Intracellular application of certain charged methanethiosulfonate (MTS) reagents modified and irreversibly inhibited Kir6.2 channels when cysteine substitutions were introduced at positions Ile-210, Ile-211, or Ser-212 within the putative cytoplasmic region. Inhibition depends on the spatial dimensions of the MTS reagents. Reaction of MTS reagents, having head diameters of 7.6–8.2 Å, with cysteines introduced at position Ser-212 must occur in more than two subunits of the tetrameric Kir6.2 complex to inhibit channel activity. MTS reagents with head diameters less than 6.6 Å modified cysteines without causing channel inhibition. An MTS reagent with a head diameter of ~10 Å could neither modify nor inhibit the channels. Channel inhibition is interpreted as blockage of the intracellular vestibule by MTS reagents that enter the channel vestibule and react with the cysteine residues at vestibule-lining positions. Data are consistent with the hypothesis that residues Ile-210–Ser-212 line a funnel-shaped vestibule of 20–25 Å in diameter, which remains unchanged during channel gating.

Inwardly rectifying K⁺ (Kir)³ channels are pivotal to many physiological processes through their role in setting the resting membrane potential, modulating action potential duration, and mediating potassium transport across membranes. Kir channels are assemblies of four identical or related subunits. Each subunit contains an N-terminal domain and a C-terminal domain, which are separated by a pore-forming transmembrane region (1). Both the N and C termini are hydrophilic enough to extend from the transmembrane region into the cytoplasm and form a cytoplasmic region (2). Growing evidence extrapolated from a prototype of the pore-forming region of a K⁺ channel (KcsA) (3) has revealed that Kir channels share a similar type of transmembrane pore structure (4–10). In contrast, knowledge about the intracellular vestibule structure of Kir channels is very limited, although it is generally believed that the transmembrane pore extends into the cytoplasmic region to form the vestibule opening to the cytoplasm. The cytoplasmic region of Kir channels has various functions that play important roles in controlling Kir channel activity (11). Therefore, most studies on the cytoplasmic region have been focused on identification of domains and residues involved in these functions. Delineation of the cytoplasmic vestibule structure, however, will allow us to understand the complete structure of Kir channels, and may also provide useful information complementary to studies on the regulatory functions of this region.

In Kir2.1, a representative Kir channel that is characterized by strong inward rectification, mutation of a negatively charged residue, Glu-224, hinders voltage-dependent pore plugging by polyvalent cations (12, 13), a mechanism causing inward rectification (1). This finding has led to the hypothesis that Glu-224 is a vestibule-lining residue at a critical site that contributes to inward rectification. The hypothesis that Glu-224 is exposed to the vestibule has been confirmed by the substituted cysteine accessibility method (SCAM; Ref. 14) in a study from Yang’s group (4), which showed that modification of an introduced cysteine at position 224 by charged, membrane-impermeable sulfhydryl-specific reagents irreversibly inhibited the channel current. The vestibule surrounding Glu-224 has such a wide diameter that it could concurrently accommodate four MTS moieties. Residues surrounding Glu-224 were also frequently found to be vestibule lining. Similar analysis, however, has not been reported for other Kir channels. In the Kir1.1 channel, a weak inward rectifier, introducing a glutamic acid at the counterpart of Glu-224 failed to reproduce the inward rectification observed in Kir2.1 channel (12). Mutation of the residue equivalent to Glu-224 in Kir3.4, a member of another Kir subfamily, also had functional consequence dissimilar to that in Kir2.1 channels (15). These observations, together with the high functional diversity of the cytoplasmic region, raise the question of whether all Kir channels share a vestibule structure similar to that of Kir2.1 channels.

The major aim of this study is to investigate the cytoplasmic vestibule architecture of the weak inward rectifier Kir6.2 channel at a site analogous to Glu-224 of Kir2.1. We chose Kir6.2 as our model for several reasons. As the channel-forming subunit of the ATP-sensitive K⁺ channel that is a critical modulator of insulin secretion and other physiological processes, Kir6.2 has been intensively investigated over the past few years (16, 17). Functional roles of many residues in the putative cytoplasmic region of Kir6.2 have been studied by mutagenesis. Kir6.2 has a characteristic weak rectification that distinguishes it from Kir2.1. It also contains an undefined nucleotide-binding site that is probably located in the cytoplasmic region. Binding of ATP to this site closes the channel. This character is particularly useful when events relating to channel gating are studied. Our strategy includes several approaches to determine

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* This work was supported by National Institutes of Health Grants HL-58133 and GM61943 and by a grant-in-aid from the American Heart Association, Southeast Affiliate (to Z. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee 38163. Tel.: 901-448-2872; Fax: 901-448-7126; E-mail: zfan@physiol.uthsc.edu.

‡ The abbreviations used are: Kir, inward rectifying K⁺ channel; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; hydrochloride; MTSSET, [2-trimethylammoniumethyl]methanethiosulfonate bromide; MTS-PtEA, 3-trimethylammoniumpropyl methanethiosulfonate bromide; MTS-TEAH, 6-(trimethylammonium)-hexyl methanethiosulfonate bromide; MTS-EDANS-CE, N-(methanethiosulfonylethylcarboxamidoethyl)-5-naphthalylamine-1-sulfonic acid, sodium salt; DTT, dithiothreitol; SCAM, substituted cysteine accessibility method.
whether Ser-212, the residue at the position analogous to Glu-224, is a pore-lining residue. Because this has proven true, we have further explored the physical dimensions of the pore at this location and compared it to the Kir2.1 channel.

EXPERIMENTAL PROCEDURES

Control Kir6.2 Channels—Most Kir channels are tetramers composed of four independent subunits. Kir6.2 also forms a channel in this way. In the absence of regulatory subunits called sulfonylurea receptors, Kir6.2 is prevented from being expressed on the surface membrane. After removal of a retention signal near the C terminus, Kir6.2 is allowed to traffic to the surface membrane (18). The channel formed by Kir6.2 has quite different gating kinetics and regulation characteristics in the absence of the sulfonylurea receptor subunits. However, either the single-channel conductance and ionic permeation are unaltered or the alterations are too subtle to detect. It is believed that removal of retention signals from Kir6.2 channels does not affect the main channel pore structures. Thus, the Kir6.2 channel is a simplified model suitable for study of pore properties. In this study, a truncated mutant of Kir6.2, Kir6.2C35, was used as a control background for the exploration of pore structure. Most subsequent mutations were constructed on this background. A modification of Kir6.2C35 containing a FLAG epitope (DYKDDDK) inserted after the first methionine was also used. Compared with Kir6.2C35, this modified channel does not show any detectable difference in major channel functions. Kir6.2ΔC35 and FLAG-Kir6.2C35 channels are both designated as Kir6.2Δ in this paper. Likewise, mutants derived from either control background are not specifically noted, unless indicated.

Site-directed Mutagenesis—Kir6.2Δ was constructed using a PCR-based site-directed mutagenesis kit (ExSite, Stratagene Inc., La Jolla, CA) as described previously (19). A double-stranded mutagenesis kit (Chameleon, Stratagene Inc.), was used to generate the desired point mutations following the manufacturer’s instructions. In most cases, a silent mutation near the mutation site was created simultaneously to facilitate the selection process. Mutations were confirmed by sequencing at a commercial sequencing facility (Davis Sequencing, LLC, Davis, CA). The resulting mutants, either amplified by maxi-scale production, or collected from mini-scale production, were used in transfection. Plasmids produced by either production method gave satisfactory results.

Construction of an S212C-Kir6.2Δ Fusion Subunit—A cDNA encoding an S212C-Kir6.2Δ subunit was constructed on a Kir6.2Δ subunit. The 3' end of a cDNA encoding a Kir6.2Δ mutant having double mutations, C42V/S212C, was linked to the 5' end of a FLAG-Kir6.2Δ construct bridged by a linker of six glycines. The final construct was subcloned into a pcR3.1-Uni mammalian expression vector (Invitrogen, Carlsbad, CA).

Cell Culture and Transfection—A COS-1 cell line was maintained in continuous culture. The method for transient transfection of COS-1 cells was as described previously (19). Briefly, Kir6.2Δ and its mutants in mammalian expression vectors were transfected with a NovaFECTOR kit (VennNova, LLC, Pompano Beach, FL). The expression of mutations, C42V/S212C, was linked to the 5'/H11032 end of a cDNA encoding a Kir6.2Δ mutant having double mutations following the manufacturer’s instructions. In most cases, a silent mutation near the mutation site was created simultaneously to facilitate the selection process. Mutations were confirmed by sequencing at a commercial sequencing facility (Davis Sequencing, LLC, Davis, CA) as described previously (19). A double-stranded mutagenesis kit (ExSite, Stratagene Inc., La Jolla, CA) was used. Compared with Kir6.2C35, this modified channel does not show any detectable difference in major channel functions. Kir6.2ΔC35 and FLAG-Kir6.2C35 channels are both designated as Kir6.2Δ in this paper. Likewise, mutants derived from either control background are not specifically noted, unless indicated.

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less than 20 s (an average of ~5 s) to exchange the bath solution. Other solution exchanges were performed using a pressurized perfusion system (DAD-12, ALA Scientific Instruments, Westbury, NY).

**Single-channel Recordings**—The patch clamp and data acquisition system have been described previously (19). Currents were usually recorded at a membrane potential of 0 mV. All experiments were performed at room temperature. Digitized current signals were manually corrected for base-line drift using pClamp8 software (Axon Instruments, Inc., Union City, CA). Average currents were measured in a 10–20-s time window. When single-channel events were analyzed, a 50% threshold criterion was used to detect events with visual confirmation. Single-channel current amplitude and kinetics were analyzed using an established method described in our previous work (19).

**Immunocytochemical Staining of Channel Expression**—Transfected cells were fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were blocked with a buffer containing 5% goat serum, 0.2% Triton X-100, and 0.05% azide in phosphate-buffered saline and then incubated with an anti-FLAG M2 antibody (Sigma) at 4 °C overnight. After being washed with phosphate-buffered saline, the cells were incubated with a secondary Alexa Fluor® 546 goat anti-mouse IgG (H+L) conjugate antibody (Molecular Probes, Inc., Eugene, OR) for 4 h. The stained cells were further incubated with a monoclonal antibody to BiP (Stressgen, Victoria, British Columbia, Canada) and subsequently stained with Alexa Fluor® 488 goat anti-rabbit IgG (H+L) conjugate antibody (Molecular Probes, Inc.). Immunofluorescence staining was viewed with a laser scanning LSM 510 confocal microscope (Zeiss, Jena, Germany). Images taken under emission/excitation wavelengths appropriate for two sets of antibodies were superimposed and compared with determine the subcellular localization of the channels.

**Statistical Analysis of Data**—Data are presented as mean ± S.E. A one-way analysis of variance followed by a post hoc Student-Newman-Keuls method was used to examine the statistical differences among all data groups. Student’s *t* test was used wherever two groups of data are compared.

**RESULTS**

**Effects of MTS Reagents on Control Kir6.2Δ Channels**—First, the effects of MTS reagents on Kir6.2Δ channel currents were examined. Because a similar protocol was used in many experiments of this study, it is described in detail here. Channel currents were recorded in inside-out patches excised from the transfected COS-1 cells. In most experiments, a patch contained 5–20 active channels at the excision of the patch. MTS reagents were added to the solution that perfused the intracellular side of the membrane. Each reagent was tested in an individual experiment. The first current trace in Fig. 2 is a typical recording from a patch having multiple Kir6.2Δ channels. Whenever possible, channel sensitivity to ATP inhibition was tested using ATP concentration steps before and after sulphydryl modification. Application of MTSET rapidly and completely inhibited the current. The current did not recover after withdrawal of MTSET. MTSEA had the same effect. This effect of MTSET and MTSEA has already been demonstrated in other studies and was absent in C42V mutant channels (trace 2 in Fig. 2) (7, 21). Thus, this inhibitory effect is most likely caused by modification of Cys-42. Results from our laboratory suggest that modification of Cys-42 by MTSET does not directly occlude the pore (see “Discussion” for more details).

In contrast to MTSET and MTSEA, application of MTS-TEAH reversibly reduced Kir6.2Δ channel activity; this activity was almost fully recovered after withdrawal of the reagent. It should be noted that a time-dependent run-down component was usually present in most experiments, which led to a lower channel activity after the treatment than before the treatment. This bias should be taken into consideration when interpreting the results of sequential measurements. We consistently observed that MTS-TEAH reversibly reduced Kir6.2Δ channel activity; this activity was almost fully recovered after withdrawal of the reagent. Examination of the single-channel current amplitude and kinetics revealed that the reversible chan-
nel current reduction was because of a decrease in the probability of a channel being open without significant change in single-channel amplitude. Repeating the same experiment at −80 mV in the presence of 140 mM symmetric [K+] across the membrane resulted in less reduction, suggesting that the effect was probably because of a voltage-dependent block by positively charged MTS-TEAH. Voltage-dependent reduction of open channel probability caused by some positively charged channel blocking reagents such as tetraethylammonium has been described previously (22, 23).

**Diverse Effects of MTS Reagents on S212C Mutant Channels**—Because MTS-TEAH, MTS-PtrEA, and MTS-EDANS-CE do not irreversibly inhibit Kir6.2 channels, they can be used to study channel structure in substituted cysteine mutants. The effects of these reagents on S212C mutant channels were therefore examined. In contrast to Kir6.2A channels, MTS-TEAH and MTS-PtrEA irreversibly inhibited S212C channel currents. Fig. 3A gives representative current recordings from these experiments. We were unable to reverse these effects with 5 mM DTT. When 50 mM DTT was applied, the effect of MTS-TEAH was partially reversed in one patch. Why DTT cannot effectively reverse the effects of MTS reagents is unclear. Perhaps access of DTT to the disulfide bonds is obstructed in a narrow space filled by MTS moieties. MTS-EDANS-CE, which has the largest space-filling moiety of all MTS reagents tested in this study, did not affect S212C channel activity.

Because MTSET and MTSEA irreversibly inhibit Kir6.2Δ channel current but not C42V channel current, a mutant containing both C42V and S212C mutations (named C42V/S212C) was constructed to eliminate the confounding effects of MTS modification of C42. The C42V/S212C mutant channels had current activity comparable with Kir6.2A and C42V channels. Interestingly, neither application of MTSET or MTSEA irreversibly inhibited the channel, in contrast to MTS-TEAH and MTS-PtrEA (Fig. 3, B and C). We also examined the effects of the other three reagents on the C42V/S212C mutant channel. The results illustrated in Fig. 3B are in good agreement with those obtained in the S212C mutant channel. The results summarized in Fig. 3C clearly indicate that irreversible channel inhibition by MTS-TEAH and MTS-PtrEA was attributable to modification of Cys-212. These experiments, however, did not distinguish whether the failure of certain reagents to inhibit the channels was because of their inability to modify Cys-212, or whether the modification of Cys-212 occurred but was insufficient to cause channel inhibition.

**MTSET, but Not MTS-EDANS-CE, Prevents the Effect of MTS-TEAH**—To determine whether the reagents unable to inhibit S212C mutants were able to react with and modify Cys-212, the inhibitory effect of MTS-TEAH was examined after pretreatment of the S212C mutants with the test reagents MTSET, MTSEA, and MTS-EDANS-CE. These tests were performed considering that if the test reagent indeed modifies Cys-212, then this residue cannot be further modified by MTS-TEAH and, as a consequence, MTS-TEAH would no longer be able to inhibit the channel. We first tested MTSET in the C42V/S212C mutant (Fig. 4A). Pretreatment of the channel with MTSET completely prevented the effect of subsequent application of MTS-TEAH. The difference between the inhibitory effect of MTS-TEAH on the untreated (referring to Fig. 3, B and C) and pretreated channels is statistically significant (Fig. 4C). MTSEA acted very similarly (repeated in three experiments; result not shown). Unlike MTSET and MTSEA, MTS-EDANS-CE did not prevent the effect of MTS-TEAH on S212C mutant channels (Fig. 4, B and C). We therefore conclude that MTSET and MTSEA can access and modify Cys-212 without causing channel inhibition; on the other hand, MTS-EDANS-CE apparently does not react with Cys-212. The data also confirmed that MTS-TEAH indeed modified this Cys residue and that this modification causes channel inhibition in S212C mutants.

**MTSEA Prevents Voltage-dependent Block of the S212C Mutant Channel by Spermine**—Variability in current response following modification of pore-lining residues by MTS reagents is expected because reagents must be of an appropriate size to occlude the channel. However, modification of a residue might also close the channel allosterically, even if it does not extend into the permeation pathway. The following experiments were designed to differentiate these two possibilities. Polyamines are known to insert themselves into the inner pore of Kir channels and produce a voltage-dependent block of ion transport (24, 25). MTS reagents that modify a pore-lining residue would project into the permeation pathway and hinder access of polyamines to the binding site(s) within the pore, thus altering their blocking effect. To test this possibility, we examined the blocking effect of spermine before and after modification of S212C mutants by MTSEA. Spermine at 0.1 mM was added to the intracellular side of a membrane that was voltage-clamped

![Image](334x442 to 528x728)

**FIG. 4. Protection against the inhibitory effect of MTS-TEAH by MTSET on S212C mutants.** A, pretreatment of C42V/S212C mutant channels with 0.6 mM MTSET for 3 min completely prevented channel inhibition by subsequent application of 0.6 mM MTS-TEAH. B, pretreatment of S212C mutant channels with 0.6 mM MTS-EDANS-CE did not prevent the effect of MTS-TEAH. In both A and B, the recording conditions and labels are the same as those described in Fig. 2. C, statistical comparison of changes in multiple-channel currents in response to MTS reagent treatments. MTSET/MTS-TEAH and MTS-EDANS-CE/MTS-TEAH stand for the protocols shown in A and B, respectively. Current change is expressed as relative current. For the double treatment, the relative current is calculated as the percentage ratio of average current measured after the second treatment to that before this treatment. The values are the mean ± S.E. of 3–13 independent experiments. Data of the experiments using MTSET to treat C42V/S212C mutant channels and using MTS-EDA-NCS-CE to treat S212C mutant channels are also plotted for comparison. Significant differences (p < 0.05) are labeled with an asterisk between the pertinent data sets.
under a ramping protocol. The initial holding potential was 0 mV. Constant ramps from the holding potential to -80 mV, then to +100 mV, and returning back to the holding potential during a period of 3.6 s were used. Because alkaline intracellular pH enhances the blocking effect of spermine in Kir6.2 channels (26), we performed these experiments in intracellular solutions of pH 8.0. We confirmed that the voltage-dependent block of Kir6.2Δ channels by spermine was much stronger at pH 8.0 than at 7.3. Higher pH also enhanced the blocking effect of spermine in C42V and C42V/S212C mutant channels to an extent similar to that in Kir6.2Δ channels. In these experiments, more than 12 traces of multiple-channel currents were averaged to obtain macroscopic current that was then plotted against the voltages. As shown in Fig. 5 (A–C), spermine blocked C42V channel currents regardless of MTSEA treatment. In C42V/S212C mutant channels, however, treatment with MTSEA significantly attenuated the voltage-dependent block by spermine (Fig. 5, D–F). The data suggest that Cys-212 is either a part of the spermine binding site or that it is located in the passage through which spermine must pass. In either case, it is clear that Cys-212 must be a pore-lining residue.

**MTS Reagents Are Insufficient to Inhibit Channels Formed by S212C-Kir6.2Δ Dimers—**If moieties of MTS reagents covalently linked to Cys-212 occlude the channel, then one may ask how many such moieties are needed to completely stop the ion flow. To explore this question, a fusion subunit containing an S212C mutant subunit and a Kir6.2Δ subunit was used. As described previously in studies that employed the same strategy to investigate the stoichiometry of channel inhibition (10), two such fusion subunits can form a functional channel composed of two mutant subunits and two Kir6.2Δ subunits at mirror positions around the channel coaxial axis. If more than two modified Cys-212 residues are required to block the channel, then MTS reagents would not irreversibly inhibit the C212S-Kir6.2Δ dimer channels. Fig. 6 (B and C) shows examples of experiments using MTS-TEAH and MTS-PtrEA. Neither reagent could irreversibly inhibit the dimer channels (Fig. 6D). However, modification of the dimer channels by MTS-TEAH attenuated spermine block (Fig. 6E), further confirming that the modified residues Cys-212 extend into the permeation pathway. Apparently, two moieties derived from either reagent are insufficient to occlude the channel completely.

**Mapping the Effects of MTS Reagents on Channels with Substituted Cysteines in the Cytoplasmic Region—**Our SCAM analysis indicated that Ser-212 is a pore-lining residue. Using the same strategy, we examined residues near Ser-212. The residues we have tested are listed in Fig. 7 along with the statistical results. At equilibrium, MTS-TEAH irreversibly reduced channel current of I210C and I211C mutants, whereas MTS-PtrEA significantly reduced channel current of the I211C mutant. It is intriguing that the reagents, at a concentration and time sufficient to inhibit all S212C mutant channels in a patch, only inactivated a fraction of I210C and I212C mutant channels. In addition, the levels of inhibition were quantitatively different between these two mutants. Interestingly, other cysteine substitution mutants listed in Fig. 7 were as insensitive to MTS-TEAH and MTS-PtrEA treatment as control Kir6.2Δ channels. Transfection of T214C did not express recordable current. Nevertheless, as shown in Fig. 7C, immunochemical display of subcellular localization of the FLAG-tagged T214C mutants (11 cells) exhibits distribution similar to that of FLAG-Kir6.2Δ channels (12 cells).

**Modification of Cysteine-substituted Mutants by MTS Reagents and Channel Gating—**An important yet unresolved question about Kir channels is whether or not the cytoplasmic vestibule is involved in gating of the channel. So far, there is no
evidence to support or refute the possibility that Ser-212 or surrounding residues have such a function. ATP inhibits the Kir6.2 channel through an ATP-sensitive gating mechanism that may involve both transmembrane and cytoplasmic regions (27–30). In the following experiments, we examined whether Ser-212 plays a role in ATP-sensitive inhibition. We first investigated whether ATP could protect Cys-212 from being modified by MTS reagents. As shown in Fig. 8A, 50 mM ATP was kept in the bath solution to completely inhibit the channel activity during the application of MTS-TEAH. The channel activity, which otherwise would recover spontaneously after removal of ATP, did not recur after MTS-TEAH treatment. Similar observation was repeated in three experiments. In the example shown in Fig. 8A, the channels inhibited by MTS-TEAH treatment were partially recovered by subsequent application of 50 mM DTT. Next, we looked at the influence of mutation and sulphydryl modification on ATP sensitivity. Qualitative measurement of ATP-sensitive inhibition followed an established protocol (19). As noted earlier, ATP-sensitive inhibition was measured before and after treatment with MTS reagents in Kir6.2Δ channels and mutants whenever possible. Fig. 8B demonstrates how the IC50 for ATP was obtained. Fig. 8C is the statistical summary and comparison of IC50 for ATP-sensitive inhibition measured from cysteine substitution mutants, before and after treatment with MTS reagents. Cysteine substitution of most residues does not cause any profound change in ATP sensitivity. ATP sensitivity was reduced significantly in G334C and C166S (backward substitution) mutant channels as reported by others (27, 29). The H186C mutant channel is slightly less sensitive to ATP than Kir6.2Δ channels. Treatment with MTS-TEAH did not change ATP sensitivity in any cysteine substitution mutants scanned in our study. It is also noticeable that ATP sensitivity of C42V/S212C mutant channel was not changed after MTSET treatment.

**DISCUSSION**

A core finding of this paper is that MTSEA and MTSET cannot irreversibly inhibit the S212C mutant channel constructed on a sulphydryl reagent-insensitive Kir6.2 background.
In each data set, except C42V/S212C mutant channel, which was treated with MTSET, MTS reagent treatment. The channels were treated with MTS-TEAH, a mutant channels by 0.6 mM MTS-TEAH in the presence of 50 mM ATP. In our experiments, we noted with interest that neither mutation nor modification of the residues under study significantly changed the unitary amplitude of the single-channel current (analysis not shown). This contradicts mutation of Glu-224, which reduces the unitary amplitude of Kir2.1 (12); modification of putative pore-lining residues in the transmembrane region of Kir6.2 also causes graded changes in the unitary amplitude (10). Although it requires further experimentation to understand this, we speculate that a wider vestibule surrounding Ser-212 of Kir6.2 may at least partly account for the difference.

We also noted that MTS-TEAH produced a reversible block of outward current (e.g. Figs. 2 and 6), but it did not block C42V/S212C channel current after MTSET modification (Fig. 4). MTS-TEAH may act as an inner pore blocker whose access to the reversible blocking site can be hindered by modified Cys-212 in a mechanism similar to that of the MTSEA prevention of spermine block.

Implications for Structure and Function of the Cytoplasmic Vestibule—Information gained from the Cys-212 accessibility reported by channel inhibition after sulfhydryl modification may also be used to estimate the vestibule size near the modified residue. The differences in accessibility shown for MTSET, MTS-TEAH, and MTS-PteEA suggest a vestibule of at least 20 Å in diameter, if we assume that four identical derivatives of the reagents can be held in this region. If it cannot accommodate four MTS-EDANS-CE derivatives simultaneously, then the vestibule is not wider than 25 Å. This estimate seems close to the estimated vestibule size of Kir2.1 at the analogous location, but the Kir2.1 inhibition by MTSEA reveals a substantial difference between these two channels. We postulate that they may be a constricted region in Kir2.1 that is lacking in the Kir6.2 channel. It should be pointed out, however, that the channel inhibition by MTS reagents reported in this study might have also been effected by other mechanisms, such as an altered electrostatic profile introduced by the charged moieties of modifying reagents, and allosteric change of channel gating caused by modification. These effects could bias the above estimation of the vestibule size.

The effects of MTS-TEAH and MTS-PteEA on cysteine mutants of the residues surrounding Ser-212 exhibit an interesting pattern; two residues next to Ser-212 on the amino side are accessible and can cause channel inhibition, whereas residues on the carboxyl side, Ala-213–Met-217, are insensitive to the reagents. Interestingly, the inhibitory effects of MTS-TEAH and MTS-PteEA were weaker at position 211 and were further

(C42V/S212C). This result is in sharp contrast to the effect of these two MTS reagents on E224C mutant channels on a Kir2.1 background (4). This difference could reflect either a global structural difference in the cytoplasmic regions of these two channels, or local variations. Our analysis using MTS reagents of larger size leads to the conclusion that, like Glu-224 of Kir2.1, Ser-212 of Kir6.2 is very possibly a vestibule-lining residue. The results support the central tenet that the cytoplasmic region of Kir channels forms a wide vestibule that contains critical elements controlling inward rectification.

Interpretation of the Effects of MTS Reagents on S212C Mutants—Results obtained from experiments that utilize irreversible change of current activity in response to sulfhydryl-specific reagents as a reporter of substituted cysteine accessibility, like the one we used in this study, must be interpreted with caution. Absence of channel inhibition by MTS reagents is insufficient to conclude that cysteines are inaccessible, simply because modification by MTS reagents may not necessarily affect the current. We determined that MTSET and MTSEA indeed modified Cys-212 by demonstrating their protective effect against MTS-TEAH (Fig. 4). Likewise, channel inhibition following cysteine modification is not sufficient evidence to identify a pore-lining residue, because inhibition may be caused either by direct blockage, or by allosteric effects. To help identify Cys-212 as a pore-lining residue, we showed that modification of S212C mutants by MTSEA (which itself does not inhibit the mutants) and modification of S212C-Kir6.2Δ dimer channels by MTS-TEAH reduced the voltage-dependent block of the pore by spermine. In contrast, modification of Cys-42 of Kir6.2Δ channel by MTS-TEAH, which does not inhibit the channel either, did not have such an effect. We postulate that the change of spermine block may reflect change of the structure or electrostatic profile of the vestibule. Taking this evidence together, we suggest that: MTSEA and MTSET can access and modify Cys-212, but the modification does not lead to channel inhibition, and MTS-PteEA and MTS-TEAH modify this cysteine and result in channel inhibition via direct pore occlusion. There are, however, two possible interpretations for the ineffectiveness of MTS-EDANS-CE. This reagent may be too large to form a blocking complex in the pore; alternatively, it may not access Cys-212. Based on these analyses and reasonable extrapolation, we conclude that Ser-212 is a putative pore-lining residue, which is most likely in the cytoplasmic vestibule of the channel, as has been proposed for its counterpart in Kir2.1 (4).

Fig. 8. Effect of ATP and modification of cysteine substitution mutants by MTS reagents. A, irreversible inhibition of S212C mutant channels by 0.6 mM MTS-TEAH in the presence of 50 mM ATP. Channel activity was partially recovered by subsequent application of 50 mM DTT. B, concentration-dependent inhibition of C42V/S212C mutant channel by ATP before and after 3-min treatment with 0.6 mM MTSET. The data are obtained from fitting the data with a Hill function. The half-inhibitory concentration (IC50) for ATP is 334 μM before the treatment and 354 μM after the treatment. C, IC50 values measured in Kir6.2A channels and cysteine substitution mutants, before and after MTSET reagent. The channels were treated with MTS-TEAH, except C42V/S212C mutant channel, which was treated with MTSET. In each data set, 3–15 experiments were pooled, except those without error bars.

2 Y. Cui and Z. Fan, unpublished observations.
reduced at position 210. Although it is difficult to interpret structural information from these results, we propose that a funnel-shaped vestibule at this location can cause this phenomenon. Protection studies combined with polyamine test of these positions may provide clues to their role in the vestibule structure. In addition, Thr-214 may deserve special attention. Mutation of this residue gave no recordable current, but immunohemical staining indicates that the expression of this mutant appears comparable with that of the Kir6.2Δ channel. Therefore, it would be interesting to investigate whether this residue is involved in supporting a conformation needed by the vestibule structure.

The cytoplasmic region of Kir6.2 may be involved in modulating channel gating (for example, see Refs. 27, 28, 31, and 32). In addition, although recent studies suggest that the transmembrane region of Kir channels contains major candidates for gating machinery (9, 33, 34), a separate intrinsic gate structure in the cytoplasmic vestibule is also suspected (35). Based on our results, the region surrounding Ser-212 seems to have little role, if any at all, in these two functions. On the other hand, our data do support the dogma that charged residues projecting into the cytoplasmic vestibule affect inward rectification as revealed by mutational analysis of strong rectifiers (4, 12, 13, 36). Although Kir6.2 is a weak rectifier, His-216 is involved in supporting a conformation needed by the vestibule function (9, 33, 34), a separate intrinsic gate structure in the cytoplasmic vestibule is also suspected (35). Based on our results, the region surrounding Ser-212 seems to have little role, if any at all, in these two functions. On the other hand, our data do support the dogma that charged residues projecting into the cytoplasmic vestibule affect inward rectification as revealed by mutational analysis of strong rectifiers (4, 12, 13, 36).

Acknowledgments—We thank Xi He and Dr. Talent I. Shevchenko for participation in construction of Kir6.2 mutants.

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