Inhibition of the Human Sodium/Bile Acid Cotransporters by Side-Specific Methanethiosulfonate Sulfhydryl Reagents: Substrate-Controlled Accessibility of Site of Inactivation

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ABSTRACT: Mammalian sodium/bile acid cotransporters (SBATs) constitute a subgroup of the sodium cotransporter superfamily and function in the enterohepatic circulation of bile acids. They are glycoproteins with an exoplasmic N-terminus, seven or nine transmembrane segments, and a cytoplasmic C-terminus. They exhibit no significant homology with other members of the sodium cotransporter family and there is limited structure/function information available for the SBATs. Membrane-impermeant methanethiosulfonates (MTS) inhibited bile acid transport by alkylation of cysteine 270 (apical SBAT)/266 (basolateral SBAT) that is fully conserved among the sodium/bile acid cotransporters. The accessibility of this residue to MTS reagent is regulated by the natural substrates, sodium and bile acid. In experiments with the apical SBAT, sodium alone increases the reactivity with the thiol reagents as compared to sodium-free medium. In contrast, bile acids protect the SBATs from inactivation, although only in the presence of sodium. The inhibition and protection data suggest that cysteine 270/266 lies in a sodium-sensitive region of the SBATs that is implicated in bile acid transport.

Sodium/bile acid cotransporters (SBATs)[1] are among the molecular components of the enterohepatic circulation in mammals responsible for reabsorption of bile acids from the intestinal lumen, the portal blood, the bile duct, and the kidney (*1*, *2*). Two homologous SBATs are found in mammals, one with apical localization in ileal/renal enterocytes and in the bile duct cholangiocytes and another that is trafficked to the basolateral sinusoidal membrane of hepatocytes. Apical and basolateral sodium/bile acid cotransporters have been cloned from a number of species including hamster, rat, rabbit, mouse, and human (*3*−*11*). They share ∼35% identity and have very similar hydrophobicity profiles indicating a common SBAT core structure that has a glycosylated N-terminus, seven or nine transmembrane segments and a cytoplasmic C-terminus (*11*−*13*). However, there are substantial differences in the range of substrates that are transported, the apical SBATs being significantly more restricted to translocation of the natural bile acids while the basolateral SBATs also transport a variety of organic anions (*14*).

The human apical SBAT was recently demonstrated to facilitate sodium/bile acid translocation in an electrogenic process with a 2:1 stoichiometry (*15*). The same stoichiometry for ion/substrate movement has previously been observed for a number of sodium cotransporters (*16*), implicating a common mechanism for energy coupling in the solute translocation process by this membrane transporter family. In addition, both the sodium/glucose cotransporter (SGLT1) and the SBAT have been suggested to bind the transported solute in a sodium-dependent manner (*17*, *18*). Regions important for recognition, binding, and translocation of the SBAT substrates have yet to be identified.

Selective amino acid reagents have previously been used to address the involvement of specific amino acid side chains in bile acid transport by the apical and basolateral SBATs (*19*, *20*). It was demonstrated that thiol-selective reagents inhibited sodium-dependent bile acid transport in rat hepatocytes and that both thiol- and amino-specific modifiers inactivated bile acid uptake in ileal brush-border vesicles. Increasing concentrations of bile acids protected the transporter from inactivation, suggesting functionally important positions for the residues being reacted. However, to date, none of these residues has been specifically identified. In the case of thiol reagents, given that there are 13 and 14 cysteines in the human apical and basolateral transporter, respectively, it is particularly important to use impermeant reagents and such reagents then allow definition of the critical

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[1] Abbreviations: SBAT, sodium/bile acid cotransporter; SGLT, sodium/glucose cotransporter; TMS, transmembrane segment; PAGE, polyacrylamide gel electrophoresis; MHBSS, modified Hanks' balanced salt solution; ChCl, choline chloride; MTS, methanethiosulfonate; MTSES, (2-sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; SPA, scintillation proximity assay; TCA, taurocholic acid; GDCA, glycodeoxycholic acid; pCMB, *p*-chloromercuribenzoate; pCMBS, *p*-chloromercuribenzenesulfonate; NEM, *N*-ethylmaleimide; DTNB, dithiobis(2-nitrobenzoic acid); DTNP, dithiobis(nitropyridine); NBD-chloride, 7-chloro-4-nitrobenz-2-oxo-1,3-diazole; PAO, phenylarsine oxide.

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cysteine(s) by site-directed mutagenesis in a transport assay.

Here we have used two charged and therefore outside-face-specific thiol-modifying reagents, MTSET and MTSES, in combination with site-directed mutagenesis of the human apical and basolateral sodium/bile acid cotransporters to identify the lumenally accessible cysteine(s) responsible for inactivation by this group of compounds. We found that an alanine substitution of cysteine 270 (human apical SBAT)/266 (human basolateral SBAT) abolished the major part of the MTS inactivation. Furthermore, the accessibility of this site to the reagents is influenced by the presence of the translocated solutes. Sodium alone increases the MTS sensitivity of the apical SBAT, while bile acids are protecting the transporters from inactivation, although only in the presence of sodium. The data suggest that these cysteines lie in a region involved in transport of bile acids by the SBATs.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Cloning Procedures, and DNA Sequencing. Site-directed mutations in the apical and basolateral human sodium/bile acid cotransporters were introduced by the use of the QuikChange kit from Stratagene. Mutant oligonucleotides for the cysteine to alanine substitutions were constructed using the Primer Select software and purchased from Gibco Life Technologies. cDNA for the SBATs was a kind gift of Dr. Paul Dawson, Wake Forest University, and the individual SBATs were cloned into the pcDNA3 vector from Invitrogen for use as a template in the PCR mutation reaction. Aliquots (1−2 μL) of the *Dna*I-digested PCR products following the QuikChange protocol were transformed into JM109 by electroporation with Bio-Rad's electropulser at 1.5 kV, 800 Ω, and 25 μF. Plasmids were purified on anion-exchange columns (Qiagen, Chatsworth, CA) and all mutations were verified by dideoxy sequencing.

Expression in COS-7/HEK 293 Cells and Measurement of Transport Activity. On day 0, COS-7 cells grown to 70−80% confluence in a 6-well tissue culture plate (Costar) in Dulbecco's modified Eagle medium (high glucose, supplemented with 10% fetal bovine serum, 100 units/L penicillin, 0.1 mg of streptomycin/L, and 2 mM L-glutamine) were transfected by 2 μg of DNA/well by use of the FuGENE6 transfection reagent from Roche Molecular Biochemicals according to the manufacturer's recommendations. On day 1 the transfected cells were trypsinized by washing the monolayer with 2 × 2 mL of 0.05% trypsin/53 mM EDTA (Gibco Life Technologies) with a subsequent 5 min incubation at room temperature. The cells were then resuspended in 5 mL of DMEM and aliquots (100 μL, ∼10^5 cells) were transferred to the desired number of wells in a 96-well Cytostar-T scintillation proximity assay (SPA) plate (Amersham/Pharmacia) shown to be suitable for measurement of solute accumulation in cell monolayers (*21*, *22*). On day 2−3 (48−72 h posttransfection), cells were 90−100% confluent in the Cytostar-T 96-well plate and uptake of [^{14}C]taurocholic acid (NEN Life Science Products, Inc.) was measured on a Microβ scintillation plate reader (EG&G/Wallac).

The HEK 293 cells were transiently transfected at 70−80% confluence with the FuGENE6 reagent directly in the Cytostar-T 96-well plate, or a clone with stable expression of the human apical SBAT was used. The stable clone was obtained by calcium phosphate transfection (Invitrogen Corp., Carlsbad, CA) of HEK 293 cells plated on 100-mm diameter tissue culture dishes (20 μg of DNA/dish) with subsequent (60 h posttransfection) selection with 750 μg of geneticin/mL. This concentration was maintained until single colonies appeared. Selected clones were then transferred to 12-well plates and grown to confluence prior to a further split into two 24-well plates for screening of taurocholate uptake in one plate and for maintenance in the other. The clone exhibiting the highest transport activity was selected and used for further studies.

Transiently transfected HEK 293 cells were monitored for taurocholate uptake 24−48 h posttransfection as above. After the transport assay, COS-7 and HEK 293 cells were lysed in 0.2 M NaOH, and after the pH was neutralized by addition of 1 volume of 1.5 M Tris-HCl, pH 6.8, the total protein was measured by the Bradford assay (Bio-Rad). The approximate expression levels of the human apical and basolateral SBATs were typically detected by Western blotting of 10 μg of total cell lysate separated on a precast 1.5 mm 10% Tris/glycine gel (Novex) and then transferred to poly(vinylidene difluoride) (PVDF) membrane (Millipore Immobilon-P, 0.22 μM). The primary antibodies for both the apical and basolateral SBAT were affinity-purified polyclonal antibodies from rabbit raised against the C-terminal sequences CEPESSFYKANGGFQPDEK (apical SBAT, QCB Inc.) and GNGTYKGEDCSPCTA (basolateral SBAT, kind gift of Drs. Ben Shneider and Meenakshisundaram Ananthanarayan, Mt. Sinai Schools of Medicine). The secondary antibody was a goat anti-rabbit horseradish peroxidase- (HRP-) with conjugated polyclonal antibody from American Qualex. Detection was made using the ECL-Plus enhanced chemiluminescence reaction from Amersham/Pharmacia.

Kinetic Characterization of Wild-Type and Mutant SBATs. Apparent K_m values for taurocholic acid and sodium were monitored by measuring initial rates of uptake at varying concentrations of the respective substrate. The sodium concentration was kept constant at ∼137 mM while the K_m was determined for [^{14}C]taurocholic acid (10 Ci/mol) and the sodium dependence was measured at 50 μM [^{14}C]taurocholic acid (0.6 μCi/mL). The data were fitted to the Michaelis−Menten equation by nonlinear regression with the enzyme kinetic software Enzfitter for MS-DOS (Robin J. Leatherbarrow, Elsevier-Biosoft, 1987).

MTS Inhibition. Stock solutions of the MTS reagents were made in distilled H_2O just prior to experiment. All experiments were performed in Cytostar-T SPA 96-well plates. These plates contain scintillant in their base, allowing real-time measurement of radioactivity accumulated in the adherent transfected COS-7 and HEK 293 cells (*21*, *22*). The effect of the methanethiosulfonate reagents on transport activity was monitored either by adding the MTS simultaneously with 50 μM [^{14}C]taurocholic acid (0.3 μCi/ml) in MHBSS/NaCl or after 5 min of preincubation at various concentrations of the MTS reagent in 50 μL of MHBSS ± NaCl or ChCl at room temperature. In the case of preincubation, inhibition was stopped by aspirating off the MTS-containing medium and the cell monolayers were washed one time with 50 μL of MHBSS/NaCl before 50 μL of 50

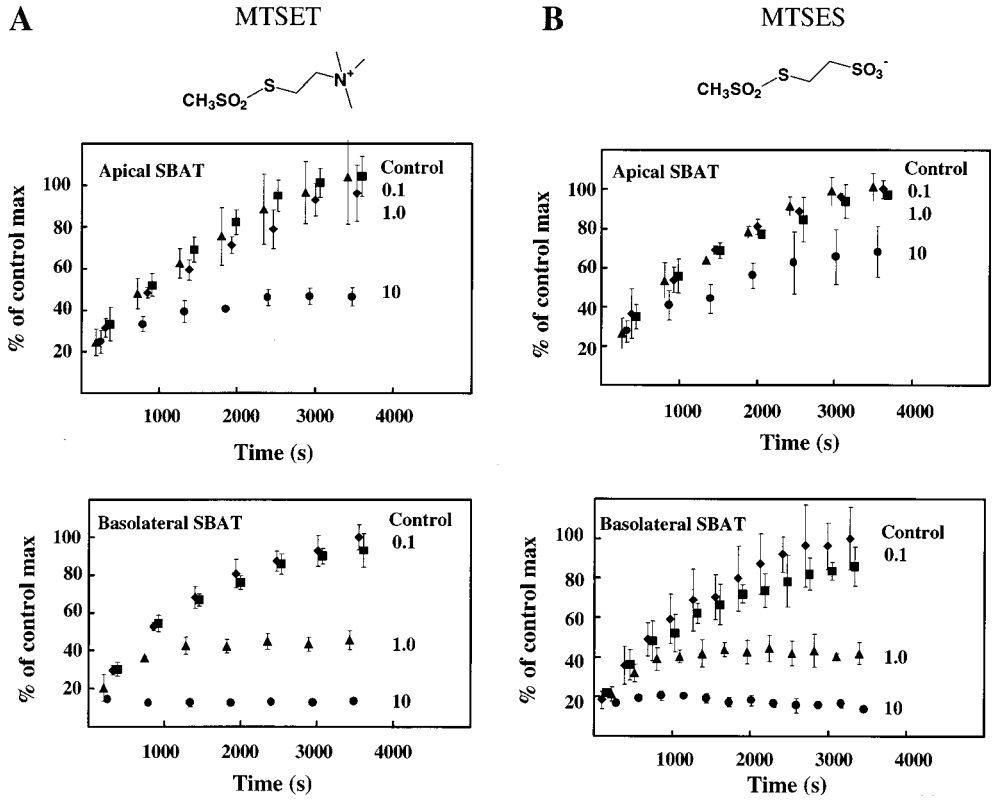

FIGURE 1: MTSET and MTSES inactivation of the human apical and basolateral sodium/bile acid cotransporters. Panels A and B show the uptake of 50 μM [^{14}C]taurocholate (0.3 μCi/mL) by transiently SBAT-transfected HEK 293 cells at (■) 0.1 mM, (▲) 1.0 mM, and (●) 10 mM MTSET and MTSES, respectively, compared to control (◆). Taurocholate accumulation in the HEK 293 monolayers was monitored in 96-well Cytostar-T SPA plates with $n = 3$ (means ± SD).

μM [^{14}C]taurocholic acid (0.3 μCi/mL) in MHBSS/NaCl was added and the uptake assay was started. The effect of the natural substrates on MTS inactivation was demonstrated by preincubation of the cells in MHBSS containing sodium or choline chloride and/or bile acid. Glycodeoxycholic acid (GDCA) at varying concentrations was allowed to equilibrate with the cells for 3 min prior to addition of the MTS reagent. In all protection experiments, the cells were washed twice with 50 μL of MHBSS/NaCl and incubated with this medium for 15 min at 37 °C after the MTS reaction. After reequilibration the MHBSS/NaCl medium was removed, 50 μL of 50 μM [^{14}C]taurocholic acid (0.3 μCi/mL) was added, and uptake was measured in the Cytostar plate.

Graphical Representation of the SBAT 2D Model. Snake-like diagrams used to display part of the human basolateral SBAT sequence were created with the Viseur program (release 2.35) designed by Campagne et al., which is available at the Viseur home page (http://www.lctn.u-nancy.fr/viseur/viseur.html). Modifications of the diagrams were made with the Adobe Photoshop software.

RESULTS

MTS Inhibition of the Wild-Type Human SBATs. Figure 1 shows the effect produced by the positively and negatively charged MTSET and MTSES, respectively, on [^{14}C]taurocholate accumulation in HEK 293 cells expressing the human apical and basolateral SBATs. The basolateral SBAT exhibited higher sensitivity with both reagents tested and 10 mM MTS resulted in complete inhibition of taurocholate uptake while the apical SBAT is only partially inhibited at this concentration. A possible explanation for this observation is the differences in affinity and specificity for substrates or inhibitors previously observed for the apical and basolateral SBATs (*14*). As the rate of hydrolysis of the methanethiosulfonates is high under the selected conditions with varying half-lifes of ~10 (MTSET) to 20 min (MTSES) (*23*), it is difficult to estimate the exact concentration of the reagents during the time course of inhibition. Therefore no attempt was made to calculate kinetic parameters for the inactivation of the SBATs by MTS. However, second-order rate constants for MTS modification of thiols as high as 10^5 M^{-1} s^{-1} have been reported for disulfide formation with easily accessible cysteines (*23*). This ensures that reaction is complete within the incubation time used here.

Cysteine to Alanine Substitutions in the Basolateral Transporter: Inactivation due to Methanethiosulfonates. The site of MTS inactivation of the human basolateral SBAT was found by generating a series of cysteine to alanine substitutions. These were selected in the highly conserved region preceding and partially including the eighth transmembrane segment in the nine-TMS topography predicted by some algorithms and recently supported by in vitro translation experiments (*13*). The sensitivity to MTSET for the cysteine to alanine mutants is shown in Figure 2 that also illustrates their positions according to the seven- and nine-TMS topography models. As can be seen in the lower panel of Figure 2, the replacement of the fully conserved cysteine 266 with an alanine abolished the inactivation of the basolateral SBAT by 1 mM MTSET while individual substitutions of cysteines 245, 250, and 260 with alanine in the region did not influence MTS inactivation. Consequently, the corresponding cysteine residue in the apical SBAT was

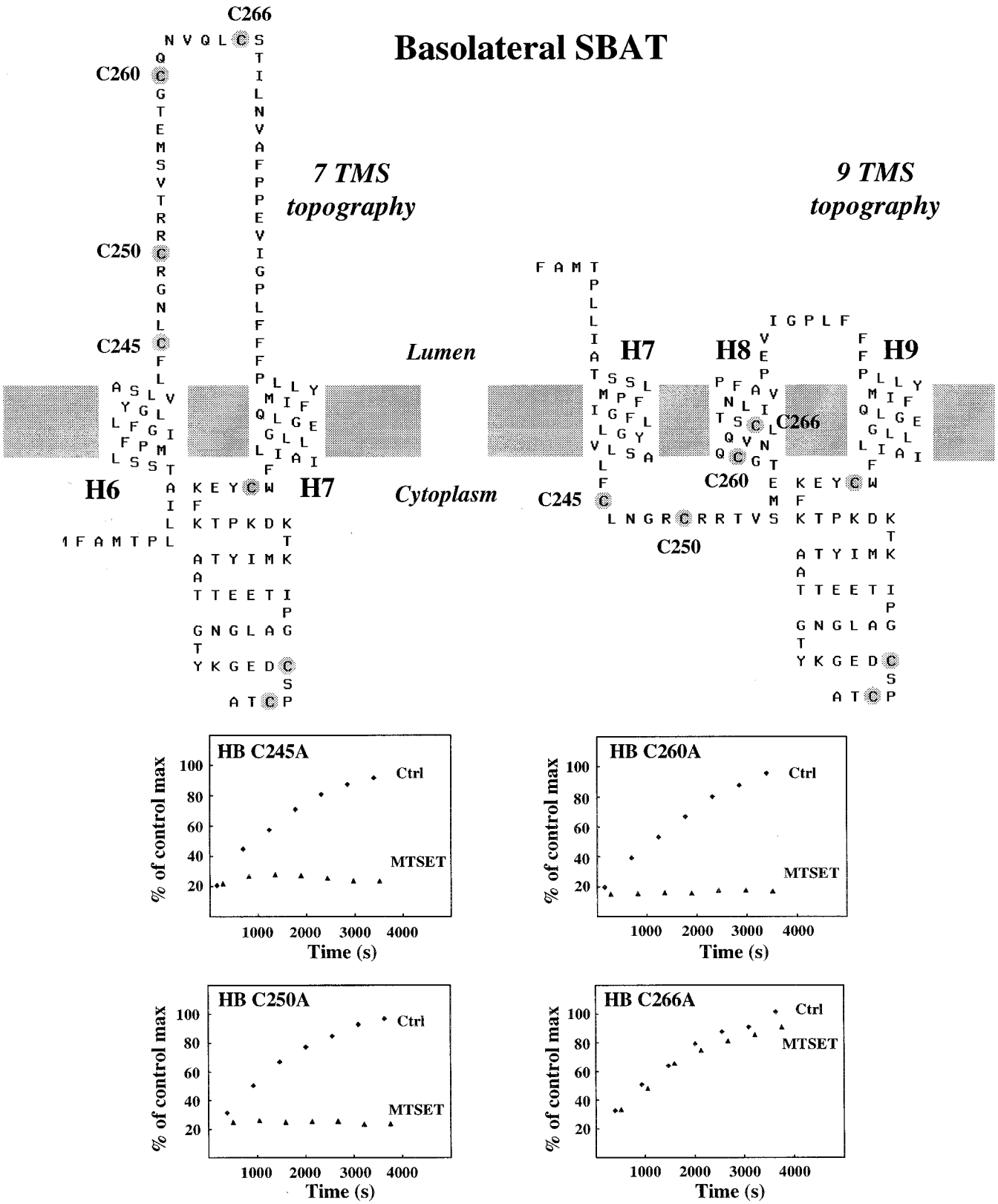

FIGURE 2: Cysteine to alanine substitutions in the highly conserved region preceding and/or including putative TMS 8. The site for inactivation by the MTS reagents was initially searched for by cysteine to alanine replacements in the basolateral transporter TMS 7−8 region. The upper panel displays the positions of the cysteines investigated according to both possible 2D models for the SBATs. The lower panel shows the effect of 1 mM MTSET on the uptake of 50 μM [^{14}C]taurocholate (0.3 μCi/mL) into HEK 293 cells transiently transfected with constructs coding for the individual cysteine to alanine substitutions in the basolateral SBAT. Taurocholate uptake was measured in Cytostar-T SPA plates with $n = 3$ (means ± SD).

also substituted by an alanine, and both the apical Cys270 and basolateral Cys266 to alanine mutants were further characterized.

$K_{m,app}$ Values for Sodium/Taurocholate Transport. The $K_{m,app}$ values for taurocholate and sodium were determined as a measure of the functional effect of the cysteine to alanine

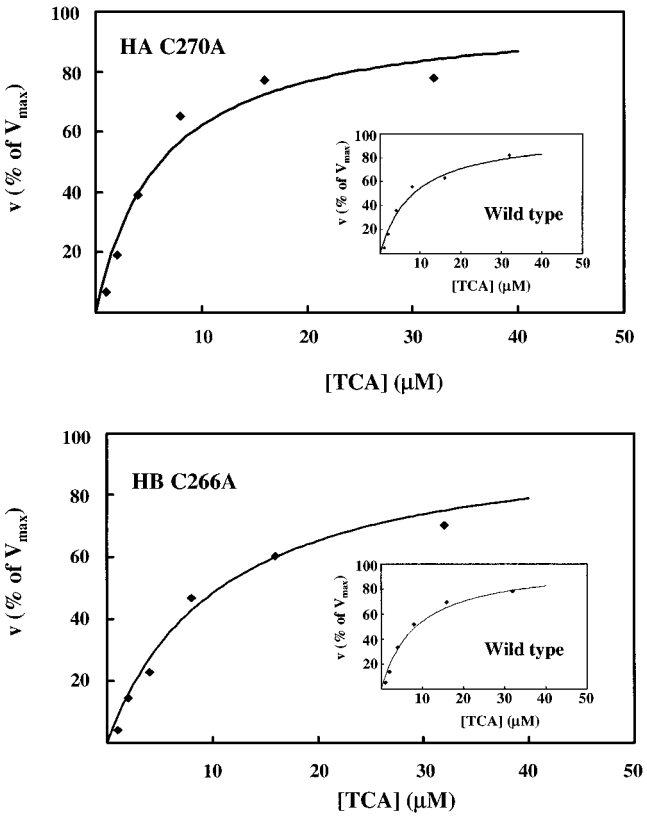

FIGURE 3: Saturation kinetics for taurocholate uptake into HEK 293 and COS-7 cells transiently transfected with wild-type and cysteine 270/266 to alanine mutant SBATs. Apparent K_m values for taurocholate translocation were determined at 20 °C in MHBSS containing 137 mM NaCl by monitoring the initial rates for accumulation of the indicated concentrations of [^{14}C]taurocholate (10 Ci/mol). Curves represent the best fit to the Michaelis−Menten equation with $K_{m,app}$ values of 8.3 and 8.5 μM for the apical (upper panel) and basolateral (lower panel) SBATs, respectively (insets). The corresponding $K_{m,app}$ values for the apical and basolateral SBAT C270A and C266A mutants were 6.1 and 10.7 μM. Experiments with the apical SBATs (upper panel) were performed with transiently transfected HEK 293 cells, while the basolateral SBATs (lower panel) were expressed in COS-7 cells. Taurocholate uptake was detected in Cytostar-T SPA plates with $n = 3$.

replacements in both transporters. Initial rates of [^{14}C]taurocholate accumulation in HEK 293 or COS-7 cells were measured for the apical and basolateral wild-type and mutant SBATs at varying concentration of sodium or taurocholic acid. Figure 3 shows the plots of initial rate versus concentration taurocholic acid measured at 137 mM Na^+. The data were fitted by nonlinear regression to the Michaelis−Menten equation and the wild-type apical and basolateral SBATs were found to have $K_{m,app}$ values for taurocholate transport of 8.3 and 8.5 μM, respectively (insets). In a similar manner, the apical and basolateral SBATs with substitutions of the fully conserved Cys270 (apical) and Cys266 (basolateral) by alanines had K_m values of 6.1 and 10.7 μM, respectively. Furthermore, the apparent K_m values for sodium activation were similar for both wild-type and mutated basolateral SBATs (data not shown) with half the maximal transport rate reached at ~20 mM Na^+. The corresponding experiment comparing the apical wild-type and mutant transporters revealed a small shift in the apparent K_m value for sodium activation from ~14 mM (wild type) to ~7 mM (C270A).

The values for maximal uptake rates obtained in various experiments with wild type and mutated SBATs correlated well with the approximate expression levels of the transporters estimated from Western analysis (data not shown). Thus, no major change in the kinetic behavior of the wild type versus the cysteine-to-alanine substituted SBATs was detected, suggesting that Cys270/Cys266 is not essential for function. This contrasted with the effect of these mutations on inhibition by the MTS reagents.

Effect of the MTS Reagents on Taurocholate Transport by the Cys270/266 to Ala Mutants. The mutants with the fully conserved Cys270/266 substituted with an alanine were expressed in COS-7 and/or HEK 293 cells and the inactivation by the MTS reagents was compared to that observed for the wild-type SBATs. Figure 4 shows the reduction in taurocholate transport found in the Cys270/266 SBATs after a 5 min preincubation with 10 mM MTSET or MTSES in MHBSS/NaCl and at room temperature. The insets in Figure 4 show the MTS sensitivity of the wild-type SBATs under the same conditions. As can be seen, both the apical and basolateral SBAT mutations generated a large decrease in the sensitivity to 10 mM MTS. The effect was more pronounced for the basolateral SBAT due to the higher MTS sensitivity seen with the wild-type transporter. There is still a reduction in the taurocholate uptake capacity of the mutants observed after exposure to the MTS reagents, perhaps due to slow access of these compounds to other cysteines or a general cellular effect. Clearly however, mutation of Cys270/Cys266 is effective in a large reduction of inhibition by the MTS reagents.

MTSET Inactivation in the Presence of the Natural Substrates. From the above, MTS alkylation of the fully conserved Cys270/266 is responsible for the major fraction of inhibition of bile acid transport by the human SBATs. It was of interest to determine if these cysteines were present in a functionally important region by a protection or sensitization assay by comparing inhibition in the presence or absence of the transported substrates, Na^+ or bile acids.

To detect an effect of the presence or absence of sodium on MTS inhibition, HEK 293 cells stably expressing the human apical SBAT were preincubated with 5 mM MTSET for 5 min in MHBSS containing 137 mM sodium or choline chloride. The results of Figure 5A show a significant increase of inhibition of taurocholate transport when the MTSET is allowed to react with the apical transporter in the presence of 137 mM sodium chloride as compared to sodium-free medium (137 mM choline chloride). Hence, sodium interaction with the transporter increases the accessibility or reactivity of cysteine 270 to MTSET.

To determine effects of the presence of bile acids on MTS inhibition, 3 min of preequilibration with 10, 50, and 100 μM GDCA in MHBSS with 137 mM NaCl prior to the addition of MTSET was carried out. The protective effect of GDCA saturated at ~50 μM and the presence of GDCA allowed only 20% inhibition of control steady-state accumulation as compared to the 60% inactivation found in the absence of bile acid. The same experiment was repeated in sodium-free medium (137 mM ChCl) and here there was no GDCA protection of the transporter from inactivation due to 5 mM MTSET (data not shown). In another set of experiments the basolateral transporter was preincubated for 5 min with 1 mM MTSET in 137 mM sodium or choline

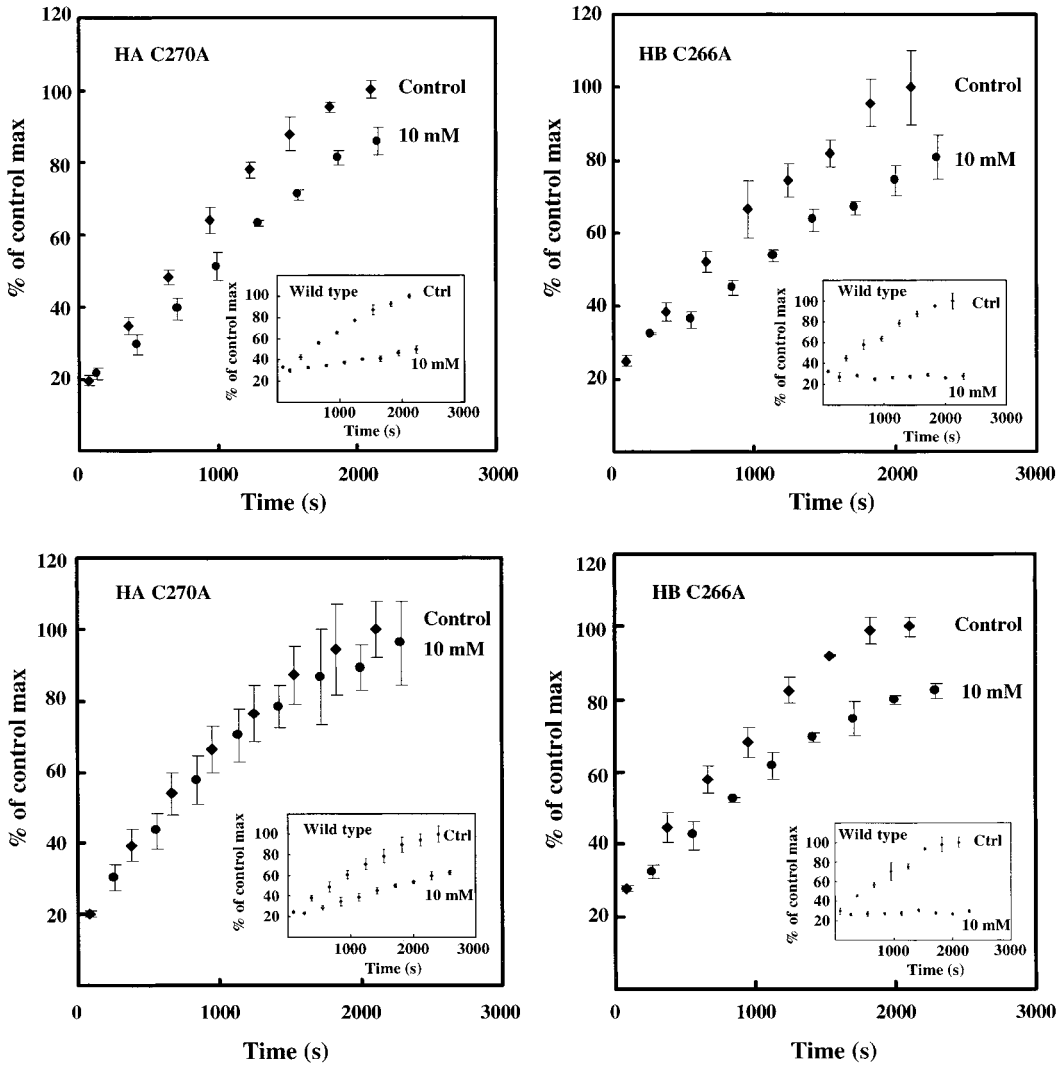

FIGURE 4: MTS inactivation of the cysteine 270/266 to alanine mutated SBATs. The effect of 10 mM MTSET and MTSES on taurocholate translocation was tested for the apical C270A and basolateral C266A mutants and compared with the wild-type transporters. The upper panel displays the offset of taurocholate accumulation into transiently transfected HEK 293 cells (apical SBAT, left) and COS-7 cells (basolateral SBAT, right) by 10 mM MTSET. The insets show the corresponding response by the wild-type SBATs. In a similar manner, the lower panel outlines the inactivation of the mutant versus wild-type transporters by 10 mM MTSES. All experiments were performed in Cytostar-T SPA plates with $n = 3$ (means ± SD).

chloride. Figure 6 shows that no significant difference in the inhibition of the basolateral SBAT due to the absence or presence of sodium could be detected under these close to saturating conditions for the MTSET reaction (panel A, no GDCA, versus panel B, no GDCA). In addition, panel A of Figure 6 demonstrates the lack of protection from alkylation of cysteine 266 when the basolateral SBAT, preequilibrated with 50 and 200 μM GDCA, is exposed to 5 mM MTSET in sodium-free medium. Figure 6B shows the corresponding experiment in the presence of 137 mM sodium chloride. The same concentrations of GDCA now result in 60% and 80% of the [^{14}C]taurocholate steady-state accumulation seen with the controls. Control reactions were preincubated with the bile acid but had water added instead of the MTS stock. Hence, increasing concentrations of glycodeoxycholic acid in the presence of external sodium protect the basolateral SBAT from inactivation by MTSET.

DISCUSSION

The human sodium/bile acid cotransporters expressed in mammalian cells have been investigated with respect to inactivation caused by the bulk and/or charge of membrane-impermeant methanethiosulfonate sulfhydryl compounds. In the SBATs, cysteine to alanine substitutions introduced by site-directed mutagenesis identified the fully conserved cysteine 270 (human apical SBAT)/266 (human basolateral SBAT) to be the major site of inactivation by these thiophilic reagents. Kinetic parameters for sodium activation and taurocholate transport were largely unaffected by the cysteine to alanine substitutions introduced, showing this residue to be nonessential but in a functionally important region of the transporter.

Such a functional location for these conserved cysteines is shown by the permissive or protective effects of sodium or bile acids. The results outlined in Figure 5A demonstrate that external sodium results in a higher reactivity as compared to sodium-free medium for the apical transporter. Bile acid protects against MTS inhibition only in the presence of sodium in the external medium (Figures 5B and 6). The position of this residue is in a ~30 amino acid long segment that is highly conserved among the sodium/bile acid cotransporters. The amphipathic nature of this segment has made it

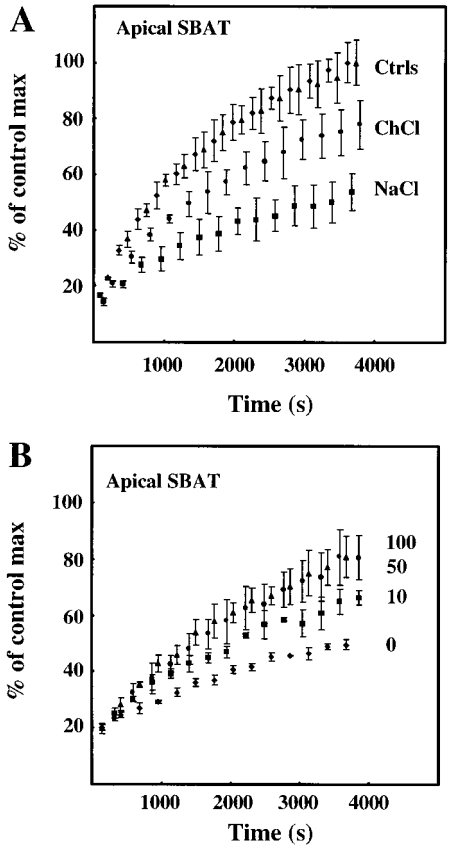

FIGURE 5: MTSET inactivation of the apical SBAT: Effect of the natural substrates. HEK 293 cells with stable expression of the apical SBAT were preincubated with 5 mM MTSET for 5 min in the presence or absence of sodium and/or glycodeoxycholic acid. Panel A shows the effect on [^{14}C]taurocholate accumulation (50 μM, 0.3 μCi/mL) after MTS inactivation in MHBSS containing 137 mM NaCl (■) as compared to sodium-free MHBSS (137 mM ChCl) (●). Control reactions were preincubated in MHBSS/NaCl (◆) or MHBSS/ChCl (▲) in the absence of MTS. The protective effect of glycodeoxycholic acid is presented in panel B. Cell monolayers were allowed to equilibrate with 0 (◆), 10 (■), 50 (▲) and 100 μM (●) glycodeoxycholic acid in MHBSS/NaCl for 3 min prior to the addition of 5 mM MTSET. Control reactions were incubated with the bile acid but with subsequent addition of dH_2O instead of MTS reagent. All experiments were performed in Cytostar-T SPA plates with $n = 3$ (means ± SD).

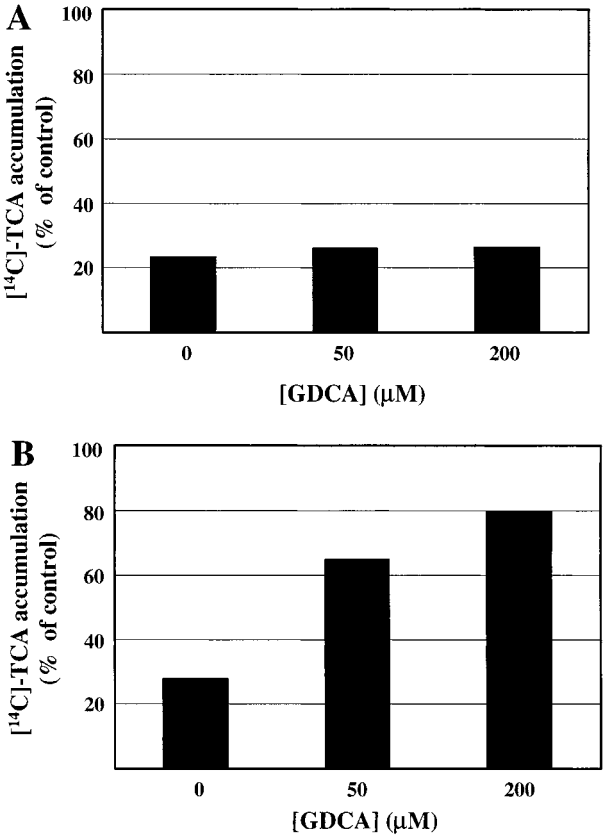

FIGURE 6: MTSET inactivation of the basolateral SBAT: Protection by glycodeoxycholic acid in the presence of ChCl (A) or NaCl (B). The protective effect of the natural substrate GDCA was tested for the basolateral SBAT expressed in COS-7 cells by preincubation with 50 or 200 μM GDCA prior to addition of 1 mM MTSET. Inactivation is displayed as percent steady-state accumulation of 50 μM [^{14}C]taurocholate (0.3 μCi/mL) after 5 min of exposure to MTSET in the absence (137 mM ChCl; panel A) or the presence (137 mM NaCl; panel B) of sodium compared to control (no MTSET present). Experiments were performed in Cytostar-T SPA plates with $n = 3$.

unclear as to whether it is an extracytoplasmic loop region between TMS 6 and 7, as presented in a seven-TMS topology model for the SBATs (*12*), or whether it instead spans the membrane as TMS 8 in a nine-TMS model. Different algorithms for topology prediction support both models but recent data from in vitro translation insertion scanning of the human apical SBAT favors a 2D structure with nine membrane-spanning segments (*13*). When the topography of the seven- or nine-transmembrane segment models is compared as illustrated for the basolateral SBAT in Figure 2, it can be seen that in the seven-TMS model cysteines 245, 250, 260 and 266 are all exposed on the lumenal surface. They would therefore all be, in principle, accessible to the MTS reagents and potential inhibitory sites. Yet, only mutation of Cys266 affected the MTS inhibition. In the nine-TMS model, Cys266 is within a membrane segment holding a number of hydrophilic residues that could be part of a substrate binding pocket and consequently allow access to the MTS compounds. The other cysteines are on the cytoplasmic face in this model and would therefore be inaccessible to the reagent. The results are more consistent with a structure encompassing nine integrated segments. These data do not exclude functionally silent alkylation of cysteines 245, 250, and 260 in an outside loop by the MTS reagents, but only modification of cysteine 266 inactivates the transporter.

The sodium and bile acid effects on SBAT modification by the MTS reagents resembles that previously demonstrated for a cysteine substitution mutant of the sodium/glucose cotransporter (*18*). It was postulated that part of the mechanism for solute translocation is a conformational transition induced by sodium binding to the glucose transporter. We also observe that sodium increases the accessibility of cysteine 270 in the apical transporter to MTS inhibition. Furthermore, the presence of both substrates protected the SGLT1 from alkylation by MTS similar to the data presented here for the SBATs. The sodium-induced bile acid binding that can be implied from the effect on MTS inhibition is also in agreement with previous data where it was demonstrated that photoaffinity derivatives of various bile acids labeled rabbit ileal (apical) SBAT in brush-border vesicles only in the presence of sodium (*17*).

The mammalian sodium/bile acid cotransporters have previously been found to be inactivated by thiophilic

molecules such as NEM, DTNB, DTNP, NBD-chloride, PAO, pCMB, and pCMBS and it has therefore been suggested that cysteines are important for substrate binding and/or translocation (*8*, *19*, *20*). In particular, pCMBS is membrane-impermeant and inhibition by this reagent implicates a lumenal cysteine as its site of inactivation. However, the cysteines modified and responsible for inhibition by any of these thiophilic reagents were not identified in these studies.

The MTS reagents have previously been proven to be useful tools for structure/function investigations of various polytopic membrane proteins (*24*−*29*). They also appear to be useful reagents for analysis of structure−function of the SBATs. These side-specific methanethiosulfonates can be used to map functionally important regions in the mammalian sodium/bile cotransporters after replacment of cysteine 270/266 with an alanine where inhibition by these covalent reagents is largely prevented. Then, introduction of cysteines in putative substrate binding/functional domains in the cysteine 270/266 to alanine transporters would be detected if MTS sensitivity were reintroduced. Saeki et al. (*8*) recently found that an alanine substitution of cysteine 51 in the mouse apical SBAT led to significant loss of activity. Thus, a cysteine-free transporter, although with favorable substitutions, may not retain enough activity to function as a template for site-directed chemical labeling of the SBATs. The simplicity of new mutagenesis protocols with subsequent expression and transport assay in mammalian cells makes cysteine scanning with membrane-impermeant methanethiosulfonates an attractive way to obtain further structural and functional information for the mammalian sodium/bile acid cotransporters.

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REFERENCES

1. Hofmann, A. F. (1993) *Gastrointestinal disease. Pathophysiology, diagnosis, management* (Sleisenger, M. H., and Fordtran, J. S., Eds.) 5th ed., W. B. Saunders Co., pp 127−150, Philadelphia, PA.
2. Love, M. W., and Dawson, P. A. (1998) *Curr. Opin. Lipidol. 9*, 225−9.
3. Wong, M. H., Oelkers, P., Craddock, A. L., and Dawson, P. A. (1994) *J. Biol. Chem. 269*, 1340−7.
4. Christie, D. M., Dawson, P. A., Thevananther, S., and Shneider, B. L. (1996) *Am. J. Physiol. 271*, G377−85.
5. Hagenbuch, B., Stieger, B., Foguet, M., Lubbert, H., and Meier, P. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10629−33.
6. Stengelin, S., Apel, S., Becker, W., Maier, M., Rosenberger, J., Wess, G., and Kramer, W. (1995) GenBank Accession No. Z54357.
7. Stengelin, S., Becker, W., Maier, M., Noll, R., and Kramer, W. (1998) GenBank Accession No. AJ131361.
8. Saeki, T., Matoba, K., Furukawa, H., Kirifuji, K., Kanamoto, R., and Iwami, K. (1999) *J. Biochem. (Tokyo) 125*, 846−51.
9. Saeki, T. (1997) GenBank Accession No. AB003303.
10. Wong, M. H., Oelkers, P., and Dawson, P. A. (1995) *J. Biol. Chem. 270*, 27228−34.
11. Hagenbuch, B., and Meier, P. J. (1994) *J. Clin. Invest. 93*, 1326−31.
12. Oelkers, P., Kirby, L. C., Heubi, J. E., and Dawson, P. A. (1997) *J. Clin. Invest. 99*, 1880−7.
13. Hallén, S., Brändén, M., Dawson, P. A., and Sachs, G. (1999) *Biochemistry 38*, 11379−88.
14. Kramer, W., Stengelin, S., Baringhaus, K. H., Enhsen, A., Heuer, H., Becker, W., Corsiero, D., Girbig, F., Noll, R., and Weyland, C. (1999) *J. Lipid Res. 40*, 1604−17.
15. Weinman, S. A., Carruth, M. W., and Dawson, P. A. (1998) *J. Biol. Chem. 273*, 34691−5.
16. Wright, E. M., Loo, D. D., Turk, E., and Hirayama, B. A. (1996) *Curr. Opin. Cell Biol. 8*, 468−73.
17. Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., and Wess, G. (1993) *J. Biol. Chem. 268*, 18035−46.
18. Loo, D. D., Hirayama, B. A., Gallardo, E. M., Lam, J. T., Turk, E., and Wright, E. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 7789−94.
19. Blumrich, M., and Petzinger, E. (1990) *Biochim. Biophys. Acta 1029*, 1−12.
20. Kramer, W., Nicol, S. B., Girbig, F., Gutjahr, U., Kowalewski, S., and Fasold, H. (1992) *Biochim. Biophys. Acta 1111*, 93−102.
21. Graves, R., Davies, R., Owen, P., Clynes, M., Cleary, I., and O'Beirne, G. (1997) *J. Biochem. Biophys. Methods 34*, 177−87.
22. Bonge, H., Hallén, S., Fryklund, J. and Sjöström, J.-E. (1999) *Anal. Biochem.* (in press).
23. Stauffer, D. A., and Karlin, A. (1994) *Biochemistry 33*, 6840−9.
24. Akabas, M. H., and Karlin, A. (1995) *Biochemistry 34*, 12496−500.
25. Akabas, M. H. (1998) *Biochemistry 37*, 12233−40.
26. Chen, J. G., Liu-Chen, S., and Rudnick, G. (1997) *Biochemistry 36*, 1479−86.
27. Nicoll, D. A., Ottolia, M., Lu, L., Lu, Y., and Philipson, K. D. (1999) *J. Biol. Chem. 274*, 910−7.
28. Xu, M., and Akabas, M. H. (1996) *J. Gen. Physiol. 107*, 195−205.
29. Yang, N., George, A. L., Jr., and Horn, R. (1996) *Neuron 16*, 113−22.

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