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The External TEA Binding Site and C-Type Inactivation in Voltage-Gated Potassium Channels

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ABSTRACT The location of the tetraethylammonium (TEA) binding site in the outer vestibule of K^+ channels, and the mechanism by which external TEA slows C-type inactivation, have been considered well-understood. The prevailing model has been that TEA is coordinated by four amino acid side chains at the position equivalent to *Shaker* T449, and that TEA prevents a constriction that underlies inactivation via a foot-in-the-door mechanism at this same position. However, a growing body of evidence has suggested that this picture may not be entirely correct. In this study, we reexamined these two issues, using both the Kv2.1 and *Shaker* potassium channels. In contrast to results previously obtained with *Shaker*, substitution of the tyrosine at Kv2.1 position 380 (equivalent to *Shaker* 449) with a threonine or cysteine had a relatively minor effect on TEA potency. In both Kv2.1 and *Shaker*, modification of cysteines at position 380/449 by 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET) proceeded at identical rates in the absence and presence of TEA. Additional experiments in *Shaker* demonstrated that TEA bound well to C-type inactivated channels, but did not interfere with MTSET modification of C449 in inactivated channels. Together, these findings rule out the possibility that TEA binding involves an intimate interaction with the four side chains at the position equivalent to *Shaker* 449. Moreover, these results argue against the model whereby TEA slows inactivation via a foot-in-the-door mechanism at position 449, and also argue against the hypothesis that the position 449 side chains move toward the center of the conduction pathway during inactivation. Occupancy by TEA completely prevented MTSET modification of a cysteine in the outer-vestibule turret (Kv2.1 position 356/*Shaker* position 425), which has been shown to interfere with both TEA binding and the interaction of K^+ with an external binding site. Together, these data suggest that TEA is stabilized in a more external position in the outer vestibule, and does not bind via direct coordination with any specific outer-vestibule residues.

INTRODUCTION

For more than three decades, tetraethylammonium (TEA) has been used to probe K^+ channel pharmacology, permeation, gating, structure, and the dynamics of protein movement at both the internal and external end of the conduction pathway. (cf. Armstrong, 1969; Miller, 1988; MacKinnon and Yellen, 1990; Choi et al., 1991; Kavanaugh et al., 1992; Bretschneider et al., 1999; Immke et al., 1999; Blaustein et al., 2000; Zaks-Makhina et al., 2004). Mutagenesis studies in the early 1990s demonstrated that the amino acid residue at *Shaker* position 449 had a dramatic impact on the blocking potency of external TEA (MacKinnon and Yellen, 1990; Kavanaugh et al., 1991; Heginbotham and MacKinnon, 1992). In several channels, amino acid substitution at the position equivalent to 449 alters TEA potency over a range from ~0.6 mM to >100 mM if the substituted amino acid is not positively charged, and eliminates TEA block if it is positively charged (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992; Pascual et al., 1995; Gomez-Hernandez et al., 1997). It was suggested that the position 449 residue does not merely exert an electrostatic influence on TEA block because a variety of neutral amino acids at this position result in different intermediate TEA potencies (MacKinnon and Yellen, 1990; (see also Fig. 1)), and substitution of a negatively charged glutamate makes the channel insensitive to TEA (Molina et al., 1997). This interpretation was recently supported by the observation that an uncharged organic channel blocker, which appears to bind to a site that overlaps the TEA binding site, is also sensitive to mutations at the position equivalent to 449 (Zaks-Makhina et al., 2004). These data, combined with the observation that all four channel subunits contribute more or less equally to TEA blocking potency (Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992), contributed to the long-held conclusion that amino acids at the position equivalent to *Shaker* 449 comprised the primary component of the TEA binding site. Indeed, it was further postulated that with tyrosines at this site, TEA was coordinated by an ''aromatic cage'' formed by the four tyrosines in the outer vestibule (Heginbotham and MacKinnon, 1992). Two recent molecular dynamics studies supported the conclusion that TEA interacts directly with these residues (Guidoni and Carloni, 2002; Luzhkov et al., 2003).

Many studies that focused on C-type inactivation in *Shaker* were also consistent with this hypothesis. In *Shaker*, the rate of C-type inactivation is dramatically altered by mutation of the position 449 amino acid from the native threonine to a variety of other amino acids (Lopez-Barneo et al., 1993). The affinity of Cd^{2+} for cysteines at position 449 increases ~45,000-fold when channels progress from open to inactivated, which indicated that these cysteines

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change exposure during C-type inactivation (Yellen et al., 1994). This change in cysteine exposure, which is thought to reflect movement of the cysteines, is associated with what appears to be a constriction just external to and/or within the selectivity filter (Liu et al., 1996; Kiss and Korn, 1998; Kiss et al., 1999). Application of external TEA slows C-type inactivation (Grissmer and Cahalan, 1989; Choi et al., 1991), and presumably the constriction. These observations fit well with the hypothesis that the constriction brings the position 449 residues closer together, and that binding of TEA between the position 449 residues slows the constriction via a ''foot-in-the-door'' mechanism (Yellen, 1998). As a result of these studies, and those described above, it became well-accepted that binding of external TEA to the channel involves the direct coordination of TEA by the four amino acids at the position equivalent to *Shaker* 449. Moreover, it followed from these observations that the position 449 residues were important components of the C-type inactivation gate, and that once this gate closed, TEA could not bind to the channel (see Yellen, 1998).

Several observations, however, are inconsistent with this hypothesis. First, TEA potency can be reasonably stable despite nonconservative changes in amino acid side chains at this position (see Fig. 1, below). Conversely, different channels can display dramatically different TEA potencies with identical residues at this position (compare, for example, Heginbotham and MacKinnon, 1992; Hadley et al., 2000). Second, in Kv2.1, modification of a cysteine at position 380 (wild-type Kv2.1 has a tyrosine at position 380, which is equivalent to *Shaker* position 449) with methanethiosulfonate (MTS) reagents changes TEA potency, but does not abolish TEA block (Pascual et al., 1995). It seems unlikely that a specific ligand binding site, which involves intimate coordination by four residues, could survive significant chemical modification, or changes in putative coordination sites by mutagenesis. Finally, a third molecular dynamics simulation study of the KcsA potassium channel suggested that block by TEA does not require a direct coordination with the residue equivalent to *Shaker* 449 (Crouzy et al., 2001). These simulations suggested that TEA can move around within the outer vestibule, and may occupy a more external position within the outer vestibule when a threonine is substituted for a tyrosine at position 449 (Crouzy et al., 2001).

Recently, we demonstrated the presence of a K^+ binding site in the outer vestibule of the Kv2.1 potassium channel that was external to and independent from the Y380 residue (Consiglio et al., 2003). This K^+ binding site may be the ''dehydration/rehydration'' site observed in KcsA (Zhou et al., 2001). In Kv2.1, a lysine at position 356, located in the outer-vestibule turret, interferes with both TEA block and the interaction of K^+ with this site (Immke and Korn, 2000; Consiglio et al., 2003). These results led us to test the hypothesis that external TEA blocked the channel by occupying the outer vestibule at a site external to the Y380/T449 residues, possibly near the outer-vestibule K^+ binding site. In addition, these studies led to critical tests of the hypotheses that 1), TEA could not bind to C-type inactivated channels; and 2), TEA slowed C-type inactivation in *Shaker* by occupying space between position 449 residues, and thus preventing them from moving closer together.

METHODS

Molecular biology and channel expression

Experiments were done on the wild-type Kv2.1 channel, several mutant Kv2.1 channels, and two mutant *Shaker* channels. Mutations to Kv2.1 were made with the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), as described previously. Mutations were confirmed by sequence analysis. *Shaker* Δ6–46 F425C was obtained from Dr. Chris Miller and *Shaker* Δ6–46 T449C was obtained from Dr. Gary Yellen. Channel cDNA was subcloned into the pcDNA3 expression vector for Kv2.1 channels, pBK-CMV for *Shaker* channels. All experiments were done on channels expressed in the human embryonic kidney cell line, HEK293 (American Type Culture Collection, Rockville, MD). Cells were maintained in DMEM plus 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) with 1% penicillin/streptomycin. Cells (2×10^6 cells/ml) were cotransfected by electroporation (Bio-Rad Gene Pulser II at 220V, 350 μF; Hercules, CA) with K^+ channel expression plasmid (0.5–10 μg/0.2 ml) and CD8 expression plasmid (0.5 μg/0.2 ml). After electroporation, cells were plated on glass coverslips submerged in maintenance media. Electrophysiological recordings were made 18–28 h later. On the day of recording, cells were washed with fresh media and incubated with Dynabeads M450 conjugated with antibody to CD8 (0.5 μl/ml; Dynal, Lake Success, NY). Cells that expressed CD8 became coated with beads, which allowed visualization of transfected cells (Jurman et al., 1994).

Electrophysiology

Currents were recorded at room temperature in the whole-cell patch-clamp configuration. Patch pipets were fabricated from N51A glass (Garner Glass, Claremont, CA), coated with Sylgard, and firepolished. Currents were collected with either an Axopatch 1D or Axopatch 200B amplifier, pClamp 9 software, and a Digidata 1322A A/D board (Axon Instruments, Foster City, CA). Currents were filtered at 2 kHz and sampled at 40–400 μs/pt. Series resistance ranged from 0.5 to 2.5 MΩ and was compensated 80–90%. The holding potential was −80 mV unless otherwise noted, and depolarizing stimuli were presented once every 10–60 s, depending on the experiment.

Data were analyzed with Clampfit 9 (Axon Instruments); curve fitting and significance testing (unpaired Student's *t*-test) were done with SigmaPlot 8.0 (SPSS, Chicago, IL). All plotted data are represented as mean ± SEM, with the number of data points denoted by n. Differences between means were considered statistically significant if p values in unpaired Student's *t*-tests were <0.05. Concentration-response curves were fit to the equation

$$Y = 100 \times [(X)^{\mathrm{n}}/((X)^{\mathrm{n}} + (\mathrm{IC}_{50})^{\mathrm{n}})],$$

where X is the drug concentration, IC_{50} is the drug concentration that produced half maximal inhibition, and n is the slope of the curve.

Electrophysiological solutions

Currents were recorded in a constantly flowing, gravity-fed bath. Solutions were changed in one of two ways. For most experiments, solutions were placed in one of six reservoirs, each of which fed via polyethylene tubing

into a perfusion manifold. Solution exited the manifold via a single quartz or polyethylene tube. Cells were lifted off of the dish before recording and placed ~20 μm from the tip of the perfusion tube. One solution was always flowing, and solutions were switched manually (solution exchange was complete within 5–10 s). Faster solution changes (complete exchange within ~19 ms; required for Figs. 8 and 9, panels *C* and *D*) were made as described previously (Andalib et al., 2002). Except where noted in the figure legend, the internal solution contained (in mM): 100 KCl, 25 *N*-methyl-glucamine (NMG)-Cl, 10 HEPES, 10 EGTA, 1 $CaCl_2$, 4 $MgCl_2$; pH with NMG to 7.3, osmolality 290 mosm. Except where noted in the figure legend, the external solution contained (in mM): 145 NMG-Cl, 20 HEPES, 10 glucose, 2 $CaCl_2$, and 1 $MgCl_2$; pH with NMG to 7.3, osmolality 330 mosm. For experiments in which drugs were added, if the drug concentration was ≤10 mM, drug was simply added to the external solution to the desired final concentration. When >10 mM drug was used, then it was substituted on an equimolar basis for NMG.

Cysteine modification experiments

In many experiments, channels that contained a substituted cysteine in the outer vestibule were modified by one of several MTS reagents (Toronto Research, Ontario, Canada). MTS solutions were made by adding dry powder to the external recording solution immediately before application, to a final concentration of 0.3–4 mM. MTS reagents were applied in one of two ways. Either MTS reagents were perfused onto the cell at the time of recording or cells were preincubated with MTS reagents for 3 min immediately before recording. In all cases where cells were preincubated, at least one equivalent experiment was done with real-time perfusion to demonstrate that both methods of modification produced similar results.

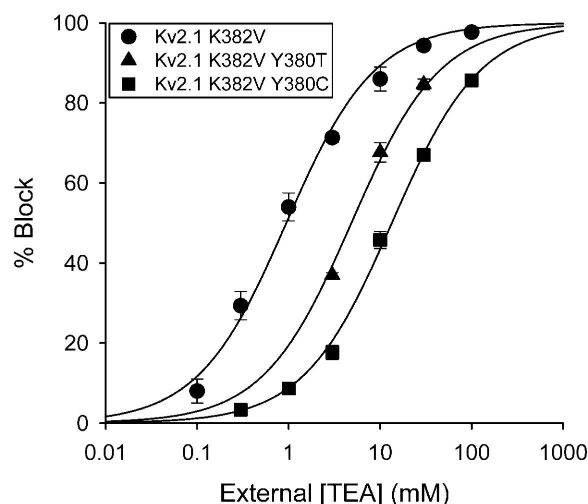

FIGURE 1 Block of Kv2.1 K382V by TEA after mutation of Y380. Concentration-dependent block by external TEA is shown for three channels: Kv2.1 K382V, which has the native tyrosine at position 380 and serves as the control channel (*circles*), and Kv2.1 K382V channels, which have the position 380 residue mutated to a threonine (Y380T; *triangles*) or a cysteine (Y380C; *squares*). Solid lines are best fits to the curves from Eq. 1, which gave calculated EC_{50} values of 0.95 mM for Y380, 5.0 mM for T380, and 13.4 mM for C380. All data points represent the mean ± SE of three cells. Recordings were made with 100 mM internal and 5 mM external K^+ (NMG made up the balance of the monovalent cation).

RESULTS

Because the location of the TEA binding site had been based largely on mutagenesis of the *Shaker* family of potassium channels, we examined TEA potency in Kv2.1 channels that had one of two mutations to the position 380 residue (equivalent to *Shaker* 449). We made these mutations in a Kv2.1 channel that already contained a well-characterized K382V mutation. This mutation to a valine, the native residue in both *Shaker* and KcsA, increased TEA potency by ~1/2 log unit, apparently due to removal of an electrostatic interference of the lysine with TEA binding (Immke et al., 1999). The K382V mutation was advantageous for two reasons: it provided an increased range of available [TEA] for complete concentration-response curves and it eliminated the possibility that the electrostatic influence would vary with different residues in the nearby 380 position.

The Y380T and Y380C mutations produced a 5- and 10-fold shift in TEA potency, respectively (Fig. 1). These results are consistent with previous studies that showed that mutation of this residue to a nonaromatic amino acid reduced TEA potency. However, these results were notable in that mutations to amino acids with dramatically different side chains, and which may have a significantly different orientation in the pore (Consiglio et al., 2003; see also Fig. 11, *A* and *B*), produced quite modest changes in TEA potency. In particular, the Y380T mutation produced a much smaller change in TEA potency than observed in *Shaker*, in which the converse, T449Y mutation, produced a >30-fold increase in TEA potency (Heginbotham and MacKinnon, 1992).

We then directly tested the hypothesis that the residues at position 380 in Kv2.1 did not coordinate TEA. To accomplish this, we asked whether the rate of modification of C380 by 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET) was altered by TEA occupancy of the outer vestibule. If TEA binding involved direct coordination by the side chains on the position 380 residue, then occupancy of the TEA binding site by TEA would slow modification by MTSET. In contrast, if cysteine modification rate were unaffected, this would provide unambiguous evidence that TEA was not bound directly to the position 380 side chains.

Application of MTSET in the absence of TEA produced a time-dependent, irreversible block of the Y380C channel (Fig. 2 *C*, *shaded symbols*). After a 40-s application, channels were blocked by 26.3 ± 2.1% ($n = 3$; Fig. 2 *C*, *shaded triangles*) . After removal of MTSET (*upward double arrows*, OFF 40), current magnitude recovered by 3% to give a final irreversible block of 23.3 ± 1.1% ($n = 3$). When MTSET was applied for 200 s, currents were irreversibly blocked by 54.0 ± 2.8% ($n = 6$; Fig. 2 *C*, *shaded circles*). In a different set of cells, 100 mM TEA was applied 50 s before, and throughout, application of MTSET (Fig. 2 *C*, *black symbols*). Initially, 100 mM TEA blocked the current by ~80%. As MTSET modified the channels, TEA block was reduced, consistent with previous observations that MTSET modification at position 380 reduced TEA potency (Pascual et al., 1995; see also Fig. 4 *A*). However, upon removal of both TEA and MTSET, currents were irreversibly reduced by identical amounts compared with

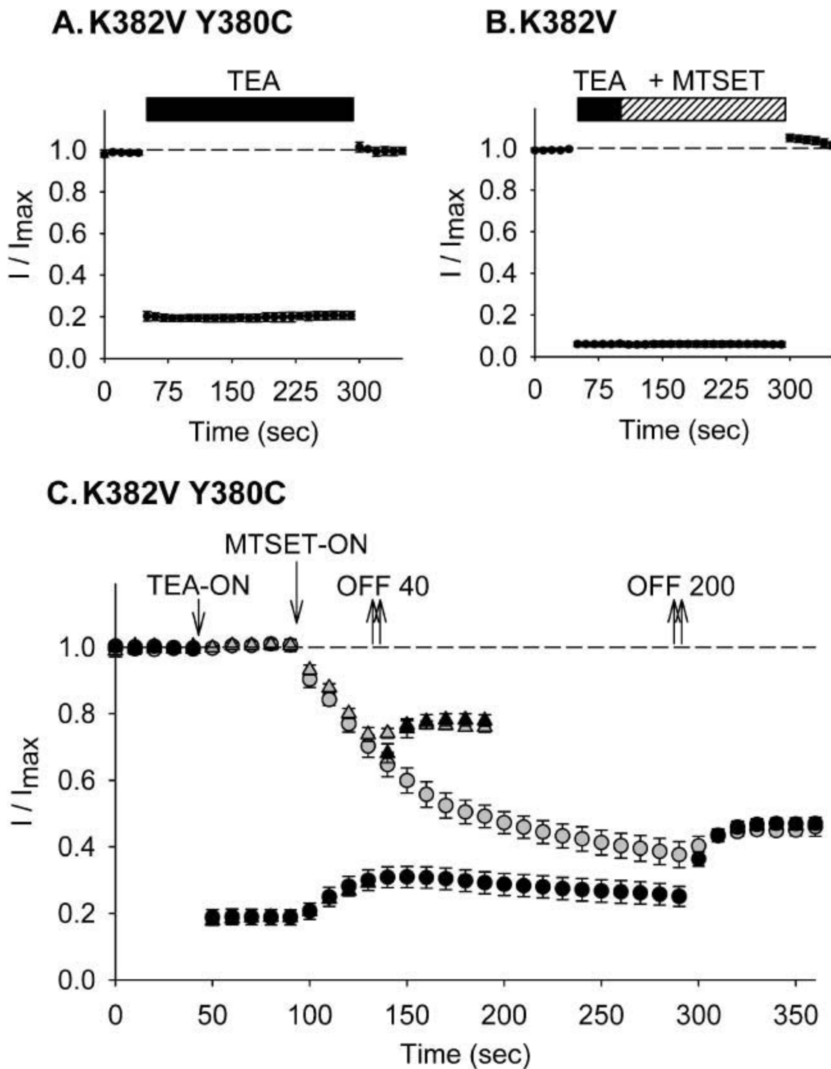

FIGURE 2 External TEA did not affect the rate of MTSET modification of C380 in Kv2.1. (*A*) Reversible block of Kv2.1 K382V Y380C by 100 mM external TEA. (*B*) After a 50-s application of 100 mM TEA alone (marked by *black bar*), 100 mM TEA plus 2 mM MTSET was applied for 200 s (marked by *hatched bar*) to a channel without the cysteine at position 380. Data points in *A* and *B* represent the mean ± SE of four cells. (*C*)Four different conditions are shown. The shaded symbols illustrate the time course of modification of C380 by 2 mM MTSET with a 40-s application (*triangles*) and 200-s application (*circles*). MTSET was removed after 40 s (OFF 40) or after 200 s (OFF 200), and irreversible block measured once current relaxed to a new steady-state level. The black symbols illustrate the time course of modification by MTSET, applied for these same two durations, in the presence of 100 mM TEA (TEA was applied for 40 s before, and throughout, exposure to MTSET). At both the 40- and 200-s time points, TEA and MTSET were removed simultaneously. Data points represent the mean ± SE of 3–6 cells.

those recorded in the absence of TEA. After 40-s and 200-s applications of MTSET in the presence of TEA, currents were irreversibly reduced by 21.8 ± 1.8% ($n = 3$; Fig. 2 *C*, *black triangles*) and 53.0 ± 1.9% ($n = 5$; Fig. 2 *C*, *black circles*), respectively. These data demonstrate that occupancy of the TEA binding site ~80% of the time had no effect on the rate of modification of the cysteines at position 380, and rule out the possibility that TEA binding involved direct coordination by the cysteine side chains. In addition, because MTSET can access C380 while TEA is in the outer vestibule, these data suggest that the cysteine side chains at position 380 are not even in the pathway through which TEA blocks the channel. (Qualitatively identical results were obtained when TEA occupied the outer vestibule ~90% of the time in a Kv2.1 Y380C K382V channel that also had the lysine at position 356 neutralized to increase TEA potency; data not shown.)

Fig. 2, *A* and *B*, illustrates control experiments. Fig. 2 *A* illustrates that in the absence of MTSET, 100 mM TEA blocked currents through Kv2.1 Y380C K382V by 80%, that the block remained steady throughout TEA application, and that current magnitude recovered fully upon removal of TEA. Fig. 2 *B* illustrates that, in Kv2.1 K382V, which lacks the cysteine at position 380, application of MTSET had no effect when applied simultaneously with TEA.

The concentration dependence of TEA block is consistent with block of a single channel by a single TEA molecule, which suggests that the outer vestibule only accommodates a single TEA molecule. However, the observation that MTSET could access C380 without interference from TEA (Fig. 2) implied the surprising possibility that two large positively charged molecules (MTSET and TEA) could simultaneously occupy the outer vestibule. Consequently, we asked whether TEA could simultaneously occupy the outer vestibule with an MTS conjugate of TEA (triethylammonium ethylmethanethiosulfonate; TEA-MTS), which contains a TEA moiety attached to a methanethiosulfonate chain. We tested this in two ways. First, we examined whether

TEA interfered with the ability of TEA-MTS to modify the channel (Fig. 3). A 3-min application of TEA-MTS irreversibly blocked the channel by 56.8 ± 1.9% ($n = 13$; Fig. 3, *shaded circles*). In another set of cells, 100 mM TEA was applied before and during TEA-MTS application (Fig. 3, *black circles*). TEA alone blocked the channel by 82%. TEA-MTS was then applied for 3 min (in the presence of TEA) and then both TEA-MTS and TEA were removed. Channels were irreversibly blocked by only 35.5 ± 2.6% ($n = 5$). Thus, TEA partially protected the channel from modification by TEA-MTS. (Note also that application of TEA-MTS produced no change in steady-state block of the channel by TEA, which indicates that binding of TEA-MTS did not influence TEA potency.) These results suggest that, in contrast to the results with MTSET, TEA could not cooccupy the outer vestibule with TEA-MTS. Importantly, the ability of TEA to interfere with modification of C380 also indicates that the MTS reagents accessed the C380 residue via the conduction pathway. (In the control experiment, application of TEA-MTS to Kv2.1 K382V, which did not contain the cysteine at position 380, produced absolutely no irreversible block; data not shown.)

The second test of this hypothesis is illustrated in Fig. 4. Both MTSET and TEA-MTS are positively charged molecules. Consequently, the above conclusions about simultaneous occupancy (or lack thereof) make the following prediction. If TEA blocks channels that contain an MTSET covalently attached within the outer vestibule, TEA potency should be reduced in MTSET-modified channels due to electrostatic repulsion. Conversely, if TEA cannot occupy TEA-MTS-modified channels, TEA will only block channels that are not modified by TEA-MTS. Therefore, TEA potency should be unchanged after exposure to TEA-MTS. Fig. 4 *A* illustrates that TEA potency was indeed reduced in channels modified by MTSET (these results are consistent with previously reported data; Pascual et al., 1995). However, TEA potency was unaffected when channels were modified by a 3-min exposure to TEA-MTS, as predicted if TEA and TEA-MTS could not cooccupy the outer vestibule.

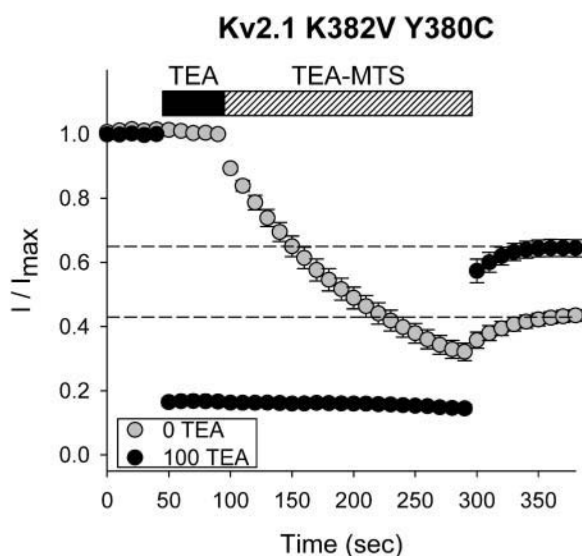

FIGURE 3 External TEA interfered with modification by TEA-MTS. The experimental protocol was the same as for Fig. 2: TEA-MTS was applied by itself (*shaded circles*; $n = 13$) or with prior and simultaneous application of 100 mM TEA (*black circles*; $n = 5$).

How far is the TEA binding site from C380?

The observations that 1), both MTSET and TEA could simultaneously occupy the outer vestibule; and 2), TEA did not interfere with modification of C380, suggested that the side chain of C380 was quite peripheral to the central core of the conduction pathway. One possible explanation for the TEA interference with TEA-MTS but not MTSET modification is that TEA-MTS, but not MTSET, interacted with the TEA binding site on its way to accessing the cysteine. Alternatively, because MTSET and TEA-MTS are relatively linear molecules, it may be that the shorter MTSET just could not reach the TEA binding site. In an attempt to distinguish these two possibilities, we examined the ability of TEA to interfere with two other positively charged MTS reagents, which were similar in structure but longer than MTSET.

Fig. 5 *A* illustrates the structures and calculated lengths (obtained using ChemDraw Pro 8.0, CambridgeSoft, Cambridge, MA) of MTSET, TEA-MTS, and two other MTS reagents, 3-(trimethylammonium)propyl methanethiosulfonate (MTSPT) and 4-(trimethylammonium)butyl methanethiosulfonate (MTSBT) (note that the terminal moiety of MTSET, MTSPT, and MTSBT are similar to tetramethylammonium, which does not block K^+ channels; see Fig. 7). Fig. 5 *B* illustrates the timecourse of modification of C380 by MTSPT in the absence and presence of 100 mM TEA. As with MTSET, 80% occupancy of the pore by TEA had absolutely no impact on the ability of the ~8.1-Å long MTSPT to modify C380. Moreover, modification by MTSPT was associated with a decrease in TEA potency (Fig. 5 *B*, *black circles*). Fig. 5 *C* illustrates the results obtained with MTSBT, which was one carbon longer than MTSPT. Similar to TEA-MTS, TEA interfered with the ability of MTSBT to modify C380. Also similar to TEA-MTS, modification by MTSBT did not influence TEA potency (Fig. 5 *C*, *black circles*). These results support the conclusion that the ability of TEA to interfere with modification of C380 by TEA-MTS, but not MTSET, was due largely to chain length, rather than a special ability of TEA-MTS to interact with the TEA binding site.

Modification of a more externally located cysteine in Kv2.1

The experiments above demonstrated that the cysteine at position 380 did not directly coordinate TEA. We next tested

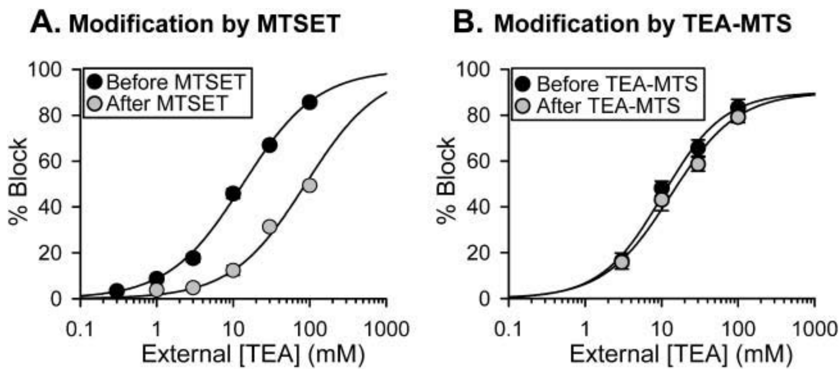

FIGURE 4 External TEA potency after modification of C380 by MTSET or TEA-MTS. (*A*) Black circles illustrate TEA concentration dependence without MTSET treatment (data are identical to those shown in Fig. 1). Shaded circles illustrate TEA concentration dependence after a 3-min preincubation with 2 mM MTSET. The IC_{50}s for block by TEA, calculated from the best fits to Eq. 1, were 13.4 mM without MTSET and 88.7 mM after MTSET preincubation. Essentially identical results were obtained when an alternative protocol, used for panel *B* experiments, was used (data not shown). (*B*) TEA concentration dependence was determined in the absence of TEA-MTS treatment (*black circles*). Then, on the same cells, 2 mM TEA-MTS was applied for 3 min, and the TEA concentration dependence was determined again. Experiments in panel *B* were done with 0 mM external K^+. All data points in panels *A* and *B* represent the mean ± SE of 3–4 cells.

the hypothesis that TEA blocked the channel by occupying a location near the outer-vestibule K^+ binding site.

In Kv2.1, a lysine at position 356, located in the turret of the outer vestibule, interferes with the ability of K^+ to interact with an outer-vestibule K^+ binding site (Consiglio et al., 2003). At high [K^+], a positive charge at this position also reduces TEA potency (Bretschneider et al., 1999; Immke et al., 1999). When Kv2.1 channels are recorded under low [K^+] conditions, this residue apparently reorients such that it completely prevents TEA from blocking the channel (Immke and Korn, 2000). Neutralization of position 356 abolishes all of these effects (Immke et al., 1999; Immke and Korn, 2000; Consiglio et al., 2003). Modification of C356 by MTSET, which produces a lysine-like side chain, produces a lysine-like interference with the outer-vestibule K^+ binding site (Consiglio et al., 2003). These results place the position 356 lysine side chain near the K^+ binding site. Consequently, we hypothesized that if TEA were occupying a location at or near the outer-vestibule K^+ binding site, TEA application would interfere with the ability of MTSET to modify the cysteine at position 356. The experiments in Fig. 6 tested this hypothesis.

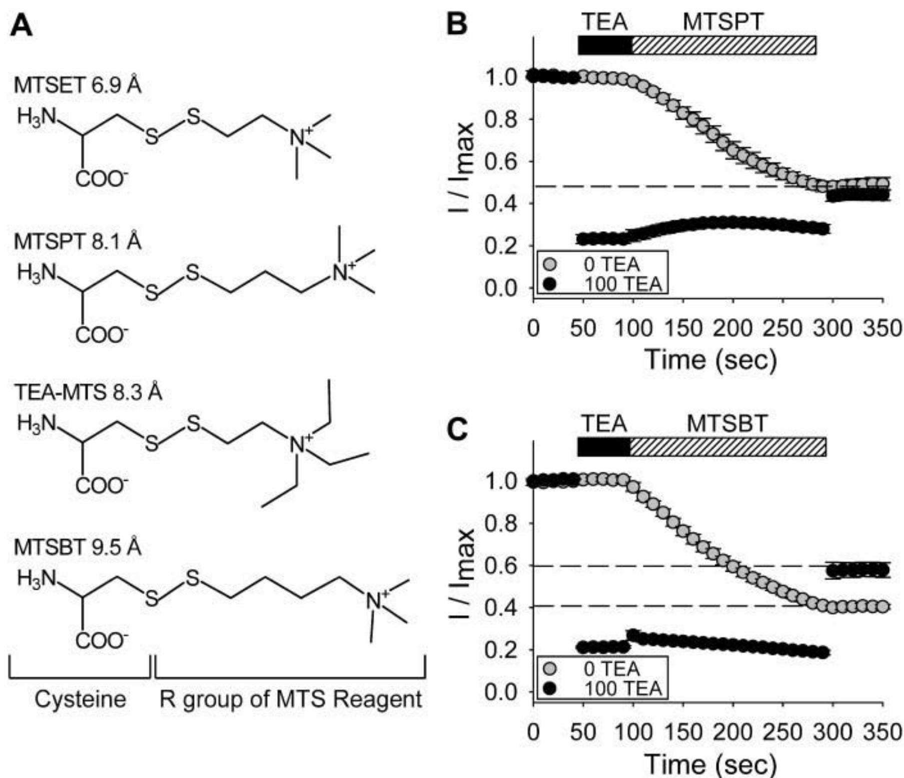

FIGURE 5 Influence of external TEA on modification of C380 by MTSPT and MTSBT. (*A*) Illustration of structures of four MTS reagents. Length of *R* group is shown after the name of the compound. (*B* and *C*) Modification of C380 by MTSPT (*B*) and MTSBT (*C*) in the absence and presence of 100 mM external TEA. Experimental protocol are as in Fig. 2. Data points represent mean ± SE of three cells.

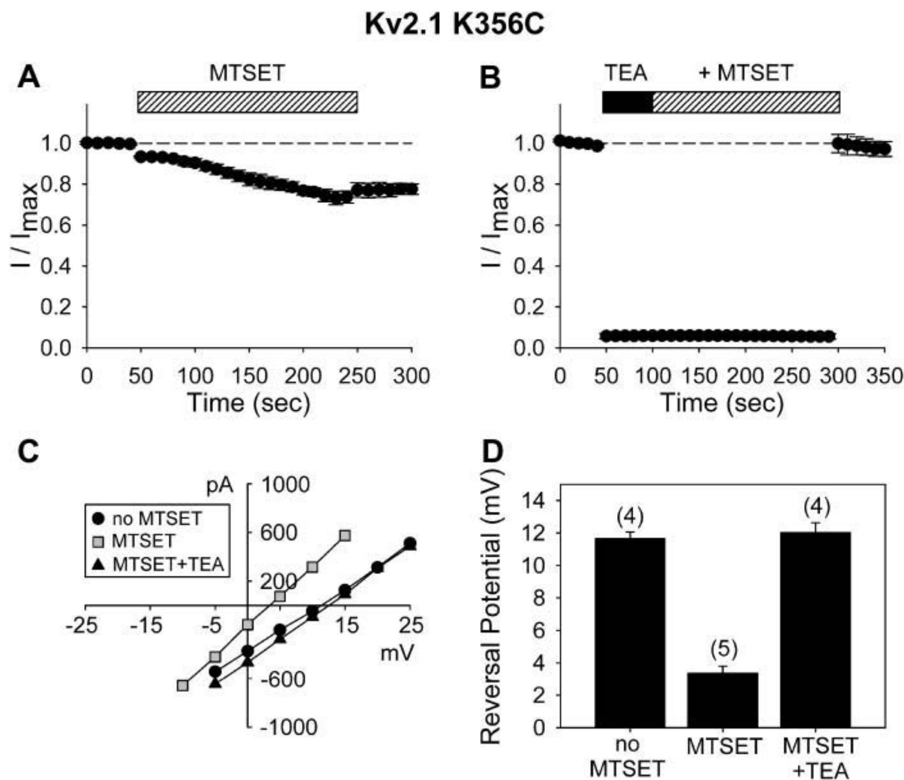

FIGURE 6 External TEA protects C356 from modification by MTSET. (*A*) Irreversible block of current after exposure of Kv2.1 K382V K356C to 2 mM MTSET ($n = 4$). (*B*) Prior and simultaneous application of 100 mM TEA completely prevented irreversible block of channel by MTSET ($n = 4$). (*C*) I-V relationships constructed under three different conditions, with 125 mM internal and external Na^+ plus 1 mM external K^+. In the absence of MTSET treatment, currents reversed at 11.4 mV. After a 3-min preincubation with 2 mM MTSET, the reversal potential shifted to 3.4 mV, which reflected modification of C356 (see Consiglio et al., 2003). Simultaneous preincubation with 100 mM TEA completely prevented the reversal potential shift associated with MTSET modification of C356 (E_{rev} = 12.3 mV). Data are illustrated from three individual cells, one in each condition. (*D*) Composite data from several cells, examined as in panel *C* (value in parentheses represents number of cells tested). The reversal potential values from cells not exposed to MTSET and from cells pretreated simultaneously with TEA and MTSET were statistically identical (11.7 ± 0.4 mV and 12.0 ± 0.6 mV, respectively).

Fig. 6 *A* illustrates time-dependent, irreversible block of Kv2.1 K356C by MTSET. Application of 4 mM MTSET for 200 s irreversibly reduced current magnitude by 23.0 ± 3.1% ($n = 3$). In a different set of cells, 100 mM TEA was applied before and during application of MTSET (Fig. 6 *B*). By itself, 100 mM TEA blocked the channels by 94.2 ± 1.4% ($n = 4$). After 3 min of MTSET application in the presence of TEA, both MTSET and TEA were removed. Current magnitude returned to the pretreatment level, which demonstrated that MTSET did not modify the channels. These data indicate that the presence of TEA completely prevented MTSET from accessing the cysteine at position 356.

Because of the slow time course and limited extent of block by MTSET in the experiment shown in Fig. 6 *A*, we performed a second assay for MTSET modification of C356. The lysine side chain at position 356 in the wild-type channel has two influences on permeation: it reduces conductance (Consiglio et al., 2003; Trapani and Korn, 2003) and produces a change in the ratio of K^+ and Na^+ permeability (Consiglio et al., 2003). Both of these effects are due to interference with the interaction of K^+ with the outer-vestibule K^+ binding site. The lysine side chain reduces the ability of K^+ but not Na^+ to access the binding site, which reduces both P_K/P_{Na} and the ability of K^+ to flow into and out of the selectivity filter. This effect can be precisely mimicked by MTSET modification of C356 (Consiglio et al., 2003).

In the presence of symmetrical 125-mM Na^+ plus 1-mM external K^+, currents through unmodified Kv2.1 K356C reversed at 11.7 ± 0.4 mV ($n = 4$; Fig. 6, *C* and *D*). After a 3-min preincubation with MTSET, the same protocol produced currents that reversed at 3.4 ± 0.4 mV ($n = 5$; Fig. 6, *C* and *D*), which indicates that the MTSET-modified cysteine interfered with the ability of K^+ to permeate the channel. To test whether TEA interfered with MTSET modification of C356, we included 100 mM TEA during the MTSET preincubation period. The reversal potential after preincubation with both TEA and MTSET was 12.0 ± 0.6 mV ($n = 4$; Fig. 6, *C* and *D*), which was statistically identical to the reversal potential in the channels not exposed to MTSET at all. Thus, in two independent assays, TEA completely prevented MTSET from accessing C356. (In the control experiment, MTSET had no influence on the reversal potential of Kv2.1 K356G K382V, which lacked substituted cysteines in the outer vestibule; data not shown.)

Tetramethylammonium (TMA)

It has been repeatedly observed that TMA does not block K^+ channels (Villarroel et al., 1988; Heginbotham and MacKinnon, 1992; Jarolimek et al., 1995). Although the difference between TEA and TMA could be attributed to differences in dehydration energy (cf. Jarolimek et al., 1995), whether TMA interacts in any way with the pore is unknown. Recently, a theoretical study utilizing the structure of KcsA suggested that TMA did indeed interact with the pore, and in fact interacted with the equivalent of the 449 residue

(Luzhkov et al., 2003). However, due to its smaller size, it was proposed to interact with only a single amino acid at a time, and apparently due to this difference in the way it bound, did not block the channel (Luzhkov et al., 2003).

We reexamined whether TMA interacted in some way with the TEA binding site. As previously observed, TMA by itself, at concentrations up to at least 100 mM, had absolutely no effect on currents through Kv2.1 (Fig. 7). In addition, block by 3 mM TEA was identical in the presence and absence of TMA (Fig. 7). Thus, there is no functional indication that TMA interacts with the outer vestibule. Moreover, these data conclusively demonstrate that, at concentrations up to 100 mM, TMA does not compete with TEA at its binding site.

Modification of C449 in *Shaker* by MTSET

In *Shaker*, introduced cysteines at position 448, just external to the selectivity filter, cross-link during inactivation (Liu et al., 1996). Along with several other observations (Yellen et al., 1994; Kiss and Korn, 1998; Kiss et al., 1999), this result solidified the idea that the pore near the selectivity filter constricts during the inactivation process. Cysteines introduced at position 449 also reorient during slow inactivation (Yellen et al., 1994). This movement is associated with an increase in Cd^{2+} affinity, which was best interpreted as Cd^{2+} being coordinated by more cysteine side chains in the inactivated state than in the open state. Together, these observations suggested that the side chains of residues 449 move closer together during inactivation. These findings fit well with a model whereby TEA slowed inactivation via a foot-in-the-door mechanism, with the door being the four position 449 residues (Yellen, 1998). Consequently, it was of interest to examine the influence of TEA on MTSET modification of C449 in *Shaker*, with three questions in mind. First, does TEA influence cysteine modification similarly in *Shaker* as in Kv2.1? This would generalize the observation that cysteine side chains at this position 1), were not directly involved in coordinating TEA; and 2), were not at a location within the conduction pathway where TEA in the outer vestibule could interfere with their modification. Second, did TEA slow inactivation by a foot-in-the-door mechanism? This mechanism predicts that TEA cannot bind to inactivated channels (cf. Yellen, 1998). Third, if, as in Kv2.1, the position 449 cysteines in *Shaker* are peripheral to the central axis of the conduction pathway (as suggested by the inability of TEA to interfere with C449 modification), do they move to a more central position during inactivation?

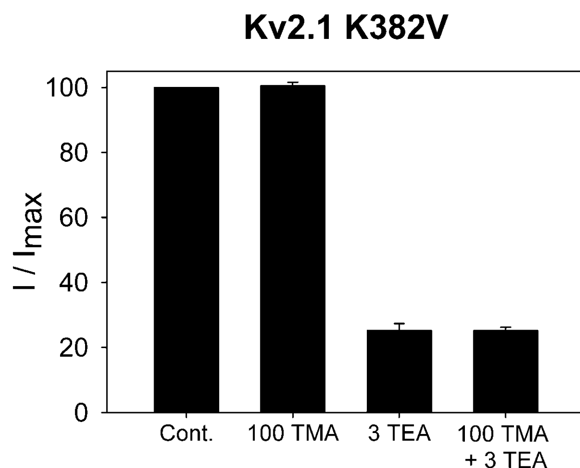

FIGURE 7 TMA has no functional effect on the channel. 100 mM TMA was applied in the absence or presence of 3 mM TEA. Data represent the mean ± SE of three cells in each condition.

Fig. 8, *A* and *B*, illustrates an experiment with *Shaker* that was analogous to that shown in Fig. 2 *C* for Kv2.1. Channels were opened briefly to minimize inactivation. Modification of C449 thus represented the access of MTSET to C449 with channels in the open (or closed) state. Application of 0.3 mM MTSET for 150 s irreversibly blocked currents by 84.1 ± 1.9% ($n = 4$; Fig. 8 *B*, *shaded circles*). In another set of cells, channels were bathed in 100 mM TEA before and during application of MTSET. TEA alone blocked channels by ~80%. In the presence of TEA, irreversible block by MTSET was statistically identical to that observed in the absence of TEA (80.8 ± 3.2%, $n = 3$; Fig. 8 *B*, *black circles*). Thus, as with C380 in Kv2.1, C449 in open *Shaker* channels was not in a position within the pore where TEA could interfere with side-chain modification.

We next examined the ability of TEA to prevent modification of C449 in inactivated channels. Fig. 8, *C* and *D*, illustrates currents evoked by 14-s depolarizations to 0 mV, at 1-min intervals. Three traces are shown. The first illustrates the evoked current with no change in external solution. In 14 s, channels inactivated by 95.8 ± 0.4% ($n = 4$). During the second evoked current (which is superimposed on the first current trace), MTSET was applied 8 s after the start of a depolarization to 0 mV (see *arrow*), at which time currents had inactivated by 94.7 ± 0.2% ($n = 3$). MTSET application lasted for 6 s, ending simultaneously with the repolarization at the end of the second trace. The third trace (smaller current magnitude) was evoked 1 min later. The 6-s application of MTSET irreversibly reduced peak current magnitude by ~60% (Fig. 8 *E*, *circles*) and increased the time constant of inactivation by ~60% (Fig. 8 *F*). The change in inactivation time constant indicates that MTSET-modified channels were carrying current. Fig. 8 *D* illustrates a similar experiment to Fig. 8 *C*, except that 100 mM TEA was applied for 1 s before MTSET application, and continued for the duration of MTSET application. TEA had no effect on the ability of MTSET to modify the channel; both current magnitude and inactivation time constant were influenced identically by MTSET when applied in the presence and absence of TEA (Fig. 8, *E* and *F*).

Two possible explanations could account for these results. One possibility was that, whether or not the cysteines moved during inactivation, they still were not at a location within the

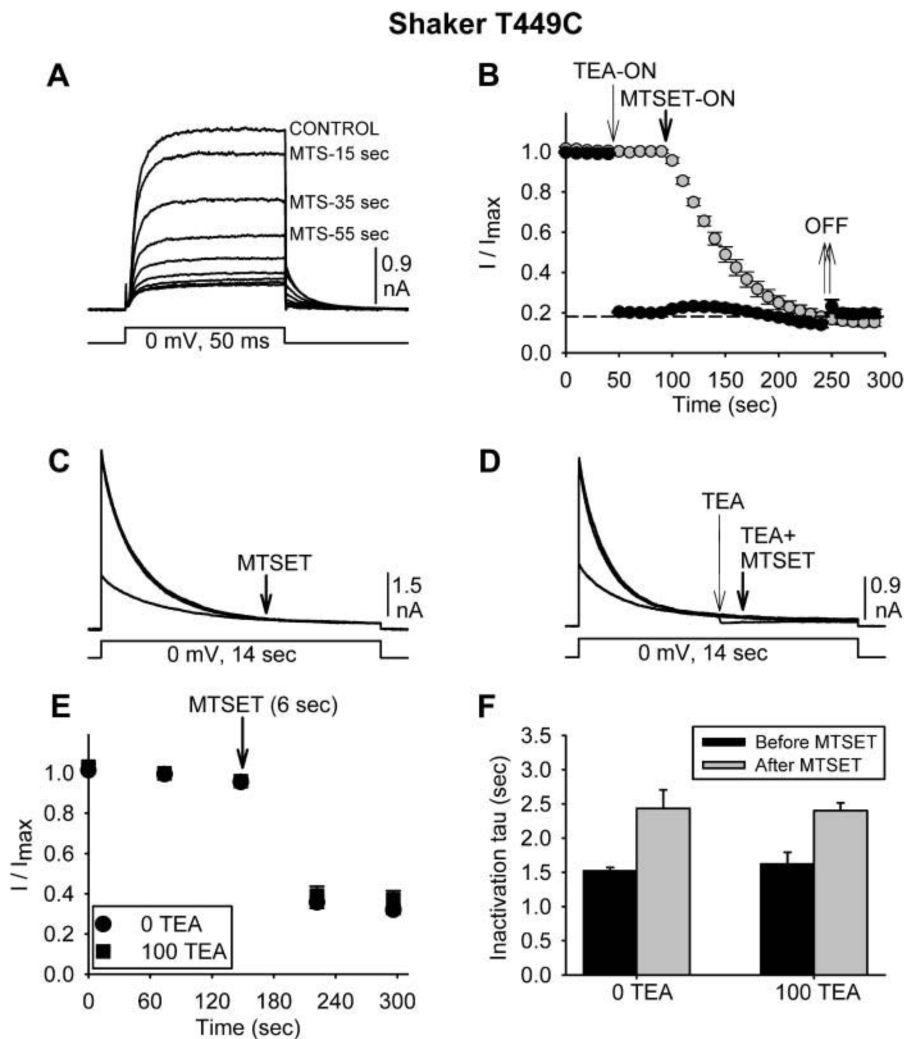

FIGURE 8 Modification of C449 in *Shaker* by MTSET. (*A*) Outward currents after 50-ms depolarizations to 0 mV in control and after application of 300 μM MTSET for various durations. (*B*) Time course of current block by MTSET in the absence (*shaded circles*; $n = 4$) and presence (*black circles*; $n = 3$) of 100 mM external TEA. Protocol was the same as in Fig. 2, except that MTSET was applied for 150 s. (*C*) Three superimposed currents are shown, evoked by depolarization to 0 mV for 14 s. In the first trace, no MTSET was applied. In the second trace, 300 μM MTSET was applied 8 s after the start of the depolarization (*arrow*) and left on the cell until the end of the stimulus, for a total application duration of 6 s. The third (smaller) current was recorded 10 s after MTSET removal. (*D*) Protocol was identical to that in panel *C*, except that 100 mM TEA was applied 1 s before and for the duration of MTSET application. (*E*) Plot of normalized peak current, measured from five consecutive traces. MTSET was applied for 6 s during the third trace, in the absence (*circles*; $n = 3$) and presence (*squares*; $n = 3$) of TEA. (*F*) Plot of the change in the inactivation time constant by MTSET in the absence and presence of TEA, obtained from the same data as in panel *E*. The effect of MTSET was statistically identical under the two conditions (2.4 ± 0.3 s and 2.4 ± 0.1 s, respectively; $n = 3$ for each).

conduction pathway where TEA could interfere with side-chain modification. Alternatively, TEA may not have occupied the outer vestibule in inactivated channels. To test this, we examined the ability of TEA to interfere with modification of C449 by TEA-MTS.

Modification of C449 in *Shaker* by TEA-MTS

Experiments with TEA-MTS (Fig. 9) were conducted identically to those with MTSET (Fig. 8). TEA-MTS was applied to open channels in the absence and presence of 100 mM TEA (Fig. 9, *A* and *B*). Application of 0.3 mM TEA-MTS for 150 s irreversibly blocked currents by 61.5 ± 1.8% ($n = 3$; Fig. 9, *A* and *B*, *shaded circles*). In a second set of experiments, cells were bathed in 100 mM TEA before and during TEA-MTS application. After 150-s exposure to TEA-MTS in the presence of TEA, currents were irreversibly blocked by just 33.6 ± 1.7% ($n = 3$; Fig. 9 *B*). This is consistent with the observations made in Kv2.1 that TEA and TEA-MTS could not simultaneously occupy the channel.

Fig. 9 *C* illustrates the effect of TEA-MTS when applied after inactivation. TEA-MTS was applied for 6 s, starting at 8 s into a 14-s depolarization (Fig. 9 *C*). On the subsequent depolarization, current magnitude was irreversibly reduced by 61.4 ± 1.8% ($n = 3$) and the inactivation time constant increased by ~58% (Fig. 9, *C*, *E*, and *F*). In Fig. 9 *D*, TEA was applied 1 s before and for the duration of TEA-MTS application. After this 6-s exposure to TEA-MTS in the presence of TEA, currents were irreversibly blocked by just 30.7 ± 4.1% ($n = 4$; Fig. 9 *E*) and the inactivation time constant was increased by just 35.2 ± 1.6% ($n = 4$; Fig. 9 *F*). These results demonstrate that TEA bound to the inactivated state of the channel with approximately the same affinity as to the open state. Consequently, the previously observed lack of interference by TEA with MTSET modification of C449 in inactivated channels (Fig. 9) was not due to the inability of TEA to bind to inactivated channels.

In the experiments of both Figs. 9 and 10, a 6-s application of MTS reagent to inactivated channels reduced current by ~60% (see panel *E*), whereas a 6-s application to channels in

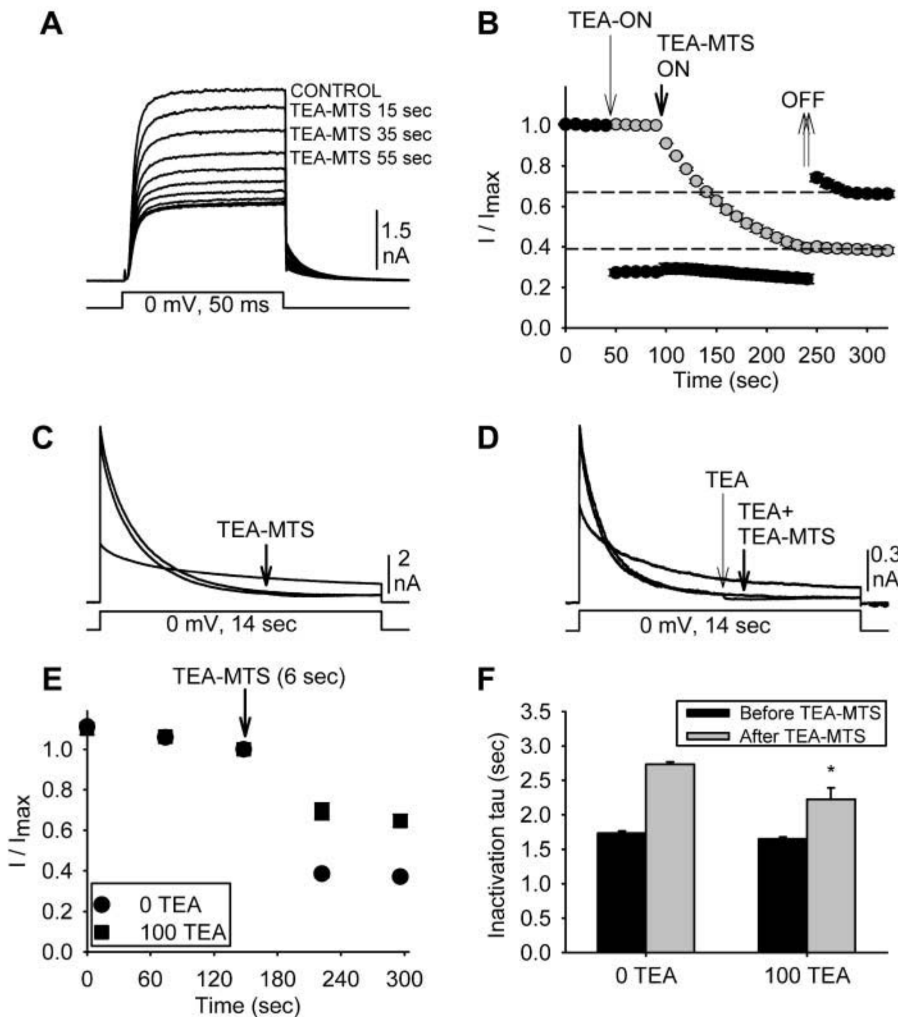

FIGURE 9 Modification of C449 in *Shaker* by TEA-MTS. Protocols and plot construction in each panel are identical to those in Fig. 8, except that C449 was modified by TEA-MTS. Current block (panels *B* and *E*) and change in inactivation time constant (panel *F*) were significantly reduced by the simultaneous presence of TEA during TEA-MTS exposure.

a combination of open and closed states (see panel *B*) resulted in much less current reduction. These data suggest that, consistent with the findings of Yellen et al. (1994), the cysteine at position 449 changed exposure during inactivation. However, the inability of TEA to protect against MTSET modification of inactivated channels indicates that the C449 side chains did not move into the central axis of the conduction pathway during inactivation. Moreover, these data suggest that TEA did not slow inactivation via a foot-in-the-door mechanism, which would require that once the door was closed, TEA could not block (see Yellen, 1998).

Modification of C425 in *Shaker*

The results above demonstrated that, as in Kv2.1, TEA binding in *Shaker* did not involve direct coordination by the C449 residue. We next sought to determine whether TEA interfered with modification of a cysteine placed at position 425 (equivalent to position 356 in Kv2.1). Without pretreatment with dithiothreitol (DTT), MTSET had no effect on *Shaker* F425C (data not shown). This represents a curious difference in the resting oxidation state of this residue in *Shaker* and Kv2.1.

After a 3-min pretreatment with 10 mM DTT, application of 300 μM MTSET irreversibly reduced current magnitude in *Shaker* F425C by 15% (Fig. 10 *A*, *shaded circles*, and *B*). When applied in the presence of 100 mM TEA, MTSET block associated with the cysteine at position 425 was completely prevented (Fig. 10 *A*, *black circles*, and *B*). Thus, as in Kv2.1, TEA occupied a site in the outer vestibule that interfered with MTSET modification of the cysteine located in the turret.

DISCUSSION

Mutation of position 449 in *Shaker*, and the equivalent mutation in several other channels, produces the largest effect on TEA potency of any mutation in the outer vestibule.

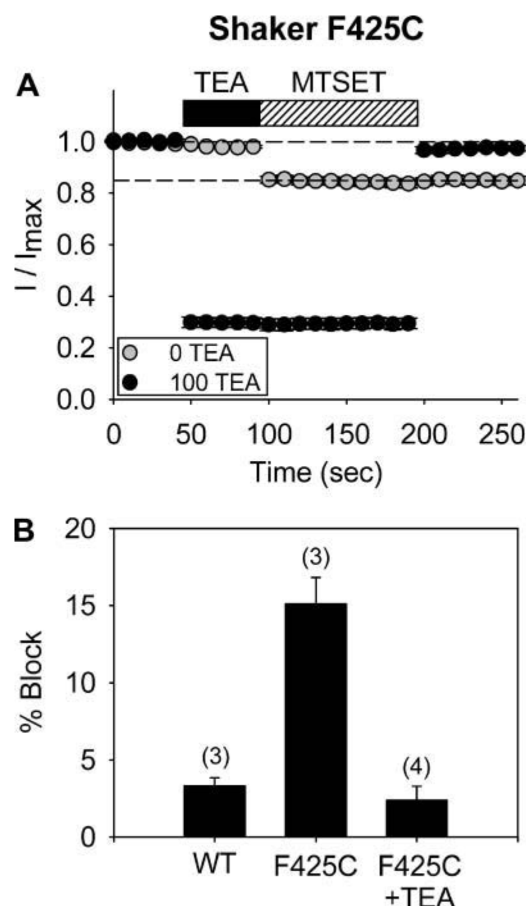

FIGURE 10 TEA interferes with MTSET modification of C425 in *Shaker*. (*A*) MTSET (300 μM) was applied in the absence (*shaded circles*) and presence (*black circles*) of 100 mM TEA. Data points represent mean ± SE of 3–4 cells. (*B*) Composite data for three conditions, including the wild-type channel (*WT*), which lacked the cysteine in the outer vestibule. All cells were preincubated for 3 min with 10 mM DTT.

Indeed, the 30-fold effect of the T449Y mutation in *Shaker*, and the contribution of this residue from all four subunits to TEA potency (Heginbotham and MacKinnon, 1992, Kavanaugh et al., 1992), were largely responsible for the concept that the position 449 residue formed the TEA binding site. In contrast to the results with *Shaker*, the reverse mutation in Kv2.1 reduced TEA potency by just fivefold. Thus, a significant change in amino acid properties at position 380 can have a relatively small impact on TEA potency. Indeed, the fivefold effect of the Y380T mutation is no greater than that produced by several other mutations in the outer vestibule, including K382V and K356G (Immke et al., 1999).

Lack of specific coordination by the side chains at position 380/449

Up to 90% occupancy of the outer vestibule of either Kv2.1 or *Shaker* by TEA produced absolutely no interference with the ability of MTSET or MTSPT to covalently modify the cysteine at position 380/449. The MTS reagents appear to access the C380 residue via the conduction pathway, as evidenced by the finding that TEA did interfere with modification of C380 by the larger MTS reagents, TEA-MTS and MTSBT. Moreover, Figs. 2 and 4 indicated that TEA and MTSET can simultaneously occupy the outer vestibule. The conclusion from these observations is that MTSET can enter the outer vestibule and access the C380 side chain unhindered even when TEA is bound to the channel. Examination of the KcsA structure, in which the primary residues were substituted with those of Kv2.1, suggests that this observation should reasonably be expected (Fig. 11 *A*). Although extrapolation from KcsA to voltage-gated channels should be treated with caution, the structural data (Fig. 11 *A*) suggest that the reactive cysteine side chain is facing somewhat down and away from the center of the conduction pathway (it is, nonetheless, exposed; the arrows in Fig. 11 *A* point to the reactive cysteine side chains). Fig. 11 *B* illustrates the structure with a tyrosine at position 380. In contrast to the cysteine side chains, the tyrosine side chains are facing up and toward the center of the conduction pathway. This figure all the more dramatically illustrates the nature of the involvement of the position 380/449 side chain in TEA binding. Certainly, the 380/449 residue can have a profound influence on TEA potency in some channels. However, despite the markedly different configuration of the tyrosine and cysteine side chains, the Y380C mutation produced just a modest change in TEA potency. The observations that the Y380T mutation had minimal impact on TEA potency (Fig. 1), that TEA blocks with reasonably high potency in both the Y380T and Y380C channels (Fig. 1), and that TEA in the outer vestibule did not interfere with MTSET modification of cysteines at this position, demonstrate that ''the TEA binding site'' does not consist of a cage formed by the four side chains of the position 380 (or 449) residue.

Where does TEA bind?

In *Shaker*, TEA block is voltage-dependent with the wild-type threonine at position 449 and voltage-independent with a tyrosine at this position (Heginbotham and MacKinnon, 1992). Together with other experiments, this led to the proposition that TEA bound closer to the selectivity filter with the threonine and farther from the selectivity filter with the tyrosine (Heginbotham and MacKinnon, 1992; Molina et al., 1997). This proposition fit well with the fact that the tyrosine side chain was longer than the threonine side chain. Following another line of inquiry, TEA was proposed to slow inactivation by occupying a site at the mouth of the selectivity filter, between the position 449 residues, and thus prevent a constriction at the outer edge of the filter (see Yellen, 1998). Together with much other data described previously, these interpretations led to a model whereby

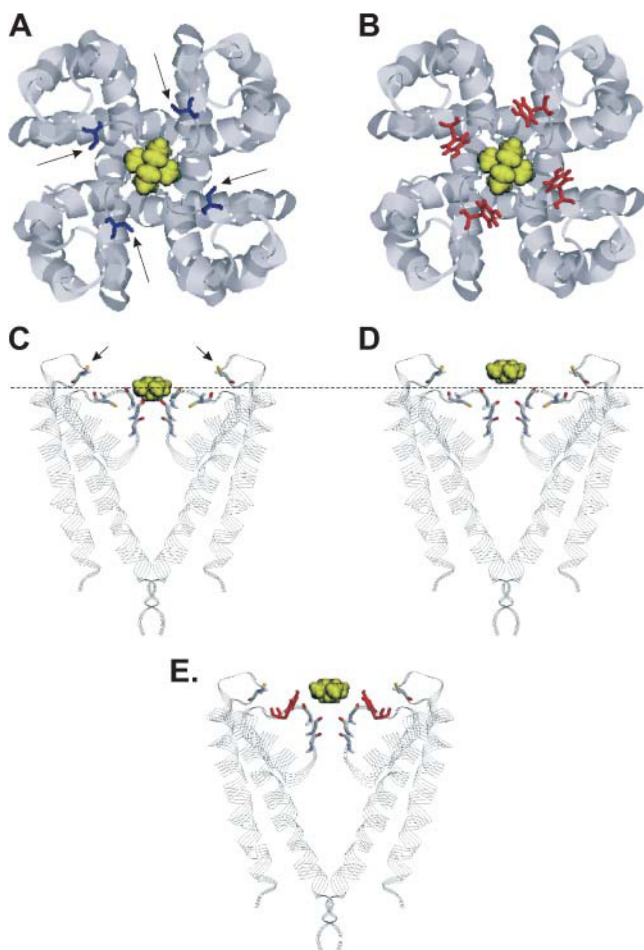

FIGURE 11 Models of TEA interaction with the outer vestibule. All models were created with Swiss-PdbViewer and edited with RasTop, using the KcsA backbone, threaded with Kv2.1 residues and minimized. (*A* and *B*) Top-down view of the channel with cysteine residues (panel *A*, *blue*) or tyrosine residues (panel *B*, *red*) illustrated at position 380. Arrows in panel *A* point to the reactive sulfur group on the cysteine side chain. TEA is shown in the conduction pathway (*yellow*). Note that the cysteine reactive side chains are pointed down and away from the central axis of the conduction pathway (*A*), whereas the tyrosine side-chain aromatics are pointed up and toward the center of the conduction pathway (*B*). (*C*) Previous model of TEA binding to the channel. View of channel is from the side, with two of four subunits shown. For illustration purposes, the channel is depicted with cysteine residues at position 356 in the turret and at position 380. The selectivity filter GYG residues are also illustrated as stick representations. Arrows point to reactive cysteine side chain at position 356. (*D*) Proposed new model, wherein TEA is stabilized more externally in the outer vestibule and placed so as not to act as a foot in the door in a constricting selectivity filter. (*E*) Same as panel *D*, except that tyrosine residues (*red*) are illustrated at position 380. The TEA molecule in these figures was reproduced from *The Journal of General Physiology*, 2001, 118:207–217, by copyright permission of The Rockefeller University Press.

TEA acted as a plug at the outer mouth of the selectivity filter (Molina et al., 1997; Yellen, 1998; see Fig. 11 *C*). However, recent simulation data using KcsA as a model suggested just the opposite. In contrast to the prevailing model, TEA appeared to bind farther from the selectivity filter when the tyrosine at position 82 (Kv2.1 position 380) was replaced by a threonine (Crouzy et al., 2001). In support of this change in interpretation, the voltage dependence of TEA block was found not to be due to the location of TEA relative to the selectivity filter (Spassova and Lu, 1999; Thompson and Begenisich, 2003). Finally, our experiments argue against the idea that TEA is the foot in the door formed at or near position 449 residues, because 1), TEA can bind to fully inactivated channels (Fig. 9); and 2), TEA does not prevent MTSET modification of C449 in fully inactivated channels. Taken together, these results suggest that TEA occupies a space more external to the selectivity filter than previously believed (see Fig. 11 *D*).

We recently demonstrated the presence of a K^+ binding site in the outer vestibule, (Consiglio et al., 2003), which seems likely to be the hydration/dehydration site described by Zhou et al. (2001). Positively charged amino acids at position 356 interfere with the ability of K^+ to interact with this site (Consiglio et al., 2003), and also interfere with the ability of TEA to block the channel (Bretschneider et al., 1999, Immke et al., 1999). Moreover, in the Kv2.1 channel, it appears that the orientation of position 356 residues can be modulated, and in one orientation, K356 can completely prevent TEA from blocking the channel (Immke and Korn, 2000).

In both Kv2.1 and *Shaker*, the presence of TEA in the outer vestibule completely prevented the MTS reagents from modifying a cysteine at the outer-vestibule position 356 in Kv2.1 (425 in *Shaker*; Figs. 6 and 10). Thus, with either a tyrosine or threonine at position 380/449, TEA is located superficially enough to prevent MTSET from accessing the position 356/425 cysteine, whose reactive side chain appears to point away from the selectivity filter (see arrows, Fig. 11 *C*). These experiments support the conclusion that TEA binds at a more external location in the outer vestibule, possibly in the vicinity of the outer-vestibule K^+ binding site (see Fig. 11 *D*).

What is the nature of the TEA binding site?

Examination of the primary amino acid sequence of Kv channels, mapped onto the KcsA backbone structure, suggests that there are no other amino acid side chains that would form a cage around TEA. The fundamental architecture of the outer vestibule, which widens in a somewhat funnel-like fashion external to the selectivity filter (Doyle et al., 1998), supports this contention. Based on all of the aforementioned data, we suggest that TEA is stabilized in the aqueous outer vestibule, and depends not on a discrete coordination by particular residues but rather on the local environment created by the topography and characteristics of the surrounding protein surface. Whereas there is compelling evidence that the nature of the TEA binding site changes somewhat with tyrosines at the 380/449 position, the results of MD simulations also suggest that TEA is not ‘‘inserted tightly’’ between the aromatic side

chains of these tyrosines (Crouzy et al., 2001). Moreover, it is clear from our data that TEA can be quite stable in the outer vestibule without these tyrosines (indeed, in Kv2.1 Y380C with both outer-vestibule lysines neutralized, TEA blocks with an IC_{50} under 4 mM; data not shown). This view of stabilizing TEA in an aqueous environment is compatible with molecular dynamics evidence that TEA undergoes rapid rotational, as well as lateral, movements (Crouzy et al., 2001). Moreover, the simulations of Crouzy et al. suggested that TEA was more hydrated when a threonine was at KcsA position 82, consistent with TEA stabilization in an aqueous environment. This model of TEA binding is also consistent with the observation that TEA is an extremely fast channel blocker (Spruce et al., 1987).

Clearly, the tyrosines at position 380/449 have an impact on TEA potency. The simulation data suggest that these four tyrosines, which project aromatic side chains into the outer vestibule, may draw TEA closer to the selectivity filter, and perhaps promote additional dehydration of TEA as it interacts with the tyrosines (Crouzy et al., 2001). However, it appears that the tyrosines do not interact with TEA via cation-pi interactions (Crouzy et al., 2001), as had been proposed (Heginbotham and MacKinnon, 1992; Kumpf and Dougherty, 1993).

Our data indicate that these four tyrosines do not create ''a TEA binding site'', but rather alter the environment in which TEA already binds in a way that promotes stronger binding. Although TEA completely blocks the pore with a variety of residues at position 380/449, it does not appear to do so by fitting as a ''tight'' plug, as depicted in several models (Molina et al., 1997; Yellen, 1998). Rather, our data, combined with that of Crouzy et al. (2001), suggest that it is stabilized in the outer vestibule, somewhat external to the selectivity filter, and that the nature of the residue at position 380/449 influences the stability by contributing to the environment of the TEA binding site. Fig. 11 *E* presents a cartoon similar to Fig. 11, *C* and *D*, except that a tyrosine is shown at position 380. Whereas it appears from the cartoon that TEA could be well-coordinated by the tyrosines, this is not necessary. TEA occupies the outer vestibule with or without the tyrosines. With the tyrosines, however, TEA is surrounded by aromatic side chains, which increase TEA stability by an amount that depends on the structural composition of each channel's outer vestibule.

An important challenge that remains is to understand how TEA is selectively stabilized in the outer vestibule. Based on the crystal structure of KcsA, Roux and MacKinnon (1999) elegantly described how a K^+ can be stabilized in the central aqueous cavity of the pore. A similar structural basis does not exist for selective stabilization of TEA in the outer vestibule. This problem is particularly intriguing for the Kv2.1 channel, which has eight exposed lysines in the outer vestibule, but which still has a respectable affinity for TEA. The solution to this puzzle would undoubtedly be valuable for the rational design of nontoxin external channel blockers.

TEA block of inactivated channels in *Shaker*

As discussed in the Introduction, a great deal of previous data was consistent with the conclusion that TEA slowed C-type inactivation by binding between the C449 residues, which would prevent the constriction presumed to occur during C-type inactivation. When the larger molecule, TEA-MTS, was used, the ability of TEA to interfere with modification of C449 in inactivated channels was as good as or better than in open channels (Fig. 9). These data demonstrate that, in contrast to previous conclusions, TEA does bind to channels in the C-type inactivated state. However, even in inactivated channels, TEA did not interfere with the ability of MTSET to modify the C449 residue (Fig. 8). These observations preclude the possibility that TEA slows C-type inactivation by becoming a foot in the door formed by position 449 side chains.

An additional conclusion is supported by these data. Yellen et al. (1994) demonstrated that the position 449 residues change exposure during the inactivation process. The observation that Cd^{2+} affinity increased 45,000-fold as a result of inactivation (Yellen et al., 1994), together with the observation that cysteines placed one position deeper toward the selectivity filter cross-link during inactivation (Liu et al., 1996), led to a model whereby the position 449 residues moved close together into the central core of the conduction pathway during inactivation (see Yellen, 1998). However, if the position 449 cysteines moved centrally during inactivation, one would expect TEA in the outer vestibule to interfere with the ability of MTSET to modify the cysteines. However, this was not observed. Thus, regardless of the exact mechanism by which Cd^{2+} affinity increases, these results argue against a model whereby the position 449 cysteines move toward the center of the conduction pathway during inactivation.

Slowing of inactivation by TEA

If TEA doesn't slow inactivation by occupying space between the position 449 residues, how does it slow inactivation? We have no direct evidence that bears on this question. However, two possibilities are evident. First, it is clear that occupancy of the selectivity filter by K^+ slows C-type inactivation, and that inactivation can only proceed when a K^+ is not at some specific selectivity-filter location (Baukrowitz and Yellen, 1996; Kiss and Korn, 1998). Thus, TEA in the outer vestibule may impede the exit of K^+ from the selectivity filter and thus slow inactivation. Alternatively, the residue at position 356, which interacts with TEA, has been shown in a number of channels to influence the rate of C-type inactivation (Perez-Cornejo, 1999; Jerng and Gilly, 2002; Kehl et al., 2002). Consequently, it is possible that TEA slows inactivation via an interaction with this residue. Resolution of this issue must await future experiments.

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