The substituted cysteine accessibility method (SCAM) was used to map the external vestibule and the pore region of the ECaC-TRPV5 calcium-selective channel. Cysteine residues were introduced at 44 positions from the end of S5 (Glu515) to the beginning of S6 (Ala560). Covalent modification by positively charged MTSET applied from the external medium significantly inhibited whole cell currents at 15/44 positions. Strongest inhibition was observed in the S5-linker to pore region (L520C, G521C, and E522C) with either MTSET or MTSES suggesting that these residues were accessible from the external medium. In contrast, the pattern of covalent modification by MTSET for residues between Pro527 and Ile534 was compatible with the presence of a α-helix. The absence of modification by the negatively charged MTSES in that region suggests that the pore region has been optimized to favor the entrance of positively charged ions. Cysteine mutants at positions −1, 0, +1, +2 around Asp542 (high Ca2+ affinity site) were non-functional. Whole cell currents of cysteine mutants at +4 and +5 positions were however covalently inhibited by external MTSET and MTSES. Altogether, the pattern of covalent modification by MTS reagents globally supports a KcsA homology-based three-dimensional model whereby the external vestibule in ECaC-TRPV5 encompasses three structural domains consisting of a coiled structure (Glu515 to Tyr529), connected to a small helical segment of 15 amino acids (525PTALFSTFELFLG539) followed by two distinct coiled structures Ile540–Pro544 (selectivity filter) and Ala545–Ile557 before the beginning of S6.

The TRP ion channels form a large class of cationic channels that are related to the product of the Drosophila TRP gene. TRP channels share a similar predicted topology of six transmembrane segments in which the amino acids that link the fifth and sixth transmembrane domains line the pore region. According to the recent IUPHAR classification of ion channels, the 21 members of the TRP family can be divided by sequence homology into three subfamilies (3, 4) as short (TRPCs), long or melastatin (TRPMx), and osm-9-like or vanilloid-like (TRPVx) channels. The molecular domains that are mostly conserved among TRP channels include part of the S6 segment, ankyrin repeats in the N terminus, and a “TRP domain” in the C terminus (EWKPFAR/5), the latter being absent from TRPV channels. The TRPC and TRPV proteins have 2–4 (1–6) functional ankryin domains at positions immediately upstream of the pore are coupled to the spectrin-based membrane cytoskeleton.

TRP channels vary significantly in their biophysical properties and gating mechanisms. In contrast to other members of the TRP family, TRPV5 and TRPV6 channels show strong inward rectification, exhibit anomalous mole-fraction effect, are activated by low [Ca2+]i, and inactivated by higher [Ca2+]i (6–8). TRPV5 and TRPV6 are also highly Ca2+-selective channels with PCa/PNa > 100. In particular, ECaC-TRPV5 displays a high Ca2+ affinity with a Kd of ~2 μM (7) that is comparable to the Kd of ~1 μM for voltage-dependent CaV channels (9). A single residue in the S5-S6 linker (Asp542) was found to account for the high Ca2+ affinity of ECaC-TRPV5 (7). The absence of the aspartate residue at the equivalent position in the pore region of TRPV1–4 channels might explain, together with the presence of a lysine residue, the ~20-fold lower Ca2+ selectivity of TRPV1–4 channels (10). TRPV5 and TRPV6 channels can also form homo- and heterotetramers suggesting that they are structurally and functionally related (11).

There is currently very little structural data available on the pore architecture of Ca2+-selective TRP channels. It is possible that the four aspartate residues form an extracellular ion binding site as it has been shown for the Glu757Asp758 residues in the KcsA crystal structure (12). It has also been proposed that the four aspartate residues project in the pore lumen as it has been suggested with the four EEEE-residues locus that accounts for the channel high Ca2+ affinity (13) of Ca2+-selective CaV1.2 channels. In two landmark studies, cysteine mutagenesis of each of the four EEEE residues locus of the CaV1.2 channel rendered the channel susceptible to irreversible inhibition by external sulfhydryl modifiers, indicating that the side chain was covalently modified by the methanethiosulfonate (MTS) reagent Cysteine substitutions at positions immediately adjacent to the EEEE locus (±1 positions) were also generally susceptible to sulfhydryl modification. Sulfhydryl modifiers had lesser effects on channels substituted one position further from the EEEE locus (±2 positions). These results suggested that the carboxylate-bearing side chains of the high affinity EEEE locus and their immediate neighbors were accessible from the water-filled extracellular medium in CaV1.2 channels.

To examine the topology of the pore region and the external...
vestibule in TRPV5 channels, we undertook a systematic analysis of pore residues accessibility using the substituted cysteine accessibility method (SCAM) (16). Mutant channels were expressed in Xenopus oocytes and their covalent modifications by externally applied membrane-impermeant methanethiosulfonate compounds of different charge and cross-section were measured. Based on the reactivity/accessibility to external sulfhydryl reagents, we report the structural features of the bacterial KcsA channel, namely the coiled region in the S5-pore linker (turret) and the pore α-helix of 15 residues that follows the turret and precedes the selectivity filter, are conserved in TRPV5 channels. Furthermore, our results show that Thr328 Ser329 Glu330, and Thr331 in the pore helix region are selectively accessible to positive MTS reagents from the external medium suggesting that they constitute part of the external vestibule in ECaC-TRPV5.

MATERIALS AND METHODS

Site-directed Mutagenesis of the Rabbit ECaC-TRPV5—The cDNAs coding for the wild-type ECaC-TRPV5 (GenBank™ AJ133128) (17) and the wild-type CaT2-TRPV5 (GenBank™ AF209196) (18) were obtained after reverse transcription of rabbit distal tubule mRNA as reported before (7). ECaC-TRPV5 and CaT2-TRPV5 were subcloned into the pT7TS vector (generously provided by Dr. Paul A. Krieg, University of Texas) using exonuclease III (19) for optimal expression in Xenopus laevis oocytes. Point mutations in ECaC-TRPV5 were performed with 39-mer synthetic oligos using the QuickChange™ XL-mutagenesis kit (Stratagene, La Jolla, CA). The C556S channel was used as a template for all cysteine mutations (see “Results”), and oligos were carefully designed to preserve that mutation. The nucleotide sequence of the S5-S6 linker including the background C556S mutation (over 600 bp) was bi-directionally analyzed using automated sequencing by BioST (Lachine, Que.). DNA constructs were linearized at the 3′-end by BamHI digestion. Run-off transcripts were prepared using methylated cap analog and T7 RNA polymerase, with the mMessage mMachine® transcription kit (Ambion, Austin, TX).

Expression of CaT2-TRPV5 Wild Type, ECaC-TRPV5 Wild Type, and Mutants in Xenopus Oocytes—Female Xenopus laevis clawed frog (Nasco, Fort Atkinson, WI) were anesthetized by immersion in 0.1% tricaine or MS-222 (3-amino-benzoic acid ethyl ester, Sigma) for 15 min before surgery as detailed before (7, 20). cDNA was injected at a concentration of 0.46–4.6 ng per oocyte depending upon the channel (wild type or mutant) being expressed. With only 0.46 ng of RNA, large whole cell inwardly rectifying currents (~50 μA) were routinely recorded with Li+ as the charge carrier (see below) for the wild-type channel less than 24 h after injection. ECaC-injected oocytes were incubated at 18°C in a calcium-free and serum-free Barth’s solution for 24–48 h before experiments.

Whole Cell Recordings—Whole cell currents were measured at room temperature with a two-electrode voltage-clamp amplifier (OC-725, Warner Instruments) as described before (7). Voltage and current electrodes (0.1–0.2 MΩ tip resistance) were filled with 3 M KCl, 1 mM EGTA; 10 mM Hepes (pH 7.4). Instantaneous current-voltage relationships were measured using voltage ramps from +80 to −150 mV at a rate of 575 mV/s from a holding potential of −50 mV. Whole cell current-voltage curves (I-V) were measured under control conditions in the presence of the nominally calcium-free Li+ solution (in mM): 120 LiCl, 5 EGTA; 2 KOH, 10 Hepes (pH 7.35 with methanethiosulfonate) was used as a possible control solution for washout and Ictrl is the whole cell peak current measured before MTS application. For the figures, current traces were averaged from n ≤ 4 separate experiments and are shown as the mean ± S.E. Hence, the thickness of the traces actually reflects the experimental variability of the MTS response. The standard errors tended to be smaller in the absence of functional modification. Inhibition was considered “significant” at p < 0.01.

Computer-predicted Structure and Homology Modeling of the Pore Region in ECaC-TRPV5—Sequence alignments were performed with the INSIGHTII HOMOLOGY module, which integrated the threading technique Profiles-3D developed in the laboratory of David Eisenberg and co-workers (27). The analysis of the three-dimensional scoring table led to the sequence ECaC1 being chosen as a possible template to be used for ECaC-TRPV5 homology modeling. The score was considerably lower for MthK, and the alignment would require the introduction of several gaps in the structure. Computer-based homology modeling was performed with Modeler V6.2 (28) using the crystal coordinates of KcsA (PDB 1BL8) as a template and involved the generation of 50 monomer models. Energy minimized, the best 50 monomer models were used in combination with CHARMM. The dimer was obtained by superposing two ECaC monomers onto the KcsA tetramer. The surface three-dimensional representation of the ECaC-TRPV5 was generated with the INSIGHTII software (Accelrys, San Diego) as described elsewhere (16).

RESULTS

Modification of Wild-type TRPV5 Channels by MTS Reagents—To examine the topology of the external vestibule and pore region in TRPV5 channels, we undertook a systematic

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Fig. 1. MTS reactivity of the wild-type ECaC- and CaT2-TRPV5 channel. Whole cell currents through ECaC- and CaT2-TRPV5 display a strong rectification at positive voltages under control conditions. A and B, bath perfusion with 5 mM MTSEA (A) or 5 mM MTSET (B) decreased currents of ECaC-TRPV5 by 30–50% within the first 30 s of perfusion (not shown) whereas further perfusion up to 5 min (2, light gray line) nearly abolished them. Only the MTSET-induced inhibition persisted after washout with the saline solution (3, gray). Perfusion with 5 mM BMS (4, dark gray line) reversed the MTSET-induced inhibition as seen with the overlapping 1–4 current traces indicating the covalent nature of the modification. Bath perfusion with 5 mM MTSES did not affect whole cell currents (not shown). C and D, in contrast, the wild-type CaT2-TRPV5 channel was not covalently modified by MTS reagents. 5-min perfusion with 5 mM MTSEA (4, light gray line) nearly completely inhibited whole cell currents (2, light gray line). Washout with the saline solution to remove unreacted MTSEA fully restored whole cell currents indicating the nonspecific nature of the inhibition. Whole cell currents were measured using a 400-ms ramp protocol from –150 to +150 mV in the presence of the 120 mM Li+ + 0 Ca2+ solution. Currents were normalized to the peak currents measured at –150 mV under control conditions and reported as the mean current trace ± S.E. (n = 4). See Table I for complete details.

analysis of pore residues accessibility using the substituted cysteine accessibility method (SCAM) with hydrophilic MTS reagents (MTSEA, MTSET, MTSES). MTS reactivity of the wild-type rabbit ECaC-TRPV5 (GenBank™ AJ133238) was investigated in the presence of Li+. As explained earlier, whole cell currents were measured in the absence of Ca2+ since ECaC undergoes Ca2+-dependent inactivation over a 5–10-min period in the presence of Ca2+ (8) whereas current levels of ECaC-TRPV5 and CaT1-TRPV6 are stable in Ca2+-free solutions with either Na+ (8) or Li+ (7, 29) as the charge carrier. As seen, whole cell currents measured under these conditions are strongly rectifying at positive voltages (Fig. 1, A and B and Table I). Perfusion with 5 mM MTSEA for up to 5 min did not modify the whole cell currents through ECaC-TRPV5 (not shown). However, external application of 5 mM MTSET (Fig. 1A) or 5 mM MTSET (Fig. 1B) significantly inhibited whole cell currents between –50 and –150 mV. Washout with the saline solution reversed the MTSEA-induced inhibition (Fig. 1A) but did not affect the MTSET-induced inhibition (Fig. 1B) suggesting that the former inhibition was nonspecific whereas the latter inhibition was truly covalent. This conclusion was further supported by the application of the reducing agent BMS that was found to fully reverse the MTSET-inhibition confirming that the MTS-induced inhibition involved the formation of a disulfide bridge between the MTS reagent and the channel protein (Fig. 1B).

In contrast to ECaC-TRPV5, the highly homologous CaT2-TRPV5 (GenBank™ AF209196) channel was not irreversibly modified by either MTS reagent (Fig. 1, C and D and Table I). As seen, whole cell Li+ currents through CaT2-TRPV5 rectified strongly at positive voltages. CaT2-TRPV5 also displayed an anomalous mole fraction effect between Ca2+ and Li+ and external Ca2+ was found to inhibit whole cell Li+ currents with a K1/2 = 2 ± 1 μM (4), which is comparable to the value reported for ECaC-TRPV5 (7). CaT2-TRPV5 was completely insensitive to MTSET (Fig. 1D) and MTSES (not shown) whereas MTSEA inhibition observed after a 5-min exposure, was completely abolished upon the reagent washout (Fig. 1C). A comparison of their primary structure indicated that 11 out of the 15 cysteine residues of ECaC-TRPV5 were strictly conserved in CaT2-TRPV5 (Fig. 2). Six conserved cysteine residues are located in putative transmembrane segments S1, S4, and S5 whereas that the remaining five residues are located either in the N terminus (Cys70, Cys112, Cys172, and Cys213) or in the C terminus (Cys518). The three non-conserved residues namely Cys4, Cys270, and Cys653 are located either in the intracellular N or C termini where they should be inaccessible to modification by external perfusion of membrane-impermeant reagents. The cysteine residue at position 556 in the pore region of ECaC-TRPV5 corresponds to His449 of CaT2 where it could potentially be accessible from the external medium.

The conservative mutation of the Cys556 residue to a serine resulted in an ECaC-TRPV5 channel completely insensitive to MTSET (Fig. 3B) and MTSES (not shown) as demonstrated by the current traces superimposed under all experimental conditions. The smaller MTSEA, documented to be somewhat membrane-permeant (24), caused current inhibition of whole cell currents after a 5-min perfusion period that did not persist upon extensive washout of the reagent (Fig. 3A). The global time course of MTS modification of C556S channels following a
TABLE I

Covalent modification of ECaC-TRPV5 wild-type and mutants in the pore helix region

Biophysical properties and covalent modification of wild-type TRPV5 channels and mutants in the putative pore helix region. Most mutant channels displayed the strong inward rectification of wild-type TRPV5 channels. Peak currents were measured in the presence of the nominally Ca\(^{2+}\)-free Li\(^+\) solution. The percentage of control currents remaining after external MTS modification with 5 mM reagent was computed after washout of the unreacted reagent (see “Materials and Methods”). A value of 100 means there was no effect. Mutant channels preserved the high-Ca\(^{2+}\) affinity as shown by the percentage of whole cell currents measured after exposure to 1 \(\mu\)M free-Ca\(^{2+}\). Data were computed at \(V_m = -150\) mV and are shown as mean ± S.E. with the number \(n\) of experiments in parentheses. N/E, non-expressor.

| Channel mutants | I-V rect. | Peak current | % control current at \(-150\) mV | 1 \(\mu\)M Ca\(^{2+}\) |
|-----------------|-----------|--------------|---------------------------------|-------------------|
| ECaC-TRPV5      | Yes       | 19 ± 2 (32)  | 78 ± 7 (7)                      | 86 ± 2 (7)        |
| C556S           | Yes       | 23 ± 3 (21)  | 90 ± 18 (9)                     | 96 ± 4 (4)        |
| CaT2-TRPV5      | Yes       | 8 ± 3 (14)   | 90 ± 11 (3)                     | 112 ± 17 (5)      |
| C556S + P527C   | Yes       | 25 ± 4 (13)  | 86 ± 5 (3)                      | 106 ± 10 (4)      |
| C556S + S528C   | Yes       | 33 ± 22 (24) | 45 ± 6 (4)                      | 101 ± 7 (10)      |
| C556S + A528C   | Yes       | 15 ± 3 (17)  | 15 ± 3 (3)                      | 78 ± 3 (4)        |
| C556S + L530C   | Yes       | 16 ± 2 (15)  | 66 ± 5 (4)                      | 91 ± 3 (3)        |
| C556S + F531C   | N/E       | N/E          | N/E                             | N/E               |
| C556S + S532C   | Yes       | 26 ± 2 (31)  | 57 ± 7 (4)                      | 96 ± 9 (5)        |
| C556S + T533C   | Yes       | 19 ± 32      | 58 ± 6 (11)                     | 81 ± 7 (4)        |
| C556S + F534C   | N/E       | N/E          | N/E                             | N/E               |
| C556S + L536C   | N/E       | N/E          | N/E                             | N/E               |
| C556S + F537C   | Yes       | 25 ± 2 (30)  | 36 ± 6 (5)                      | 90 ± 8 (4)        |
| C556S + E535C   | Yes       | 24 ± 3 (8)   | 106 ± 11 (4)                    | 105 ± 4 (5)       |
| C556S + L538C   | Yes       | 24 ± 11 (18) | 36 ± 8 (4)                      | 105 ± 4 (3)       |
| C556S + T539C   | Yes       | 30 ± 3 (28)  | 108 ± 6 (9)                     | 109 ± 7 (9)       |

Fig. 2. Putative secondary structure of the epithelial ECaC-TRPV5 Ca\(^{2+}\) channel. The deduced primary sequences of ECaC1 (GenBank\(^{TM}\) AJ133128) and CaT-2 (GenBank\(^{TM}\) AF209196) are highly homologous with 84% overall identity. The 15 endogenous cysteine residues of ECaC-TRPV5 are shown as circles with the empty circles showing residues conserved between ECaC and CaT2 whereas filled circles highlight cysteine residues absent in CaT2. The inset underscores the pore region in the S5-S6 linker region with 46 out of 52 amino acids being strictly conserved between the 2 channels in that region. A single cysteine residue (shaded box) differs in that region, the cysteine previously identified as the key molecular determinant of high Ca\(^{2+}\) affinity in ECaC-TRPV5 is shown with an asterisk.

10-min period was reported for MTSEA (Fig. 3C) and MTSET (Fig. 3D) at two voltages +20 mV and −100 mV. MTSEA steadily decreased currents measured at −100 mV over that period but currents could be recovered at 92 ± 3% (4) after washout of the reagent (Fig. 3C). Whole cell currents at ±20 mV were not affected indicating that the MTSEA-induced modification did not affect the channel rectification. Longer incubation periods up to 20 min with MTSET or MTSES did not significantly affect whole cell currents (not shown). Hence C556S channels did not undergo covalent modification by either MTSEA, MTSET, or MTSES. The key biophysical features of the wild-type channel namely the steep inward rectification and the high affinity for Ca\(^{2+}\) ions (Table I) were preserved in the C556S channel. The C556S channel was thus used as the template channel for the cysteine mutations in order to extend the characterization of the pore properties of the ECaC-TRPV5 channel undertaken in a previous work (7). Cysteine mutations were introduced one by one into the C556S channel in the S5-S6 linker region from Asp\(^{152}\) to Tyr\(^{555}\). In all cases, whole cell currents were measured after 5-min exposure to 5 mM MTS (MTSEA, MTSET, or MTSES). The MTS-induced modification was reported after extensive washout of the reagent solution to
ensure that channel modification did not result from nonspecific effects.

**External MTS Reactivity of the S5-to-Pore Linker Region Asp515-Tyr526.** The MTS reactivity was first studied in the region referred to as the S5-to-pore linker region spanning 11 amino acids from Asp515 to Tyr526. With the exception of L520C, all mutant channels in that region featured the typical inward rectification and the high Ca$^{2+}$ affinity of TRPV5 channels. Three consecutive mutant channels L520C, G521C, and E522C were strongly inhibited by positively and negatively charged MTS. Inhibition was nearly completed within 30 s and persisted through extensive washout periods with the control solution. Average I-V data are shown in Fig. 4 for E522C. As seen, perfusion with 5 mM BMS, a potent reducing agent almost completely restored whole cell currents in MTSEA- and MTSET-modified E522C channels confirming that the channel had formed a covalent disulfide bridge with the MTS reagents. BMS nonetheless failed to restore under the same conditions the whole cell currents of MTSES-modified channels. Altogether, these data suggest that L520C, G521C, and E522C were readily accessible from the aqueous external medium.

Such robust MTS reactivity was nonetheless limited to these three residues in that region. The SCAM data for the S5-pore linker are summarized in Fig. 5. Nothing can be inferred from positions 515, 518, 519, and 523 since E515C, N518C, N519C, and F523C mutants failed to express significant whole cell currents. Of the remaining mutants in that region, only D525C was covalently modified by the three MTS reagents and this, to a moderate extent with residual currents ranging from 60 ± 7% (5) for MTSEA (p < 0.01), and 61 ± 8% (4) for MTSET (p < 0.01) to 77 ± 8% (4) for MTSES (p < 0.1) as measured at -150 mV. Mutants P517C and S524C were only partially inhibited by MTSEA with residual currents of 64 ± 8% (4) and 45 ± 10% (3) respectively, suggesting a limited access to larger reagents at these positions.

**SCAM Analysis of the Pro527-Ile540 Region—Cysteine substitution in the P527-I540 region resulted in 11 of 14 channels with typical inward rectification properties and high Ca$^{2+}$ affinity (Table I). Only two mutants, F531C and L536C failed to express whole cell currents larger than -1 μA at -150 mV. The overall pattern of MTS modification in that region is summarized in Fig. 6A. Covalent modification by positively charged MTSEA and MTSET reagents resulted in a significant inhibition (p < 0.01) of whole cell currents at positions T528C, A529C, S532C, and E535C that persisted upon extensive washout (Table I). Average I-V data for T528C (Fig. 7, A-C) and E535C (Fig. 7, D) are shown in the presence of MTSEA (Fig. 7, A and B) and MTSET (Fig. 7, C and D). As seen, MTSEA and MTSET inhibition of T528C currents occurred within the time frame of bath perfusion (< 30 s). The E535C mutant channel was also significantly inhibited by MTSEA (Fig. 7B) but inhibition by MTSET (Fig. 7D) was reduced considerably and required a longer perfusion period. It follows that MTSET reactivity was strong within the first half of the region (528C, 529C,
532C, and 535C) but absent after position 535 whereas the smaller MTSEA reagent induced robust inhibition down to position 539 (Table I). Indeed, F537C and T539C were functionally modified by MTSEA with residual currents of 59 ± 4% (5) and 36 ± 8% (4) respectively, after a 5-min perfusion period. Mutants L538C and I540C were completely insensitive to modification by MTSEA and MTSET.

Although residues Thr533 and Ile540 are located a few residues away from the Asp542 residue responsible for the high affinity Ca"2+ binding site (7), the MTS response of either mutant was not altered when experiments were conducted in the presence of 1 μM free Ca"2+. The concentration required to inhibit 50% of the whole cell Li"+/H11001 currents (results not shown). This suggests that Ca"2+ binding/transit through the channel did not significantly alter the pore structure to such an extent that it would modify the side chain accessibility to MTSEA and MTSET.

Finally, MTSES reactivity was absent in the entire Pro547–Ile540 region. A slight increase in whole cell currents was however observed at some positions upon washout of the unreacted MTSES reagent although in both cases there was no discernible change in the whole cell currents in the presence of MTSES. The absence of MTSES-induced modification suggests the presence of an intrinsic electrostatic field, which would prevent negatively charged ions to penetrate into the pore.

Cysteine Mutations of the Ile541–Pro544 Region Yielded Non-functional Channels—Cysteine substitutions of residues Ile541–Pro544 surrounding the high affinity Ca"2+ binding site located at Asp542, namely I541C, D542C, G543C, and P544C all yielded non-functional channels. Although this absence of functional expression could result from a dysfunction of the permeation pathway, mutations of Asp542 to Ala (A), Gly (G), Glu (E), and Asn (N) in the wild-type ECaC-TRPV5 channels were shown to produce functional channels with whole cell currents exhibiting the typical inward rectification properties (7).

External MTS Reactivity in the Pore-to-S6 Linker Region Ala545–Ala566—As shown in Fig. 2, the last structural region studied spanned from Ala545 up to Ala566 in the S6 segment. Fig. 6B shows the histogram summarizing the MTS data for the 11 cysteine mutations in the pore-to-S6 linker (A545C to C556). Three consecutive positions in that region (A545C, N546C, and Y547C) produced whole cell currents with milder rectification properties. Five (545C, 546C, 547C, 549C, 550C) out of 11 positions in that region reacted strongly with external MTS and MTSET with MTSET-modification resulting in residual currents of 39 ± 5% (8) (p < 0.001); 47 ± 7% (8) (p < 0.001); 34 ± 6% (4) (p < 0.001); 53 ± 5% (4) (p < 0.001); and 63 ± 2% (4) (p < 0.01) at −150 mV, respectively. Furthermore, the negatively charged MTSES inhibited 545C, 546C, and 549C channels at p < 0.001 suggesting that access to these residues was not determined by the charge of the reagent. This region also included a series of mutants that were either non-functional (L551C, P552C, and Y555C) or completely insensitive to MTS modification (S548C and M554C) whereas F553C was only partially inhibited by external MTSEA with residual currents of 53 ± 7% (5) (p < 0.001) measured at −150 mV.

The external accessibility of S6 residues was tested intermittently at positions Ala560C, Ala561C, and Ala566C. The A566C channel was non-functional whereas the A560C, A561C, and A566C channels remained completely insensitive to modification by MTSET and MTSES (results not shown) but
Effects of MTS reagents on the Cys mutants spanning the Glu 515 to Tyr 526 region in the ECaC-TRPV5 channel. Mutants were perfused with 5 molar of the MTS solution for 5 min in the 120 mM LiCl (5 KCl) solution using the protocol described previously. The histogram reports the percent of whole cell currents remaining after washout of the MTS reagents with the saline solution such that a ratio of 100 indicates that whole cell currents were not modified by MTS reagents as compared with control current traces. Whole cell currents through E515C, N518C, G521C, and E522C were not significantly different than currents measured in non-injected oocytes. MTSEA, MTSET, and MTSES rapidly inhibited whole cell currents for L520C, G521C, E522C, and S524C at p < 0.001 (**). MTSEA inhibited whole cell Li^- currents of P517C and S524C at p < 0.01 (data not shown).

A560C was moderately inhibited by MTSEA with residual currents of 67 ± 8% (7) as measured at −150 mV.

**DISCUSSION**

A systematic analysis of some of the structural features of the external vestibule of ECaC-TRPV5 was performed using SCAM. Positively charged MTS reagents were generally the most reactive as expected for cation selective channels with the external vestibule of ECaC-TRPV5 was performed using SCAM. Partial inhibitions could be produced by MTS reagents in the selectivity filter or closer to the pore. Positively charged MTS reagents were generally the most reactive as expected for cation selective channels with the external vestibule of ECaC-TRPV5 was performed using SCAM. Partial inhibitions could be produced by MTS reagents in the selectivity filter or closer to the pore. Finally cysteine mutations that yielded non-functional channels are mostly found in the selectivity filter or close to the pore helix region. As seen in Fig. 9A, the selectivity filter between Ile540 and Pro541 predicted to be longer than in KcsA. The three-dimensional-based structure alignment finally suggests that the S6 a-helix should start at Thr558 whereas the current secondary structure model (17) based on hydrophobicity plots predicts S6 should begin at Cys556.

The surface three-dimensional representation (Fig. 9B) of the model ECaC-TRPV5 channel displays a comprehensive color-coded picture of the residues modified by MTSET and their relative accessibility from the aqueous medium. Residues that displayed the strongest MTSET reactivity are colored in red whereas partial inhibitions are shown in pink. Yellow-colored residues formed non-functional cysteine channels. Residues that failed to react with MTSET are presented in blue. As seen, residues that displayed the strongest reactivity appear to be located at the external interface and surround the pore area. Residues that caused a partial inhibition (pink) or failed to inhibit ion fluxes appear to be positioned further away from the pore. Finally cysteine mutations that yielded non-functional channels are mostly found in the selectivity filter or close to the pore helix region. It is however important to bear in mind that the implication of nonexistent or partial functional modification cannot be immediately translated into structural information. The absence of modification (residues shown in blue in Fig. 9B) could result either from the inaccessibility of the residue of else from a “silent” covalent modification that does not affect ion fluxes. Partial inhibitions could be produced by the limited accessibility of the residue or from a decrease in the channel single channel conductance coupled with an increase in the channel mean open time (16) whereby the MTS reagent behaves like an open channel blocker. Despite these limitations, the surface three-dimensional representation globally supports our SCAM data to the extent that the strong reactivity of some residues was correlated with their accessibility from the external medium whereas non-reactive residues appear to be buried within the protein.

**The Asp515–Tyr526 Region Could Form a Coiled Structure in ECaC-TRPV5**—The three-dimensional model obtained by homology (Fig. 9A) predicts that the region spanning 11-amino acids from Asp515 to Tyr526 in ECaC-TRPV5 should form a coiled structure. This region projects in the aqueous external medium and is called the “turret” in the crystal structure of KcsA (36), MthK (37), and KirBac1.1 (38) channels. The residues located in such a structure are expected to be readily accessible without any apparent periodicity in MTS-induced modification. The robust reactivity of three consecutive residues in that region (L520C, G521C, and E522C) argues for the
presence of a coiled region that is easily accessible from the aqueous external medium. The SCAM data for the remaining S5-pore linker residues showed however milder reactivity for since only D525C was moderately modified by the three MTS reagents. Overall, the strong reactivity of three consecutive residues irrespectively of the charge of the MTS reagent agree with the surface representation of the S5-pore-S6 region as these residues correspond to the red-colored spots surrounding the channel pore (Fig. 9B).

The Pore Helix in ECaC-TRPV5—The structural region in ECaC-TRPV5 spanning from Pro527 to Ile541 could correspond to the “pore helix” brought to light by the x-ray structures of the bacterial KcsA (12, 36), MthK (37), and KirBaC1.1 (38) channels. It is assumed that the pore helix provides a structural support to the selectivity filter. Its net negative dipole should contribute to the stabilization of $\text{K}^+$ in the water-filled central cavity of the closed KcsA channel (39) and could account for charge selectivity in $\text{Ca}^{2+}$-permeable glutamate receptors.
KcsA: 35-LLVIVLLAGSYLAVLARSGDGAQILITYFES-ESWSNEFATGCVYDL-GSPW---WGPYQ----YVVKVG21-100
ECaC: 499-VVILGFASAPHTFPHEDPNDAGEQFDDYKPFSTERTFPFSSNPAYSDPFWY-1VPAATTGL1-568

**Fig. 8.** Computer-predicted secondary structures generated by PROFILES-3D (INSIGHT II) using KcsA (PDB 1BL8) as the template. Structure homology was performed for the 499–568 region of ECaC-TRPV5 with only part of the actual S5 and S6 transmembrane regions. The domains were identified as they appear in KcsA. The coiled structure following the selectivity filter is predicted to be longer in ECaC-TRPV5 than in KcsA such as the α-helix TM2 (shown as a dotted box) starts at Thr537 in KcsA whereas it starts at Thr539 in ECaC-TRPV5.

**Fig. 9.** A, ribbon three-dimensional representation of two ECaC-TRPV5 monomers obtained by homology modeling using Modeller 6.2 as viewed from a perspective parallel to the membrane. The side chains of MTSET-modified positions in the pore helix region (Thr528, Ser532, Glu535, and Thr539) and in the wild-type channel at Cys556 are shown. Aasp-42 is projecting toward the selectivity filter. B, surface three-dimensional representation of ECaC-TRPV5 obtained by homology modeling using Modeller 6.2 as viewed from a perspective parallel to the membrane. Residues are color-coded according to their reactivity toward MTSET. Residues that were very significantly inhibited by MTSET at p < 0.01 are shown in red whereas pink-colored residues were inhibited by MTSET at p < 0.01. Blue residues remained insensitive to MTSET and non-functional residues are shown in yellow. White residues were not tested. Asp442 is shown in green to indicate the region of the selectivity filter. The arrow indicates the direction of the ion fluxes.

AMPAR (40). In the three-dimensional model obtained by homology (Fig. 9A), the side chains of hydrophilic residues Thr528, Ser532, Glu535, and Thr539 are projecting toward the selectivity filter. This orientation is compatible with the observation that these hydrophilic positions were covalently modified by MTSEA and MTSET. In addition, the location of the hydrophilic Thr533 residue could also explain its relative insensitivity to MTSET and MTSEA. A529C is the only residue that was covalently modified by MTSEA and MTSET despite being apparently inaccessible in the three-dimensional model. It could be speculated that the relative accessibility of Ala529 derives from its relative proximity to the external medium being located at the same height as Asp544. The three-dimensional model patterned after the dimensions of KcsA should also be refined to take into account the observation that MTSET (26 Å) was able to reach position E535C whereas MTSEA (10 Å) could go further down to T539C at the junction where the pore helix finishes and the selectivity filter starts suggesting that the pore region is larger in ECaC-TRPV5 than KcsA. Finally, the three-dimensional model suggests that the access to the pore has been designed to select positively charged ions. Given that the side chains of Glu535 and Asp542 appear to project in the same direction, we can speculate that these negatively charged residues provide together a potent negative field that could prevent MTSES to reach its targeted cysteine residue in the pore helix.

The Structural Features of the Pore-to-S6 Linker Region in ECaC-TRPV5—The selectivity filter is predicted to encompass Ile540 to Pro544, a region that was devoid of functional cysteine mutant channels and shown in yellow in Fig. 9B. Furthermore, the Pro544 to Ile557 region that links the selectivity filter to S6 is predicted to form a coiled structure (Fig. 9A) considerably longer than the equivalent region in the KcsA. The robust MTSEA and MTSET reactivity of the first three consecutive residues (A545C, N546C, Y547C) is indeed compatible with the presence of a coiled structure. Furthermore, A545C, A564C, and V549C channels were significantly inhibited by the negatively charged MTSES indicating that access to these residues was not limited as we observed in the pore helix region. The SCAM data were not however helpful in determining the beginning of the α-helix in S6 since most mutants after Asp550 were either non-functional (L551C, P552C, and Y555C) or else non-reactive (M554C). The Cys556 residue found to be completely insensitive to modification by external MTSET-reactivity to the wild-type channel is found at the end of this coiled structure (Fig. 9A) where it could be accessible from the extracellular medium. We can only speculate that MTSEA was too small to block efficiently ion fluxes through the selectivity filter since the dimensions of the three-dimensional representation are very approximate. Finally, the A560C, A561C, and A566C residues present in the S6 segment were found to be completely insensitive to modification by external MTSET and MTSES reagents whereas A563C was non-functional. As predicted by the three-dimensional model (Fig. 9B), these hydrophobic residues should be buried within the channel.

**Comparison with the SCAM Analysis of Models K+ Channels**—There exists little data on the cysteine reactivity/accessibility of the external vestibule of crystallized K+ channels (KcsA, MthK, KirBac1.1) besides the observation that Y82C located upstream to the GYGD (77–80) signature sequence, can be functionally modified by external MTSET in the KcsA channel (41). SCAM was used to investigate the structural features of the extra long extracellular S5-P linker that is absent from ECaC-TRPV5 channels and was also used to study the external vestibule of voltage-gated K,2.1 (42) channels. In this last case, cysteine-substituted residues in the Ile365–Gly377 region that includes the lower part of the pore helix and the
selectivity filter were either non-functional or insensitive to external MTS modification (42) just as we have shown in ECaC-TRPV5. The alignment produced by PROFILES-3D would correlate A545C in ECaC-TRPV5 with T380C in Kv2.1 (42). These residues were shown to be functionally modified by MTSEA, MTSET, and MTSES in both channels. Thr539 in ECaC-TRPV5 would also be aligned with Thr373 in Kv2.1. Since Thr373C in Kv2.1 was unaffected by MTSEA (42) whereas Thr539 in ECaC-TRPV5 was significantly inhibited by MTSEA, it is suggested that the pore region is wider in ECaC-TRPV5 than in K+ channels. Definite answers to these questions will await the x-ray crystal structure of TRPV channels.

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