of Nortriterpene Kv1.3 Inhibitors. The nortriterpene correolide series of compounds was discovered as part of an ongoing search for novel natural product inhibitors of Kv1.3 (*16*). There were four products isolated from roots and bark of the Costa Rican tree, *Spachea correae* that inhibited $^{86}Rb^+$ efflux from CHO/Kv1.3 cells: correolide, C11−C12 dehydrocorreolide, C4 desacetyl correolide and C4 desacetyl, and C11−C12 dehydrocorreolide (Figure 1). A biologically inactive correolide analogue, C4 desacetyl correolide, THF C3 acid, was prepared by transforming the A-ring lactone of C4 desacetyl correolide to a tetrahydrofuran C3 carboxylic acid (*19*).

Correolide and C11−12 dehydrocorreolide display similar potencies as inhibitors of $^{86}Rb^+$ efflux from CHO/Kv1.3 cells with IC_{50}s of 86 and 75 nM, respectively (Figure 1A). These compounds are 14- and 66-fold, respectively, more potent than their C4 desacetyl congeners. Titration of inhibitory activity reveals monophasic profiles and Hill coefficients of 1 for all correolide analogues. The inhibitory effects of correolide or C11−12 dehydrocorreolide can be reversed by washing cells. Compounds were preincubated with CHO/Kv1.3 cells for 10 min, and cells were washed several times before depolarization with high-potassium buffer to begin efflux. Under these conditions, about 50% of the block produced by saturating concentrations (850 nM) of correolide or C11−12 dehydrocorreolide could be reversed.

For specificity studies, correolide was tested in 108 receptor ligand binding and enzymatic assays in a standard PanLabs screen. There was no significant inhibitory activity (>25%) against any of these targets when tested at 4 μM, a concentration approximately 40-fold higher than its Kv1.3 IC_{50} value (data not shown). It was most potent in blocking ligand binding to NMDA receptors (23%), sodium channels (21%), 2b adrenergic receptors (21%), IL-6 receptors (24%), and 5-HT1 receptors (21%). Correolide had little or no effect in various other binding assays monitoring neurotransmitter or hormone receptors, enzyme assays measuring kinase, phosphatase or synthetase activities, or on drug/toxin binding to ion channels. The latter targets include assays directed against ligand-gated channels, L and N-type calcium channels and voltage-gated, ATP-dependent and small conductance calcium-activated (apamin-sensitive) potassium channels.

The effects of this structural class on a number of different ion channels were studied in further detail. Correolide has no effect on the functional activity of either $GABA_A$ or NMDA channels expressed in Ltk cells at 30 and 10 μM, respectively. Neither correolide nor C11−12 dehydrocorreolide has any effect on isradipine or diltiazem binding to the L-type calcium channel in purified porcine cardiac sarcolemmal membranes (up to 100 μM). In voltage-clamp measurements of L- and T-type calcium channels in guinea pig atrial myocytes, correolide at 2 μM did not block channel activity. Although correolide displayed some activity in Pan Labs screens where batrachatoxin binding to rat brain sodium channels was monitored, it is inactive at 150 μM on veratridine-stimulated $^{22}Na^+$ flux through rat brain IIA sodium channels stably expressed in CHO cells (*25*). This compound at 10 μM has weak blocking effects on the high conductance, calcium-activated potassium channel of primary bovine aortic smooth muscle cells, and at 0.5 μM does not affect the small conductance, calcium-activated potassium channel present in human T lymphocytes. When measured in isolated guinea pig ventricular myocytes at a concentration of 10 μM, correolide did not block I_{Kr}, I_{Ks}, or I_{K1}.

While correolide shows significant selectivity against a variety of ion channels, it is far less selective against Kv1 series channels. In $^{86}Rb^+$ efflux assays employing cell lines stably expressing Kv1.1, Kv1.2, Kv1.5, and Kv1.6 channels, the IC_{50}s for correolide-mediated channel inhibition are 430, 700, 1150, and 450 nM, respectively. Conditions for these assays are identical to those for the Kv1.3 $^{86}Rb^+$ efflux assay, except higher concentrations of potassium are required to maximally open the channels. Flux assays for Kv1.4 were problematic due to the very short open time of this channel and no meaningful titration of correolide was possible. Of the other Kv series channels studied, correolide (10 μM) did not interact with Kv3.2 or Kv4 and was 20-fold weaker against Kv2.1 than Kv1.3 in diTC binding experiments (see below).

Characterization of the Interaction of Correolide with Kv1.3. Potency of inhibition of $^{86}Rb^+$ efflux from CHO/Kv1.3 cells increases with time of preincubation. The correolides were preincubated for either 10 or 240 min followed by an efflux period of 15 min in depolarizing buffer. In the experiment shown in Figure 1A, the potency of correolide increases 7-fold (IC_{50} of 12 nM). Similarly, the potency of C11−C12 dehydrocorreolide increases 2-fold. However, the potency of the more hydrophilic C4 desacetyl correolide does not increase upon extended exposure to CHO/Kv1.3 cells. Although some correolide analogues display increased potencies with longer incubations, standard assay protocols were set at the 10 min preincubation to facilitate high-throughput assays.

The potency of correolide also increases with time after opening of the channel. When titrations of correolide inhibition are monitored after a standard 10 min preincubation in low potassium buffer, but terminated at short times (10 and 30 s) after depolarization, $^{86}Rb^+$ efflux is less potently inhibited as indicated by the high IC_{50} values shown in Figure 1B. Titrations performed at successively longer depolarization times show progressively lower IC_{50} values. This increased potency of block with time after depolarization may indicate that correolide is a better inhibitor of a conformational state of the channel which develops after channel opening. In marked contrast, MgTX, which is expected to block different states of Kv1.3 equally well, does not vary in potency under this protocol (Figure 1B).

It might be expected that an inhibitor of Kv1.3 would affect binding of inhibitory peptides to this channel since other small molecule inhibitors have been found to do so (*6*). However, neither correolide nor C11−C12 dehydrocorreolide had any affect on $[^{125}I_2]$MgTX binding to membranes prepared from either CHO/Kv1.3 cells (up to 30 μM) or Jurkat cells. Similarly, data from the Pan Labs screen revealed that correolide had no effect on ChTX binding to Kv1.X channels in rat brain synaptosomal membrane vesicles.

$[^3H]$C20−29 Dihydrocorreolide Binding Characteristics. A tritium-labeled analogue, $[^3H]$C20−29 dihydrocorreolide (diTC; diHC with ^1H; Figure 1), was prepared by reduction of the exocyclic C20−C29 double bond in the presence of a Wilkinson's catalyst (*17*). Under physiological NaCl and KCl concentrations, diTC associates with CHO/Kv1.3 membranes in a specific, saturable fashion with a large total to nonspecific binding ratio (Figure 2A). No specific binding was detected with membranes prepared from nontransfected CHO cells. The binding characteristics of diTC in CHO/Kv1.3 membranes are conventional as indicated by a monophasic saturation curve (Figure 2A) and a linear Rosenthal−Scatchard analysis (not shown). The equilibrium K_d determined for diTC binding is 11 nM.

Association rates of diTC with CHO/Kv1.3 plasma membranes were determined under pseudo-first-order conditions at 97 nM, approximately 10 times the K_d value of diTC (half time, 5.4 min; k_1 of 1.19×10^{-3} min^{-1} nM^{-1}; Figure 2A, inset). Dissociation of diTC from CHO/Kv1.3 plasma membranes is monophasic, has a half-time of 51.1 min, and yields a first-order rate constant, k_{-1} of 1.36×10^{-2} min^{-1}. Dissociation of diTC from intact CHO/Kv1.3 cells has a similar half-time of 57 min, under essentially identical binding conditions (data not shown). The K_d determined for diTC binding in membranes from the combination of association and dissociation constants is 11.4 nM, which is very close to the 11 nM value determined in equilibrium saturation binding studies.

To determine the effect of K^+ on the interaction of diTC with Kv1.3 channels, ligand binding was monitored in the presence of KCl concentrations ranging from 10 μM to 300 mM (Figure 2B). Except for 300 mM KCl, the total concentration of KCl and NaCl was maintained at 135 mM. Stimulation of diTC binding occurs from 10 μM to 10 mM KCl, and decreases at higher KCl concentrations.

From the data presented above, it is apparent that diTC and MgTX bind to different sites on Kv1.3. One MgTX molecule is thought to bind per channel tetramer (*26*). To determine the relative ratio of MgTX and diTC binding sites,

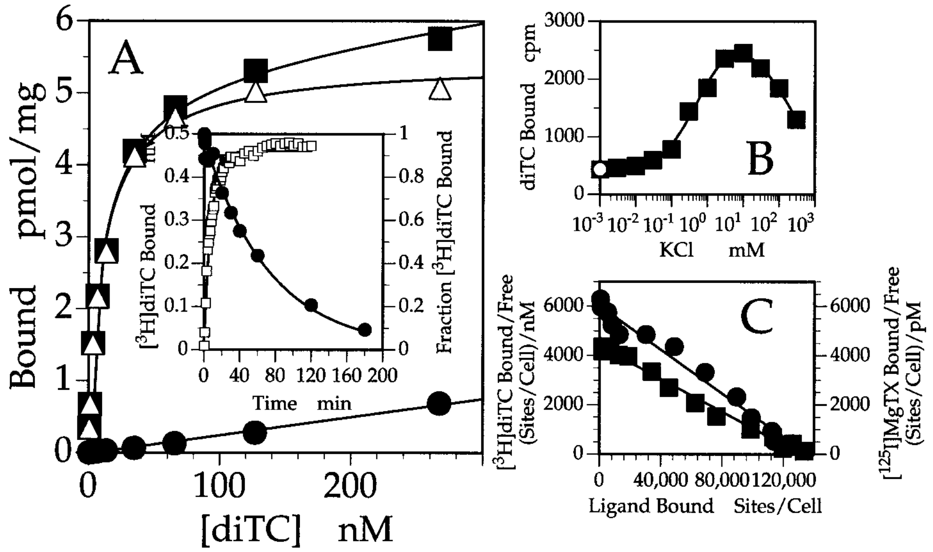

FIGURE 2: DiTC binding to plasma membranes prepared from CHO/Kv1.3 cells. (A) Equilibrium binding. Membranes were incubated with increasing concentrations of diTC in the absence (■) or presence (●) of 10 μM diHC at room temperature for 1 h as described in the Experimental Procedures. Specific binding (△), determined as the difference between total and nonspecific binding, was fit as described in the Experimental Procedures. (Inset) Kinetics of binding. Both association (□) and dissociation (●) experiments were performed at room temperature. For association, membranes were incubated with 97 nM diTC for the indicated periods of time. Nonspecific binding, determined in the presence of 10 μM diHC, is time invariant and has been subtracted from the experimental points. After preincubation with diTC, dissociation kinetic reactions were initiated by addition of 10 μM diHC. The fit of specific binding data, as described in the Experimental Procedures, yields a pseudo-first-order association rate constant (k_{+1}) of 0.001 19 nM^{-1} min^{-1}, with a $t_{1/2}$ of 5.4 min, and a first-order dissociation rate constant (k_{-1}) of 0.0136 min^{-1}, with a $t_{1/2}$ of 51.1 min, giving a K_d (k_{-1}/k_{+1}) of 11.4 nM. (B) KCl dependence of diTC binding to Kv1.3. HEK 293/Kv1.3 plasma membranes were incubated with diTC in a medium consisting of 135 mM NaCl and 20 mM TrisHCl, pH 7.2, in the absence (○) or presence (■) of increasing concentrations of KCl. (C) Binding of [$^{125}I_2$]MgTX and diTC to CHO/Kv1.3 cells. [$^{125}I_2$]MgTX (●) or diTC (■) was incubated with CHO/Kv1.3 cells for 2 h at room temperature. The incubation medium described in the Experimental Procedures was identical for both ligands. Binding parameters were determined by Rosenthal-Scatchard analysis. DiTC: B_{max} = 132 000 sites/cell, K_d = 30 nM. [$^{125}I_2$]MgTX: B_{max} = 135 000 sites/cell, K_d = 22.5 pM.

intact CHO/Kv1.3 cells were employed because vesicles are of mixed membrane polarity and Kv1.3 inhibitory peptides penetrate plasma membrane vesicles poorly, unlike the correolides (see Figure 4A and below). Maximal binding was determined by Rosenthal−Scatchard analysis and shows approximately 130 000 binding sites/cell for both ligands (Figure 2C). This equivalence of B_{max} indicates that the triterpenes, like the peptidyl inhibitors, bind to the channel tetramer with a 1:1 stoichiometry. The K_d for diTC was 30 nM, a value that is 3-fold higher than that measured for binding of ligand to plasma membranes prepared from the same cell type. The K_d for [$^{125}I_2$]MgTX at 22.5 pM is in the same range as has been reported for toxin binding to Kv1.3 in plasma membranes (*9*).

Purified rat brain synaptic membrane preparations have previously been used to characterize the binding of Kv1.X selective peptides and are predominantly heteromultimeric complexes of Kv1.1 and Kv1.2 channels (*11*, *12*). In addition, this preparation contains channels from the Kv2, Kv3 and Kv4 families (*27*). However, Kv1 channels in human brain membranes have not been similarly characterized and diTC was used as a probe for this purpose. Binding of diTC to synaptic membranes was observed to be specific and saturable and to occur to a single population of sites. As shown in Figure 3A, diTC binds to this membrane preparation with an affinity (K_d = 5.2 nM) similar to that measured for Kv1.3. However, the association and dissociation kinetics of diTC binding to synaptic membranes are markedly different from those measured with Kv1.3 (Figure 3A, inset). The association rate constant (2.5 × 10^{-4} min^{-1} nM^{-1}) is 5-fold slower than for Kv1.3 (Figure 3A, inset). Consequently, equilibrium experiments with synaptic membranes were performed with overnight incubations. In addition, dissociation of ligand occurs with a half time of 12 h and yields a k_{-1} of 9.3 × 10^{-4} min^{-1}. The K_d calculated from these values is 3.7 nM, which is in good agreement with the K_d measured in equilibrium saturation binding. Incubation of diTC overnight with CHO/Kv1.3 plasma membranes did not change the observed K_d in this preparation. Nearly identical equilibrium and kinetic-binding parameters for diTC are observed with rat brain synaptic membranes (data not shown).

To determine whether diTC binding in brain occurs to Kv2 or Kv3 series channels, membranes were prepared from COS cells transiently transfected with Kv2.1 or Kv3.2 in which a *myc* tag was incorporated at the C terminus. Although both channels were well expressed according to Western analysis for *myc* expression, binding of diTC was detected only to membranes containing Kv2.1 channels. The affinity of diTC for Kv2.1 as compared to Kv1.3 was reduced 20-fold (K_d of 200 nM). No binding was observed with 10 nM diTC to human cardiac ventricular muscle sarcolemmal membrane vesicles, known to contain Kv2 and Kv4 series channels (*28*, *29*) (protein ≤ 5 mg/mL). DiTC did not bind to purified sarcolemmal membrane preparations derived from either bovine aortic or tracheal smooth muscle, rich sources of the high conductance calcium activated (maxi K) channels (*30*, *31*), nor did it bind to plasma membranes derived from COS-1 cells transiently transfected with α and β subunits of the maxi K channel (*32*).

The specificity of diTC for Kv1 channels in human brain synaptic membranes was determined in immunoprecipitation

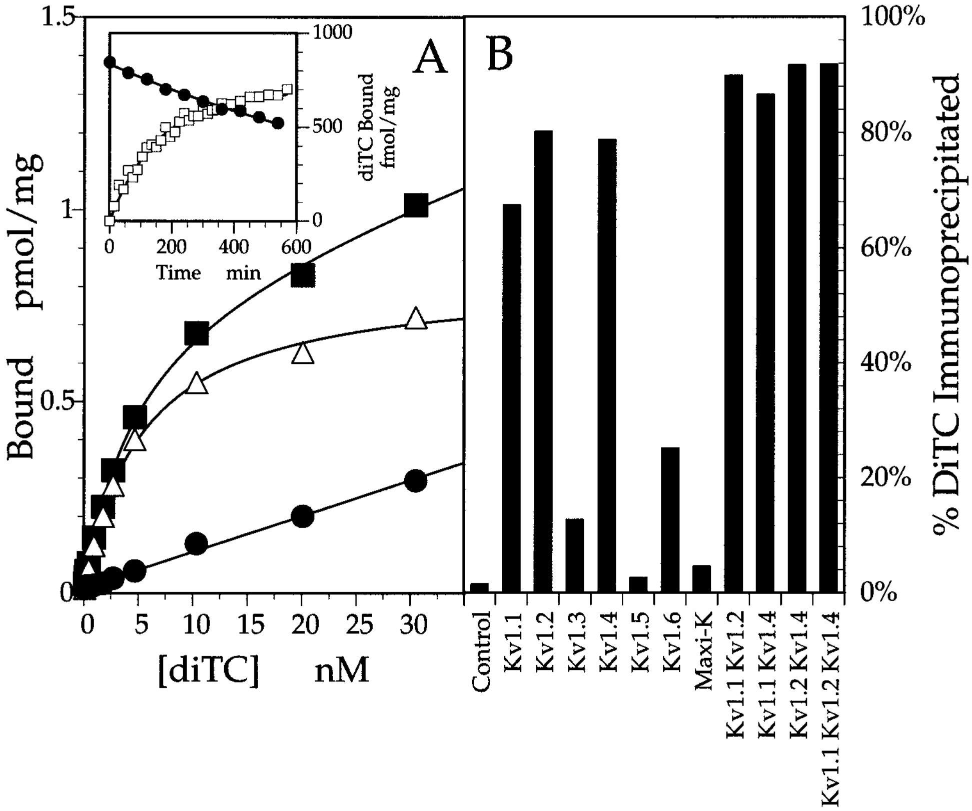

FIGURE 3: DiTC binding to human brain synaptic membranes. (A) Equilibrium binding. Human brain synaptic membranes were incubated with increasing concentrations of diTC for 20 h at room temperature. Nonspecific binding (●), determined in the presence of 10 μM diHC is subtracted from total binding (■) to determine specific binding (△). (Inset) Kinetics of binding. For association (□), membranes were incubated with 10 nM diTC for the indicated periods of time at room temperature. After incubation with diTC, dissociation (●) was initiated by addition of 10 μM diHC and samples were incubated at room temperature for the indicated periods of time. Fit of specific binding data as described in the Experimental Procedures yields a pseudo-first-order association rate constant (k_{+1}) of 0.000 255 nM^{-1} min^{-1}, with a $t_{1/2}$ of 111 min and a first-order dissociation rate constant (k_{-1}) of 0.0093 min^{-1}, with a $t_{1/2}$ of 745 min. The K_d, calculated as k_{-1}/ k_{+1}, is 3.7 nM. (B) Immunoprecipitation of solubilized diTC binding sites from human brain membranes. Digitonin-solubilized human brain diTC receptors were precipitated using saturating concentrations of the indicated anti-Kv1 or anti-maxi-K channel antibodies, as indicated in the Experimental Procedures. The percent of total receptors precipitated, defined by the sum of receptors present in the pellet and those in the supernatant, under each specific condition, is indicated.

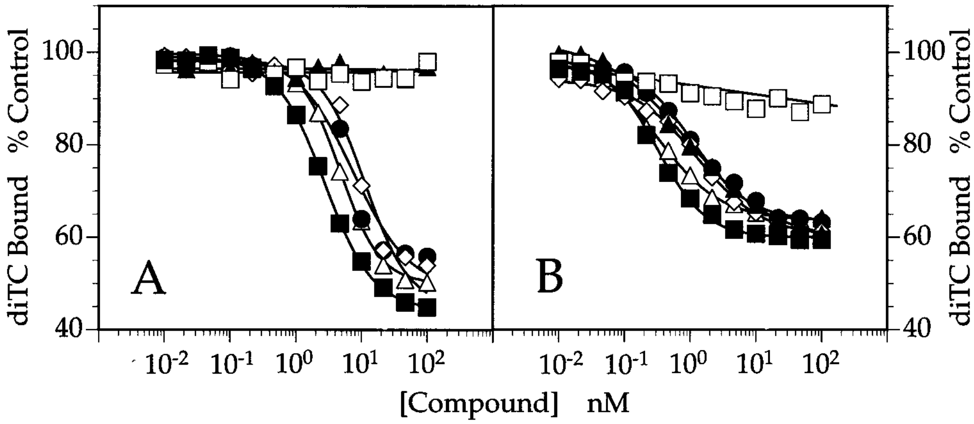

FIGURE 4: Pharmacology of diTC binding to Kv1.3 or human brain synaptic membranes. Membranes derived from CHO/Kv1.3 cells (A) or human brain synaptosomes (B) were incubated with 10 nM diTC in the absence or presence of increasing concentrations of either AgTX-1 (△), AgTX-2 (●), ChTX (◇), α-DaTX (▲), IbTX (□), or MgTX (■). Incubations in A were carried out for 2 h and in B for 20 h at room temperature. Inhibition of binding was assessed relative to an untreated control. Half-maximal inhibitory concentrations in nanomolarity: (A) MgTX, 2.7; ChTX, 11.4; AgTX-1, 4.8; AgTX-2, 7.4; (B) MgTX, 0.32; ChTX, 1.3; AgTX-1, 0.31, AgTX-2, 1.1; α-DaTX, 0.92. For comparison, diHC caused complete inhibition of diTC binding with IC$_{50}$ values of 11 and 9.1 nM in panels A and B, respectively.

experiments (Figure 3B). Between 70 and 80% of solubilized diTC binding sites can be precipitated by either Kv1.1, Kv1.2, or Kv1.4 antibodies, whereas a much smaller amount was precipitated by anti-Kv1.3 or anti-Kv1.6 antibodies. Combinations of Kv1.1, Kv1.2 and Kv1.4 antibodies all provided about the same amount of additional immunoprecipitation, which was greater than 90%. Antibodies raised against either the α subunit of the maxi K channel or Kv1.5 were unable to immunoprecipitate diTC-binding sites. These data indicate that most of the diTC binding in human brain

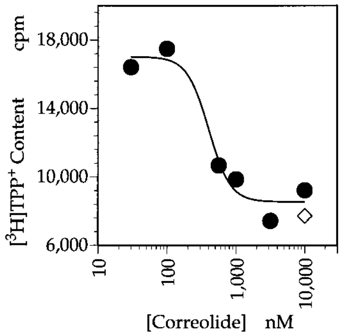

FIGURE 5: Effect of correolide on membrane potential in human peripheral blood T lymphocytes. Purified human T cells were incubated for 4 h with $[^3H]TPP^+$ in the absence or presence of increasing concentrations of correolide (●). Cells were assayed for $[^3H]TPP^+$ content. The effect of saturating concentrations (50 nM) of MgTX is indicated (◇).

is to heterotetramers consisting of Kv1.1, Kv1,2, and Kv1.4 subunits with small contributions of Kv1.6 or Kv1.3.

Molecular Pharmacology of diTC Binding. Several peptides have been shown to be potent inhibitors of Kv1.3 (*9*, *26*, *33*−*36*). A series of these peptides were analyzed for their ability to affect diTC binding to CHO/Kv1.3 plasma membranes. As shown in Figure 4A, four known Kv1.3 inhibitory peptides, MgTX, ChTX, AgTX-1, and AgTX-2, exhibit potent block of diTC binding. Their rank order is similar to that observed for inhibition of $[^{125}I]$MgTX binding (*9*). Two peptides have no effect on diTC binding; IbTX, a selective blocker of the maxi K channel (*37*), and α-DaTX, an inhibitor of Kv1.1, Kv1.2, and Kv1.6 (*36*, *38*, *39*). The Kv1.3 blocking peptides, which are known to bind in the outer vestibule of the channel, only inhibit half of diTC binding. In contrast, diHC blocks diTC binding completely with an IC_{50} of 11 nM. Complementary experiments were performed by monitoring diTC binding to human brain synaptic membranes (Figure 4B). Similar to the results shown above, the five peptides known to block brain Kv1.X channels (*10*, *13*) inhibited only half of the diTC binding in these membranes. Compounds that were strongly inhibitory in both sets of experiments, MgTX, ChTX, AgTX-1, and AgTX-2, are more potent in their inhibition of diTC binding to synaptic membranes than to Kv1.3, consistent with their potency against brain receptors (*9*). The maxi K blocker IbTX also has no affect on diTC binding to synaptic membranes. Unlike the observation with Kv1.3 membranes, diTC binding to synaptic membranes is inhibited by α-DaTX, consistent with the reported selectivity of α-DaTX for Kv1.1 and Kv1.2 channels (*10*, *39*).

Effects of Correolide on Human Peripheral Blood T Lymphocytes. Similar to the effect of peptidyl blockers of Kv1.3, correolide depolarizes human peripheral blood T lymphocytes as measured by the reduction of $[^3H]$tetraphenylphosphonium ion partitioning across the plasma membrane (Figure 5). The concentration dependence is steep as has been observed with the peptide channel blockers (*2*). Correolide exhibits an ED_{50} of 400 nM and depolarizes the T cells to the same extent as a saturating concentration of MgTX.

DISCUSSION

The nortriterpene correolide is the first potent, small molecular weight inhibitor of Kv1 series potassium channels to be isolated from a natural product source. This compound blocks Kv1.3 in flux experiments, and a radiolabeled derivative, diTC, is a high-affinity ligand for this channel. While correolide is 4−14-fold weaker in inhibiting $^{86}Rb^+$ efflux through other Kv1 series channels, it is highly selective against all other channel and receptor assays in which it was tested. Correolide has a large complex structure with several potentially reactive functionalities. However, the reversibility of both channel block and ligand binding indicates that correolide does not react covalently with Kv1.3. Equivalence of maximal binding of diTC and MgTX implies that one correolide molecule binds per channel tetramer and is sufficient to block the channel. The binding constant for diTC is of higher affinity than its potency in blocking $^{86}Rb^+$ efflux through Kv1.3, but this difference decreases with increased preincubation time in the flux assay. Increase in potency of correolide in the efflux assay with time after addition of high potassium suggests that correolide interacts better with Kv1.3 in a depolarized state than at resting potential. DiTC also binds to Kv1 channels in human brain synaptic membranes, but immunoprecipitation and binding experiments indicate that diTC identifies other Kv1 channels besides Kv1.3 in this preparation. The ability of correolide to depolarize human peripheral blood T lymphocytes to the same level as MgTX predicts that correolide will block T-cell activation in a manner similar to that of the peptidyl Kv1.3 inhibitors.

In the evaluation of different structural motifs, the equivalency of the C ring unsaturated C11−12 dehydrocorreolide with correolide suggests that the structural constraints within this portion of the C ring are not important for channel blocking activity. However, the C4 substitution appears critical for potency as indicated by the loss of activity of the C4 desacetyl versions of correolide and C11−12 dehydrocorreolide. The C4 desacetyl correolide THF C3 acid only weakly inhibits $^{86}Rb^+$ efflux under standard conditions.

Correolide analogues have several potentially reactive functionalities such as the E-ring epoxide, the exocyclic C20−29 double bond, and an unsaturated carbon−carbon bond positioned α/β to the lactone carbonyl, which is a potential site for Michael addition. Several lines of evidence suggest that these groups do not participate in covalent modification of Kv1.3. First, C4 desacetyl correolide, THF C3 acid, with most of the potentially reactive moieties, is very weak in the $^{86}Rb^+$ efflux assay (Figure 1A). Note that other members of the THF A ring series of compounds exist that are quite active as Kv1.3 inhibitors (in preparation). The high selectivity of correolide against non-Kv1 family channels, the partial depolarization of human T cells, and the reversibility of both binding and block of flux all indicate that the potentially reactive structural moieties of this molecule are largely benign.

The increased potency of correolide in the $^{86}Rb^+$ efflux assay with time of preincubation indicates that equilibrium is not reached under the standard 10 min preincubation protocol. Correolide also increases in potency with time after initiating the efflux assay. Increasing the potency of correolide at longer post depolarization times suggests that a significant amount of block occurs after most of the channels have gone through opening or inactivation cycles. These data predict that correolide prefers to bind to some state of the channel other than the closed state.

Correolide exhibits a complex interaction with peptide binding to the outer vestibule of the pore of Kv1.3. This molecule shows no measurable effect on [$^{125}I_2$]MgTX binding to plasma membranes prepared from either Jurkat or CHO/Kv1.3 cells. However, reverse coupling is shown by peptide toxin inhibition of diTC binding to plasma membranes. Only half of the diTC binding is inhibited, presumably because plasma membranes are vesicles of mixed sidedness (roughly 50:50, outside out:inside out) that are permeable to diTC, but not to the peptides. Potencies of various inhibitory peptides are approximately what would be expected under the near physiological salt conditions employed for the assay (*9*). These data suggest that binding of peptide to the pore of Kv1.3 induces a channel conformation that alters the structure of the nortriterpene receptor to inhibit the interaction of correolide.

An additional coupling between correolide and K^+-binding sites on Kv1.3 is indicated by the potentiation of diTC binding to plasma membranes as a function of potassium concentration. It has previously been shown that low levels of K^+ (optimum at 3−10 mM) are absolutely required for the binding of peptide ligands to Kv1.3 (*9*). The same K^+ concentration range enhances diTC binding, but is not absolutely necessary for the interaction of this ligand with the channel. One possible explanation is that K^+ binds to a site in the pore whose occupancy is necessary to stabilize a specific conformation of the channel. The decreased stimulation observed at higher concentrations of KCl may be an indication of inhibitory action at a different site on the channel.

Only a single MgTX is thought to interact with the tetrameric Kv1.3 channel and is believed to bind across all four of the identical α subunits through multiple attachment sites in the outer vestibule of the pore (*26*). Thus, the equivalence of maximal diTC and MgTX binding to CHO/Kv1.3 cells, coupled with the Hill coefficient of 1 observed in the $^{86}Rb^+$ efflux experiments, is consistent with occupancy of a single site by the correolides to effect block of the channel. As discussed above, the correolide- and peptide-binding sites are likely to be different, but the limitation of a single correolide-binding site implies some interaction across multiple subunits. Although available data cannot distinguish among different models, the interactions of correolide with multiple subunits could involve direct binding to several subunits, block of access to other binding sites, or indirect allosteric interactions mediated through a single subunit interaction. Nonetheless, it is postulated that correolides share with MgTX the ability to affect several subunits simultaneously.

The specificity of correolide for Kv1 channels is further demonstrated in immunoprecipitation experiments with solubilized human brain diTC-binding sites. Most of the diTC binding can be immunoprecipitated by a combination of Kv1.1, Kv1.2, and Kv1.4 antibodies. These data suggest that other types of voltage-gated K^+ channels such as Kv2, Kv3, and Kv4 do not contribute to the high-affinity receptor for diTC. Although peptidyl blockers of Kv1 channels have been useful in determining the subunit composition of such channels in rat brain (*11*, *12*), some channels such as Kv1.4 and Kv1.5 are not sensitive to those inhibitors. Therefore, the possible existence of homomultimeric Kv1.4 and Kv1.5 channels has not been determined. Since diTC is expected to be a high-affinity probe for Kv1.4 and Kv1.5 channels, the contribution of these proteins to Kv1 channel complexes can now be assessed. Interestingly, Kv1.5 does not appear to be present in human brain synaptic membranes, while Kv1.4 appears to be present in complexes with Kv1.1 and Kv1.2 channels. Similar results have been obtained for immunoprecipitation of diTC bound to rat brain synaptic membranes (data not shown).

The only other small molecular weight inhibitor of Kv1.3 that has been well characterized in the literature is WIN 17317-3, an iminodihydroquinoline from Sterling−Winthrop (*6*) that was discovered as an inhibitor of ChTX binding to Jurkat cells ($IC_{50} = 80$ nM). WIN 17317-3 was reported to selectively block only Kv1.3 and Kv1.4 among all other Kv1 series channels tested (*40*). WIN 17317-3 is a use-dependent blocker and inhibits Kv1.3 with an IC_{50} of 350 nM when measured by patch voltage-clamp techniques (*6*). In addition, this compound was reported to have immunosuppressant activity in in vitro protocols with human T cells. However, WIN 17317-3 was found to be a potent blocker of voltage-gated sodium channels (*41*, *42*), which would compromise its ability to be analyzed for immunosuppressant activity in whole animal models. Furthermore, WIN 17317-3 does not block human T cell activation in the same fashion as the Kv1.3 inhibitory peptides, suggesting some nonspecific interactions in these cell based assays (G. Koo et al., unpublished observations).

Kv1.3 sets the resting potential in human peripheral blood T lymphocytes at approximately −50 mV (*2*). It has been shown that peptides that block Kv1.3 depolarize human T cells to approximately −30 mV where other conductances maintain this depolarized resting potential. It is predicted, therefore, that selective small molecule Kv1.3 inhibitors would also depolarize T cells to this same potential. The observation that the extent of depolarization by correolide is the same as that produced by a saturating concentration of MgTX suggests that the correolides should be expected to act as immunosuppressants in the same manner as has been demonstrated with MgTX (*1*, *5*).

This observation that correolide depolarizes human T cells to the same level as MgTX is significant, in that it is consistent with depolarization being affected only through block of Kv1.3 and not through any other mechanism. Our experience indicates that less selective Kv1.3 inhibitors depolarize human T cells to 0 mV and probably have effects on additional targets in these cells. Thus, the correolide class of compounds appears to have appropriate characteristics for development of an immunosuppressant. Since homotetrameric Kv1.3 is the only known Kv series channel present in human T lymphocytes (*9*, *34*, *43*, *44*) and since correolide is selective as a voltage-gated potassium channel inhibitor in these cells, it is predicted to work the same way as peptidyl inhibitors in both in vitro and in vivo immunological assays. Data to be reported completely support this expectation (G. Koo et al., in preparation). At −50 to −60 mV, a few open Kv1.3 channels will maintain the resting potential. Cell depolarization can occur upon preferential binding of inhibitors to channels after they have opened, suggesting a feed-forward mechanism for correolide action. This is an ideal mechanism for Kv1.3 inhibitors being developed as immunosuppressants.

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