Correolide, a novel nortriterpene natural product, potently inhibits the voltage-gated potassium channel, Kv1.3, and [3H]dihydrocorreolide (diTC) binds with high affinity \( (K_d \approx 10^{-11} \text{M}) \) to membranes from Chinese hamster ovary cells that express Kv1.3 (Felix, J. P., Bugarianes, R. M., Schmalhofer, W. A., Borris, R., Goetz, M. A., Hensens, O. D., Bao, J.-M., Kayser, F., Parsons, W. H., Rupprecht, K., Garcia, M. L., Kaczorowski, G. J., and Slaughter, R. S. (1999) *Biochemistry* 38, 4922–4930). Mutagenesis studies were used to localize the diTC binding site and to design a high affinity receptor in the diTC-insensitive channel, Kv3.2. Transferring the pore from Kv1.3 to Kv3.2 produces a chimera that binds peptidyl inhibitors of Kv1.3 with high affinity, but not diTC. Transfer of the Sg region of Kv1.3 to Kv3.2 reconstitutes diTC binding at 4-fold lower affinity as compared with Kv1.3, whereas transfer of the entire S5-S6 domain results in a normal Kv1.3 phenotype. Substitutions in Sg-S6 of Kv1.3 with nonconserved residues from Kv3.2 has identified two positions in Sg and one in S6 that cause significant alterations in diTC binding. High affinity diTC binding can be conferred to Kv3.2 after substitution of these three residues with the corresponding amino acids found in Kv1.3. These results suggest that lack of sensitivity of Kv3.2 to diTC is a consequence of the presence of Phe382 and lle387 in Sg, and Met458 in S6. Inspection of K1.1-1.6 channels indicates that they all possess identical Sg and S6 domains. As expected, diTC binds with high affinity \( (K_d \text{ values 7–21 nM}) \) to each of these homotetrameric channels. However, the kinetics of binding are fastest with Kv1.3 and Kv1.4, suggesting that conformations associated with C-type inactivation will facilitate entry and exit of diTC at its binding site. Taken together, these findings identify Kv1.1 channel regions necessary for high affinity diTC binding, as well as, reveal a channel conformation that markedly influences the rate of binding of this ligand.

Voltage-gated potassium channels participate in a number of important cellular functions (1). Therefore, specific modulation of the activity of these proteins may lead to the development of novel therapeutic agents. Peptidyl blockers of potassium channels purified from venom of different organisms have been useful in defining various structural and functional properties of potassium channels (2). Two of these peptides, margatoxin (MgTX)\(^1\) and charybdotoxin (ChTX), have been critical in identifying Kv1.3 as a target for development of novel immunosuppressants (3–6). In the course of screening for small molecule Kv1.3 inhibitors to support these efforts, a novel nortriterpene, correolide, was isolated from the plant *Spaecia correa* (7) and shown to be a potent Kv1.3 blocker (8). Correolide is a selective inhibitor of the Kv1 family of potassium channels, displaying very low affinity for other ion channels and membrane proteins. However, its selectivity for Kv1.3 appears to be limited and in functional assays it blocks other Kv1 channels with 4–20-fold lower potency. A tritium-labeled derivative of correolide, [3H]dihydrocorreolide (diTC; Table I), binds reversibly and saturably to Kv1.3; the stoichiometry of the binding reaction suggests that one molecule of correolide binds per channel tetramer; the binding sites for correolide and peptidyl blockers are distinct (8). Because of the large structural diversity of voltage-gated potassium channels (9–11), knowledge of specific molecular determinants that determine high affinity binding of modulators among different channel proteins is crucial for development of potent and selective therapeutic agents.

In this study, we investigate the molecular basis for interaction of diTC with Kv1.3 and design a high affinity receptor in the diTC-insensitive channel, Kv3.2. Our results indicate that residues located in the Sg and S6 region are responsible for the high affinity interaction of diTC with Kv1.3 and that the lack of sensitivity of Kv3.2 to diTC is due to the presence of Phe382 and lle387 in Sg, and Met458 in S6. Although all Kv1 channels display conserved residues in the Sg and S6 domains and diTC binds with high affinity to all of these channels, the kinetics of ligand association and dissociation are much faster with Kv1.3 and Kv1.4 than with other Kv1 channels. The energy barrier for diTC to enter or leave its binding site may be less for Kv1.3 and Kv1.4 as a consequence of a change in channel conformation induced by C-type inactivation, which is a common feature of these two channels. A preliminary report of these findings has been made in abstract form (12).

**Experimental Procedures**

**Materials**—Restriction enzymes and the pCI-neo vector were bought from Promega. The pEGFP-N1 vector was from CLONTECH, and Pfu DNA polymerase from Stratagene. Taq-A-201 cell line, a subclone of the human embryonic kidney cell line, HEK293, which expresses the SV40 T antigen, was a gift of Dr. Robert DuBridge. All tissue culture media were from Life Technologies, Inc., serum was from Hyclone, and the FuGENE\(^\text{TM}\) transfection reagent was from Roche Molecular Biochemi-

\(^1\) The abbreviations used are: ChTX, charybdotoxin; Kv, voltage-gated potassium channel; diTC, [3H]dihydrocorreolide; diHg, dihydrocorreolide; HgTX1, hongotoxin-1; MgTX, margatoxin; K, equilibrium dissociation constant; \( k_y \), dissociation rate constant; \( k_x \), association rate constant.
Binding of Correolide to Potassium Channels

RESULTS

Construction of K_{3.2}/1.3 Channel Chimeras—Although diTC binds to K_{3.2} with high affinity, this ligand does not interact with members from other families of voltage-gated potassium channels such as K_{3.2} (8). Therefore, chimeras were constructed using regions of K_{3.2} and K_{3.2} to identify those domains that confer high affinity binding of diTC. This is an approach similar to that used to identify determinants responsible for block of potassium channels by peptide inhibitors (17, 18).

K_{1.3} channels are inhibited with high affinity by peptidyl blockers such as ChTX (6, 19, 20), hongotoxin-1 (HgTX_{1}) (13), MgTX (21), kalotoxin (22), and stichodactyla toxin (23). Furthermore, high affinity binding sites for radiolabeled ChTX (24), MgTX (25), and HgTX_{1} (13) have been identified in membranes prepared from cells containing K_{3.2} channels. K_{3.2} channels, however, are not sensitive to any of the identified peptidyl blockers of potassium channels (2). To introduce a marker into K_{3.2} that could be used to quantify levels of channel expression after transient transfection of TsA-201 cells, a chimera was constructed in which the pore region of K_{3.2} was transferred to K_{3.2} (Fig. 1). Expression of this and other chimeras was also monitored by immunoblotting membrane preparations using an antibody against the c-Myc sequence that was added to the C terminus of these proteins. Such experiments indicate that wild type channels and all chimeric channels analyzed in these studies give similar levels of protein expression (data not shown).

To determine whether the chimeric proteins associate to form tetrameric structures known to be required for binding of peptides, binding of ^{125}I-HgTX_{1}A19Y/Y37F was measured to membranes derived from TsA-201 cells transiently transfected with either K_{1.3} or K_{3.2}. The results of these experiments are shown in Fig. 2A. ^{125}I-HgTX_{1}A19Y/Y37F binds to a single class of sites that display K_{Dvalues} of 0.29 and 3.8 pm for K_{1.3} and K_{3.2}, respectively. Thus, transferring the pore region of K_{1.3} yields a high affinity receptor for HgTX_{1} in K_{3.2} that can be used to monitor levels of channel expression. The differences in HgTX_{1} affinity noted between K_{1.3} and this chimera are similar to those found in studies where the pore of K_{1.3} was transferred to K_{2.1} to confer agitoxin 2 sensitivity to the later channel (18). In this study, we have not investigated whether these differences are due to effects on peptide association or dissociation kinetics.

Several other K_{3.2} chimeras were constructed (Fig. 1) and characterized initially for functional channel tetramerization. In these cases, either transmembrane domain S_{5}-S_{6} or the complete S_{5}-S_{6} region of K_{1.3} was transferred to the K_{3.2} (P..K_{1.3}) chimera. Binding of ^{125}I-HgTX_{1}A19YY37F was monitored to these chimeras, and the results are shown in Fig. 2A. In all instances, high affinity binding of ^{125}I-HgTX_{1}A19YY37F occurred to a single class of sites as indicated by the fact that the Hill coefficients of all slopes shown in Fig. 2A are close to unity. The highest affinity for peptide was observed with the chimera in which the S_{5}-S_{6} region of K_{1.3} had been transferred. In fact, this chimera possesses the same affinity for HgTX_{1} as does native K_{1.3}. Binding of ^{125}I-HgTX_{1}A19YY37F to K_{3.2} was further characterized. Peptidyl blockers of K_{1.3} such as ChTX, MgTX, and stichodactyla toxin cause concentration-dependent inhibition of ^{125}I-HgTX_{1}A19YY37F binding (data not shown), and inhibitory potencies of these peptides for the various chimeras relative to the wild type channel scale with the ability of ^{125}I-HgTX_{1}A19YY37F to bind to these proteins. These data suggest that all K_{3.2} chimeras that were constructed can assemble to form tetrameric structures and that the molecular pharmacology of these chimeras with respect to the pore resembles closely that of native K_{1.3} in terms of their sensitivity to a number of different peptidyl inhibitors.

Binding of diTC to K_{3.2}/1.3 Channel Chimeras—To deter-
mine whether the high affinity interaction of diTC can be conferred to the correolide-insensitive channel, Kv3.2, diTC binding was evaluated in membranes prepared from the same K\textsubscript{3.2}/K\textsubscript{1.3} chimeras. diTC binds to a single class of sites present in this K\textsubscript{1.3} membrane preparation with a \( K_d \) of 9.4 nM (Fig. 2B). This value is nearly identical to the \( K_d \) of diTC measured in other types of membranes where K\textsubscript{1.3} is expressed (see Ref. 8 and this study). diTC binding cannot be detected with the K\textsubscript{3.2}(P,K\textsubscript{3.2}) chimera at a ligand concentration of 20 nM (data not shown), indicating that some other channel domain(s), and not the pore region, is necessary for the high affinity interaction of diTC with K\textsubscript{1.3}. In addition, the K\textsubscript{3.2} chimera containing only the S\textsubscript{4} region from K\textsubscript{1.3} did not display any detectable diTC binding (data not shown). Because the number of tetrameric channels present in these membranes can be determined independently from maximal levels of \( ^{125}\text{I}-\text{HgTX}, \text{A19Y/Y37F} \) binding, it is possible to estimate the \( K_d \) of diTC for these constructs to be >1 \( \mu \)M. However, the K\textsubscript{3.2} chimeras containing either the S\textsubscript{4} or the S\textsubscript{4}/S\textsubscript{5} region of K\textsubscript{1.3} did bind diTC potently with \( K_d \) values of 48.8 and 18 nM, respectively (Fig. 2B). Thus, the presence of S\textsubscript{5} from K\textsubscript{1.3} appears to be necessary for conferring a high affinity interaction of diTC with K\textsubscript{3.2}. Attempts to demonstrate a loss of diTC binding in K\textsubscript{1.3} by transferring S\textsubscript{5} from K\textsubscript{3.2}, however, were unsuccessful. The resulting construct did not yield any detectable \( ^{125}\text{I}-\text{HgTX}, \text{A19Y/Y37F} \) binding, despite the fact that expression of the protein could be easily detected in Western blots. It is interesting that the chimera that contains the S\textsubscript{5}/S\textsubscript{6} region of K\textsubscript{1.3} displays about 4-fold higher affinity for diTC than when S\textsubscript{5} is transferred to K\textsubscript{3.2} by itself. This finding is consistent with the observation from competition binding experiments that a 4-fold loss in diHC affinity is observed when S\textsubscript{5} from K\textsubscript{3.2} is transferred to K\textsubscript{1.3} (data not shown), and suggests that differences in S\textsubscript{5} between the two channels are not the major contributor to correolide sensitivity.

Comparison of the sequences of K\textsubscript{1.3} and K\textsubscript{3.2} in S\textsubscript{5} and S\textsubscript{6} indicate that 16 nonidentical residues are present; 11 in S\textsubscript{5} and 5 in S\textsubscript{6} (Fig. 3). To determine whether any of these residues contribute to the differences observed in diTC binding, individual mutations were introduced in K\textsubscript{1.3} to incorporate those amino acids present in K\textsubscript{3.2}. In these studies, binding parameters were determined with each mutant for both \( ^{125}\text{I}-\text{HgTX}, \text{A19Y/Y37F} \) and diTC, and the results are presented in Fig. 4. In these plots, a ratio of 1 indicates that the behavior of the mutant is identical to wild type, whereas a ratio of >1 reflects decreased binding as a result of the amino acid substitution. All mutants give similar levels of channel expression, as indicated by the maximum density of \( ^{125}\text{I}-\text{HgTX}, \text{A19Y/Y37F} \) binding sites, and all but one, V364I, displayed an identical affinity for \( ^{125}\text{I}-\text{HgTX}, \text{A19Y/Y37F} \) as wild type (Fig. 4A). These data indicate that, with the V364I exception, no major structural changes were conferred in the pore of the channel as a consequence of these mutations. In marked contrast, three mutations, L346F and F351I, both in S\textsubscript{5}, and L422M in S\textsubscript{6} had significant effects on diTC binding, causing 25-, 3-, or 5-fold decreases in ligand affinity, respectively (Fig. 4B). There is also a modest effect of a 2-fold increase in \( K_d \) with the mutation S362T. These data are consistent with previous observations regarding the K\textsubscript{3.2}/K\textsubscript{1.3} chimeras, and suggest that lack of high affinity interaction of diTC with K\textsubscript{3.2} could be due to the combined effect of 3 nonconserved residues in K\textsubscript{1.3}, 2 located in S\textsubscript{5} and 1 in S\textsubscript{6}.

To test this idea further, two K\textsubscript{3.2} mutants, K\textsubscript{3.2}F382L/M458L, and K\textsubscript{3.2}F382L/I387F/M458L, were constructed so that the corresponding residues in K\textsubscript{1.3} were introduced into K\textsubscript{3.2}, and their diTC binding properties were compared with those of K\textsubscript{1.3}. Results of these experiments are presented in Fig. 5. diTC binds to a single class of sites that display \( K_d \) values of 5.7, 20.9, and 10.2 nM for K\textsubscript{1.3}, K\textsubscript{3.2}F382L/M458L, and K\textsubscript{3.2}F382L/I387F/M458L, respectively (Fig. 5A). Thus, introduction of Leu, Phe, and Leu into K\textsubscript{3.2} at positions 382, 387, and 458, respectively, reconstitutes a high affinity receptor for diTC; in the absence of these mutations, the \( K_d \) for K\textsubscript{3.2} is >1 \( \mu \)M. As suggested from mutagenesis experiments with K\textsubscript{1.3}, the double mutant K\textsubscript{3.2}F382L/M458L has a 4-fold lower affinity for diTC than does K\textsubscript{1.3}. These results were also confirmed in competition experiments (Fig. 5B). diHC inhibits binding of diTC to these channels with \( K_d \) values of 6, 19.2, and 9.4 nM for K\textsubscript{1.3}, K\textsubscript{3.2}F382L/M458L, and K\textsubscript{3.2}F382L/I387F/M458L, respectively. All these data, taken together, strongly suggest that lack of high affinity interaction of diTC with K\textsubscript{3.2} is due to the presence of only three amino acids; Phe\textsuperscript{382} and Ile\textsuperscript{387} in S\textsubscript{5}, and Met\textsuperscript{458} in S\textsubscript{6}.

**Interaction of DiTC with K\textsubscript{1} Channels**—Because the S\textsubscript{3} and S\textsubscript{4} domains are conserved among all K\textsubscript{1} channels, it might be expected that diTC would bind to these proteins with similar \( K_d \) values. Previously reported data indicate that diTC binds with high affinity to a single class of sites in human and rat brain membranes, a receptor most likely comprised of heteromultimers of K\textsubscript{1.1}/K\textsubscript{1.2}/K\textsubscript{1.4} channels (8, 13), and that \( K_d \) values

**Fig. 1. K\textsubscript{3.2}/K\textsubscript{1.3} chimeras.** Schematic representation of the different channels or chimeras used in transient transfection of TsA-201 cells. Although not indicated in the figures, all subunits contain a c-Myc sequence added to their C termini.
determined from either equilibrium binding or ratio of rate constants are equivalent for both brain and K,1,3 membranes. However, there are marked differences in the kinetics of ligand binding. Thus, rates of ligand association and dissociation are about 20-fold faster for K,1,3 than with brain synaptic membranes. Because brain membranes do not contain a homogeneous population of any given K,1 channel, the properties of diTC binding to HEK293 membranes containing either K,1,1, K,1,2, K,1,3, K,1,4, K,1,5, or K,1,6 homotetrameric channels were investigated next. Under equilibrium conditions, diTC binds in a saturable fashion and with similar K_d values (7–21 nM) to a single class of sites present in each of these membrane preparations (Table I). These findings are consistent with the postulate that high affinity diTC binding would be nearly equivalent for all members of the K,1 channel family.

The kinetics of diTC association to homomultimeric K,1 channels were investigated next. At a ligand concentration close to K_d, diTC associates with channels in each of these membrane preparations in a time-dependent fashion; binding kinetics are pseudo-first order (Fig. 6). However, the association rate constant (k_1), varies greatly depending on the K,1.1 channel under consideration. Thus, association rates of diTC with either K,1,3 or K,1,4 channels are about 10–50-fold faster than those determined for the other K,1 channels (Table I). The kinetics of ligand dissociation were also determined from each of these channels. In all cases, dissociation of diTC is a time-dependent event that follows monoexponential kinetics, indicative of a first-order reaction (Fig. 7). Dissociation rate constant values (k_1) for the different K,1 channels are listed in Table I. K_d values determined from the ratio of k_1 versus k_1 are similar to those determined under equilibrium binding conditions, suggesting that diTC binds to homotetrameric K,1 channels through a simple bimolecular reaction. As observed for k_1, K,1,3 and K,1,4 are the channels that display the fastest dissociation kinetics. Differences in k_1 values between these and other K,1 channels are about 5–25-fold. Thus, the affinity of diTC for all K,1 channels, as determined under equilibrium binding conditions, is similar because relative differences in association and dissociation rate constants cancel each other. These data suggest that with some K,1 channels, ligand must overcome a much larger energy barrier than is present in either K,1,3 or K,1,4 during binding and unbinding reactions. Interestingly, K,1,3 and K,1,4 are the only channels in the K,1 family that undergo significant C-type inactivation, a process involving a conformational change of some residues in the outer vestibule of the channel that constricts the pore and causes blockade of the ion conduction pathway (26). It is possible that accessibility to the binding site for diTC becomes more facile after C-type inactivation occurs.

**Kinetics of diTC Binding to K,1,3 Channel Mutants with altered C-type Inactivation**—The idea that C-type inactivation induces a conformational change in K,1,3 that modifies the accessibility of diTC to its binding site was tested by investigating two channel mutants, K,1,3/G,375Q and K,1,3/H,399N, which display altered inactivation kinetics. In K,1,3, residues Gly,375 and His,399 are located in the external vestibule of the channel and appear to be directly involved in the conformational change that leads to channel inhibition (27). The mutant K,1,3/G,375Q is 8-fold slower in achieving C-type inactivation than wild type K,1,3, whereas K,1,3/H,399N is 50-fold faster in this respect. Importantly, under equilibrium conditions, both K,1,3 mutants and the wild type channel bind diTC with identical affinities when the reaction is monitored in membranes prepared from TsA-201 cells transiently expressing these proteins (data not shown). However, kinetics of diTC dissociation are markedly different among these various channels (Fig. 8). Ligand dissociation follows first-order kinetics with t_1/2 values of 59, 18, and 305 min for K,1,3, K,1,3/H,399N, and K,1,3/G,375Q, respectively. Thus, diTC dissociation kinetics are 5-fold slower from a protein where the rate of C-type inactivation is diminished, whereas it is 3-fold faster from a K,1,3 mutant displaying enhanced inactivation.
studies demonstrate that the affinity of diTC for all these Kv1.3 channels is the same, it is predicted that the kinetics of ligand association will also be modified accordingly. These data are consistent with the idea that diTC accessibility to and from its binding site is influenced by the conformational change eliciting C-type inactivation.
DISCUSSION

Correolide is the first potent, small molecule, natural product inhibitor of a voltage-gated potassium channel to be described. It displays remarkable specificity among ion channels in that it only interacts with Kv1 family channels. The results presented in this manuscript address two important aspects concerning the interaction of diTC with potassium channels. The first deals with the molecular basis for high affinity interaction of diTC with Kv1 channels, and in this context it has been possible to confer high affinity diTC binding to the diTC-insensitive channel, Kv3.2. Second, with respect to Kv1 channels, which possess a conserved diTC binding domain and to which diTC binding is of equivalent high affinity, the kinetics of ligand binding track with the ease by which these channels enter the C-type inactivated state.

Studies with chimeric channels constructed from domains of Kv1.3 and the diTC-insensitive channel, Kv3.2, indicate that it is possible to confer correolide sensitivity to Kv3.2 by transferring only the S5 region of Kv1.3. These data suggest that differences between Kv1.3 and Kv3.2 in S5 are a major determinant in the lack of sensitivity of the latter channel to this inhibitor. Site-directed mutagenesis studies have identified three nonconserved residues that may account for the lack of sensitivity of Kv3.2 to diTC. High affinity interaction of diTC with Kv3.2 has been achieved by mutating these residues to the corresponding amino acids found in Kv1.3. Two of these residues are located in S5, whereas the third is in S6. Interestingly, differences in the S6 regions of these two channels appear to play a minor role in diTC binding, despite the fact that residues of S6 have been shown to contribute to formation of the pore (28, 29). Two important questions arise from these studies. Is the binding domain for diTC restricted primarily to the S5 region? Alternatively, are residues of S5 critical for conferring a special conformation to a binding domain that is located in another region of the channel? Comparison of the S5 and S6 regions of all Kv1 channels indicates that there are no differences among any of these proteins. However, ligand binding kinetics are markedly different between channels possessing C-type inactivation and those channels that do not display this property. It appears that conformational changes induced by C-type inactivation make drug access to the diTC binding domain easier. Thus, it is possible that the specific residues that contribute to the binding site for diTC are common to many channels, but that the three-dimensional structure of the re-

| Table 1 |

| Value of $k_1$ or $k_2$ for diTC binding to homotetrameric Kv1 channels were calculated as described in the text. $K_d$ values for diTC were also determined under equilibrium binding conditions as described. These values are the average of 2–4 determinations carried out with different membrane preparations. |

| $k_1 \times 10^5$ | $k_2 \times 10^4$ | $K_d$ value |
|-----------------|-----------------|-------------|
| $min^{-1} ns^{-1}$ | $min^{-1}$ | $K_d$ value |
| $k_2/k_1$ | Equilibrium |
|-----------------|-----------------|-------------|
|-----------------|-----------------|-------------|
| Kv1.1           | 6.2 ± 0.35      | 21 ± 5      | 34 21 ± 5 |
| K1.2            | 9.4 ± 3.5       | 12 ± 2      | 13 10 ± 4 |
| K1.3            | 263 ± 84        | 133 ± 12    | 5 10 ± 1 |
| K1.4            | 120 ± 30        | 90 ± 20     | 8 10 ± 1 |
| K1.5            | 9.1 ± 1.8       | 10 ± 0.7    | 11 7 ± 1 |
| K1.6            | 4.8 ± 1.5       | 5.4 ± 1.4   | 11 19 ± 8 |

**FIG. 6.** Binding of diTC to Kv1 channels: association kinetics. Membranes prepared from HEK293 cells stably transfected with either Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, or Kv1.6 channels were incubated with diTC for the indicated periods of time at room temperature. Nonspecific binding, determined in the presence of 10 μM diHC, is time-invariant and has been subtracted from the experimental points. Specific binding data have been fit to the pseudo-first order association reaction as described under “Experimental Procedures.”
ceptor differs between these channels. On the other hand, the pathway for drug entering its receptor site could be the rate-determining step for binding. For some Kv1 channels, this energy barrier represents an additional 2 kcal/mol, but for other channels, such as Kv3.2, it could be much larger so as to prevent drug binding at all. Site-directed mutagenesis studies along S5 and S6 in Kv1.3 would be required to identify those specific residues that contribute to the diTC binding pocket. With use of the recently disclosed high resolution structure of the KcsA channel (28), it may be possible to model the structure of Kv1.3. Such a model could be useful in general for probing the molecular determinants that control diTC binding to potassium channels.

Correolide and several structural analogs were isolated from the plant Spachea correa (7) based on their ability to block Kv1.3 potently in a functional efflux assay (8). These compounds also inhibit lymphocyte activation mediated by Ca$^{2+}$-dependent stimulation of T cells in in vitro assays, as well as in a mini-pig in vivo delayed-type hypersensitivity test. The mechanism by which correolide and its analogs suppress T cell activation is directly related to blockade of Kv1.3. Like peptide inhibitors, correolide partially depolarizes T cells (8). This depolarization leads to diminution of the Ca$^{2+}$ signaling that occurs upon activation of the T cell receptor complex. As expected, the properties of correolide and its analogs resemble those of peptidyl inhibitors of Kv1.3, such as MgTX, in immunological assays (4, 5). Given these observations, correolide-derived agents might be candidates for development as novel immunosuppressant drugs for treatment of graft rejection and autoimmune diseases.

The development of a safe immunosuppressant requires a minimum side effect profile. For agents targeting voltage-gated potassium channels, this is a monumental task given the large diversity of these proteins and their wide distribution throughout the body. Peptidyl inhibitors of Kv1 channels are not state-dependent channel blockers and are generally neurotoxic. Although the safety profile of correolide and its analogs requires further investigation, preliminary results from evaluation of 

FIG. 7. Binding of diTC to Kv1 channels: dissociation kinetics. Membranes prepared from HEK293 cells stably transfected with either Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, or Kv1.6 channels were incubated with 10 nM diTC. Dissociation kinetics were initiated by adding 10 μM diHC and incubating at room temperature for different periods of time. Specific binding data have been fit to a single monoeXponential curve corresponding to a first-order reaction.

FIG. 8. Binding of diTC to mutant Kv1.3 channels. Membranes prepared from TsA-201 cells transiently transfected with either Kv1.3 (■), Kv1.3/H399N (○), or Kv1.3/G375Q (▲) were incubated with 10 nM diTC. Dissociation kinetics were initiated by addition of 10 μM diHC followed by incubation at room temperature for different periods of time. Specific binding data have been fit to a single monoeXponential decay corresponding to a first-order reaction.

2 G. Koo, J. T. Blake, K. Shah, M. J. Staruch, F. Dumont, D. Wunderler, M. Sanchez, O. B. McManus, A. Sintina-Meisher, R. C. Böltz, M. A. Goetz, R. Baker, J. Bao, F. Kayser, K. M. Rupprecht, W. H. Parsons, X.-C. Tong, I. E. Ita, J. Pivvichay, S. Vincent, P. Cunningham, D. Hora, Jr., W. Feeney, G. Kaczorowski, and M. S., Springer, submitted for publication.
two correolide analogs that, after acute administration, block a delayed-type hypersensitivity reaction in mini-pigs, do not reveal a significant toxic side effect profile.\textsuperscript{2} Perhaps correolide achieves functional selectivity \textit{in vivo} as a blocker of T cell Kv1.3 channels. Such functional selectivity may be related to the mechanism by which correolide blocks Kv1.3. Preliminary studies indicate that this agent interacts with high affinity with either open or C-type inactivated, but not with the closed state of the channel (8, 30). Kv1.3 exists primarily in the inactivated state in T cells with a resting potential of approximately \(-50 \text{ mV}\). If correolide would preferentially associate with the inactivated form of the channel, it may be possible to shift the channel equilibrium to the drug-bound form that is nonconductive. In firing neurons, however, the drug-sensitive inactivated form of the channel may be present for only a very brief period of time; drug binding might be negligible under these conditions, given the slow onset of correolide action observed with brain native channels (8). Although more detailed studies are necessary to discern the mechanism by which correolide interacts with potassium channels, it is tempting to speculate that the state-dependent interaction of this agent, together with its kinetics of binding, may be important for limiting toxicity \textit{in vivo}.

\textbf{Acknowledgments—}We thank Dr. Bernardo Rudy for his gift of Kv3.2, and Dr. David Melillo for radiochemical synthesis support.

\textbf{REFERENCES}

1. Hille, B. (1992) \textit{Ionic Channels of Excitable Membranes}, 2nd Ed., Sinauer Associates, Inc., Sunderland, MA
2. Garcia, M. L., Hanner, M., Knaus, H.-G., Koch, R., Schmalhofer, W., Slaughter, R. S., and Kaczorowski, G. J. (1997) \textit{Adv. Pharmacol.} \textbf{39}, 425–471
3. Lin, C. S., Boltz, R. C., Blake, J. T., Nguyen, M., Talento, A., Fischer, P. A., Springer, M. S., Sigal, N. H., Slaughter, R. S., Garcia, M. L., Kaczorowski, G. J., and Koo, G. C. (1993) \textit{J. Exp. Med.} \textbf{177}, 637–645
4. Leonard, R. J., Garcia, M. L., Slaughter, R. S., and Reuben, J. P. (1992) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{89}, 10994–10998
5. Koo, G. C., Blake, J. T., Talento, A., Nguyen, M., Lin, S., Sirotina, A., Shah, K., Mulvany, K., Jr., D. H., Cunningham, P., Wunderler, D. L., McManus, O. B., Slaughter, R., Bugianesi, R., Felix, J., Garcia, M., Williamson, J., Kaczorowski, G., Sigal, N. H., Springer, M. S., and Feeley, W. N. (1997) \textit{J. Immunol.} \textbf{158}, 5120–5128
6. Price, M., Lee, S. C., and Deutsch, C. (1989) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{86}, 10171–10175
7. Goetz, M. A., Hensens, O. D., Zink, D. L., Borris, R. P., Morales, F., Tamayo-Castillo, G., Slaughter, R. S., Felix, J., and Ball, R. G. (1998) \textit{Tetrahedron Lett.} \textbf{39}, 2895–2898
8. Felix, J. P., Bugianesi, R. M., Schmalhofer, W. A., Borris, R., Goetz, M. A., Hensens, O. D., Bao, J.-M., Kayser, F., Parsons, W. H., Rupprecht, K., Garcia, M. L., Kaczorowski, G. J., and Slaughter, R. S. (1999) \textit{Biochemistry} \textbf{38}, 4922–4930
9. Salinas, M., Duprat, F., Heurteaux, C., Hugnot, J.-P., and Lazdunski, M. (1997) \textit{J. Biol. Chem.} \textbf{272}, 24371–24379
10. Chandy, K. G., and Gutman, G. A. (1995) in \textit{Handbook of Receptors and Channels} (North, R. A., ed., CRC Press, Inc., Boca Raton, FL
11. Pettig, J., Heinemann, S. H., Wunder, F., Lorra, C., Parej, D. N., Dolly, J. O., and Pongs, O. (1994) \textit{Nature} \textbf{369}, 289–284
12. Hanner, M., Schmalhofer, W. A., Green, B., Liu, J., Sanchez, M., Slaughter, R. S., Kaczorowski, G. J., and Garcia, M. L. (1999) \textit{Biophys. J.} \textbf{76}, (abstr.) 77
13. Koschak, A., Bugianesi, R. M., Mitterdorfer, J., Kaczorowski, G. J., Garcia, M. L., and Knaus, H.-G. (1998) \textit{J. Biol. Chem.} \textbf{273}, 2639–2644
14. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) \textit{Gene} \textbf{77}, 61–68
15. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) \textit{Gene} \textbf{77}, 51–59
16. Hanner, M., Vianna-Jorge, R., Kamassah, A., Schmalhofer, W. A., Knaus, H.-G., Kaczorowski, G. J., and Garcia, M. L. (1996) \textit{J. Biol. Chem.} \textbf{271}, 16289–16296
17. Izhii, T. M., Maylie, J., and Adelman, J. P. (1997) \textit{J. Biol. Chem.} \textbf{272}, 23195–23200
18. Gross, A., Abramson, T., and MacKinnon, R. (1994) \textit{Neuron} \textbf{13}, 961–966
19. Sands, S. B., Lewis, R. S., and Cahalan, M. D. (1989) \textit{J. Gen. Physiol.} \textbf{93}, 1061–1074
20. Swanson, R., Marshall, J., Smith, J. S., Williams, J. B., Boyle, M. B., Folandar, K., Luneau, C. J., Antanavage, J., Olivera, B., Buhrow, S. A., Bennett, C., Stein, R. B., and Kaczmarek, L. K. (1990) \textit{Neuron} \textbf{4}, 929–939
21. Garcia-Calvo, M., Leonard, R. J., Novick, J., Stevens, S. P., Schmalhofer, W., Kaczorowski, G. J., and Garcia, M. L. (1993) \textit{J. Biol. Chem.} \textbf{268}, 18866–18874
22. Aiyar, J., Withka, J. M., Rizzi, J. P., Singleton, D. H., Andrews, G. C., Lin, W., Boyd, J., Hanson, D. C., Simon, M., Dethlefs, B., Lee, C.-L., Hall, J. E., Gutman, G. A., and Chandy, K. G. (1995) \textit{Neuron} \textbf{15}, 1169–1181
23. Pennington, M. W., Byrnes, M. E., Zaydenberg, I., Khaytin, I., Chastonay, J. D., Krafte, D. S., Hill, R., Mahair, V. M., Volberg, W. A., Gorczyca, W., and Rem, W. R. (1995) \textit{Int. J. Pept. Protein Res.} \textbf{46}, 354–358
24. Slaughter, R. S., Shevell, J. L., Felix, J. P., Lin, C. S., Sigal, N. H., and Kaczorowski, G. J. (1991) \textit{Biophys. J.} \textbf{59}, 213 (abstr.)
25. Helms, L. H. H., Felix, J. P., Bugianesi, R. M., Garcia, M. L., Stevens, S., Leonard, R. J., Knaus, H.-G., Koch, R., Wanner, S. G., Kaczorowski, G. J., and Slaughter, R. S. (1997) \textit{Biochemistry} \textbf{36}, 3737–3744
26. Liu, Y., Jurman, M. E., and Yellen, G. (1996) \textit{Neuron} \textbf{16}, 859–867
27. Nguyen, A., Kath, J. C., Hansen, D. C., Biggers, M. S., Canniff, P. D., Donovan, C. B., Mather, R. J., Bruns, M. J., Rauer, H., Aiyar, J., Lepple-Wienhues, A., Gutman, G. A., Grissmer, S., Cahalan, M. D., and Chandy, K. G. (1996) \textit{Mol. Pharmacol.} \textbf{50}, 1672–1679
28. Doyle, D. A., Cahral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) \textit{Science} \textbf{280}, 69–77
29. Liu, Y., Holmgren, M., Jurman, M. E., and Yellen, G. (1997) \textit{Neuron} \textbf{19}, 175–184
30. Wunderler, D., Leonard, R. J., Sanchez, M., and McManus, O. B. (1999) \textit{Biophys. J.} \textbf{76}, 186 (abstr.)