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Molecular Determinants of KCNQ1 Channel Block by a Benzodiazepine

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ABSTRACT

KCNQ1 channels underlie the slow delayed rectifier K^+ current, mediate repolarization of cardiac action potentials, and are a potential therapeutic target for treatment of arrhythmia. (*E*)-(+)-*N*-[(3*R*)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(2,4-dichlorophenyl)-2-propenamide [L-735821 (L-7)] is a potent blocker of KCNQ1 channels. Here we describe the structural determinants of KCNQ1 that are critical for high-affinity block by L-7 using site-directed mutagenesis to alter specific residues and voltage clamp to record channel currents in *Xenopus laevis* oocytes. Chimeric channels were constructed by combination of regions from L-7–sensitive KCNQ1 and L-7–insensitive KCNQ2 channel subunits. This approach localized the drug interaction site to the pore and S6 domains of KCNQ1. Substitution of single amino acids identified Thr-312 of the pore domain and Ile-337, Phe-339, Phe-340, and Ala-344 of the S6 domain as the most important molecular determinants of channel block. Some mutations also altered the inactivation properties of KCNQ1, but there was no correlation between extent of inactivation and sensitivity to block by L-7. Modeling was used to simulate the docking of L-7 to the KCNQ1 channel pore. The docking was consistent with our experimental data and predicts that L-7 blocks K^+ conductance by physically precluding the occupancy of a K^+ ion to a pore helix-coordinated site within the central hydrated cavity, a crucial step in ion permeation.

KCNQ1 (*KVLQT1*) was discovered by positional cloning (Wang et al., 1996) and is the founding member of the KCNQ voltage-gated K^+ channel family. KCNQ1 channels are characterized by fast activation and a delayed, voltage-dependent inactivation. Coassembly with the β-subunit KCNE1 generates a very slowly activating delayed-rectifier K^+ current, I_{Ks}, with no apparent inactivation (Barhanin et al., 1996; Sanguinetti et al., 1996). KCNQ2 subunits coassemble with KCNQ3 subunits to form channels that conduct the neuronal M-current (Wang et al., 1998). Physiological functions for KCNQ1/KCNE channels include repolarization of cardiac action potentials, modulation of H^+ secretion into the stomach, Cl^- secretion into the colon, and secretion of K^+ into the stria media of the inner ear (Neyroud et al., 1997; Mall et al., 1998; Grahammer et al., 2001).

A potent class of I_{Ks} blockers was discovered serendipitously. Substituted 1,4-benzodiazepines were developed as potent and selective antagonists of cholecystokinin-B receptors (Evans et al., 1987). A side effect of some of these compounds was prolongation of QT interval caused by block of I_{Ks} channels in the heart. Optimization of I_{Ks} block by chemical modification of the cholecystokinin-B antagonists led to the discovery of L-7 (Fig. 1) and related compounds (Selnick et al., 1997). Selective blockers of I_{Ks} were initially developed as class III antiarrhythmic drugs (Busch et al., 1996; Salata et al., 1996) because of their ability to prolong ventricular refractoriness. Other potential therapeutic applications for KCNQ1 blockers include peptic ulcer disease or diarrhea (Grahammer et al., 2001). However, the subsequent finding that mutations in either *KCNQ1* (Wang et al., 1996) or *KCNE1* (Splawski et al., 1997a,b) cause inherited LQTS understandably diminished interest for development of I_{Ks} blockers as antiarrhythmic agents. An understanding of the molecular features of the L-7 binding site of KCNQ1 might facilitate the design of newer drugs that block the channel in a fashion that has less inherent proarrhythmic risk.

The goal of this study was to determine the structural basis for functional interaction of L-7 with the KCNQ1 channel. We constructed KCNQ1/KCNQ2 chimera channels to identify important domains of KCNQ1 that mediate block. This was followed by site-directed mutagenesis of the identified domains to determine whether specific residues might mod-

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ABBREVIATIONS: L-7, L-735821 ((*E*)-(+)-*N*-[(3*R*)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(2,4-dichlorophenyl)-2-propenamide); MES, 2-[*N*-morpholino]ethanesulfonic acid; I_{Ks}, slow delayed rectifier K^+ current; wt, wild type

ulate the sensitivity of KCNQ1 channels to L-7. Finally, molecular modeling was used to predict a favorable docking of L-7 to the central cavity of KCNQ1 that was consistent with our experimental findings.

Materials and Methods

Molecular Biology. The procedures used to generate *KCNQ1/KCNQ2* chimeras in psGEM were described previously (Seebohm et al., 2001). Silent mutations were introduced to produce unique restriction endonuclease sites at corresponding positions in human *KCNQ1* and *KCNQ2*, namely a *Sac*I site (at KCNQ1 amino acids Glu-361/Leu-362), a *Nsi*I site (at KCNQ1 amino acids Asp-301/Ala-302/Leu-303), a *Kpn*I site (at KCNQ1 amino acid Val-319/Pro-320), a *Xho*I site (at KCNQ1 amino acids Ser-276/Ser-277), and a *Bam*HI site (at KCNQ1 amino acids Gly-348/Ser-349). Chimeras were created by digest and subsequent ligation of the respective regions from *KCNQ1* and *KCNQ2*, resulting in the chimeras C1–C4 (Fig. 3). For C-1, *Sac*I and *Bam*HI sites were used to introduce the S5-S6 domains of KCNQ1 into KCNQ2. For C-2, *Xho*I and *Nsi*I sites were used to introduce portions of S5 and the pore of KCNQ2 into KCNQ1. For C-3, *Nsi*I and *Kpn*I sites were used to introduce the pore of KCNQ2 into KCNQ1. For C-4, *Kpn*I and *Bam*HI sites were used to introduce part of the pore loop and the S6 of KCNQ2 into KCNQ1. Site-directed mutagenesis was performed by polymerase chain reaction using the megaprimer method (Sarkar and Sommer, 1990) and native *Pfu* polymerase or a 5:1 *Pfu/Taq* polymerase mix. All constructs were confirmed by automated DNA sequencing. The constructs were linearized with *Nhe*I, and cRNA was transcribed in vitro with Capscribe (Roche Applied Science, Indianapolis, IN) using T7 polymerase.

Oocyte Isolation and Two-Electrode Voltage Clamp. Ovarian lobes were removed from *Xenopus laevis* frogs anesthetized with tricaine. Follicle cells were removed from oocytes by treatment for 90 to 150 min with 1 mg/ml type II collagenase (Worthington Biochemicals, Freehold, NJ) in ND96-Ca^{2+}-free solution containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6. Oocytes were subsequently stored at 18°C in Barth's solution containing 88 mM NaCl, 1 mM KCl, 0.4 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4, plus gentamycin (50 mg/liter). Each oocyte was injected with ~10 ng of wt or mutant *KCNQ1* cRNA.

A Dagan TEV-200 amplifier (Dagan Corp., Minneapolis, MN) and standard two-electrode voltage clamp techniques (Stuhmer, 1992) were used to record currents at 24°C in oocytes 2 to 3 days after injection with cRNA. Data acquisition was performed using a Dell Optiplex GX150 computer, a Digidata 1200 A/D interface, and pClamp 6 software (Axon Instruments, Union City, CA). Homomeric wt KCNQ1 channel currents were elicited every 20 s with 2-s depolarizing pulses that were applied in 10-mV increments to potentials between −70 and +50 mV. Heteromeric wt KCNQ1/KCNE1 channel currents were elicited every 30 s with 4-s depolarizing pulses to the same potentials. The effects of L-7 on KCNQ1/KCNQ2 chimera channels and mutant KCNQ1 channel currents were determined by repetitive pulses to +10 mV applied once every 20 s. All currents were elicited from a holding potential of −80 mV.

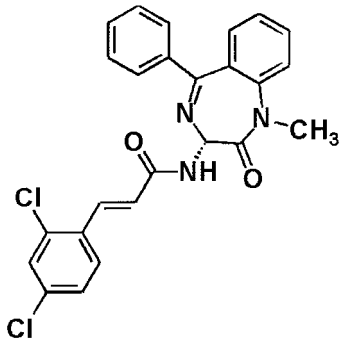

Fig. 1. Chemical structure of L-7.

L-7 (Merck Research Laboratories, West Point, PA) was freshly prepared each day from a frozen 100 mM stock dimethyl sulfoxide solution. The concentrated drug solution was dissolved in a low-Cl^- saline solution containing 96 mM NaOH, 2 mM KOH, 2 mM $Ca(OH)_2$, 1 mM $MgCl_2$, 101 mM MES, and 5 mM HEPES, pH 7.6. L-7 was tested at concentrations up to 10 μM, which was the highest concentration that remained completely soluble in the physiological saline solution.

Data Analysis. Analysis of data were performed with pClamp 8 (Axon Instruments) and Origin 7 software (OriginLab Corp, Northampton, MA). The concentration of drug required for 50% block of current (IC_{50}) was calculated from current amplitudes measured at the end of test pulses with a Hill equation: $I/I_{max} = 1/(1 + [C/IC_{50}]^{nH})$, where I is current amplitude at the end of a depolarizing pulse, I_{max} is the maximal current amplitude, C is concentration of the drug, and n_H is the Hill coefficient.

The fraction of inactivated channels was calculated by tail current analysis (Tristani-Firouzi and Sanguinetti, 1998). The deactivating component of the tail currents was extrapolated back to the end of the depolarizing pulse and the amplitude (x) was measured. The initial tail current amplitude was subtracted from the extrapolated amplitude x to give the amplitude of recovery, y. The fraction of inactivated channels during the preceding depolarization was estimated by the ratio y/x.

Numerical values are reported as mean ± S.E.M. (n = number of experiments). Student's t test was used to test for statistical significance between groups. A value of $p < 0.05$ was considered statistically significant.

Molecular Modeling of KCNQ1. The KcsA structure (Doyle et al., 1998) was retrieved from the Protein Data Bank (code 1BL8). A three-dimensional structural model of the S5/H5/S6 domains of KCNQ1 was constructed based on the crystal structure of the corresponding domains of KcsA, with which it shares 46% homology and 36% identity. The KCNQ1 model was generated using the Modeler module of Insight II (ver. 98.0; Accelrys, San Diego, CA). Docking of L-7 in the KCNQ1 homology model was performed using the flexible ligands oriented on grid (FLOG) procedure (Miller et al., 1994). Similar dockings (not shown) were obtained using a geometric approach with the Gramm ver. 1.03 program (Katchalski-Katzir et al., 1992).

Results

KCNQ1/KCNE1 Channels Are More Sensitive Than KCNQ1 Channels to Block by L-7. Homomeric KCNQ1 and heteromeric KCNQ1/KCNE1 (I_{Ks}) channels expressed in *X. laevis* oocytes were blocked by L-7 in a concentration-dependent manner (Fig. 2, A and B). Channel block was quantified by measuring whole-cell currents at the end of 2-s (KCNQ1) or 4-s (KCNQ1/KCNE1) depolarizing pulses to +10 mV, applied from a holding potential of −80 mV. Oocytes were treated with L-7 for 10 min to obtain steady-state inhibition. KCNQ1 channel current was less sensitive to block by L-7 (IC_{50} = 173 nM) than heteromeric KCNQ1/KCNE1 channel current (IC_{50} = 37 nM). The concentration-response relationships are shown in Fig. 2C. The Hill coefficient for block of KCNQ1 and KCNQ1/KCNE1 channel currents was 0.5 and 0.3, respectively, suggesting possible negative cooperativity for drug binding to the channel.

The effect of L-7 on the KCNQ1 current-voltage relationship was estimated by normalizing current amplitudes measured at the end of 2-s pulses to the peak current measured at +50 mV. This relationship was not altered by 0.1 or 1 μM L-7 (Fig. 2D), indicating that drug block was independent of

voltage. This is not surprising because L-7 is mostly uncharged (calculated pK_a < 2.5) at physiological pH. Block of KCNQ1/KCNE1 channel current was also voltage-independent (data not shown). However, the block of KCNQ1 channels was time-dependent during a depolarizing pulse as illustrated in Fig. 2E for an oocyte treated with 10 μM L-7. Control current at 10 mV was >90% activated within ~0.6 s, then increased very gradually throughout the 5-s pulse. In the presence of drug, current reached a peak value within 0.3 s and then decreased throughout the pulse. In summary, block of KCNQ1 by L-7 was concentration- and time-dependent but voltage-independent.

KCNQ1/KCNQ2 Chimera Channels. To determine the domains of KCNQ1 that bind L-7, we took advantage of the differential sensitivity to L-7 of KCNQ1 and KCNQ2 channels. L-7 is a potent blocker of KCNQ1, whereas KCNQ2 channel current was not affected by 10 μM drug (n = 4; Fig. 3A). The first chimera (C-1) was constructed by introducing S5-S6 of KCNQ1 into the KCNQ2 background. This chimera channel retained sensitivity to L-7 (Fig. 3B), with an IC_{50} value of 0.69 ± 0.08 μM (n = 3). Chimera 2 (C-2) was constructed by introducing portions of S5 and part of the pore loop of KCNQ2 into a KCNQ1 background. This chimera channel was even more sensitive to L-7 (Fig. 3C), with an IC_{50} value of 0.40 ± 0.02 μM (n = 8). Chimera 3 (C-3) was constructed by introducing the pore loop from KCNQ2 into a KCNQ1 background. This chimera channel was relatively insensitive to L-7, with an IC_{50} value of 16.7 μM ± 0.08 (n = 5). Chimera 4 (C-4) was constructed by introducing a portion of the pore loop and the entire S6 domain of KCNQ2 into the KCNQ1 background. This channel was unaffected by 10 μM of L-7 (Fig. 3D; n = 5). Finally, we attempted to introduce sensitivity to L-7 into KCNQ2 channels by inserting specific domains of KCNQ1 into the KCNQ2 background. Three regions of KCNQ1 defined by pairs of restriction enzyme cutting sites (*Nsi*1-*Kpn*I, *Kpn*I-*Bam*HI, and *Nsi*I-*Bam*HI) were individually introduced into the KCNQ2 background. Unfortunately, none of these chimeric constructs were functional when expressed in oocytes and thus could not be used to test sensitivity to L-7. A summary of the results obtained with the KCNQ1/KCNQ2 chimera channels is shown in Fig. 3E. These data suggest that the pore helix and the S6 domain are regions that mediate high-affinity block of KCNQ1 by L-7.

Single Amino Acid Mutations within the Pore and S6 Domains Identify Potential Residues for Drug Interaction. Alanine-scanning mutagenesis (or Ala→Cys) of the S6 domain was performed to identify specific residues that influenced sensitivity of KCNQ1 channels to block by L-7. Only the amino acids of S6 predicted to form the walls of the central cavity (Phe-332–Leu-347) were mutated to Ala or Cys (Fig. 4A). In addition, because mutation to Ala resulted in several nonfunctional channels (e.g., T311A; Wollnik et al.,

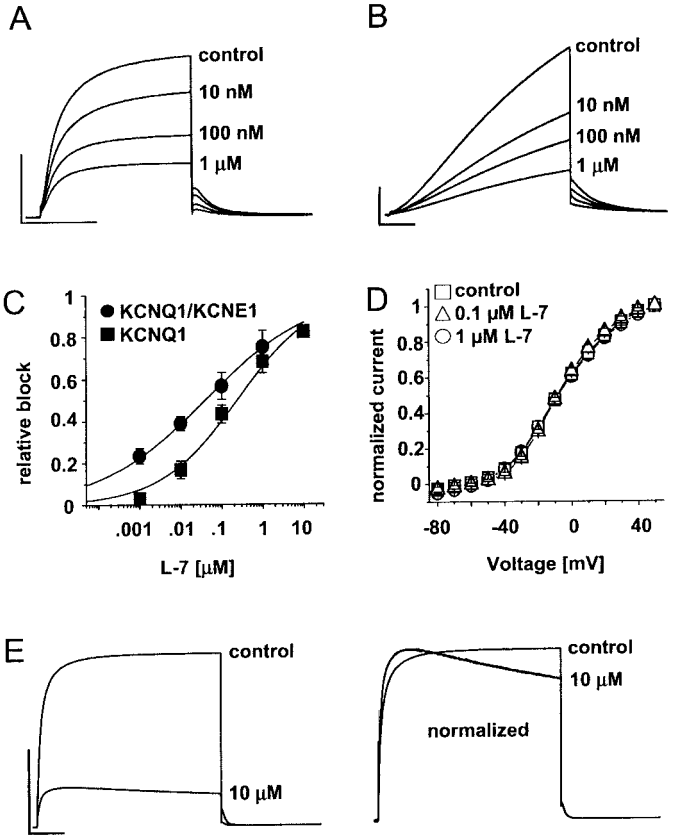

Fig. 2. L-7 blocks KCNQ1 and KCNQ1/KCNE1 channels. A, KCNQ1 channel current elicited with a 2-s pulse to +10 mV before (control) and after block by the indicated concentrations of L-7. B, KCNQ1/KCNE1 channel currents elicited with a 4-s pulse to +10 mV. C, concentration-effect relationship for block of currents by L-7. The IC_{50} for KCNQ1/KCNE1 current was 37 nM (n_H = 0.3; n = 8). The IC_{50} for KCNQ1 current was 173 nM (n_H = 0.5; n = 6–11). D, current-voltage relationship for KCNQ1 channel current. Currents were normalized to amplitudes recorded at +50 mV. E, effect of 10 μM L-7 on KCNQ1 current elicited with a 5-s pulse to +10 mV. Right shows currents normalized to their peak values. For A, B, and E, the horizontal calibration bar represents 1 s and the vertical bar represents 1 μA.

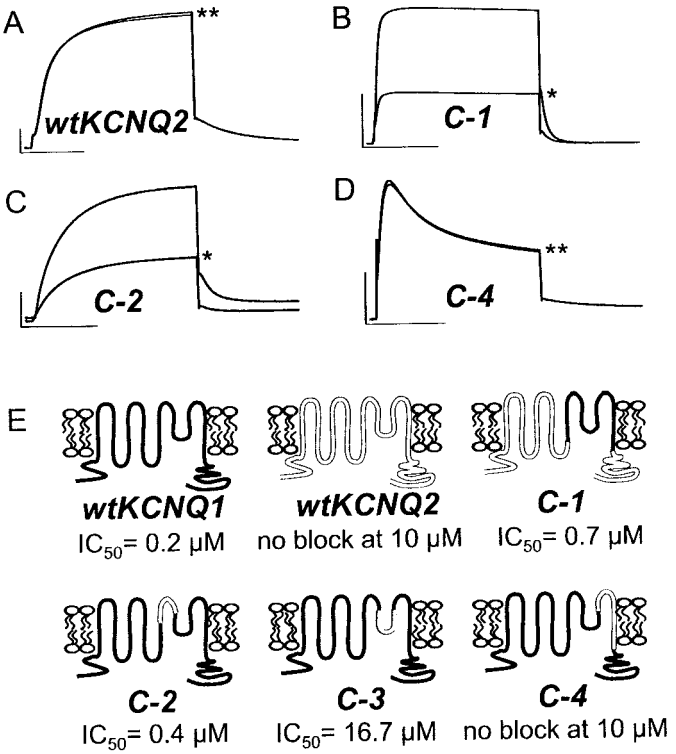

Fig. 3. Effect of L-7 on KCNQ1/KCNQ2 chimera channels. A, L-7 did not decrease wt KCNQ2 channel current. B to D, effect of 1 μM (*) or 10 μM (**) L-7 on KCNQ1/KCNQ2 chimera channel current. For A to D, horizontal calibration bar represents 1 s and vertical bar represents 1 μA. E, diagrams of chimera channel subunits, indicating location of splice points and sensitivity to block by L-7.

1997), a portion of the pore loop (Val-307 to Ile-313; Fig. 4A) was scanned by mutation to relatively conserved residues. The effect of 3 μM L-7 on each functional point mutant channel was determined. This concentration caused a large (70%) decrease, but incomplete block of wt KCNQ1 current. T309S, P343A, G345A, I346A and L347A KCNQ1 subunits either did not form functional channels or were expressed too poorly to evaluate drug sensitivity.

The effects of 3 μM L-7 on wt and several mutant KCNQ1 channel currents are shown in Fig. 4B. Wild-type KCNQ1 was reduced 70 ± 4% by this concentration of L-7 (n = 6). Some mutant channels, such as F335A, retained relatively normal biophysical properties and sensitivity to L-7, whereas other mutations altered channel properties or were less sensitive to L-7. For example, V310L KCNQ1 activated more slowly and was less blocked by drug than wt KCNQ1. T312S and I337A KCNQ1 channels deactivated more slowly than wt channels but were inhibited only slightly by 3 μM L-7. F339A and F340A channels inactivated more than wt channels and were relatively insensitive to 3 μM L-7. In the absence of drug, A344C channel current was similar to wt KCNQ1. However, treatment with 0.1 or 1 μM L-7 caused a −15 mV shift in the voltage dependence of KCNQ1 activation (n = 5, data not shown) and consequently had an agonist effect when currents were measured at +10 mV (Fig. 5, dashed curve). At 3 μM, L-7 reduced A344C by 33 ± 4%. The blocking activity of L-7 on pore and S6 domain mutant channels is summarized in Fig. 4C.

The specific amino acid used to substitute for the native residue may affect sensitivity to drug. Therefore, we also tested the effects of drug on channels that contained a conserved mutation of Phe-340 or Ile-337, two of the most important amino acids we identified in the Ala scan. Both F340Y (n = 3) and I337V (n = 5) KCNQ1 channels were insensitive to 10 μM L-7, the highest concentration of drug that remained soluble in the saline solution. Based on the effects of 3 μM L-7 on mutant channel currents, the residues that most affected drug interaction were, in order of potential importance: Phe-340, Thr-312, Phe-339, Ile-337 and Ala-344. The concentration-dependent effect of L-7 on channels containing point mutations of these residues is summarized in Fig. 5.

Lack of Correlation between Inactivation and Block of KCNQ1 Channels. Block of Na^+, Ca^{2+}, and some K^+ channels by drugs has often been attributed to stabilization of or preferential binding to inactivated states of the channel. Evidence for this mechanism includes a positive correlation between channel inactivation and block. Therefore, the extent of inactivation and sensitivity to block by L-7 was assessed for wt and mutant KCNQ1 channels. The fraction of inactivated channels during a 5-s pulse to +10 mV was determined by analysis of tail currents at −60 mV as described under *Materials and Methods*. The extent of inactivation was compared with the percentage block of current at +10 mV by 3 μM L-7 (Fig. 6). There was no apparent correlation between these two parameters (correlation coefficient for linear fit, r = 0.09).

Many, but not all of the residues found to be important for block of KCNQ1 channels by L-7 are the same in KCNQ2 (Fig. 4A, boxed residues), raising the question as to why KCNQ2 channels are not drug sensitive. In a final attempt to modify KCNQ2 channels to acquire sensitivity to L-7, we introduced three mutations (G301A, V302I, L275V) into KCNQ2 to match the residues native to KCNQ1. However, KCNQ2 channels with these three mutations did not functionally express.

Discussion

The Benzodiazepine L-7 Blocks KCNQ1 Channels. Benzodiazepines are best known for their ability to activate γ-aminobutyric acid-A receptors and block gastrin or cholecystokinin receptors (Evans et al., 1987; Bock et al., 1989). However, despite structural similarities to other benzodiazepines, the 3-substituted 5-phenyl-1,4-benzodiazepine L-7 is a relatively specific blocker of I_{Ks} (Selnick et al., 1997), an action that prolongs cardiac action potential duration (Salata et al., 1996), and QT interval (Selnick et al., 1997). Chromanol 293B is another I_{Ks} blocker first developed as an antiarrhythmic agent that prolongs action potentials and QT interval (Varro et al., 2000).

KCNQ1/KCNE1 channels were more sensitive to block by L-7 than KCNQ1 channels. This is not a unique finding. KCNE1 was previously reported to increase the sensitivity of

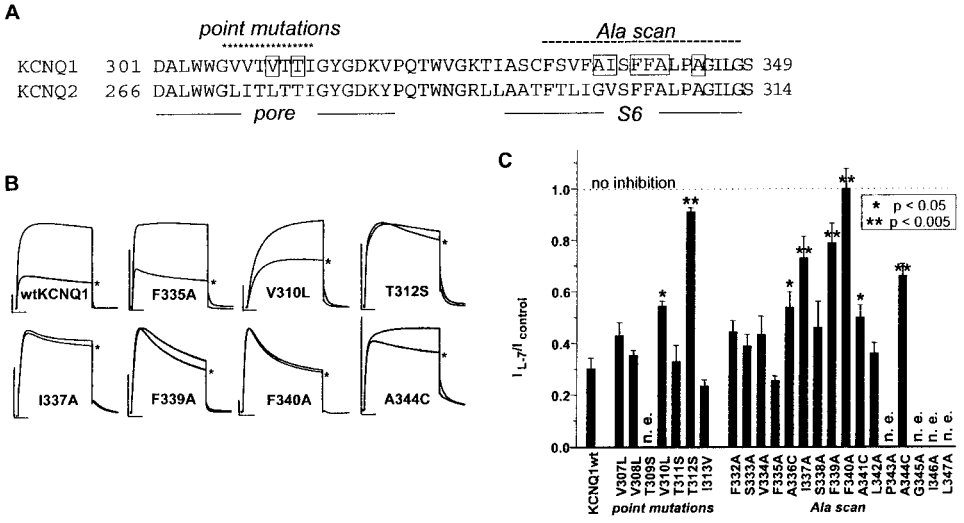

Fig. 4. Residues that potentially interact with L-7 defined by scanning mutagenesis of KCNQ1. A, sequence alignment of KCNQ1 and KCNQ2 indicating residues in the pore helix and S6 domains that were mutated. Specific residues in KCNQ1 that altered drug block are boxed. B, representative examples of wt and mutant KCNQ1 channel currents before and after treatment of oocytes with 3 μM L-7 (*). Horizonal calibration bar represents 1 s and vertical bar represents 1 μA. C, normalized KCNQ1 channel current (IL-7/$I_{control}$) measured after steady-state block by 3 μM L-7 (n = 3–8). n.e., no functional channel expression detected by voltage clamp analysis.

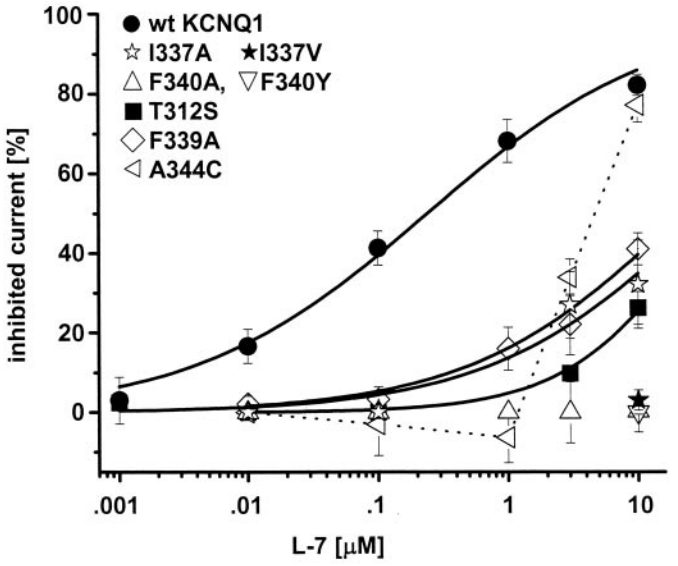

Fig. 5. Concentration-dependent block of wt and mutant KCNQ1 channels by L-7. The IC_{50} was >10 μM for all the mutants shown except A344C (n = 4–10 for each mutant). At a concentration of 0.1 and 1 μM, L-7 increased the current magnitude of A344C KCNQ1 channel current; therefore, the concentration-effect relationship (dashed curve) was not fitted with a Hill equation.

KCNQ1 channels to chromanol 293B (Lerche et al., 2000). The mechanism of this effect is not known but may be related to the fact that longer pulses were used to activate KCNQ1/KCNE1 channels, allowing greater time for drug to gain access and bind to the open state of the channel. L-7 preferentially blocks open channels, as indicated by the slow rate of I_{Ks} (Salata et al., 1996) or KCNQ1 channel block during a pulse to +10 mV (Fig. 2E).

The concentration-effect relationship for block of KCNQ1 and KCNQ1/KCNE1 currents by L-7 had Hill coefficients of 0.5 and 0.3, respectively. In binding assays, a Hill coefficient <1 can sometimes be attributed to heterogeneity in the receptor population or the presence of nonequivalent sites on an oligomeric protein. However, because we measured currents conducted by homotetrameric channels, it is plausible that there are two L-7 binding sites on each KCNQ1 channel and that binding of drug to one site partially inhibits binding of a second drug molecule to another site. It is also possible that the Hill coefficient was <1 because current block was not assessed under steady-state conditions. As shown in Fig. 2E, KCNQ1 current decreased throughout a 2-s pulse in the presence of 10 μM L-7. However, when percentage block of KCNQ1/KCNE1 current was calculated after 1-s or the end of a 5-s pulse, there was no change in the slope of the concentration-response curve (data not shown). Radiolabeled L-7 binding studies are needed to determine whether KCNQ1 channels contain one or two binding sites for benzodiazepines.

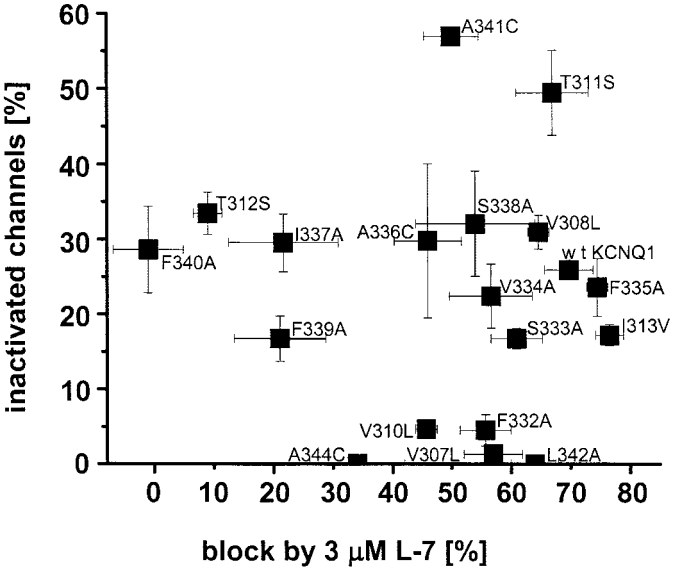

Fig. 6. KCNQ1 channel inactivation and sensitivity to block by L-7 are not correlated. The percentage of channels that inactivated at +10 mV was estimated using tail current analysis as described under *Materials and Methods* (n = 5–15).

L-7 Binds to the S6 Domain of KCNQ1 Channels. Using a site-directed mutagenesis/voltage clamp approach, we identified amino acid residues that make up the putative binding site for the potent benzodiazepine L-7 on KCNQ1. This indirect approach did not directly monitor changes in binding affinity as could be done with a radioligand binding assay. Nonetheless, the amino acids in the S6 domain we identified as important for drug interaction are predicted to face the central cavity of the KCNQ1 channel, as is expected for a drug that preferentially blocks channels in the open state. Based on a KCNQ1-KCNQ2 chimera analysis, chromanol 293B also binds to the S6 domain of KCNQ1 and it is possible that it binds to some or all of the residues identified here for L-7. The putative binding site for other drugs that block voltage-gated ion channels has also been localized to residues of the S6 domain, including blockers of Kv channels (Yeola et al., 1996; Lees-Miller et al., 2000; Mitcheson et al., 2000a; Hanner et al., 2001), Ca^{2+} channels (Hockerman et al., 1995, 1997a,b; Peterson et al., 1997) and Na^{+} channels (Ragsdale et al., 1994, 1996). Many of these compounds seem to preferentially bind to or stabilize channels in the inactivated state, as indicated by a drug-induced shift in the apparent voltage dependence of inactivation of Na^{+} channels (Hille, 1977) or Ca^{2+} channels (Bean, 1984; Sanguinetti and Kass, 1984) or by a decrease in affinity for inactivation-removed mutant K^{+} channels (Ficker et al., 2001; Herzberg et al., 1998; Wang et al., 1997). However, we found no correlation between the extent of KCNQ1 channel inactivation and reduction of current by L-7. Moreover, KCNQ1/KCNE1 channels that do not appreciably inactivate during the 4-s pulse used to elicit currents were more sensitive to L-7 than wt KCNQ1 channels. Drugs, toxins, or cations can also reduce K^{+} channel current by causing a positive shift in the voltage dependence of activation (Johnson et al., 1999; Sanguinetti et al., 1997; Swartz and MacKinnon, 1997). However, L-7 did not alter the voltage dependence of KCNQ1 activation. Taken together, these findings suggest that L-7 physically occludes the pore by binding to the S6 domain of KCNQ1 and does not decrease conductance by modulating channel gating.

Phe-340 was very sensitive to amino acid substitution because mutation to Ala or a conserved Tyr led to a similar reduction in drug potency. Substitution of Phe-340 with a Tyr residue may cause a reorientation of the side group sufficient to significantly reduce the postulated (see below) π-stacking interaction between Phe-340 and the benzyl group of L-7.

This is somewhat surprising, but illustrates the strict requirement for a Phe at position 340 to enable high-affinity interaction with L-7. An equally unexpected finding was that substitution of Ile-337 with a smaller hydrophobic residue (Val or Ala) rendered the mutant channels considerably less sensitive to block by L-7. This finding suggests that an interaction between the 5-phenyl group of L-7 and Ile-337 is a strict requirement for high-affinity binding that can be disrupted by even a minor change in hydrophobicity or volume of the amino acid at position 337.

The voltage dependence of A344C channel activation was shifted to more negative potentials by L-7, causing an apparent activation when current magnitude was monitored at a potential (+10 mV) below that required for full activation. Activation was observed only for an L-7 concentration < 1 μM; higher concentrations of drug decreased current magnitude. The mechanisms of the drug-induced changes in A344C KCNQ1 channel gating are unknown; however, they are probably related to the special location of Ala-344 in the S6 domain. Mutation to Ala of the residue on either side of Ala-344 led to a loss of function. Both of these residues, Pro-343 and Gly-345, are potential α-helix breakers and may be important components of the activation gate. The PAG sequence of KCNQ1 is in the same position as the PVP sequence common to most other Kv channels. PVP has been proposed to produce a kink in the S6 domain of Kv channels and, based on solvent accessibility experiments, is further proposed to constitute the narrowest part of the pore contributed by the crossing of the S6 domains (del Camino et al., 2000).

Docking of L-7 to a Homology Model of KCNQ1. To gain insight into the specific interactions between L-7 and KCNQ1, a model of the S5-S6 region of KCNQ1 was constructed using the solved crystal structure of the KcsA K^+ channel from *Streptomyces lividans* (Doyle et al., 1998) as a template. Docking of L-7 was performed using the FLOG procedure (Miller et al., 1994). Figure 7, A and B, shows the model structure of KCNQ1 with a single L-7 molecule docked in the most preferred orientation based on energy minimization. Mutations of Ile-337 or Phe-340 caused the greatest decrease in drug potency. These two residues are located one helical turn apart from another in the middle of the S6 domain and, based on our homology model, are predicted to face the central cavity of the KCNQ1 channel. Together with Phe-339 and Ala-344 of the S6 domain and Thr-312 of the pore helix, these residues are proposed to comprise the most important sites for L-7 interaction with the channel. Modeling predicts L-7 to be in close contact with each one of these five amino acids on different KCNQ1 subunits. Our docking model suggests the following interactions: 1) Phe-340 (and perhaps Phe-339) of one subunit π-stacks with the benzyl group of L-7; 2) Ile-337 (and perhaps Thr-312) of an adjacent subunit makes hydrophobic contact with the 5-phenyl group of L-7; 3) Ala-344 of one or two subunits has hydrophobic contact with the 2,4-dichlorophenyl group of L-7. Thus, the orientation of L-7 within the central cavity predicted by the most favored docking to the KCNQ1 homology model is consistent with our experimental data.

The solved crystal structure of KcsA characterized by straight inner helices (Doyle et al., 1998) most probably represents the closed state of the channel. The crystal structure of the Ca^{2+}-activated MthK channel suggests that K^+ channel activation involves bending of the inner helices away from the central cavity, facilitated by a conserved Gly residue located in the middle of the α-helix that forms a "gating hinge" (Jiang et al., 2002a,b). It has also been suggested that

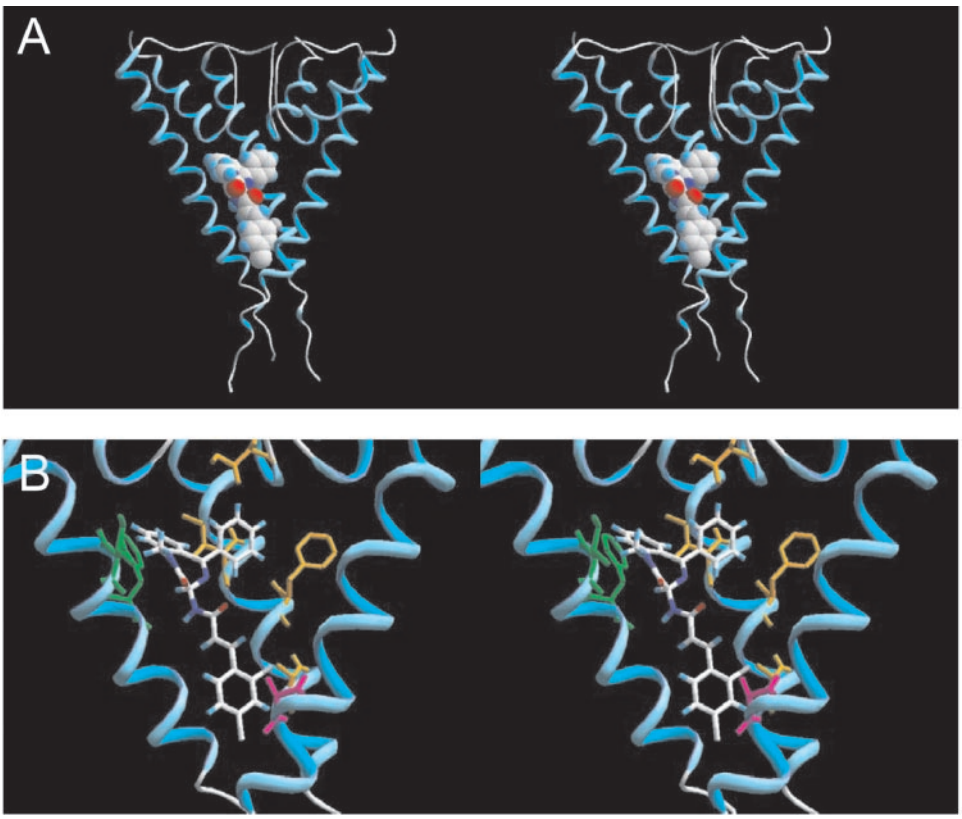

Fig. 7. Docking of L-7 to S6 domains of the KCNQ1 channel in the closed state. A, stereoview of L-7 docked to the KCNQ1 homology ribbon model of the pore and S6 domains for 3 subunits. L-7 is shown in a CPK-colored spacefill model. B, enlarged stereoview of the central cavity of the KCNQ1 channel with L-7 depicted in a CPK-colored stick model. Residues identified in the Ala/Cys-scan to affect block by L-7 are portrayed in stick mode. The left subunit highlights Ile-337 and Phe-340 colored in green; the middle subunit highlights Thr-312, Ile-337, Phe-340, and Ala-344 in orange; Ala-344 is colored pink in the right subunit.

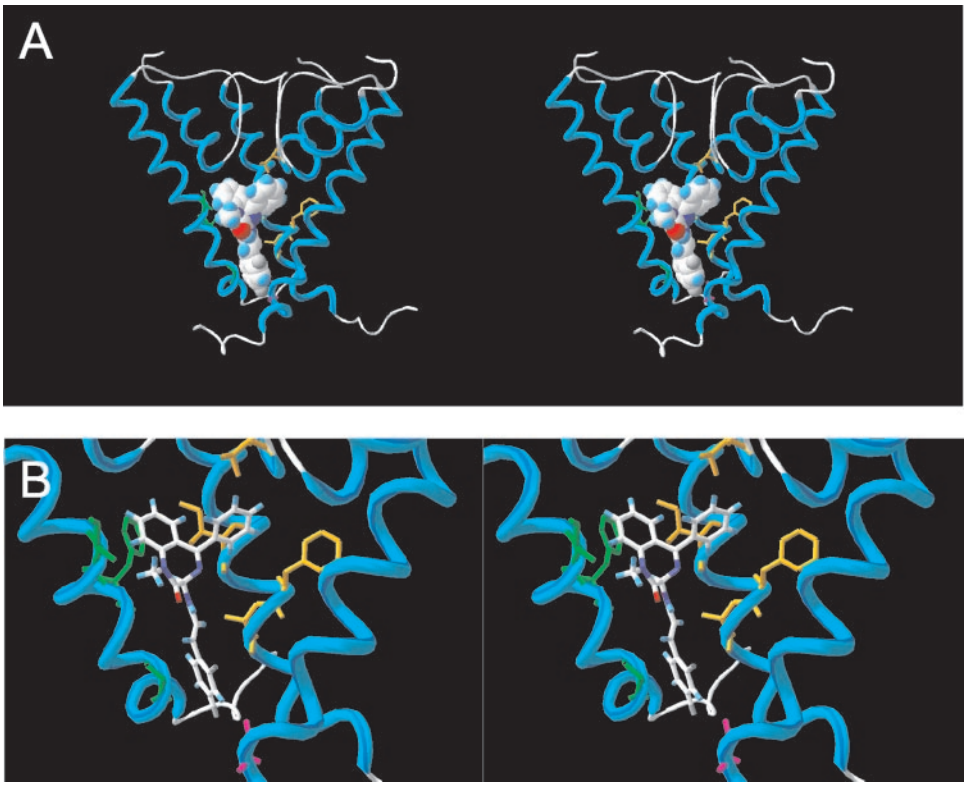

Fig. 8. Docking of L-7 to S6 domains of the KCNQ1 channel in the putative open state. A, stereoview of L-7 docked to the KCNQ1 homology ribbon model of the pore and S6 domains for 3 subunits. L-7 is shown in a CPK-colored spacefill model. B, enlarged stereoview of the central cavity of the KCNQ1 channel with L-7 depicted in a CPK-colored stick model. Residues identified in the Ala/Cys-scan to affect block by L-7 are portrayed in stick mode. The left subunit highlights Ile-337 and Phe-340 colored in green; the middle subunit highlights Thr-312, Ile-337, Phe-340 and Ala-344 in orange; Ala-344 is colored pink in the right subunit.

a relatively conserved PVP sequence in Kv channels would favor a bend in the S6 (inner helix) domain (del Camino et al., 2000). KCNQ1 has a PAG sequence in place of PVP, and the putative gating hinge residue is an Ala instead of a Gly. Therefore, it is difficult to model the open conformation of the KCNQ1 channel. As a compromise, we modeled the docking of L-7 to a putative open conformation of KCNQ1 (Fig. 8, A and B) using an approach described by Hanner et al. (2001) for Kv1.3 channels that emphasizes the importance of the PAG motif. Despite the significant change in channel structure and shift in drug orientation, L-7 is predicted to interact with the same residues as shown in the closed state.

Molecular Mechanisms of Pore Block. The structure of the KcsA K^+ channel (Doyle et al., 1998; Roux and MacKinnon, 1999; Zhou et al., 2001b) has revealed that a single K^+ ion is normally coordinated in the center of the hydrated cavity of the channel. All four pore helices of the tetrameric channel are oriented toward the center of the inner cavity and provide dipole forces that stabilize the K^+ ion. Transient occupation of this coordination site by K^+ is a required step in the ion conduction process for the KcsA channel (Roux and MacKinnon, 1999; Zhou et al., 2001b), and by inference, in other K^+ channels. Based on the most favored docking in the KCNQ1 homology model, L-7 extends into the central cavity of the channel and encroaches on the space normally occupied by the central hydrated K^+ ion. Thus, L-7 may block KCNQ1 channels by disrupting the normal relay of single K^+ ions through the central cavity. A similar mechanism could explain the block of other Kv channels such as HERG by MK-499 (Mitcheson et al., 2000a,b), Kv1.4 channels by tetrabutylammonium (Zhou et al., 2001a), and Kv1.3 by correolide (Hanner et al., 2001).

Limitations of the Study. There are several limitations of the approach used to identify the putative binding site for L-7. First, we measured the effect of mutations on block of KCNQ1 channel current rather than directly assaying for altered ligand binding. Second, we studied only single amino acid substitutions for most residues and did not attempt to detect additive or synergistic effects caused by mutation of more than a single residue at a time. For some mutations, altered drug sensitivity may have been caused by an allosteric effect.

In summary, we have localized the putative KCNQ1 channel binding site for a potent benzodiazepine to specific residues located on the S6 transmembrane domain and the base of the pore helix. The mechanism of block is proposed to be disruption of a specific step in the permeation process rather than a nonspecific occlusion of the pore or a stabilization of the inactivated or closed state of the channel.

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