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 $K_v$ 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 $K_v$ 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 mKv1.3; H401 in rKv1.3) 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...
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 \([\text{TEA}^+]\), Y379 in Kv1.1, V406 in Kv1.2, H404 in mKv1.3 (H401 in hKv1.3), 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 6.2 and containing either MES or HEPES at pH 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): 160 NaCl, 2 MgCl₂, 10 HEPES, adjusted to pH 7.2 with KOH, with an osmolarity of 290–320 mOsm.

\(\text{TEA}^+\) was purchased from FLUKA Chemie AG (Buchs, Germany) as tetraethylammonium chloride. To get solutions with different \(\text{TEA}^+\) concentrations, the normal mammalian Ringer’s solution was mixed with appropriate amounts of a \(\text{TEA}^+\)-stock solution containing (in mM): 160 \(\text{TEA}^+\), 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES. Diethyl pyrocarbonate (DEPC) was purchased from FLUKA Chemie AG (Buchs, Germany). DEPC was added to mammalian Ringer’s solution at pH 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 \(\text{TEA}^+\) effect was reevaluated at pH 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 \(\text{TEA}^+\) 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 mKv1.1 wild-type (wt) cDNA (Chandy et al., 1990), and the pSP64T plasmids (Krieg and Melton, 1984) containing the sequences for the mutant mKv1.3 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 (mKv1.1) and SP6 (mKv1.3 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 (MW 100 kDa) 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 mKv1.1 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 \([\text{TEA}^+]_0\) on current through wt Kv1.1 channels

To characterize the effect of extracellularly applied \(\text{TEA}^+\) 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 \(\text{TEA}^+\) concentrations (Fig. 2). The control current in the absence of \([\text{TEA}^+]_0\) activated fast, in the millisecond range, and showed only little inactivation during the 50-ms pulse. This

FIGURE 2 Effect of external \(\text{TEA}^+\) on current through Kv1.1 channels at pH 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 \(\text{TEA}^+\) 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 0.3 mM TEA\(^+\). The channels were activated, with maximum conductance at potentials more positive than ~50 mV and reached a maximum conductance at ~0 mV (Fig. 3 B). Maximum conductance, \(g_{K_{\text{max}}}\), 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 \((k = 11 \pm 3\,\text{mV} \quad \text{and} \quad E_{1/2} = -34 \pm 8\,\text{mV})\). 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\(^+]_o\) (Fig. 3 B). In both cases 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-

![Image](https://example.com/image.png)

**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: \(g_K(E) = g_{K_{\text{max}}}([1 + \exp(-E/E_\text{1/2})])\), with parameter values for the control (▼) and the TEA\(^+\) (▼) data of \(g_{K_{\text{max}}} = 6.1\) 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 \(g_{K_{\text{max}}} = 4.1\) 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\(^+\) (▼) were divided by the peak currents in the absence of TEA\(^+\) (▼) from (A) and (C), respectively. The resulting ratios \((I/I_{\text{max}})\) 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\(^+\) (▼) were divided by the peak currents in the absence of TEA\(^+\) (▼). The resulting ratios \((I/I_{\text{max}})\) 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 \(I/I_{\text{max}} = 1/[1 + ((\text{[TEA}^+]_o)/K_h)^h]\) with \(h\) in both cases 0.99 < \(h\) < 1.01 and \(K_h\) 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 \([\text{TEA}^+]\)). 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 \([\text{TEA}^+]\), in comparison to results described by Ludewig et al. (1993) who described a voltage-dependent block of current through \(\alpha\text{Kv}1.1\) channels (RCK1) by internal \(\text{Mg}^{2+}\). 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 \([\text{TEA}^+]\), indicating that \([\text{TEA}^+]\) block as well as the \(p\text{H}_o\)-dependence of \([\text{TEA}^+]\) block (see below), were unaltered (data not shown).

In additional experiments we investigated the effect of \([\text{TEA}^+]\), on current through \(\alpha\text{Kv}1.1\) channels at \(p\text{H}_o\) 6.2 (Fig. 3 C). Decreasing \(p\text{H}_o\) 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 \(p\text{H}_o\) resulted in a reduced conductance decrease after application of 0.3 mM \([\text{TEA}^+]\) (Fig. 3 D) compared to the reduction obtained at \(p\text{H}_o\) 7.4 (compare Fig. 3 D with Fig. 3 B). \(g_{\text{Kmax}}\) in the presence and absence of 0.3 mM \([\text{TEA}^+]\) at \(p\text{H}_o\) 6.2 was 2.6 and 4.1 nS, respectively. Thus, 0.3 mM \([\text{TEA}^+]\), at \(p\text{H}_o\) 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 \([\text{TEA}^+]\), This reduction is significantly weaker when compared to the reduction at \(p\text{H}_o\) 7.4 (to 53.1%; \(p < 0.01\)).

Fig. 3 E clearly shows the voltage-independence of current reduction after 0.3 mM \([\text{TEA}^+]\), at \(p\text{H}_o\) 6.2 (■) and 7.4 (□), respectively. Maximum current after the application of \([\text{TEA}^+]\) 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 \([\text{TEA}^+]\) effect seemed to be independent of the applied voltage for depolarizations between 0 and 100 mV at both \(p\text{H}_o\) (see Fig. 3, C–E), in our analysis we used the ratio of peak currents at 40 mV to evaluate the \([\text{TEA}^+]\) effect. We therefore constructed dose-response curves from experiments similar to Fig. 2 at \(p\text{H}_o\) 7.4 and 6.2, as shown in Fig. 3 F. Ratios of peak currents in the presence (\(I\)) and absence (\(I_{\text{max}}\)) of \([\text{TEA}^+]\) were plotted against the applied \([\text{TEA}^+]\) concentration and a Hill equation was fitted to the data with a Hill coefficient close to 1, suggesting that one \([\text{TEA}^+]\) molecule is sufficient to block the channel. From the fit we also obtained \(K_d\) values for \([\text{TEA}^+]\) block at \(p\text{H}_o\) 7.4 and 6.2 of 0.34 and 0.5 mM, respectively. Thus the effect of \([\text{TEA}^+]\) on current through \(\alpha\text{Kv}1.1\) channels is weaker at \(p\text{H}_o\) 6.2 by a factor of ~1.5 compared to the blocking effect at \(p\text{H}_o\) 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 \(p\text{H}_o\) 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 \([\text{TEA}^+]\) at low \(p\text{H}_o\), 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 \(p\text{H}_o\)-dependent reduction of the \([\text{TEA}^+]\) effect. DEPC reacts with histidyl residues to yield an N-carboxyhistidyl 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 \(\mu\text{M}\) DEPC on the \([\text{TEA}^+]\) blocking effect in \(p\text{H}_o\) 6.2. Whereas 0.3 mM \([\text{TEA}^+]\), blocks current through \(\alpha\text{Kv}1.1\) channels to 52% and 61% of controls in \(p\text{H}_o\) 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 \(p\text{H}_o\) 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 \([\text{TEA}^+]\), to 51.6 ± 1.5% and 52.8 ± 2% at \(p\text{H}_o\) 7.4 before and \(p\text{H}_o\) 6.2 after DEPC treatment. The mean current reduction was not statistically different in both cases. DEPC did not only abolish the \(p\text{H}_o\)-dependent \([\text{TEA}^+]\) effect but also slowed activation of \(\alpha\text{Kv}1.1\) current, as has been described for \(K^+\) currents in squid giant axons (Spires and Begenisich, 1990). The loss of the \(p\text{H}_o\)-depen-
dent TEA\textsuperscript{+} effect after DEPC treatment indicated that indeed histidines might be responsible for this effect.

Effect of [TEA\textsuperscript{+}]\textsubscript{o} on current through H355G and H355K mutant Kv1.1 channels

What residue could be responsible for the increase in $K_d$ at pH\textsubscript{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\textsuperscript{+}]\textsubscript{o}. Lowering pH\textsubscript{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\textsuperscript{+} and more or less protonated histidines might explain the increased $K_d$ at low pH\textsubscript{o}. To test whether H355 plays a role in the pH\textsubscript{o}-dependent [TEA\textsuperscript{+}]\textsubscript{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\textsuperscript{+}]\textsubscript{o} blocked currents through the H355G mutant Kv1.1 channels at pH\textsubscript{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\textsuperscript{+}]\textsubscript{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\textsuperscript{+}]\textsubscript{o} identical to Kv1.1 wt channels at pH\textsubscript{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\textsuperscript{+} 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\textsubscript{o} effect, lowering pH\textsubscript{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\textsubscript{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\textsubscript{o}) of both mutants have slopes not different from zero, thereby excluding any pH\textsubscript{o} dependency of TEA\textsuperscript{+} 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\textsubscript{max}, see also Fig. 5 A) and a minimum $K_d$ (K\textsubscript{min}) 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 $K_d$\textsubscript{max} and $K_d$\textsubscript{min} (see legend to Fig. 5) and a value for half-maximum protonation (pK\textsubscript{a}) of 5.9. Similar experiments as shown in Fig. 5 were performed in bathing solutions with low ionic strength at different pH\textsubscript{o} (data not shown). The $K_d$ values of H355K and H355G mutants were reduced but still independent of pH\textsubscript{o}. The $K_d$ value of the wt channel (H355) at pH\textsubscript{o} 7.4 was similar to the $K_d$ value of the H355G mutant and still pH\textsubscript{o}-dependent, as expected for an electrostatic interaction between the charge at position 355 and TEA\textsuperscript{+} (Aiyar et al., 1995).

Effect of [TEA\textsuperscript{+}]\textsubscript{o} on current through H404Y mutant Kv1.3 channels

To further substantiate our hypothesis that electrostatic interactions between residue H355 and TEA\textsuperscript{+} are responsible for TEA\textsuperscript{+}’s pH\textsubscript{o} dependency, we investigated another volt-

![Figure 5](image-url)
The absence of a glycine at homologous 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 protonatable 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 (rKv1.3 channels, Y401 homologous to position 404 in mKv1.3 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 mKv1.3 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$^+$], 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. Therefore we 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 mKv1.3 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$^+$]$_{bulk}$) 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):

$$[\text{TEA}^+]_{\text{eff}}/[\text{TEA}^+]_{\text{bulk}} = K_{dpH7.4}/K_{dpHY} \quad (1)$$
Similar calculation can be done for the Kv1.1 mutant channels:

\[ [\text{TEA}^+]_{\text{obs}} / [\text{TEA}^+]_{\text{bulk}} = \frac{K_{\text{d355G}}}{K_{\text{d355K}}} \]  

(2)

If a potential is responsible for the change in the effective [\text{TEA}^+]_o, one can calculate this potential \( \Psi \) from the following equation:

\[ [\text{TEA}^+]_{\text{obs}} / [\text{TEA}^+]_{\text{bulk}} = \exp(z\Psi F/RT) \]  

(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\(_7.4\) and 0.5 mM at pH\(_6.2\), the potential \( \Psi \) amounts to \( \approx 10 \) 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 \( \Psi \approx 23 \) 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 \( \Psi \) created at position 355 should decrease with distance \( x \) from the charge according to the simplified equation from Gouy-Chapman (for surface potential \( \Psi \)):

\[ \Psi(x) = \frac{qe}{4\pi\varepsilon_0\varepsilon\gamma} \exp(-x/D) \]  

(4)

where \( \varepsilon_0 \) is the dielectric constant, \( \varepsilon \) is the dielectric 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 \( \Psi \) increases close to a protein/water interface (Stocker and Miller, 1994; Aiyar et al., 1995). \( \gamma \) 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 \( \approx 7.37 \) Å.

For the estimation of the distance between the charges at position H355 and TEA\(^+\) through the potential \( \Psi \) 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. 5B). 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 \( \Psi \). 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 [\text{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 [\text{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\(_7.4\), 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 \( \approx 0.3 \) 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. 5C) 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 [\text{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. 5B) 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 (\( 4 \times \approx 0.5 \)). 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 \( \approx 10 \) 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 \( \approx 14 \) and \( \approx 20 \) 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 Kv1.3, with a histidine at the TEA\(^+\) binding site (H401 in rKv1.3 equivalent to Y379 in Kv1.1). These authors described a change in \( K_d \) for TEA\(^+\) to block current through rKv1.3 channels from \( \approx 11 \) mM at pH\(_o\) 7.5 to \( \approx 50 \) mM at pH\(_o\) 6.5. If we perform the identical calculation as described above we obtain a distance between
H401 in rKv1.3 and TEA\(^+\) of \(\sim 5.5\) 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 shown in blue. Y82 of the KcsA channel, the equivalent of Y379 in Kv1.1 (T449 in *Shaker*) is shown in red.

**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\(^+\)], 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.

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 \(\sim 25–30\) Å differences between KcsA and Kv1.1, one can easily picture Y379 interacting intimately with TEA\(^+\), assuming a radius for TEA\(^+\) of \(\sim 4\) 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).
(Stockler 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 pHo (a change from 9.1 Å at pHo 6.2 to 9.8 Å at pHo 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 pKₐ for protonation of the second H355, and so on. No apparent change in the “real” pKₐ 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 pHo 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 (Stockler 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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