Truncated K^+ Channel DNA Sequences Specifically Suppress Lymphocyte K^+ Channel Gene Expression

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ABSTRACT We have constructed a series of deletion mutants of Kv1.3, a *Shaker*-like, voltage-gated K^+ channel, and examined the ability of these truncated mutants to form channels and to specifically suppress full-length Kv1.3 currents. These constructs were expressed heterologously in both *Xenopus* oocytes and a mouse cytotoxic T cell line. Our results show that a truncated mutant Kv1.3 must contain both the amino terminus and the first transmembrane-spanning segment, S1, to suppress full-length Kv1.3 currents. Amino-terminal-truncated DNA sequences from one subfamily suppress K^+ channel expression of members of only the same subfamily. The first 141 amino acids of the amino-terminal of Kv1.3 are not necessary for channel formation. Deletion of these amino acids yields a current identical to that of full-length Kv1.3, except that it cannot be suppressed by a truncated Kv1.3 containing the amino terminus and S1. To test the ability of truncated Kv1.3 to suppress endogenous K^+ currents, we constructed a plasmid that contained both truncated Kv1.3 and a selection marker gene (mouse CD4). Although constitutively expressed K^+ currents in Jurkat (a human T cell leukemia line) and GH3 (an anterior pituitary cell line) cells cannot be suppressed by this double-gene plasmid, stimulated (up-regulated) *Shaker*-like K^+ currents in GH3 cells can be suppressed.

INTRODUCTION

Shaker-like K^+ channels are composed of four identical α-subunits (Tempel et al., 1987; MacKinnon, 1991), each of which contains a cytoplasmic amino terminus, six transmembrane-spanning regions, a pore region, and a carboxy terminus. Recent work suggests that the cytoplasmic amino-terminal region is responsible for inter-subunit recognition and assembly (Li et al., 1992; Shen et al., 1993; Lee et al., 1994; Hopkins et al., 1994). This region is referred to as the "tetramerization 1" (T1) domain (Shen et al, 1993). A truncated K^+ channel subunit containing T1, but not all six transmembrane segments, is incapable of forming a functional channel. However, such a truncated subunit suppresses the expression of full-length ("dominant negative suppression") K^+ channels, as has been demonstrated for a *Shaker* B K^+ channel in oocytes (Li et al., 1992). A presumed mechanism for this suppression is that the truncated K^+ channel subunit can bind to full-length K^+ channel subunits and competitively inhibit formation of a functionally competent channel. The importance of T1 in the mechanism by which subunits recognize one another and assemble is different for different K^+ channel isoforms. In some cases T1 is essential for assembly and functional expression (e.g., *Shaker* B (Li et al., 1992) and Kv1.1 (Shen et al., 1993; Hopkins et al., 1994)). For other isoforms, extensive amino-terminal deletion does not abolish functional expression in oocytes (e.g., Kv1.3 (Aiyar et al., 1993), Kv1.4 (Lee et al., 1994), and Kv2.1 (VanDongen et al., 1990)), suggesting that transmembrane segments and intersegment loops must also stabilize associations between subunits. Similar conclusions have been drawn by Lee et al. (1994) and Babila et al. (1994), each of whom implicates a role for the S1 transmembrane segment of *Shaker*-related peptides in subunit assembly.

We have extended the oocyte studies of Li et al. (1992) to ask the following three questions. (1) Are the regions of the peptide responsible for suppression in *Shaker* B K^+ channels the same in Kv1.3, the major T lymphocyte channel? (2) Can this suppression strategy be applied to heterologously expressed K^+ currents in mammalian cells? (3) Can a variant of this strategy be used to selectively eliminate endogenously expressed K^+ channels? To these ends we have heterologously expressed both full-length and truncated *Shaker*-like and *Shaw*-like K^+ channel genes both in *Xenopus* oocytes and in CTLL-2 cells, a mouse T cell devoid of K^+ channels (Deutsch and Chen, 1993). We then implemented this strategy in Jurkat (a human T cell leukemia line) and in GH3 (an anterior pituitary line) cells, both of which have endogenous K^+ channels.

We developed the CTLL-2 as an expression system (Deutsch and Chen, 1993) because these cells allow us to study function and assembly of specific T lymphocyte K^+ channel isoforms in the absence of other channels, yet in a lymphocyte. In the studies presented in this paper, we have co-transfected CTLL-2 cells with K^+ channel genes and a selection surface marker, human CD4. Transfected cells can then be selectively adhered (i.e., panned) for patch-clamp study using anti-human CD4 antibody (Deutsch and Chen, 1993). The CTLL-2 line is an ideal expression system because these T cells contain no voltage-gated K^+ currents. Moreover, there is no detectable mRNA for Kv1.3 (Deutsch and Chen, 1993). Both Kv1.3 and Kv3.1 express well, typically >1 nA per cell (i.e., >600–700 channels per cell) at voltages more positive than +20 mV. The heterologously

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expressed currents exhibit characteristics that are virtually identical to the native T cell currents with respect to gating, selectivity, kinetics, and pharmacology. The experimental conditions yield a panned population of CD4-adhered cells that are >95% positive for K^+ currents. This method is therefore suitable for studying mechanisms of expression and suppression of K^+ channels and the consequent impact of these channels on T cell physiology.

MATERIALS AND METHODS

CTLL-2 transfection

Cytotoxic murine T cells (CTLL-2) were transiently co-transfected with a plasmid using a cytomegalovirus (CMV) promoter (pRc/CMV; 5.4 kb) and a human Kv1.3 insert (1.8 kb) or a rat Kv3.1 insert (1.8 kb; gift from Teresa Perney, Yale University), or a variety of truncated K^+ channel inserts (see Fig. 1) along with a Ccd4neo plasmid (5.0 kb; pUC-based plasmid with a CMV promoter driving CD4; gift from Ray Sweet, SmithKline Beecham) containing human surface membrane CD4 (1.7 kb).

Using sterile techniques, cells were collected and suspended in Opti-MEM plus recombinant interleukin 2 (rIL-2) (8.3 ng/ml; Cetus) at 2×10^7 cells/ml with Ccd4neo (10 μg/ml) and pRc/CMV/Kv1.3 or pRc/CMV/Kv3.1 (80 μg/ml). Control cells were transfected with Ccd4neo and CMV/β-galactosidase (mole ratio of 1:8). For suppression experiments, three separate plasmids were used in a mole ratio of 1:8:16 for CD4 plasmid to full-length K^+ channel plasmid to truncated K^+ channel plasmid. All suspensions were incubated on ice for 10 min in a 0.4 cm electrode gap cuvette (400 μl) and then electroporated using a BTX-electroporator (San Diego, CA) with settings determined previously to give ~50% viability (220–320V; 800 μF; 72 Ω). The resultant time constants were 24–30 ms. Cells were incubated for an additional 10 min on ice, transferred back to culture medium in the incubator, and grown for 48 h.

Jurkat transfection

Jurkat cells are a human T cell leukemia line. We used a Jurkat line that was transformed with SV40 large T antigen (Northrop et al., 1993), given to us by G. Crabtree (Stanford University). These cells were cultured at $<1 \times 10^6$/ml and passaged every 2–3 days. For transient transfections these cells were collected and suspended at 14×10^6/ml in a Hanks-20 mM HEPES Balanced Salt Solution at pH 7.2 plus 10% fetal bovine serum, pRc/CMV/mouse CD4 (3.2 nM), pRc/CMV β-galactosidase (Clontech, Palo Alto, CA,; 3.2 nM), or 3.2 nM of double-gene plasmid pRc/CMV/Kv1.3(+866)/CMV/mCD4, which contained the first 866 bases of Kv1.3 (upstream) and mouse CD4 (see Fig. 8).

All suspensions were incubated on ice for 10 min in a 0.4 cm electrode gap cuvette (400 μl) and then electroporated using a BTX-electroporator (San Diego, CA) with settings determined previously to give 0.4–0.5 cell survival at time 0 and 0.3–0.4 cell survival at 24 h posttransfection (320 V, 950 μF, 48 Ω, τ = 22 ms). Cells were incubated for an additional 10 min on ice, transferred back to culture medium in the incubator, and grown for 72–96 h.

GH3 transfection

GH3 cells were plated onto tissue culture dishes (100 mm) freshly coated with poly-D-lysine (Sigma Chemical Co., St. Louis, MO) at 30% confluency. 7 h later, these cells were transiently co-transfected (Ca-phosphate method as described in Margolskee et al., 1993) with a plasmid containing mouse CD4 alone or with a double-gene plasmid, pRc/CMV/Kv1.3(+866)/CMV/mCD4.

Electrophysiology

CTLL-2 cells were collected from culture and incubated with monoclonal anti-human CD4 antibody (AMAC, Westbrook, ME; 0.5 μg/10^6 cells) and adhered to petri dishes (35 mm) coated with goat anti-mouse IgG as described previously (Matteson and Deutsch, 1984). Dishes were washed gently 5 times with 1 ml of normal extracellular bath medium (see below) plus rIL2 (8.3 ng/ml) for the patch-clamp experiments. All experiments were carried out at room temperature. Standard patch-clamp techniques were used as described previously (Matteson and Deutsch, 1984). Pipettes were made from SG10 glass (Richland Glass Co., Richland, NJ), coated with Sylgard 184 (Dow Corning, NY), and fire-polished to give electrodes of ~3 MΩ. The bath solutions were (in mM): 147 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, 10 HEPES, and rIL2 (8.3 ng/ml) (pH 7.35, 305 mOsm). The pipette solution was (in mM): 130 KF, 11K_2EGTA, 1 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (pH 7.20, 283 mOsm). Because of the large amplitude of K^+ currents in transfected cells, we used 60–70% compensation of the series resistance. In some cases the currents were so large at +50 mV that substantial voltage errors occurred despite series resistance compensation. Because the maximal conductance (G_{max}) of Kv1.3 and Kv3.1 saturates at voltages more positive than −20 and +20 mV (Deutsch and Chen, 1993), respectively, we estimated G_{max} for depolarizations to a command voltage of +50 mV. The actual membrane potential was determined at the peak current, after correction for the contribution of series resistance, and this voltage was used to calculate driving force. Holding potential was −100 mV.

Jurkat cells were collected from culture and incubated for 30 min on ice with PBS/5% fetal bovine serum and 2% each of rabbit and goat sera to block nonspecific Fc receptor binding to petri dishes. Cells were adhered to petri dishes (35 mm) coated with rat anti-mouse CD4 antibody for 45 min at room temperature. Dishes were washed gently 8 times with 1 ml of phosphate-buffered saline and used in patch-clamp experiments as described for CTLL-2 cells. Holding potential was −70 mV. Nonspecific binding was assessed by determining adhesion of cells transfected with pRc/CMV/β-galactosidase as a control gene, and incubating cells with PBS/5% fetal bovine, rabbit and goat sera, and anti-CD4 antibody, as described above. No nonspecific binding was detected.

GH3 cells were collected from culture using a phosphate-buffered saline (PBS) solution containing 0.5 mM EDTA and no added divalent cations. Cells were washed with PBS and transferred to petri dishes (35 mm) coated with mouse anti-human CD4 (L3T4, Pharmingen, San Diego, CA; 0.2 mg/dish) as described previously (Matteson and Deutsch, 1984). Dishes were washed gently 5 times with 1 ml of normal extracellular bath medium and recorded in whole-cell mode. The bath solutions were (in mM): 135 NaCl, 1.8 KCl, 1.35 $CaCl_2$, 0.9 $MgCl_2$, 9 Hepes, 10 TEA, pH 7.4. The pipette solution was (in mM): 130 KF, 10 KCl, 5 EGTA, 10 K-HEPES, pH 7.3. A 7 mV liquid junction potential correction was used. Data were filtered at 2 kHz. Recording was begun 60 s after breaking in. Holding potential was −80 mV.

Oocyte expression and electrophysiology

Oocytes were isolated from *Xenopus laevis* females (*Xenopus* I, Ann Arbor, MI) as described previously (Chahine et al., 1992). Stage V–VI oocytes were selected and micro-injected with 15–40 ng of K^+ channel encoding cRNA. The mole ratio of cRNA injected for truncated K^+ channel gene to full-length K^+ channel gene was 2:1. K^+ currents from cRNA-injected oocytes were measured with two-microelectrode voltage clamp using a TEC 01C oocyte clamp (npi electronic Gmblt, Tamm, Germany) after 24–72 h. Electrodes (<1 MΩ) contained 3 M KCl. The bath Ringer solution contained (in mM): 116 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES (pH 7.6). The holding potential was −100 mV.

Recombinant DNA techniques

Standard methods of plasmid DNA preparation, restriction enzymes analysis, agarose gel electrophoresis, and bacterial transformation were used. All isolated fragments were purified with "Geneclean" (Bio 101 Inc.), recircularized using T4 DNA ligase, and then used to transform DH5α competent cells (BRL, Inc.). The nucleotide sequences at the 5′-ends of all N-terminal deletion mutants and at the 3′-ends of all C-terminal deletion mutants were confirmed by restriction enzyme analysis or by DNA sequence

analysis (Sequenase Version 2.0 DNA sequencing Kit, USB, Inc.). The parent vector pGEM9zf(−) and pRc/CMV contained the full-length (1.8 kb) open reading frame of Kv1.3 plus 0.18 kb of untranslated sequence.

Construction of pRc/CMV mouse cell surface CD4 plasmid

XpUC/L3T4 (mouse CD4) was a gift from Jane Parnes (Stanford University). CD4 was isolated by *Eco*RI/*Xba*I-digestion and triple ligated with an *Eco*RI/*Pvu*I-digested fragment and an *Xba*I/*Pvu*I-digested fragment, each of which was previously isolated from pRc/CMV.

Construction of truncated mutants of Kv1.3 and Kv3.1

N- and C-terminal deletion mutants were constructed using the native restriction endonuclease sites shown in Fig. 1. To obtain the truncated Kv1.3 (+217), a *Pvu*I/*Pvu*II-digested fragment was isolated from pRc/CMV/truncated Kv1.3 (+671), and ligated into *Xba*I blunt-ended/*Pvu*I-digested pRc/CMV. Truncated Kv1.3 (+551) was created by deleting the digestion fragment of pGEM9zf(−)/Kv1.3 with *Sac*I and *Hin*dIII. The clone was filled in using the Klenow reaction, and the blunt ends were religated. Truncated Kv1.3 (+671) was made by deleting a *Aat*II/*Hin*dIII-digested fragment from pGEM 9zf(−)/Kv1.3, which was then blunt-ended using the Klenow reaction and religated to give pGEM9zf(−)/Kv1.3 (+671). An *Eco*RI/*Not*I-digested fragment was then isolated from this clone and triple-ligated with an *Eco*RI/*Pvu*I-digested fragment and a *Not*I/*Pvu*I-digested fragment, each of which were previously isolated from pRc/CMV, to generate the pRc/CMV/Kv1.3(+671). A *Mlu*I/*Bal*I-digested fragment was isolated from pRc/CMV/Kv1.3 and ligated into *Xba*I blunt-ended/*Mlu*I-digested pRc/CMV/Kv1.3 to give pRc/CMV/truncated Kv1.3 (+866).

Kv1.3(T1−) was made by deleting an *Eco*RI/*Nco*I-digested fragment from pGEM9zf(−)/Kv1.3, which was then filled in using the Klenow reaction and the blunt ends religated. A similar strategy was used to construct the other deletion mutants: (i) truncated Kv1.3 (S5-COOH) was made using an *Eco*RI/*Pst*I digestion, and (ii) truncated Kv1.3 +671 (T1−) was made using an *Eco*RI/*Nco*I digestion of pGEM9zf(−)/truncated Kv1.3 (+671). Truncated Kv3.1 (+683) was made by ligating a *Bst*bI blunt-ended/*Pvu*I-digested fragment isolated from pRc/CMV/Kv3.1 into *Xba*I blunt-ended/*Pvu*I-digested pRc/CMV to give pRc/CMV/Kv3.1 (+683).

Construction of double-gene plasmid containing truncated Kv1.3 and mouse CD4

To clone pRc/CMV/mCD4 plus truncated Kv1.3(+671), a *Bgl*II partially filled in (A, G)/*Pvu*I-digested fragment was isolated from pRc/CMV/truncated Kv1.3 (+671) and ligated into *Xho*I partially filled in (T, C)/*Pvu*I-digested pRc/CMV/mCD4. To clone pRc/CMV/truncated Kv1.3 (+866) plus mCD4, an *Xho*I blunt-ended/*Pvu*I-digested fragment was isolated from pRc/CMV/truncated Kv1.3 (+866) and ligated into *Nru*I/*Pvu*I-digested pRc/CMV/mCD4.

Flow cytometry measurements

Jurkat cells transfected with double-gene plasmid pRc/CMV/Kv1.3(+866)/CMV /mCD4, were collected at 72 and 96 h posttransfection, resuspended at 20 × 10^6 cells/ml in PBS plus 2% fetal bovine serum (PBS/2%FBS), and incubated with 10 μl of anti-mouse CD4 antibody conjugated to phycoerythrin (AMAC, Westbrook, ME) on ice, in the dark, for 30 min. A parallel control sample was incubated with isotype-phycoerythrin-labeled IgG. Both samples were washed 2 times with 2 ml each of cold PBS/2%FBS, resuspended in 0.5 ml of PBS/2%FBS, and analyzed immediately using a Becton-Dickinson FACSort.

RESULTS

Suppression in oocytes

The truncated Kv1.3 sequences used in these studies are shown in Fig. 1. Although dominant negative suppression in oocytes has been shown for *Shaker* B channels (Li et al., 1992) and for Kv1.1 and Kv1.5 (Babila et al., 1994), we did not know whether similar suppression could be achieved with full-length human Kv1.3 and a series of truncated Kv1.3 sequences in oocytes and, furthermore, which of these truncated sequences would be most efficacious. We therefore made cRNA truncated Kv1.3 by deleting sequences at NH_2-terminal restriction sites 217, 551, or 671 (Fig. 1 *A–C*) and co-injected each of them along with cRNA for full-length Kv1.3. The 217- and 551-truncated sequences lack transmembrane segments and contain the first one-third, or all, respectively, of the T1 segment. The 671-truncated sequence contains T1 plus the first transmembrane-spanning segment, S1. As shown in Fig. 2, functional expression of full-length Kv1.3 was inhibited 100% by 671-, but *not* by 217- or 551-truncated NH_2-terminal cRNA sequences of Kv1.3. Suppression is specific as an equal amount of cell surface CD4 cRNA, a non-channel-related species, co-injected with full-length Kv1.3, did not suppress Kv1.3 expression (data not shown). Fig. 2, *B* and *C* show that co-injection of 551-truncated NH_2-terminal cRNA did not alter the current versus voltage relationship of full-length Kv1.3, nor did it alter activation or inactivation kinetics. Truncated constructs, regardless of whether they suppress channel expression, do not alter channel properties. These results show that S1 is required for suppression.

Kv1.3 is able to form heteromultimers with other *Shaker*-like isoforms (Hopkins et al., 1994), reflecting an ability of Kv1.3 subunits to recognize and assemble with, for example, Kv1.1. Therefore it is possible that the truncated Kv1.3 peptides can also associate with *Shaker*-like subunits and lead to suppression of expressed currents. To determine whether the 671-truncated Kv1.3 sequence can suppress other *Shaker*-like currents (intra-subfamily specificity), we co-injected cRNA for Kv1.4 and for 671-truncated Kv1.3 in oocytes.

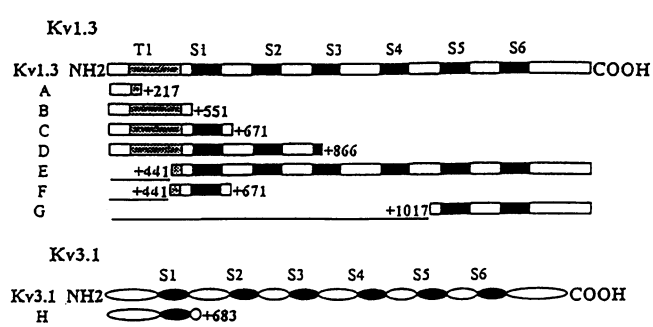

FIGURE 1 Full-length and truncated K^+ channel DNA sequences. Diagram of full-length Kv1.3 and Kv3.1, and truncated fragments of Kv1.3 and Kv3.1. The solid boxes and ovals represent the six transmembrane segments. The shaded boxes represent the T1 segment. The numbers represent the restriction enzyme site in the full-length, wild-type Kv1.3 clone, starting from the ATG start site (+18).

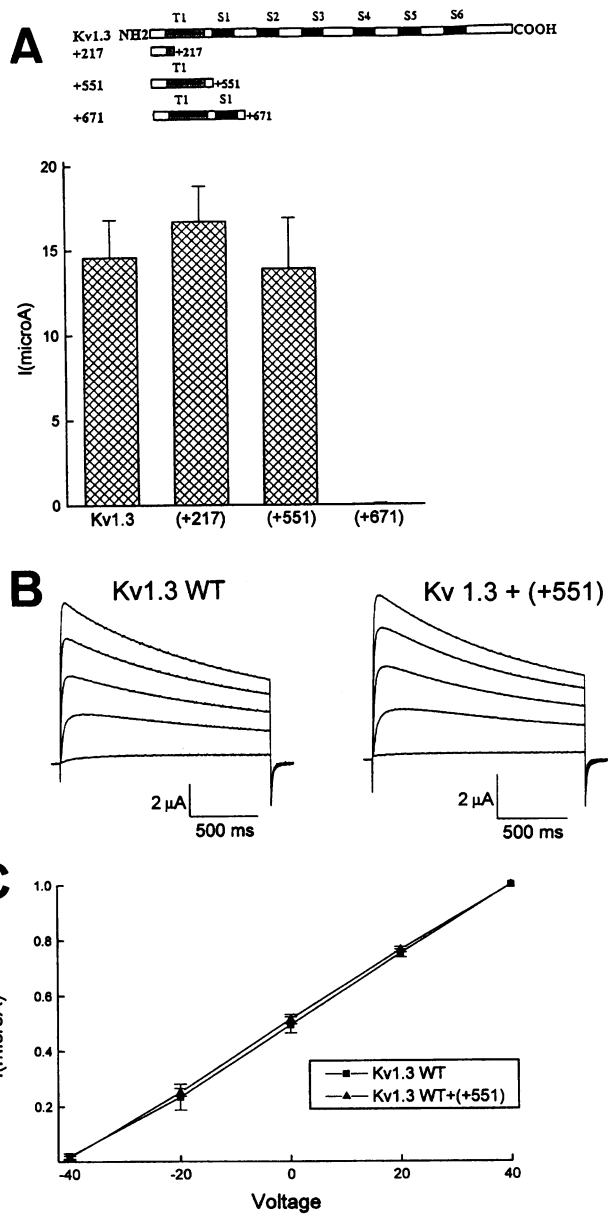

FIGURE 2 Inhibition of Kv1.3 in oocytes in the presence of truncated Kv1.3 DNA sequences. *Xenopus* oocytes were injected with cRNA for either full-length Kv1.3 alone or Kv1.3 plus truncated Kv1.3 containing the first 217, 551, or 671 bases of Kv1.3. Oocyte recordings were made 24 h postinjection, at room temperature, using a standard two-electrode technique. (*A*) Peak current at +50 mV was measured. Data are plotted as means ± SEM (n = 5). (*B*) Currents elicited from oocytes injected with Kv1.3 alone (*left* traces) or Kv1.3 and truncated 551 (*right* traces) by steps from a holding potential of −100 mV to voltages between −40 and +40 mV in 20-mV increments. (*C*) Current-voltage curves each normalized to I_{max} for Kv1.3 alone at +40 mV for the currents shown in *B*.

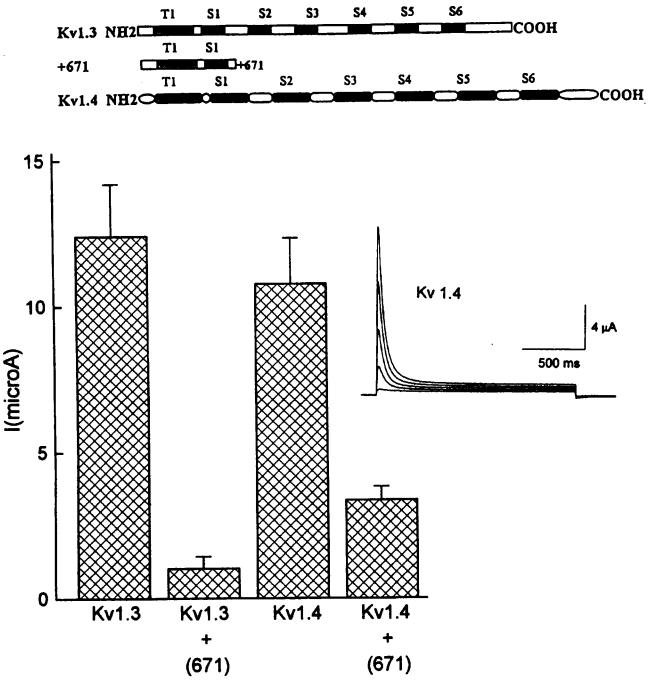

FIGURE 3 Suppression of Kv1.3 and Kv1.4 by truncated Kv1.3 DNA sequences. *Xenopus* oocytes were injected with cRNA for either full-length Kv1.3 alone, Kv1.3 plus 671-truncated Kv1.3, full-length Kv1.4 alone (40 ng/oocyte), or Kv1.4 plus 671-truncated Kv1.3 (Kv1.4 was preheated at 52°C for 5 min). In each case, the mole ratio of cRNA injected for truncated gene to full-length gene was 2:1 and peak current at +50 mV was measured. Data are plotted as means ± SEM (n = 5). (*inset*) Current was elicited from oocytes injected with Kv1.4 alone by steps from a holding potential of −100 mV to voltages between −40 and +40 mV in 20-mV increments.

Kv1.4 is another *Shaker*-like, voltage-gated K^+ channel, capable of forming heteromultimers with Kv1.3. Expression of Kv1.4 was also inhibited, approximately 69%, by 671-truncated Kv1.3 (Fig. 3).

To determine whether other tetramerization domains besides T1 in Kv1.3 exist and are able to promote homomultimerization, we used mutants of Kv1.3 lacking the first 440 bases. These are referred to as T1(−) mutants (Kv1.3(T1−); Fig. 1 *E*). Kv1.3(T1−) forms channels (Fig. 4) with electrophysiological characteristics similar to those of Kv1.3. For Kv1.3(T1−), the activation time constant is 6.1 ± 0.6 ms (n = 4, mean ± SEM), inactivation time constant is 880 ± 23 ms (n = 4) at +50 mV, and the activation threshold is ~−55 mV. The respective values for Kv1.3 expressed in oocytes are 5.6 ± 0.1 ms (n = 4), 966 ± 53 ms (n = 4), and ~−50 mV. As shown in Fig. 4, the 671-truncated sequence of Kv1.3 does not suppress Kv1.3(T1−), yet does suppress wild-type, full-length Kv1.3 (see Fig. 2). Although the 671-truncated Kv1.3 sequence appeared to block expression of full-length Kv1.3, and the T1 segment alone was not sufficient to block expression, we did not know whether S1 alone was sufficient to block expression. To test the possibility that S1 is sufficient for suppression, we constructed an S1 segment that contained a 671-truncated Kv1.3 sequence minus the T1 region (671(T1−); Fig. 1 *F*) and co-injected it along with either full-length Kv1.3 or full-length Kv1.3 lacking the T1 segment (Kv1.3 (T1−)). The 671(T1−) peptide does not suppress either wild-type Kv1.3 or Kv1.3 (T1−). These results suggest that both T1 and S1 are necessary for dominant negative suppression of full-length Kv1.3.

Other transmembrane-spanning regions may also contribute to stabilization interactions, which could also be exploited for dominant negative suppression. Specifically,

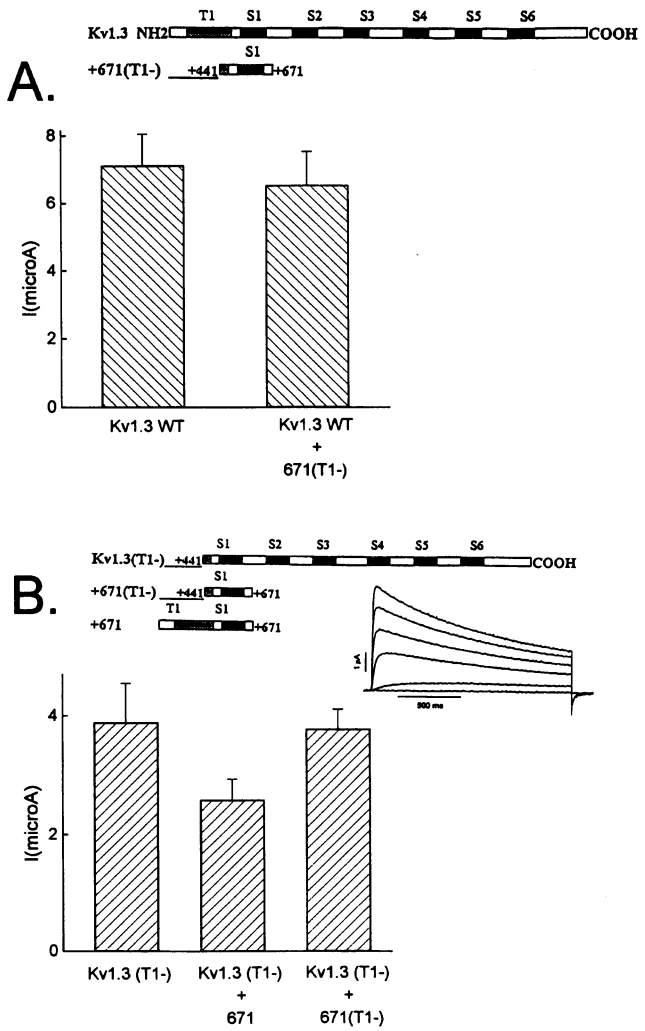

FIGURE 4 Effect of S1 segment (T1-deletion of 671) on expression and suppression of Kv1.3 in *Xenopus* oocytes. Oocytes were injected with cRNA for (*A*), Kv1.3 alone or Kv1.3 plus truncated 671 (T1−) or (*B*), Kv1.3(T1−) alone, Kv1.3(T1−) plus truncated 671, or Kv1.3(T1−) plus truncated 671-(T1−). Experimental details were as described in Fig. 2. Data (n = 8) are plotted as means ± SEM for (*A*), and trimmed means ± SEM (α = 0.15) for (*B*). (*inset*) Current was elicited from oocytes injected with Kv1.3 (T1−) alone by steps from a holding potential of −100 mV to voltages between −0 and +40 mV in 20-mV increments.

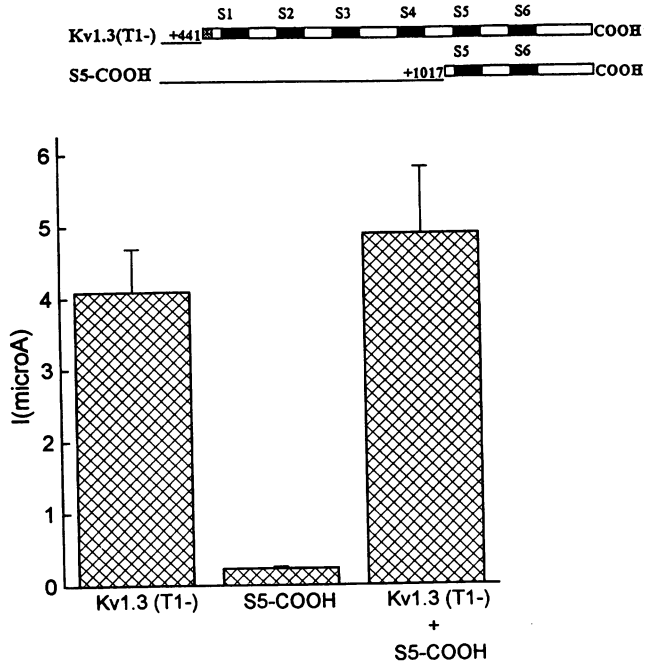

FIGURE 5 Truncated S5-COOH Kv1.3 cRNA injected into oocytes. Oocytes were injected with either Kv1.3(T1−) alone, truncated S5-COOH alone, or with Kv1.3 (T1−) plus truncated S5-COOH, and peak current was measured at +50 mV as described in Fig. 2. Data are plotted as means ± SEM (n = 5). The small currents observed with S5-COOH alone were not different from currents obtained from uninjected oocytes.

regions other than those in the NH_2-terminal portion of Kv1.3 may be important. To determine whether a truncated Kv1.3 sequence containing only S5, H5, S6, and the C-terminus could suppress assembly of Kv1.3, we constructed DNA sequence *G* in Fig. 1, which contains only the 3′-end of the clone, namely, from base 1017 to the end of the reading frame. This truncated S5-COOH sequence of Kv1.3 does not form functional channels; nor does it suppress Kv1.3(T1−) (Fig. 5).

Suppression in mammalian cells

The biophysical properties of K^+ channels depend critically on the cells in which they are expressed and on the method of recording their currents. For example, the inactivation kinetics of the K^+ currents of Kv1.3 are indistinguishable for endogenous channels in native T lymphocytes and for heterologously expressed channels in a lymphocyte cell line. However, currents of Kv1.3 expressed in *Xenopus* oocytes have slower inactivation kinetics. The possible reasons for this difference are severalfold, and include (i) differences in posttranslational modification between oocytes and lymphocytes, e.g., glycosylation and phosphorylation, and (ii) the possible presence of endogenous subunits in lymphocytes that could interact with expressed channels.

Having shown dominant negative suppression in oocytes for Kv1.3 using truncated Kv1.3 sequences, and having defined segments that contain important interaction sites, we next determined whether dominant negative suppression could also be achieved in mammalian cells. We chose a lymphoid expression system, CTLL-2, a mouse cytotoxic T cell line, which we previously used for expression of Kv1.3 and Kv3.1 (Deutsch and Chen, 1993). We co-transfected CTLL-2 with full-length Kv1.3 and 671-truncated Kv1.3 (Fig. 1 *C*). Functional expression of Kv1.3 current, as determined by whole-cell recording, was inhibited by 89% (Fig. 6 *A*). This inhibition was specific because there was no statistically significant inhibition of heterologously expressed full-length Kv3.1, a K^+ channel isoform from a different subfamily, the *Shaw*-like subfamily. Similarly, in CTLL-2 transfected with full-length Kv3.1 and 683-truncated Kv3.1 (Fig. 1 *H*), functional expression of Kv3.1 current was inhibited by 90% (Fig. 6 *B*). Likewise, inhibition

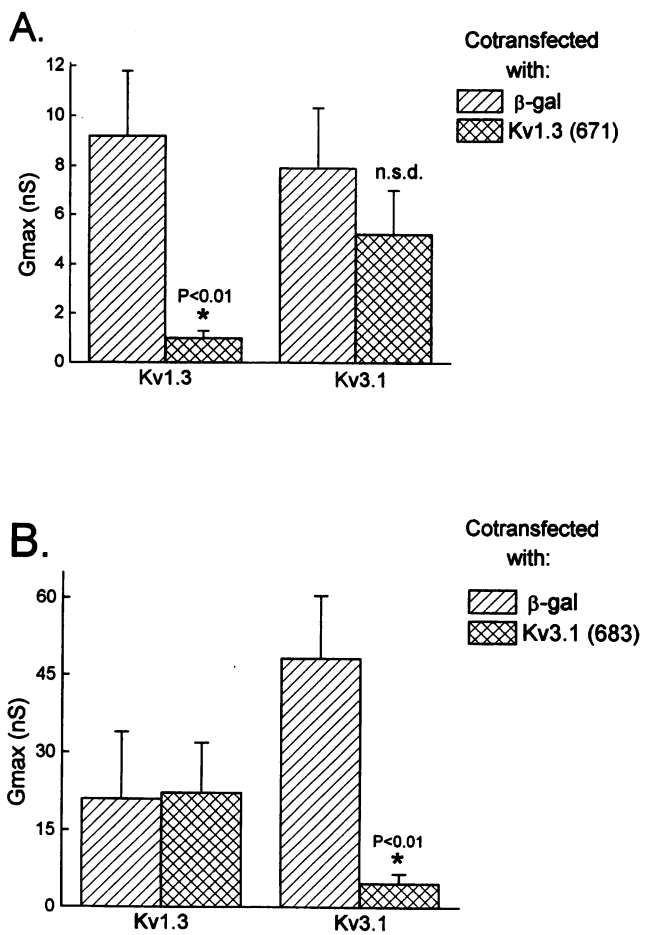

FIGURE 6 CTLL-2 transfected with full-length and truncated K channel sequences. (*A*) CTLL-2 transfected with three plasmids: cell surface human CD4 and either full-length Kv1.3 or Kv3.1 and either truncated Kv1.3 or β-galactosidase gene (control). (*B*) CTLL-2 transfected with three plasmids: cell surface human CD4 and either full-length Kv1.3 or Kv3.1 and either truncated Kv3.1 or β-galactosidase gene (control). Cells were harvested 24 h after transfection and patch-clamped as described in Deutsch and Chen (1993). Currents were similar to those reported previously (Fig. 1, Deutsch and Chen, 1993). G_{max} (nS) is the calculated conductance for steps to +50 mV from a holding potential of −100 mV. Data are plotted as means ± SEM in panel *A* for β-galactosidase control (n = 10) or 671-truncated Kv1.3 (n = 7), each co-transfected along with full-length Kv1.3, and for β-galactosidase control (n = 9) or 671-truncated Kv1.3 (n = 11) each co-transfected along with full-length Kv3.1, and in panel *B* for β-galactosidase control (n = 7) or 683-truncated Kv3.1 (n = 6) co-transfected along with Kv1.3, and for β-galactosidase control (n = 6) or 683-truncated Kv3.1 (n = 9) co-transfected along with full-length Kv3.1. A nonparametric rank sum test was performed to give the indicated p values.

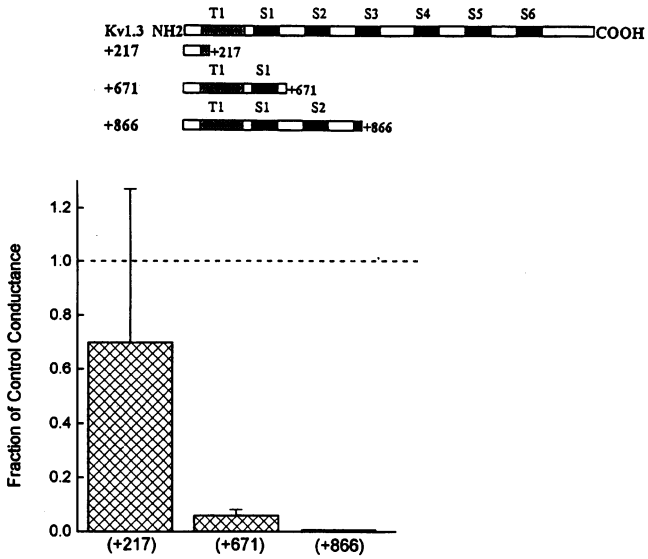

FIGURE 7 Fractional inhibition of Kv1.3 conductance in CTLL-2 by truncated DNA sequences. CTLL-2 co-transfected with either full-length Kv1.3 and β-galactosidase or full-length Kv1.3 and truncated Kv1.3 containing the first 217, 671, or 866 bases of the translated portion of the Kv1.3 gene. Conductances (nS, adaptively trimmed means ± SEM) are 15.0 ± 2.7 (n = 11) and 10.5 ± 8.3 (n = 8) for control (β-galactosidase) and 217-truncated Kv1.3, respectively; 15.8 ± 2.4 (n = 9) and 0.91 ± 0.3 (n = 8) for control and 671-truncated Kv1.3, respectively; and 12.0 ± 6.0 (n = 8) and 0.0 ± 0.0 (n = 8) for 866-truncated Kv1.3, respectively. Experimental conditions were as described for Fig. 6. This figure shows fractions of control.

was highly specific because no inhibition was observed with CTLL-2 co-transfected with full-length Kv1.3 and 683-truncated Kv3.1. Furthermore, as shown in Fig. 7, a similar rank order of potency was observed in CTLL-2 as was seen in oocytes. These results suggest that the amount of inhibition and the specificity of dominant negative suppression are similar in the oocyte and the CTLL-2 lymphoid expression systems, and that dominant negative suppression is a viable suppression strategy in mammalian cells.

Having shown that *heterologously* expressed K^+ currents can be suppressed by expression of truncated K^+ channel sequences in mammalian cells, and specifically in T cells, we tested whether this strategy could be effective in suppressing *endogenous* K^+ currents. To do this we optimized the transfection conditions. All of the above-mentioned transfection strategies rely on the efficiency of co-transfection of two separate plasmids. An alternative strategy entails developing double-gene plasmids, specifically, a single plasmid containing both a selection marker gene and an appropriate truncated K^+ channel gene. Each insert on these plasmids has its own promoter and 3′-terminal polyadenylation sequence. If both inserts of the plasmids are transcribed, we should obtain 100% efficiency of co-transfection. Thus far, we have made (from 5′-to-3′): (i) CMV/mouse CD4 plus CMV/truncated Kv1.3 (+671) (8.8 kb) and, shown in Fig. 8, (ii) CMV/truncated Kv1.3 (+866) plus CMV/mouse CD4 (8.8 kb). Plasmid (i) has been tested for suppression of heterologously co-expressed Kv1.3 in CTLL-2 using transfected human CD4 selection. It suppressed >90% of the current in all of the transfectants (panned cells). Plasmid (ii) has been tested in tsA201 cells (a human epithelial cell line derived from HEK 293) for CD4 expression. This plasmid is expressed at extremely high levels in these cells, resulting in $>10^5$-fold enhancement of selective adhesion over control cells. It has also been tested in Jurkat cells.

Jurkat cells, a human T cell leukemia line, express endogenous Kv1.3. We transfected a Jurkat line transformed with

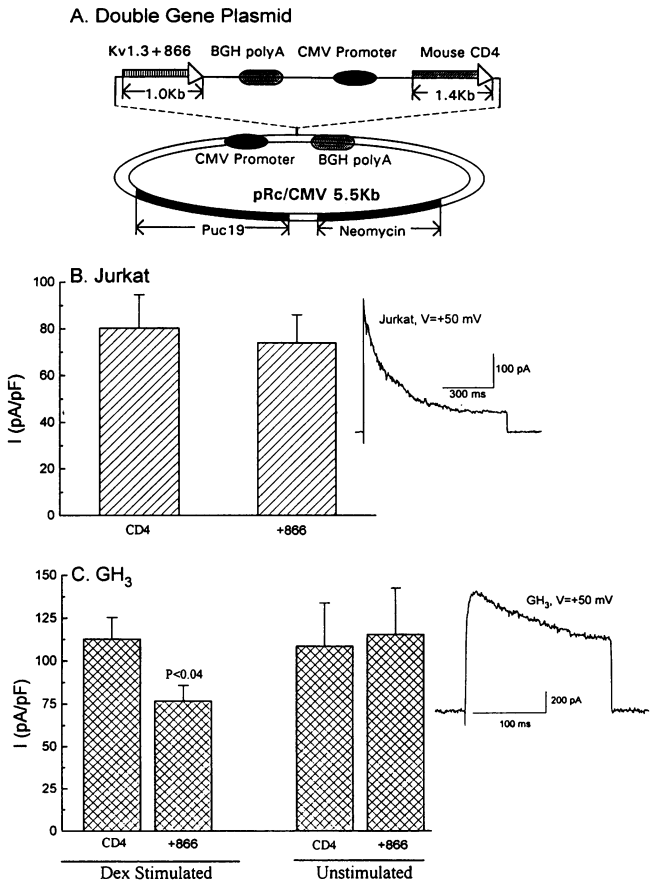

FIGURE 8 Jurkat and GH3 cells transfected with truncated Kv1.3 (+866). (*A*) Double-gene plasmid, pRc/CMV/+866Kv1.3/CD4. Truncated +866 Kv1.3, which contains the N-terminus, S1, and S2 segments, is upstream of the mouse CD4 plasmid. (*B*) Jurkat cells were transfected with either mouse CD4 plasmid alone (control) or a double-gene plasmid containing truncated +866 Kv1.3 (+866), grown for 96 h, and patch-clamped in whole-cell mode. Currents were elicited for a step to +50 mV from a holding potential of −70 mV. (*inset*) Cell transfected with CD4 alone: cell capacitance was 3.2 pF; series resistance was 3.1 MΩ. (*C*) GH3 cells were transfected with either mouse CD4 plasmid alone (control) or a double-gene plasmid containing truncated +866Kv1.3 (+866), grown for 30 h, incubated with dexamethasone (1 μM; *left* panel, dex-stimulated) or no additives (*right* panel, unstimulated) for 15 h, collected, and patch-clamped in whole-cell mode. The experiments on stimulated and unstimulated cells were performed on separate days. Currents were elicited for a step to +50 mV (200 ms) from a holding potential of −80 mV. (*inset*) Stimulated GH3 cell transfected with CD4 alone; cell capacitance was 12 pF; series resistance was 3.9 MΩ. For Jurkat and GH3 experiments, peak current was measured and normalized to cell capacitance and plotted as current density (means ± SEM).

SV40 large T antigen (Northrop et al., 1993) with the double-gene plasmid containing mouse CD4 and truncated Kv1.3 (+866) to determine whether constitutively expressed *Shaker*-like currents could be suppressed. In the Jurkat cells, overall transfection efficiency was 5–7% on day 3 posttransfection and 13–19% on day 4, as measured using fluorescence-activated cell sorting and an anti-mouse CD4 antibody conjugated with phycoerythrin. These cells were panned for patch clamp studies (as described above) using anti-mouse CD4 antibody-coated petri dishes and compared with cells transfected with the same molar concentration of CD4 alone and panned in an identical manner. In both cases there was no nonspecific binding and the panned population for electrical recording contained only positive transfectants. As shown in Fig. 8, peak current measured for a step to +50 mV gave current densities of 80.3 ± 14.3 (mean ± SEM, n = 8) for CD4-transfectants and 74.1 ± 11.9 (mean ± SEM, n = 7) for double-gene transfectants. There was no suppression in these experiments.

The inability to achieve dominant negative suppression could be caused by insufficient turnover of endogenous channel protein. To explore this possibility, we used a well characterized mammalian cell line, GH3, that expresses both constitutive and stimulated *Shaker*-like K^+ channels with high turnover rates (Levitan et al., 1991; Takimoto et al., 1993). GH3 cells are an anterior pituitary cell line that constitutively express *Shaker*-like Kv1.4 and Kv1.5, *Sha*b-like Kv2.1, and Ca^{2+}-activated K^+ currents (Levitan et al., 1991). Kv1.5 currents in GH3 cells are up-regulated within hours of stimulation by dexamethasone (Levitan et al., 1991; Takimoto et al., 1993). The increase in currents is caused by an increased transcription of Kv1.5, which leads to an increase in channel protein expression. Therefore, this cell offered a good opportunity for the rapid turnover of channel messenger RNA and channel protein requisite for the dominant negative suppression experiments. We tested whether endogenously up-regulated channels could be suppressed using the dominant negative strategy. GH3 cells were transfected with CD4 alone or with a double-gene plasmid, grown for 30 h, and then stimulated with dexamethasone (1 μM) for 15 h. Positive transfectants were selectively adhered 45 h posttransfection using anti-CD4 antibody, and patch-clamped as described above. In these experiments, Kv2.1 was blocked by extracellular TEA at a concentration (10 mM) that does not affect Kv1.4 or Kv1.5 currents, and activation of Ca^{2+}-activated K^+ currents was precluded by using intracellular solutions containing EGTA as well as extracellular TEA. The cells were studied in whole-cell mode, and the results are shown in Fig. 8 for depolarizations to +50 mV. Peak current measured for a step to +50 mV gave current densities (pA/pF) of 112.8 ± 12.8 (mean ± SEM, n = 7) for CD4-transfectants and 76.8 ± 9.2 (mean ± SEM, n = 8) for double-gene transfectants. Endogenous *Shaker*-like current was inhibited by 32% compared with CD4-transfected controls ($p < 0.04$).

Having shown that truncated Kv1.3 (+866) suppressed up-regulated endogenous channels in GH3 cells, we determined whether the expressed truncated Kv1.3 (+866) could also suppress constitutively made endogenous *Shaker*-like current in these cells. Cells were transfected as described above, but were not stimulated with dexamethasone. Peak current measured for a step to +50 mV gave current densities (pA/pF) of 108.7 ± 25.3 (n = 9) for CD4-transfectants and 115.7 ± 26.9 (n = 7) for double-gene transfectants, which are not significantly different (Fig. 8).

DISCUSSION

In this paper, we show for the first time in mammalian cells that heterologously and endogenously expressed voltage-gated K^+ channels can be suppressed by heterologous N-terminal truncated K^+ channel DNA sequences. This suppression is specific among subfamilies, i.e., truncated sequences from one subfamily suppress K^+ channel expression of members of only the same subfamily, and is quite potent in mammalian cells. For Kv1.3, the cytoplasmic T1 region and the transmembrane S1 segment are both required for dominant negative suppression. Our results are in agreement with the hypothesis that the N-terminal domain confers selectivity and specificity in K^+ channel co-assembly (Li et al., 1992), whereas the S1 segment may stabilize subunit interactions (Babila et al., 1994; Shen et al., 1993).

K^+ channel region involved in assembly

Voltage-gated K^+ channel polypeptides contain a hydrophobic core between the hydrophilic NH_2- and CO_2H-termini. This inner core contains several putative transmembrane segments and connecting extramembraneous linkers. The proposed structure for this class of K^+ channels is a tetramer of identical α-subunits (Tempel et al., 1987; MacKinnon, 1991) and may also include peripheral β-subunits, similar to that recently cloned from rat brain (Rettig et al., 1994). Thus far, a low-density electron diffraction image of *Shaker* K^+ channels indicates that the α-subunits form a fourfold symmetric tetramer, with a large, central vestibule that presumably constitutes a part of the pathway for ions (Li et al., 1994). How each monomeric subunit recognizes and co-assembles with other subunits is unknown, and which segments of the K^+ channel monomers provide stabilization interactions between subunits has only recently been investigated (Isacoff et al., 1990; Li et al., 1992; Shen et al., 1993; Lee et al., 1994; VanDongen et al., 1990; Hopkins et al., 1994; Babila et al., 1994). Two categories of experimental data address these issues: biochemical assays of peptide multimerization and electrophysiological measurements of channels. From the former type of experiment, it appears that K^+ channel tetramers are held together by noncovalent interactions, and that translated subunit proteins mix in the membrane and associate in subunit-to-subunit interactions to form multimeric channels (Shen et al., 1993). A *Drosophila Shaker* B K^+ channel peptide that contains only the hydrophilic NH_2 terminus can form homotetramers, albeit in small proportion to the monomeric peptide pool size (Li et al., 1992). Li et al. suggest that 114 amino acids define a recognition sequence required for multimerization and propose a model for *Shaker* B assembly in which the homophilic NH_2 termini tetramerize at the endofacial side of the membrane, thereby forming a vestibule at the cytoplasmic end of the channel. Similarly, for Kv1.1 from *Aplysia* neurons (Shen et al., 1993) and from rat brain (Babila et al., 1994), an N-terminal sequence has been identified that interacts with the full-length Kv1.1 channel. This domain has been called the "T1 domain" for tetramerization 1 domain (Shen et al., 1993).

It is likely that the four subunits of Kv1.3, the predominant isoform present in T lymphocytes, tetramerize in a fashion similar to that of the *Shaker* channel subunits. Kv1.3 also has an N-terminal domain homologous to the T1 domain of non-mammalian K^+ channels and other *Shaker*-like K^+ channels (Kv1.4 and Kv1.5; Lee et al., 1994; Babila et al., 1994). However, this domain is not necessary for homomultimer channel formation. Identical currents are produced regardless of whether Kv1.3 contains a T1 domain. Moreover, we have expressed Kv1.3(T1−) in two mammalian cells, CTLL-2 and tsA201, albeit at lower expression efficiency (data not shown). Lee et al. (1994) have found similar results for Kv1.4 and Kv1.5 in oocytes. Moreover, they show that T1 plays a role in preventing heteromultimeric formation between subfamilies and that removal of the T1 domain prevents heteromultimeric assembly within a subfamily. Our results with Kv1.3(T1−) are in contrast to those of Isacoff et al. (1990), who showed that ShB(T1−) expression in oocytes did not give rise to homomultimeric K^+ currents. Our findings show that sites in the central core of the homotetrameric human *Shaker*-like voltage-gated K^+ channel polypeptide, in addition to N-terminal cytoplasmic interactions, are involved in stabilizing subunit interactions and are even sufficient to permit correct subunit assembly. Moreover, these interactions appear to be conserved across subfamilies (Lee et al., 1994; VanDongen et al., 1990).

The results of our dominant negative suppression studies of Kv1.3 indicate that both the NH_2 terminus and the first transmembrane-spanning segment, S1, are required for suppression. Truncated *Shaker*-like peptides associate with lower efficiency if either segment is missing, as measured electrophysiologically (Attali et al., 1993; Matsubara et al., 1991) or biochemically (Shen et al., 1993, Babila et al., 1994). These studies suggest that S1 is important for homomultimerization of certain isoforms and also for specific heteromultimer formation (Babila et al., 1994). It is not clear whether the importance of S1 is due to a requirement for specific stabilizing transmembrane domain interactions or to its enhancement of subunit insertion into membranes, i.e., an inability of soluble T1 alone to interact with membrane proteins (Shen et al., 1993; Babila et al., 1994).

Although other transmembrane segments besides S1 are likely to contribute to stabilization of a multimeric channel, they have not yet been examined in detail. However, for Kv1.3 we have shown that the last two transmembrane-spanning segments, S5 and S6, and the cytoplasmic C-terminal region are not sufficient to suppress Kv1.3(T1−).

Dominant negative suppression

Can the strategy of dominant negative suppression be used (i) to dissect assembly mechanisms (i.e., subunit-subunit interactions) in vivo, and (ii) to determine the physiological role of K^+ channels in mammalian cells both in vitro and in vivo? Moreover, can the diversity of important stabilization interactions among K^+ channel isoforms be exploited so as

to confer specificity to targeted suppression? It appears that different domains are important for each isoform, even within a single subfamily. For instance, for Kv1.1 the T1 domain is required for homomultimeric expression of K^+ currents in oocytes (Shen et al., 1993; Hopkins et al., 1994) as well as for heteromultimeric expression (Hopkins et al., 1994). However, T1 is not sufficient for expression of Kv1.1 current, as systematic deletion in S1–S6 sequences showed that all six segments are also necessary (Hopkins et al., 1994). In contrast, T1 deletion mutants of Kv1.3, Kv1.4, and Kv1.5 do produce homomultimeric K^+ currents identical to their respective wild-type currents when expressed in oocytes (vide supra; Lee et al., 1994). The efficiency of suppression appears to be influenced by the presence of S1, and different isoforms may be more sensitive than others to this parameter because of their having variable lengths of N-termini (e.g., Kv1.5 vs. Kv1.1; Babila et al., 1994). Despite low efficiency of inhibition by S1-deleted truncated sequences, 10-fold higher concentrations of truncated peptide can sometimes effect similar levels of suppression (Babila et al., 1994).

An understanding of channel assembly mechanisms would be extremely useful for determining the physiological role of individual channel subtypes in cell function. To this aim we have carried out suppression studies in mammalian cells as well as in oocytes. Heterologous K^+ channel expression can be suppressed in mammalian cells (Figs. 6–8), as well as in *Xenopus* oocytes (Figs. 2–5) by expressed truncated DNA sequences from the NH_2-terminus of K^+ channel genes. Where comparable DNA has been tested, the results have been similar, regardless of the expression system. Suppression of Kv1.3 requires sequences included within both the NH_2-terminus and the first membrane-spanning segment, S1.

In addition to the dominant negative suppression achieved with heterologously expressed full-length K^+ channels and truncated peptides, we have also succeeded in suppressing *endogenous* stimulated (up-regulated) *Shaker*-like K^+ currents in GH3 cells using truncated Kv1.3 DNA (Fig. 8). However, suppression of constitutively expressed K^+ currents in Jurkat and GH3 could not be achieved (Fig. 8). The ability of truncated K^+ channel polypeptides to suppress full-length Kv1.3 may depend on (1) the rate of transcription and/or translation, or (2) whether assembly of subunits occurs co-translationally or posttranslationally. In the first case, a large discrepancy in the rates of net synthesis of wild-type (endogenous) Kv1.3 and truncated deletion mutant (heterologous) Kv1.3 might result in preferential homomultimeric association of either the Kv1.3 or truncated peptide. In the second case if, for example, constitutive wild-type Kv1.3 is assembled co-translationally but a truncated deletion mutant is assembled posttranslationally; then dominant negative suppression will not be observed. These possibilities may explain the results we obtained for Jurkat and unstimulated GH3. The dominant negative suppression achieved in CTLL-2, oocytes, and stimulated GH3 cells may reflect matched kinetics of net synthesis and/or identical times and compartments for assembly. Furthermore, the 32% inhibition measured for stimulated GH3 cells may be determined by the rate of turnover of the channel protein. Hormone stimulation increases expression of the 76 kDa Kv1.5 protein threefold within 12 h without altering its half-life of 4 h (Takimoto et al., 1993). However, higher turnover rates could lead to greater suppression.

The mechanism of suppression is unknown. For instance, it is possible, but not yet proven, that competition of full-length peptide subunits with truncated subunits leads to formation of nonfunctional tetramers, as suggested from the data of Li et al. (1992) and Shen et al. (1993). However, it is also possible that a truncated channel peptide can co-assemble with a tetramer of full-length subunits and cause impaired gating and/or permeation. Multimerization of a Kv1.1 (T1−) deletion mutant with four full-length Kv1.1 subunits has been detected biochemically (Shen et al., 1993), but not tested functionally.

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