External Tetraethylammonium As a Molecular Caliper for Sensing the Shape of the Outer Vestibule of Potassium Channels

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ABSTRACT External tetraethylammonium (TEA^+) blocked currents through Kv1.1 channels in a voltage-independent manner between 0 and 100 mV. Lowering extracellular pH (pH_o) increased the K_d for TEA^+ block. A histidine at position 355 in the Kv1.1 channel protein (homologous to *Shaker* 425) was responsible for this pH-dependent reduction of TEA^+ sensitivity, since the TEA^+ effect became independent of pH_o after chemical modification of the Kv1.1 channel at H355 and in the H355G and H355K mutant Kv1.1 channels. The K_d values for TEA^+ block of the two mutant channels (0.34 ± 0.06 mM, $n = 7$ and 0.84 ± 0.09 mM, $n = 13$, respectively) were as expected for a vestibule containing either no or a total of four positive charges at position 355. In addition, the pH-dependent TEA^+ effect in the wt Kv1.1 channel was sensitive to the ionic strength of the solution. All our observations are consistent with the idea that lowering pH_o increased protonation of H355. This increase in positive charge at H355 will repel TEA^+ electrostatically, resulting in a reduction of the effective $[TEA^+]_o$ at the receptor site. From this reduction we can estimate the distance between TEA^+ and each of the four histidines at position 355 to be ~10 Å, assuming fourfold symmetry of the channel and assuming that TEA^+ binds in the central axis of the pore. This determination of the dimensions of the outer vestibule of Kv1.1 channels confirms and extends earlier reports on K^+ channels using crystal structure data as well as peptide toxin/channel interactions and points out a striking similarity between vestibules of Kv1.1 and KcsA channels.

INTRODUCTION

Many kinds of potassium channels can be blocked by extracellular tetraethylammonium (TEA^+) with concentrations that block half the channels ranging from 0.2 to >100 mM (Hille, 1992). The most critical residue conferring sensitivity to block by $[TEA^+]_o$ was identified (MacKinnon and Yellen, 1990; Kavanaugh et al., 1991, 1992) to be a tyrosine at the C-terminal end of the P-region of Kv channels (see Fig. 1). Replacement of this tyrosine in Kv1.1 with valine makes the channel resistant to $[TEA^+]_o$, whereas the introduction of a tyrosine in Kv1.2 in place of a valine made this normally $[TEA^+]_o$-insensitive channel now sensitive to block by $[TEA^+]_o$ (Kavanaugh et al., 1992; Chandy and Gutman, 1995). Similarly, replacing the threonine in the *Shaker* channel (position 449) by a tyrosine greatly enhanced sensitivity of the channel to block by $[TEA^+]_o$. In addition, there is also a direct relationship between the numbers of subunits containing tyrosine and the sensitivity of the channels to be blocked by $[TEA^+]_o$, indicating that four subunits interact simultaneously with $[TEA^+]_o$ to form a high-affinity binding site (Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992; Chandy and Gutman, 1995). In that case where TEA^+ can bind and interact with the tyrosines, TEA^+ block is without effect on C-type inactivation (Molina et al., 1997). If histidines interact directly with $[TEA^+]_o$, as is the case for Kv1.3 (Grissmer et al., 1990), TEA^+ block prevents C-type inactivation (Grissmer and Cahalan, 1989) and the effect of $[TEA^+]_o$ to block current through this channel depends on pH_o (Kavanaugh et al., 1991), suggesting that the protonation of this histidine (H404 in *m*Kv1.3; H401 in *r*Kv1.3) can influence TEA^+ block presumably through electrostatic repulsion. Similarly, we present evidence in this paper that the protonation of another histidine (H355) in Kv1.1, at the entrance of the external vestibule of the channel, can effect TEA^+ block. From these findings we conclude that the distance between TEA^+ and each of the four histidines at position 355 is ~10 Å. Some of the results have been reported in preliminary communications (Bretschneider et al., 1997a,b; 1998).

MATERIALS AND METHODS

Cells

All experiments were carried out on single cells of a rat basophilic leukemia cell line, RBL-cells (Eccleston et al., 1973). Cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in a culture medium of EMEM supplemented with 1 mM L-glutamine and 10% heat-inactivated fetal calf serum in a humidified, 5% CO_2 incubator at 37°C. Cells were plated to grow nonconfluently onto glass one day before use for injection and electrophysiological experiments (Rauer and Grissmer, 1996).

Solutions

The experiments were done at room temperature (21–25°C). Cells measured in the whole-cell configuration were normally bathed in mammalian Ringer's solution containing (in mM): 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 X (with X either HEPES, MES, or citrate), with an osmolarity of 290–320 mOsm. The pH_o was adjusted to 5.0, 5.5, 6.2 (X: citrate); to 6.2, 6.6, 6.8, 7.0 (X: MES), and to 7.0, 7.4 (X: HEPES) with

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                    P
                 ◄──────►
Kv1.1   EAEEAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGK    386
Kv1.2   --D-RD-Q-P--------------------V-T-----    413
mKv1.3  --DDPS-G-N-----------T---------H-------    411
Shaker  --GSEN-F-K-----------T---------T--GFW--    456
KcsA    -RGAPGAQLITY-R-L--S-ETA-------L----LW-R    89
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FIGURE 1 Putative pore (P) region of voltage-gated *Shaker*-related K^+ channels and the KcsA channel. The bold residues toward the right represent the putative binding sites for $[TEA^+]_o$, Y379 in Kv1.1, V406 in Kv1.2, H404 in *m*Kv1.3 (H401 in *h*Kv1.3), and T449 in *Shaker*. The other bold residue in Kv1.1 is H355.

NaOH. No differences in current were seen comparing mammalian Ringer's solution containing either citrate or MES at pH_o 6.2 and containing either MES or HEPES at pH_o 7.0. To reduce ionic strength 130 NaCl was isoosmotically substituted by glucose. A simple syringe-driven perfusion system was used to exchange the bath solutions in the recording chamber. The internal pipette solution for the whole-cell recordings contained (in mM): 134 KF, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, adjusted to pH 7.2 with KOH, with an osmolarity of 290–320 mOsm.

TEA^+ was purchased from FLUKA Chemie AG (Buchs, Germany) as tetraethylammonium chloride. To get solutions with different TEA^+ concentrations, the normal mammalian Ringer's solution was mixed with appropriate amounts of a TEA^+-stock solution containing (in mM): 160 TEA^+, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES.

Diethyl pyrocarbonate (DEPC) was purchased from FLUKA Chemie AG (Buchs, Germany). DEPC was added to mammalian Ringer's solution at pH_o 6.2 immediately before use to get final concentrations of 0.5–1 mM. Cells were perfused with DEPC containing solution for ~5 min. Immediately after this modification the TEA^+ effect was reevaluated at pH_o 6.2 in a solution containing no DEPC.

Electrophysiology

Experiments were carried out using the whole-cell recording mode of the patch-clamp technique (Hamill et al., 1981) as described before (Rauer and Grissmer, 1996). Electrodes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) in three stages, coated with Sylgard (Dow Corning, Seneffe, Belgium), and fire-polished to resistances measured in the bath of 2.5–4 MΩ. Membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA elektronik, Lambrecht, Germany) interfaced to a Macintosh computer running acquisition and analysis software (Pulse and PulseFit). Capacitative and leak currents were subtracted using the P/10 procedure. Series resistance compensation (>80%) was employed if the current exceeded 1 nA. Filter frequency was normally 2.9 kHz. The holding potential in all experiments was either −120 or −80 mV. We did not observe any differences in the pH-dependence of the TEA^+ block when using these two holding potentials.

Nonlinear approximations and presentation of data were performed using the program Sigmaplot (Jandel, Corte Madera, CA). Averaged data are given as mean values ± SD with n = number of investigated cells. K_d without SD are derived from the equation described in the legend to Fig. 3 *F*, whereas K_d with SD represent means of K_d of individual measurements of different cells derived from the same equation but taking the exponent $h = 1$ (see legend to Fig. 3 *F*). Statistical significance was verified by an unpaired Student's t-test with $p < 0.01$.

Expression

PBSTA plasmids containing the entire coding sequence of the *m*Kv1.1 wild-type (wt) cDNA (Chandy et al., 1990), and the pSP64T plasmids (Krieg and Melton, 1984) containing the sequences for the mutant *m*Kv1.3 H404Y channels (a generous gift from Dr. K. George Chandy, University of California, Irvine, CA) were linearized with Pst1 and EcoR1, respectively, and transcribed in vitro with the T7 (*m*Kv1.1) and SP6 (*m*Kv1.3 H404Y) Cap-Scribe System (Boehringer Mannheim, Mannheim, Germany). The resulting cRNA was phenol/chloroform-purified and could be stored at −75°C for several months.

Injection

The cRNA was diluted with a fluorescent FITC-dye (0.5% FITC-Dextran in 100 mM KCl) to a final concentration of 1 μg/μl. RBL cells were injected with the cRNA/FITC solution filled in injection capillaries (Femtotips) using an Eppendorf microinjection system (Micromanipulator 5171 and Transjector 5246). In the visualized cells specific currents could be measured 3–6 h after injection.

Generation of the H355G and H355K mutant Kv1.1 channel

For substituting histidine either with glycine or lysine at position 355 in *m*Kv1.1 channels, the QuikChange site-directed mutagenesis kit (Stratagene GmbH, Heidelberg, Germany) was used and the mutations were confirmed by sequencing single-stranded DNA with the Cy5-AutoRead Kit (Pharmacia, Uppsala, Sweden).

RESULTS

Effect of $[TEA^+]_o$ on current through wt Kv1.1 channels

To characterize the effect of extracellularly applied TEA^+ on current through voltage-gated Kv1.1 channels, we measured current in response to depolarizing voltage steps to 40 mV in the absence and presence of different TEA^+ concentrations (Fig. 2). The control current in the absence of $[TEA^+]_o$ activated fast, in the millisecond range, and showed only little inactivation during the 50-ms pulse. This

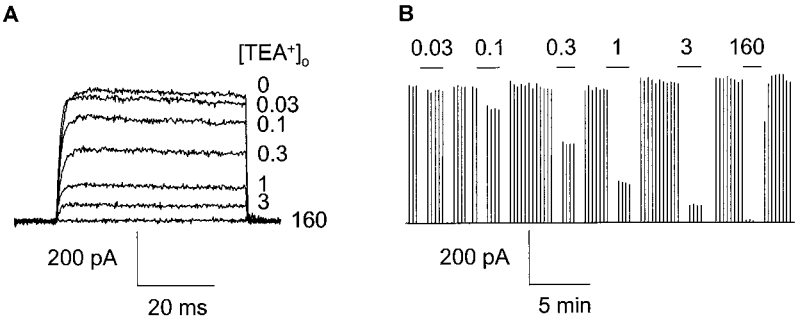

FIGURE 2 Effect of external TEA^+ on current through Kv1.1 channels at pH_o 7.4. (*A*) Whole-cell currents elicited by 50-ms depolarizing voltage steps from −80 mV (holding potential) to 40 mV are shown in the absence and presence of different external TEA^+ concentrations. (*B*) Peak K^+ currents of the same cell as shown in (*A*) are plotted against the absolute time during the experiment.

current through wt Kv1.1 channels obtained through cRNA injection of RBL cells was similar to current through Kv1.1 channels stably expressed in a mammalian cell line described earlier (Grissmer et al., 1994). Addition of TEA^+ to the bathing solution resulted in a dose-dependent reduction in current amplitude (Fig. 2 *A*). TEA^+ had no apparent effect on activation or inactivation. The latter finding was confirmed by measuring Kv1.1 current in response to depolarizing voltage steps of up to 1 s duration in the absence and presence of different TEA^+ concentrations (data not shown). This is in agreement with Molina et al. (1997) who suggested that if tyrosines interact with $[TEA^+]_o$, which is the case for Kv1.1 (Y379), $[TEA^+]_o$ could not reach the site for interacting with the C-type inactivation mechanism.

The current reduction by $[TEA^+]_o$ was fully reversible upon washout, even in a bathing solution containing 160 mM TEA^+. This can be seen in Fig. 2 *B*, where peak currents after repetitive depolarizations (every 30 s) were plotted against the absolute time during the experiment. It is obvious from the records shown in Fig. 2, *A* and *B* that 0.3 mM $[TEA^+]_o$ is sufficient to block ~50% of the current through Kv1.1 channels at 40 mV.

To investigate whether TEA^+ blockade is voltage-dependent we depolarized cells to different voltages every 30 s, first in the absence then in the presence of 0.3 mM $[TEA^+]_o$. The resulting currents obtained in each solution were superimposed and can be seen in Fig. 3 *A*. Peak currents from these records were taken and converted into the corresponding conductances by dividing the peak current amplitude with the driving force for $[K^+]$ ($E - E_K$, with $E_K = -85$ mV determined from tail currents, data not shown). Kv1.1 channels became activated with depolarizations more positive than −50 mV and reached a maximum conductance at ~0 mV (Fig. 3 *B*). Maximum conductance, g_{Kmax}, was independent of the applied voltage at potentials more positive than 0 mV. To quantify the voltage-dependence of the current, the data were fitted with a Boltzmann equation (Fig. 3 *B*) with values for the steepness of the voltage-dependence, k (11 mV), and the voltage where half the channels were activated, $E_{1/2}$ (−34 mV) similar to earlier reports (Grissmer et al., 1994). Additional determinations in 26 cells confirmed these values ($k = 11 \pm 3$ mV and $E_{1/2} = -34 \pm 8$ mV). Application of 0.3 mM $[TEA^+]_o$ resulted in a potential-independent reduction in conductance (Fig. 3 *B*). This is different from published data from Newland et al. (1992) who found a slight but measurable voltage-dependence ($\delta = 0.11$). A fit of a Boltzmann equation to the data obtained in the presence of 0.3 mM $[TEA^+]_o$ yielded values for k and $E_{1/2}$ similar to those obtained in the absence of $[TEA^+]_o$. g_{Kmax} in the presence and absence of 0.3 mM $[TEA^+]_o$ was 3.2 and 6.1 nS, respectively. Thus, 0.3 mM $[TEA^+]_o$ resulted in a voltage-independent reduc-

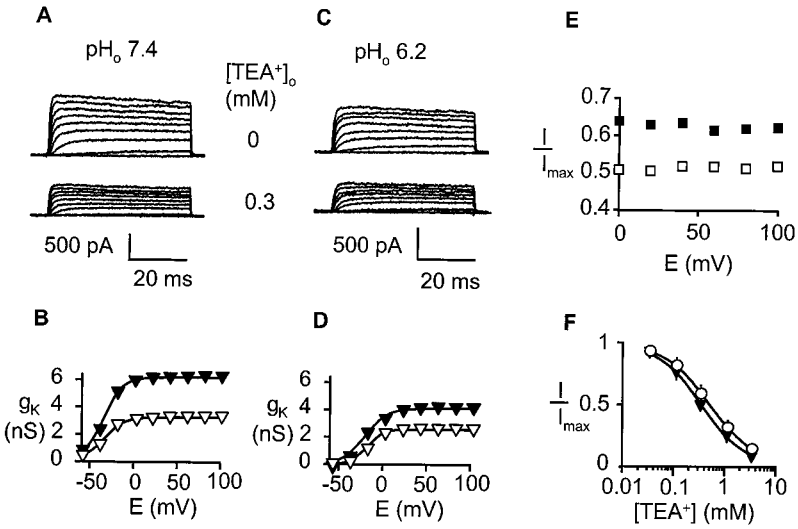

FIGURE 3 Influence of pH_o on the effect of external TEA^+ to block current through Kv1.1 channels. (*A*) Whole-cell currents, elicited in response to a set of 50-ms depolarizing voltage steps between −60 and 100 mV in 20 mV increments every 30 s from a holding potential of −80 mV, are shown in the absence and presence of 0.3 mM TEA^+ at pH_o 7.4. (*B*) Corresponding peak K^+ conductance-voltage relations for the K^+ currents shown in (*A*) are plotted against the applied membrane potential. The lines through the points were fitted with the Boltzmann equation: $g_K(E) = g_{Kmax}/\{1 + \exp[(E_{1/2} - E)/k]\}$, with parameter values for the control (▼) and the TEA^+ (▽) data of $g_{Kmax} = 6.1$ and 3.2 nS, respectively. Values for k (11 mV) and $E_{1/2}$ (−34 mV) were not significantly different between the two curves. (*C*) Whole-cell currents were elicited as described in (*A*) only at pH_o 6.2. (*D*) Corresponding peak K^+ conductance-voltage relations for the K^+ currents shown in (*C*) are plotted against the applied membrane potential. The lines through the points were fitted as described in (*B*) with parameter values for the control and the TEA^+ data of $g_{Kmax} = 4.1$ and 2.6 nS, respectively. Values for k (11 mV) and $E_{1/2}$ (−18 mV) were not significantly different between the two curves. (*E*) Peak currents obtained in the presence of TEA^+ (I) were divided by the peak currents in the absence of TEA^+ (I_{max}) from (*A*) and (*C*), respectively. The resulting ratios (I/I_{max}) were plotted against the applied membrane potential. Data from (*A*) at pH_o 7.4 (□) and data from (*C*) at pH_o 6.2 (■). (*F*) Peak currents elicited as in Fig. 2 in pH_o 7.4 (▼) and 6.2 (○) in the presence of TEA^+ (I) were divided by the peak currents in the absence of TEA^+ (I_{max}). The resulting ratios (I/I_{max}) and standard deviations of 4–13 cells were plotted against $[TEA^+]_o$ on a logarithmic scale. (In some cases standard deviations are smaller than symbols.) The lines through the points were fitted to modified Hill equations of the form $I/I_{max} = 1/[1 + ([TEA^+]/K_d)^h]$ with h in both cases $0.99 < h < 1.01$ and K_d in pH_o 7.4 of 0.34 and in pH_o 6.2 of 0.5 mM.

tion of the maximum conductance to 52% of the control value. Similar results were obtained in 13 other cells with 53.1 ± 2.5% conductance or current reduction after 0.3 mM $[TEA^+]_o$. Interestingly, in most of our experiments, we did not observe a conductance decrease at potentials more positive than 60 mV in the absence or presence of $[TEA^+]_o$ in comparison to results described by Ludewig et al. (1993) who described a voltage-dependent block of current through *r*Kv1.1 channels (RCK1) by internal Mg^{2+}. In those cases where we did observe such a conductance decrease at positive potentials (>60 mV), the conductance decrease was similar in solutions without and with $[TEA^+]_o$, indicating that $[TEA^+]_o$ block as well as the pH_o-dependence of $[TEA^+]_o$ block (see below), were unaltered (data not shown).

In additional experiments we investigated the effect of $[TEA^+]_o$ on current through Kv1.1 channels at pH_o 6.2 (Fig. 3 *C*). Decreasing pH_o from 7.4 to 6.2 by itself shifted the voltage-dependence of activation 21 ± 7 mV ($n = 5$) toward more depolarized potentials, an effect usually attributed to the neutralization of negative surface charges by protons (Hille, 1992). In addition to this shift in voltage-dependence, lowering pH_o resulted in a reduced conductance decrease after application of 0.3 mM TEA^+ (Fig. 3 *D*) compared to the reduction obtained at pH_o 7.4 (compare Fig. 3 *D* with Fig. 3 *B*). g_{Kmax} in the presence and absence of 0.3 mM $[TEA^+]_o$ at pH_o 6.2 was 2.6 and 4.1 nS, respectively. Thus, 0.3 mM $[TEA^+]_o$ at pH_o 6.2 resulted in a voltage-independent reduction of the maximum conductance to 63% of the control value. This value could be confirmed in seven additional measurements with a mean conductance reduction of 62.8 ± 3.1% after 0.3 mM $[TEA^+]_o$. This reduction is significantly weaker when compared to the reduction at pH_o 7.4 (to 53.1%; $p < 0.01$).

Fig. 3 *E* clearly shows the voltage-independence of current reduction after 0.3 mM $[TEA^+]_o$ at pH_o 6.2 (■) and 7.4 (□), respectively. Maximum current after the application of $[TEA^+]_o$ was divided by control current (data from Fig. 3, *A* and *C*) and the resulting ratio was plotted against the applied membrane potential.

Since the $[TEA^+]_o$ effect seemed to be independent of the applied voltage for depolarizations between 0 and 100 mV at both pH_o (see Fig. 3, *C–E*), in our analysis we used the ratio of peak currents at 40 mV to evaluate the $[TEA^+]_o$ effect. We therefore constructed dose-response curves from experiments similar to Fig. 2 at pH_o 7.4 and 6.2, as shown in Fig. 3 *F*. Ratios of peak currents in the presence (I) and absence (I_{max}) of TEA^+ were plotted against the applied TEA^+ concentration and a Hill equation was fitted to the data with a Hill coefficient close to 1, suggesting that one TEA^+ molecule is sufficient to block the channel. From the fit we also obtained K_d values for $[TEA^+]_o$ block at pH_o 7.4 and 6.2 of 0.34 and 0.5 mM, respectively. Thus the effect of $[TEA^+]_o$ on current through Kv1.1 channels is weaker at pH_o 6.2 by a factor of ~1.5 compared to the blocking effect at pH_o 7.4. Means of K_d values (see Methods) of 0.34 ± 0.05 ($n = 13$) and 0.50 ± 0.06 ($n = 6$) obtained at pH_o 7.4 and 6.2, respectively, were significantly different ($p < 0.01$).

To characterize and identify the amino acid in the channel protein that is responsible for the weaker blocking effect of TEA^+ at low pH_o we investigated this effect in more detail. If the protonation of histidines is responsible for this effect, chemical modification of histidines with DEPC should abolish the pH_o-dependent reduction of the TEA^+ effect. DEPC reacts with histidyl residues to yield an N-carbethoxyhistidyl derivative, thereby preventing the protonation of the imidazole side chain of histidines (Miles, 1977). Fig. 4 illustrates the results of such an experiment where we investigated the effect of 500 μM DEPC on the TEA^+ blocking effect in pH_o 6.2. Whereas 0.3 mM $[TEA^+]_o$ blocks current through Kv1.1 channels to 52% and 61% of controls in pH_o 7.4 and 6.2, respectively (superimposed traces of whole cell currents of one cell on the left side and in the middle of the figure), it blocks current to 52% of controls after pretreatment with DEPC in pH_o 6.2 (same cell). Similar results were obtained in five other cells with DEPC concentrations up to 1 mM with a current reduction after application of 0.3 mM $[TEA^+]_o$ to 51.6 ± 1.5% and 52.8 ± 2% at pH_o 7.4 before and pH_o 6.2 after DEPC treatment. The mean current reduction was not statistically different in both cases. DEPC did not only abolish the pH_o-dependent TEA^+ effect but also slowed activation of Kv1.1 current, as has been described for K^+ currents in squid giant axons (Spires and Begenisich, 1990). The loss of the pH_o-depen-

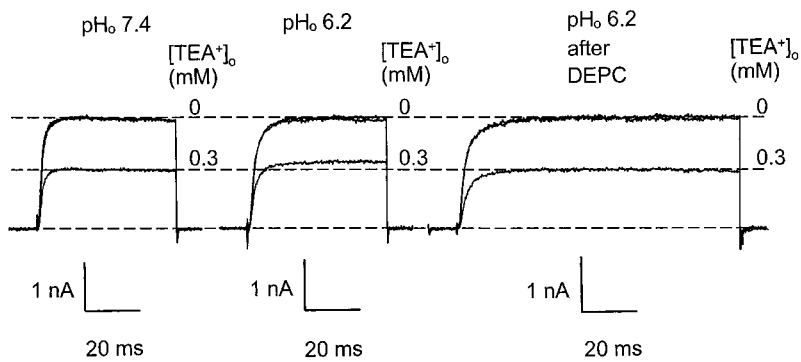

FIGURE 4 Influence of DEPC treatment on $[TEA^+]_o$ block of current through Kv1.1 channels. Whole-cell currents of one cell were elicited by 50- or 100-ms depolarizing voltage steps to 40 mV and are shown before, during, and after the application of 0.3 mM TEA^+ at pH_o 7.4 (*left*), at pH_o 6.2 (*middle*), and at pH_o 6.2 after washout of 500 μM DEPC (cell was treated with DEPC for 5 min at pH_o 6.2; *right*).

dent TEA^+ effect after DEPC treatment indicated that indeed histidines might be responsible for this effect.

Effect of $[TEA^+]_o$ on current through H355G and H355K mutant Kv1.1 channels

What residue could be responsible for the increase in K_d at pH_o 6.2? The investigations with DEPC gave a first indication that histidyl residues could be involved in this effect. From the KcsA crystal structure data (Doyle et al., 1998) as well as several works using peptide toxins (Goldstein and Miller, 1993; Ranganathan et al., 1996), it is known that position 355 is on the surface of Kv1.1 at the extracellular side of each homomer of Kv1.1 channels, would therefore line the outer vestibule of the channel, and could interact with $[TEA^+]_o$. Lowering pH_o from 7.4 to 6.2 could change the degree of protonation of these histidine imidazol rings, thereby changing their surface charge and the different electrostatic interactions between TEA^+ and more or less protonated histidines might explain the increased K_d at low pH_o. To test whether H355 plays a role in the pH_o-dependent $[TEA^+]_o$ effect we substituted H355 in Kv1.1 channels with glycine through site-directed mutagenesis and measured currents in this H355G mutant, as described before for current through wt Kv1.1 channels. The mutation by itself did not change the electrophysiological properties of the current with respect to activation and inactivation (data not shown).

$[TEA^+]_o$ blocked currents through the H355G mutant Kv1.1 channels at pH_o 7.4 with identical potency to currents through wt Kv1.1 channels, as can be seen in Fig. 5 *A*, which shows the dose-response relation for $[TEA^+]_o$ to block current through H355G mutant Kv1.1 channels. The half-maximum block was obtained with 0.34 ± 0.06 mM ($n = 7$) $[TEA^+]_o$ identical to Kv1.1 wt channels at pH_o 7.4. Furthermore, imitating a maximum protonation of H355 by substituting it with lysine (H355K) gave currents with similar characteristics with respect to activation and inactivation compared to wt channels and a K_d for TEA^+ block of 0.84 ± 0.09 mM ($n = 13$) (Fig. 5 *A*). If the degree of protonation of H355 is solely responsible for the pH_o effect, lowering pH_o should not influence the K_d values of the H355G and the H355K mutant channels. This is indeed the case, as can be seen in Fig. 5 *B*. The K_d values for both mutant channels either at pH_o 7.4, 6.8, or 6.2 are identical: ~0.8 mM for H355K and ~0.3 mM for H355G (see Fig. 5 *B*). Linear regressions through data points (K_d versus pH_o) of both mutants have slopes not different from zero, thereby excluding any pH_o dependency of TEA^+ blocking effect in this pH range. If we assume that the K_d values of H355K and H355G mutant channels represent the fully protonated and unprotonated H355 in Kv1.1 channels, successive protonation of H355 should give K_d values ranging between the borders of the mutant channels with a maximum K_d of 0.84 (K_{dmax}, see also Fig. 5 *A*) and a minimum K_d (K_{dmin}) of 0.34 mM (Fig. 5, *A* and *B*). The fit through data points of wt channels (Fig. 5 *B*) gave the expected values for K_{dmax} and K_{dmin} (see legend to Fig. 5) and a value for half-maximum protonation (pK_a) of 5.9. Similar experiments as shown in Fig. 5 were performed in bathing solutions with low ionic strength at different pH_o (data not shown). The K_d values of H355K and H355G mutants were reduced but still independent of pH_o. The K_d value of the wt channel (H355) at pH_o 7.4 was similar to the K_d value of the H355G mutant and still pH_o-dependent, as expected for an electrostatic interaction between the charge at position 355 and TEA^+ (Aiyar et al., 1995).

Effect of $[TEA^+]_o$ on current through H404Y mutant Kv1.3 channels

To further substantiate our hypothesis that electrostatic interactions between residue H355 and TEA^+ are responsible for TEA^+'s pH_o dependency, we investigated another volt-

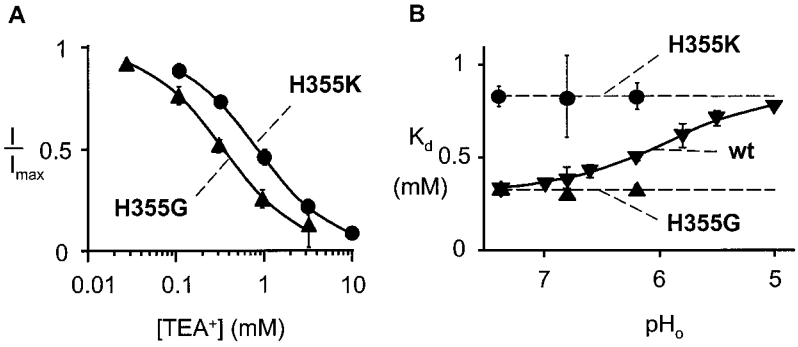

FIGURE 5 Dose-response curves for the inhibition of currents through mutant Kv1.1 channels and pH_o dependency of block by $[TEA^+]_o$. (*A*) Peak current ratios obtained as described in Fig. 3 *E* in Kv1.1 H355K or H355G mutant channels were plotted against the TEA^+ concentration (5–13 cells). Data were fitted as described in Fig. 3 *E* with $0.99 < h < 1.01$ and K_d for H355G (▲) of 0.34 and for H355K (●) of 0.84 mM. (*B*) Mean K_d values and standard deviations of Kv1.1 wt (▼), H355G (▲), and H355K (●) channels were plotted against pH_o. K_d values were obtained from the ratio of peak K^+ currents using the Hill equation (see legend to Fig. 3 *E*) with $h = 1$ (5–13 cells). Straight dashed lines represent linear regressions of data points (▲, ●) with slopes not different from zero. The regression coefficient is >0.99. The curve represents a fit through data points (▼) with the equation $K_d = K_{dmin} + \{[K_{dmax} - K_{dmin}]/[1 + (10^{pHo}/10^{pKa})^h]\}$ with $K_{dmin} = 0.33$, $K_{dmax} = 0.84$, pK_a 5.9, and $h = 0.96$.

age-gated K^+ channel, with a glycine at the homologous position to H355 in Kv1.1 (G380 in Kv1.3). To be able to directly compare the TEA^+ sensitivities between the two channels we used a mutant Kv1.3 channel that had a tyrosine at position 404 (homologous to Y379 in Kv1.1 channel), the putative TEA^+ binding site, and therefore high TEA^+ affinity. Depolarization-evoked currents were similar to currents through Kv1.1 channels (data not shown). We calculated K_d values at pH_o 7.4 (*white bars* in Fig. 6) and 6.2 (*black bars* in Fig. 6) from current reduction in 0.3 mM $[TEA^+]_o$ at different depolarizations and found K_d values close to 0.3 mM independent of the applied voltage. To visualize the lack of pH_o-dependency in this mutant channel (with no protonable histidine in the S5–S6 linker) we plotted the ratios $K_{dpH6.2}/K_{dpH7.4}$ against the applied membrane potential (*right diagram* in Fig. 6). These ratios were not different from 1 as tested for voltages where >5 cells had been examined ($p < 0.01$). Furthermore, there was no difference in the K_d value compared with those for the Kv1.1 channels at pH_o 7.4. These results confirmed our hypothesis that H355 in Kv1.1 channels determined the pH_o-dependence of the TEA^+ blocking effect. The lack of a pH_o sensitivity on current through the H404Y mutant Kv1.3 channels was similar to results by Kavanaugh et al. (1991) who investigated currents in the range of pH_o = 8–6.5 through RGK5 H401Y channels (*r*Kv1.3 channels, Y401 homologous to position 404 in *m*Kv1.3 channels) expressed in oocytes.

Estimation of distance between H355 and TEA^+ in Kv1.1 channels

From the data obtained with the mutant H355G and H355K Kv1.1 channels and the mutant H404Y *m*Kv1.3 channels we conclude that H355 in Kv1.1 channels can influence TEA^+ binding presumably by the pH_o-dependent protonation of the imidazole ring in H355. How can we explain this effect? One possible explanation could be that the protonation of H355 would create a local potential that would repel the also positively charged TEA^+, therefore reducing the effective $[TEA^+]_o$ at the receptor site. No change of the "true" affinity to the TEA^+ receptor site (Y379 in Kv1.1; Y404 in the mutant Kv1.3) is necessary for this explanation. This assumption is supported by the following findings. 1) The effect of TEA^+ depends only on pH_o in the wt Kv1.1 channel, not in the H355G and H355K mutant Kv1.1 channels or the H404Y mutant Kv1.3 channel with no histidine in the pore region. 2) The K_d values for TEA^+ blockade are almost identical for the wt Kv1.1 channel at pH 7.4, with little protonation at H355, and for the H355G mutant Kv1.1 channel and the H404Y mutant Kv1.3 channel (with a glycine at the position equivalent to H355 in Kv1.1 channel). In all three cases K_d values for TEA^+ blockade are ~0.3 mM, indicating that the mutations did not change the affinity for TEA^+ to its receptor site. We therefore conclude that a K_d of ~0.3 mM represents the "true" affinity of TEA^+ to its binding site at Y379 in Kv1.1 channels. Reducing pH_o did not influence K_d in *m*Kv1.3 H404Y, Kv1.1 H355G, and Kv1.1 H355K mutant channels, indicating that affinity of TEA^+ to the binding site is independent of pH_o. 3) Substituting the uncharged amino acid glycine in the H355G mutant Kv1.1 channel into a positively charged lysine (H355K) reduced TEA^+ affinity ~2.5-fold. This is the same factor as calculated from the K_{dmax} and K_{dmin} end points of the titration curve of Kv1.1 wt channels (see Fig. 5 *B*). Therefore, the weaker block in the H355K mutant Kv1.1 channel compared to the H355G or unprotonated wt Kv1.1 channel could be solely explained by charge repulsion.

If our hypothesis is correct and the reduced blocking effect is caused by a reduction in the effective $[TEA^+]$ at its binding site ($[TEA^+]_{eff}$) compared to the bulk concentration ($[TEA^+]_{bulk}$), this decrease in $[TEA^+]$ can then be calculated through the ratios of the K_d values obtained in pH_o 7.4 and pH_o Y (Y: 7.0, 6.8 6.6, 6.2, 5.5, and 5.0):

$$[TEA^+]_{eff}/[TEA^+]_{bulk} = K_{dpH7.4}/K_{dpHY} \quad (1)$$

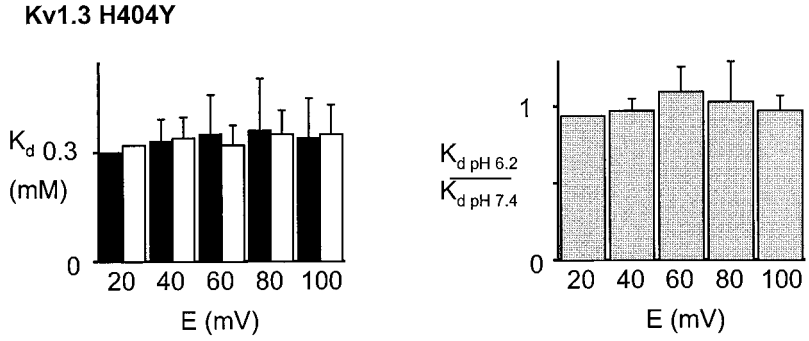

FIGURE 6 TEA^+ blocking effect on current through H404Y mutant *m*Kv1.3 channels in pH_o 7.4 and 6.2. Currents through Kv1.3 H404Y mutant channels were elicited as described in Fig. 2 at different voltages in the absence and presence of 0.3 mM $[TEA^+]_o$. K_d values were obtained at each voltage as described in the legend to Fig. 5 *B*. Shown are mean values with standard deviations for two (E = 60, and 80 mV), or three cells (*left*). The K_d value at 20 mV was obtained from a single measurement. $K_{dpH\ 6.2}/K_{dpH\ 7.4}$ ratios are plotted against the applied membrane potential at the right side. Shown are mean values with standard deviations obtained from two to three cells except for the ratio at 20 mV, where only one cell was under investigation.

Similar calculation can be done for the Kv1.1 mutant channels:

$$[TEA^+]_{eff}/[TEA^+]_{bulk} = K_{dH355G}/K_{dH355K} \quad (2)$$

If a potential is responsible for the change in the effective $[TEA^+]_o$ one can calculate this potential Ψ from the following equation:

$$[TEA^+]_{eff}/[TEA^+]_{bulk} = \exp(z\Psi F/RT) \quad (3)$$

with *R, F,* and *T* with their usual meaning and $z = 1$ (charge of TEA^+). For instance, with the K_d values of 0.34 mM at pH_o 7.4 and 0.5 mM at pH_o 6.2, the potential Ψ amounts to ~10 mV. This potential is presumably created by the protonation at the four histidines at position 355. Calculation of the potential difference in H355K compared to H355G (Eq. 2) gave a Ψ ~23 mV. We tried to estimate the distance between the charges at H355 (protonated histidines and positive charge of lysines in H355K, respectively) and TEA^+ since a potential Ψ created at position 355 should decrease with distance *x* from the charge according to the simplified equation from Gouy-Chapman (for surface potential $<kT/e$):

$$\Psi(x) = \frac{qe}{4\pi\epsilon\epsilon_0 x\gamma}\exp(-x/D) \quad (4)$$

where ϵ_0 is the dielectricity constant, ϵ is the dielectricity constant of water, taken as 80, *D* is Debye length, *q* is the degree of charge (see below), *e* is the elementary charge, and *x* is the distance from the center of charge. A correction factor $\gamma = 0.8$ was included in the equation to take into account that Ψ increases close to a protein/water interface (Stocker and Miller, 1994; Aiyar et al., 1995). γ is distance-dependent and should reflect the proximity and shape of the dielectric interface between water and protein (Stocker and Miller, 1994). The Debye length, *D*, in Eq. 4 depends on the ionic strength of the solution and can be calculated for our solutions to be ~7.37 Å.

For the estimation of the distance between the charges at position H355 and TEA^+ through the potential Ψ created by the protonation of the four H355 we need to know the pH_o-dependent degree of protonation at H355. The degree of charge at H355 is described by the pK_a for this histidine. For our calculations of *q* (degree of charge) we used the experimentally derived pK_a value of 5.9 for H355 (see Fig. 5 *B*). We can now solve Eq. 4 for different *q* in different pH_o for *x* assuming that each histidine at position 355 contributed equally to the generation of Ψ. We estimated distances between the charge at each of the four H355 and TEA^+ to be 9.1–9.8 Å.

DISCUSSION

Currents through Kv1.1 channels are blocked by $[TEA^+]_o$ in a pH_o-dependent manner. Our data suggest that a histidine in the outer vestibule of the channel (H355) is responsible for this pH_o-dependency. We have used this pH_o-sensitivity of the $[TEA^+]_o$ block to apply a method for determining the three-dimensional shape of this membrane protein through electrostatic interactions between charged amino acid residues in the channel protein on one side and the small blocker molecule (tetraethylammonium) with the same charge on the other side. It is obvious that these two charges will repel each other depending on how far apart those two charges are. For the estimation of the distance between the positive charges at position H355 in Kv1.1 and TEA^+, we made the assumption that by reducing pH_o we did not change the affinity of TEA^+ for its binding site, presumably Y379. The finding that we observed a pH_o-dependency of TEA^+ block only in the wt Kv1.1 but not in the H355G mutant Kv1.1 as well as the H404Y mutant Kv1.3 channel supports this assumption. In addition, the affinity for TEA^+ was almost identical for the wt Kv1.1 channel at pH_o 7.4, with little protonation at H355 and for the H355G mutant Kv1.1 channel and the H404Y mutant Kv1.3 channel. In all three cases K_d values for TEA^+ blockade are ~0.3 mM. The identical K_d values for TEA block of the H355K mutant Kv1.1 channel and the fully protonated H355 wt channel (0.84 mM in both cases, see Fig. 5) can also be used to argue that the H355K mutant channel 1) did not change the affinity of TEA^+ for its binding site, 2) did not sterically hinder the docking of TEA^+ to its receptor site, and 3) only reduced the effective $[TEA^+]_o$ at the site. We therefore concluded that the reduced TEA^+ blocking effect at low pH_o on wt Kv1.1 channels is caused by a reduction in the effective TEA^+ concentration at its binding site. This reduction was caused by the creation of a potential at the TEA^+ binding site through protonation at all four H355. We assumed that all four H355 are equally protonated with a pK_a of 5.9 for histidine (see Fig. 5 *B*) similar to estimations used by others (Creighton, 1984; Aiyar et al., 1995). Under our conditions with a pH_o of 5.8, all four H355 will then be approximately half-protonated, i.e., each H355 carries on average approximately half a charge. That means at the TEA^+ binding site the potential had been created by a total of approximately two charges ($4 \times \sim 0.5$). With a simplified Gouy-Chapman equation we could then calculate and estimate the distance between the charge at each of the four H355 and TEA^+ to be ~10 Å (see Fig. 7). This calculation of the distance between H355 and TEA^+ would still be below 10 Å if we used a pK_a of 6.2 for this histidine, as has been done by others (Aiyar et al., 1995). If we assume a fourfold symmetry of the channel with TEA^+ at the central axis of the channel pore, this places H355 in adjacent and opposing subunits ~14 and ~20 Å apart, respectively.

The distance of Y379 to TEA^+ can be estimated using the data by Kavanaugh et al. (1991) on the pH_o-dependent TEA^+ effect on *r*Kv1.3, with a histidine at the TEA^+ binding site (H401 in *r*Kv1.3 equivalent to Y379 in Kv1.1). These authors described a change in K_d for TEA^+ to block current through *r*Kv1.3 channels from ~11 mM at pH_o 7.5 to ~50 mM at pH_o 6.5. If we perform the identical calculation as described above we obtain a distance between

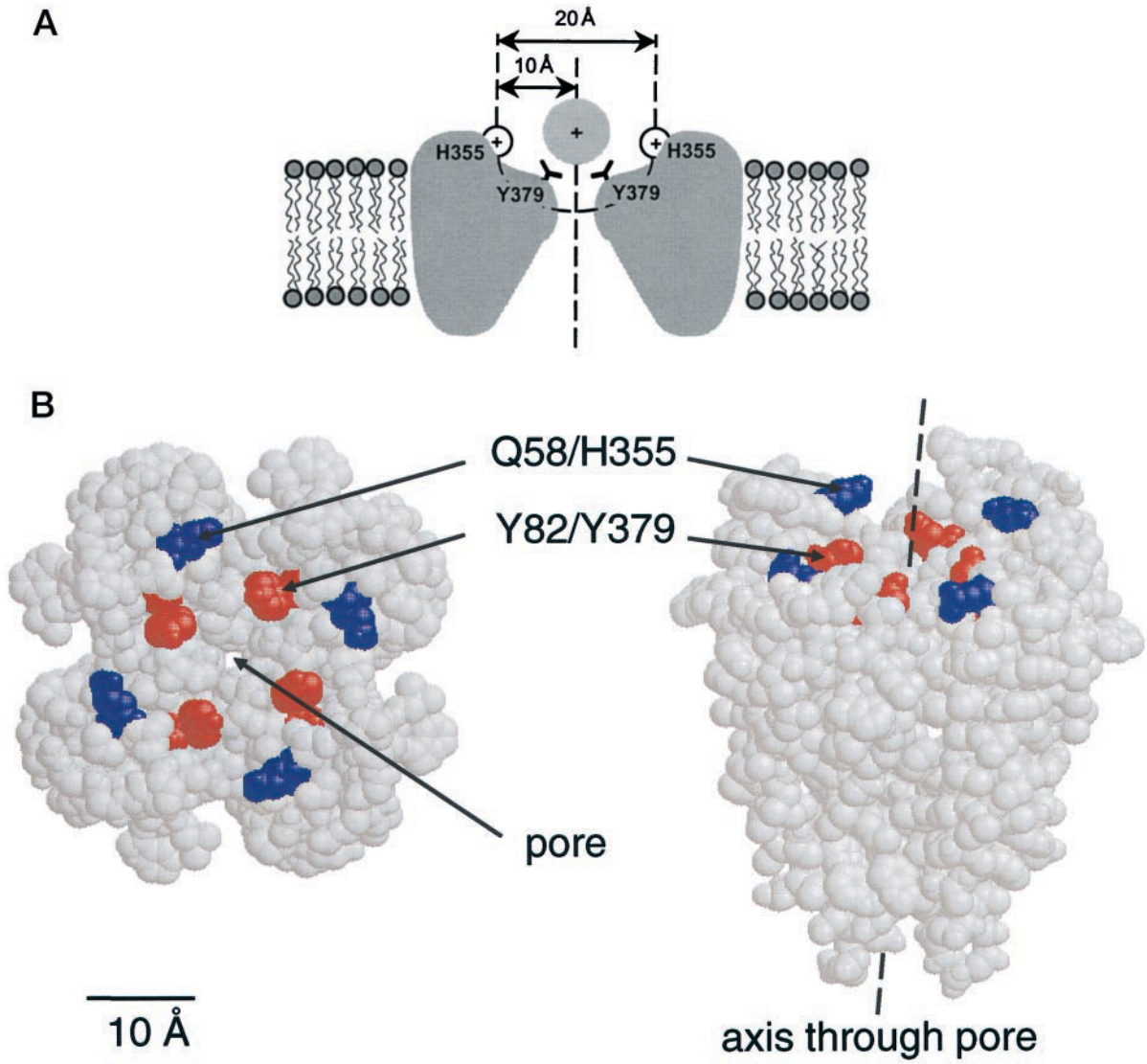

FIGURE 7 Proposed interaction of amino acid residues and TEA^+. (*A*) Diagram of the external vestibule of a Kv1.1 channel illustrating the interactions between $[TEA^+]_o$ and H355. (*B*) Surface view of the KcsA channel from the top (*left*) and slightly tilted (*right*) using RasMol (version 2.6). Q58 of the KcsA channel, equivalent to H355 in Kv1.1 (F425 in *Shaker*), is shown in blue. Y82 of the KcsA channel, the equivalent of Y379 in Kv1.1 (T449 in *Shaker*) is shown in red.

H401 in *r*Kv1.3 and TEA^+ of ~5.5 Å. This distance is in agreement with data obtained by others (Aiyar et al., 1995) on the dimensions of the inner part of the outer vestibule of voltage-gated K^+ channels.

A direct comparison of our data from the Kv1.1 channel with the crystallization data from the *Streptomyces lividans* channel, KcsA, by Doyle et al. (1998) can readily be made since the homology between KcsA and Kv1.1 is remarkable. Fig. 7 *B* visualizes the top surface of KcsA with the relevant residues highlighted. Q58 of KcsA in blue, equivalent to H355 in Kv1.1 (F425 in *Shaker*), is located ~12–13 Å from the central pore axis. Y82 of KcsA, in red, the equivalent of Y379 in Kv1.1 (T449 in *Shaker*) is positioned closer to the pore than Q58, ~6–7 Å from the central pore axis. Allowing for slight side chain movements and minor differences between KcsA and Kv1.1, one can easily picture Y379 interacting intimately with TEA^+, assuming a radius for TEA^+ of ~4 Å, and H355 interacting electrostatically with TEA^+. Another argument for the overall similarity in structure between KcsA and voltage-gated K^+ channels can be made by the creation of a mutant KcsA channel with an improved agitoxin2 sensitivity compared to the wt KcsA channel (MacKinnon et al., 1998).

In addition to the similarities between KcsA and Kv1.1, our results on the three-dimensional structure of the external outer margin of the vestibule confirm and extend those observed from the interactions between other channel proteins and larger peptide toxins. Different groups reported dimensions of the outer margin of the Kv1.3 channel of 28–34 Å (Aiyar et al., 1995) in diameter and ~25–30 Å

(Stocker and Miller, 1994), ~26–30 Å (Goldstein et al., 1994), or 22–30 Å (Hidalgo and MacKinnon, 1995) in diameter of the *Shaker* channel, respectively. One possible explanation for the small differences could be that the channels might change their conformations differently when binding peptide toxins compared to when interacting with small blocking molecules. Alternatively, there could be differences in the overall dimension and topology of the outer vestibule of closely related ion channels, as has been recognized earlier (Aiyar et al., 1995).

Position 380 in Kv1.3 channels (analogous to position 355 in Kv1.1) was thought to sterically, not electrostatically, hinder only the docking of peptide toxins to the channels (Goldstein and Miller, 1993; Ranganathan et al., 1996; see Fig. 6 of Aiyar et al., 1995). In a recent publication, however (Perez-Cornejo et al., 1998), it was shown that if position F425 (analogous to H355 in Kv1.1 and G380 in Kv1.3) in the *Shaker* K^+ channel was mutated into a histidine, CTX block became pH-dependent. The authors found that the protonation of the histidines reduced the ability of CTX to block the F425H mutant *Shaker* channel similar to our observation with $[TEA^+]_o$ on wt Kv1.1 channels. They conclude that the histidines at position 425 in the *Shaker* mutant interact with three positive charges in the CTX molecule including K11, K31, and possibly K27. Our results with H355 in wt Kv1.1 and $[TEA^+]_o$ could confirm and support both findings. It is obvious that we have demonstrated an electrostatic interaction between the charge at position 355 in Kv1.1 and $[TEA^+]_o$. In addition, the tendency in our calculation, although small, that the distance from H355 to TEA^+ gets larger by lowering pH_o (a change from 9.1 Å at pH_o 6.2 to 9.8 Å at pH_o 5.0) could indicate that the charges at H355 could sense the protonation of the other histidines, the charges of the histidines could repel each other, and the histidyl side chains could therefore be pushed apart, making the outer vestibule somewhat wider. Another indication for the sensing of the protonation of H355 between different subunits could be in the Hill coefficient obtained in Fig. 5 *B* to be <1. This behavior is expected if the protonation of H355 in a single subunit could influence the local $[H^+]_o$, thereby changing the apparent pK_a for protonation of the second H355, and so on. No apparent change in the "real" pK_a of the histidines is necessary for this explanation.

We have no way of proving whether the speculative view of the change of the dimension in the outer vestibule is actually happening; however, it could explain the small changes in the calculated distances between the charge at position 355 in Kv1.1 channels and TEA^+. The calculated distance between K355 in the H355K mutant (mimicking a fully protonated H355 at very low pH_o in the wt) and TEA^+ of 10 Å would also support this view.

In conclusion, we have mapped the dimension of the outer margin of the vestibule of the Kv1.1 channel to be ~20 Å. Our data on the outer vestibule of Kv1.1 channels confirm and extend findings for closely related K^+ channels (Kv1.3 and *Shaker*) by others (Stocker and Miller, 1994; Goldstein et al., 1994; Aiyar et al., 1995; Hidalgo and MacKinnon, 1995) using peptide toxin mapping and crystallization (Doyle et al. 1998). Our finding has direct structural implications on Kv1.1 or related ion channels and will therefore influence therapeutic drug design, especially in the context of the search for nonpeptide small molecule blockers/modulators of ion channels.

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