Development of a Novel Thiol Reagent for Probing Ion Channel Structure: Studies in a Model System[†]

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ABSTRACT: We have synthesized a novel thiol reagent, 2-[(methylsulfonyl)thio]ethyl [*N*-(*N*,*N*-dimethylamino)ethyl]carbamate (MTSAC), that contains a carbamate functional group as well as a (positively charged) terminal amino group. The carbamate C−N bond isomerizes on a millisecond time scale and significantly alters the three-dimensional shape of the reagent. The behavior of this reagent was contrasted with that of the commonly used thiol reagent, [(methylsulfonyl)thio]ethylamine MTSEA [Akabas, M. H., & Karlin, A. (1995) *Biochemistry 34,* 12496−12500], with respect to its effect on single-channel currents passing through modified gramicidin channels. While both reagents decreased single-channel currents, the MTSAC-treated channels also showed a pattern of steps in the current recordings on the time scale of the carbamate bond isomerization. Moreover, the pattern and size of these steps were sensitive to the location of the thiol-reactive site in relation to the channel entrance. Thus, MTSAC may prove useful as a reagent for establishing the proximity to the pore in studies of ion channel proteins of unknown structure.

Membrane protein ion channels are the subject of intensive study because of their central role in regulation of ion flow across cell membranes. Despite their importance, little is known about their structure and how they function because they reside within a lipid bilayer; this bilayer environment complicates the use of conventional techniques for structural determination such as X-ray crystallography and NMR spectroscopy. Alternative means of probing protein channel structure are therefore required.

A powerful technique for studying ion channel structure is the recording of currents through a membrane containing the channels. Such electrophysiological recordings can provide single-molecule resolution and, when used in conjunction with site-directed mutagenesis, can be used to elucidate structure−function relationships.

For large complex ion channels like the sodium channel, much attention has been directed toward elucidation of the pore region; the pore region is believed to be responsible for the ion selectivity of the channel (MacKinnon, 1995). A currently popular method for studying the pore region and the ion pathway through the protein is "scanning-cysteine accessibility mutagenesis" [*e.g.* Akabas et al. (1994), Sun et al. (1996), and Karlin and Akabas (1996)]. Amino acids believed to be in or near the pore of the channel are mutated to cysteine (via site-directed mutagenesis). The expressed protein is then exposed to a thiol-reactive compound, and the effect (if any) on channel behavior is recorded. A number of different thiol reagents are in use. Metal ions such as silver and cadmium which form complexes with thiol groups have been used (Lu & Miller, 1995; Tomaselli et al., 1995; Backx et al., 1992), and (methylsulfonyl)thio (MTS)[1] reagents are widely used (Akabas et al., 1992). MTS reagents include MTS-ethylammonium ($MTSEA^+$), MTS-ethyltrimethylammonium ($MTSET^+$), and MTS ethyl sulfonate ($MTSES^-$). These reagents covalently link a charged group to the channel. The MTS reagents are employed most commonly in whole cell current measurements to identify changes (usually an overall decrease in current) from wild type behavior (Akabas et al., 1994; Sun et al., 1996; Stauffer & Karlin, 1994; Kürz et al., 1995). Examples of single-channel recording of MTS-modified channels have also been reported (Mindell et al., 1994).

While these approaches have provided new structural insight in a number of cases, they are not without shortcomings. Generally, the reagent must be applied for a relatively long time (minutes) relative to the time frame of protein motion. Because of this exposure time, the reagent may react with even a very minor channel conformation (Lu & Miller, 1995). Attempts to overcome the problem using very brief applications of reagents have been reported (Cheung & Akabas, 1996).

Another more basic difficulty with the thiol-labeling approach is the assumption that a change in total current is in fact due to the presence of the thiol reagent in the pore. One cannot rigorously exclude the possibility that binding of a thiol reagent might inactivate a channel even at some distance from the pore region. In the whole cell case, thiol modification may even affect the interaction of other molecules with the channel; that is, the effect of the thiol

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[1] Abbreviations: BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; DCC, dicyclohexylcarbodiimide; DMAP, (dimethylamino)pyridine; DMF, dimethylformamide; DTT, dithiothreitol; gram, gramicidin; gram′-SH, gramicidin-(OCO)-CH_2SH; gram″-SH, gramicidin-(OCO)-CH_2CH_2SH; gram′-S-MTSAC, gramicidin-(OCO)-CH_2S-MTSAC; gram″-S-MTSAC, gramicidin-(OCO)-CH_2CH_2S-MTSAC; gram″-S-MTSEA, gramicidin-(OCO)-CH_2CH_2S-MTSEA; MS-EI, mass spectrometry, electron impact; MS-ES, mass spectrometry, electrospray; MS-FAB, mass spectrometry, fast atom bombardment; MTSEA, [(methylsulfonyl)thio]ethylamine/ammonium; MTSAC, 2-[(methylsulfonyl)thio]ethyl-*N*-[(*N*,*N*-dimethylamino)ethyl]carbamate; THF, tetrahydrofuran; TLC, thin-layer chromatography; TEA, triethylamine; TFA, trifluoroacetic acid.

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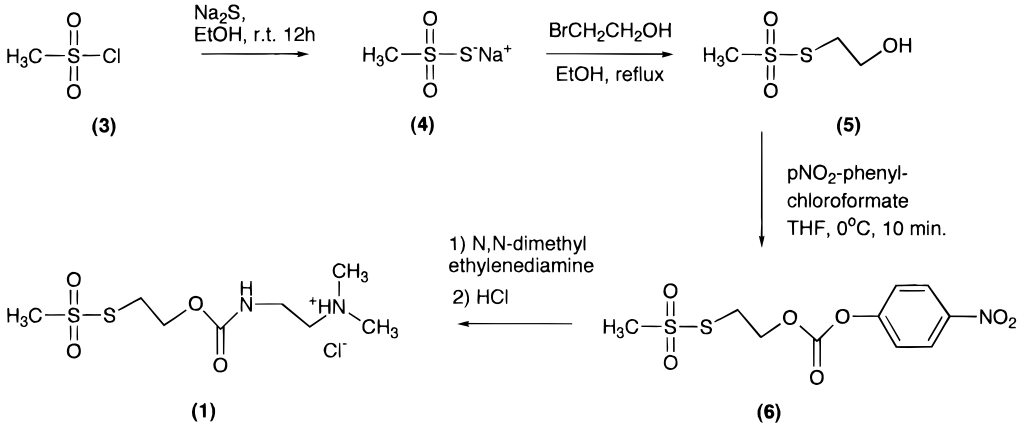
Scheme 1



reagent may be long range. A demonstrated change in single-channel conductance after application of the reagent would be more direct evidence for the presence of the reagent in or near the ion pathway. However, even here, the conductance change may be due to a change in protein structure. For example, Mindell et al. (1994) have proposed an alteration of protein structure as an explanation for the observed changes in single-channel current after treatment of a cysteine-modified diphtheria toxin channel with $MTSES^{-}$. A thiol reagent that provided a unique "signature" in current recordings, when it was present near the pore, but not when it was distant, would allow one to draw more definite conclusions about the location of the reagent.

Carbamate bonds undergo cis−trans thermal isomerization at a frequency of 1−100 Hz at room temperature (Jaikaran & Woolley, 1995; Kessler & Molter, 1976). A thiol reagent incorporating a carbamate bond in addition to a charged amino group would be expected to change its effective shape (*i.e.* its electrostatic charge distribution) as the isomerization occurred. If the reagent were located near the pore in the ion channel protein, this change should alter ion flux, thus acting as an "internal current switching device" causing current steps or a "flicker" in the single-channel current, on a time frame specified by the activation energy of carbamate bond isomerization (Woolley et al., 1995; Jaikaran & Woolley, 1995). If current steps were seen on the expected time scale when this thiol reagent was added to a cysteine-modified channel, it must be concluded that the thiol group is in fact near the pore (and the coupled reagent is moving freely). A long range effect is very unlikely since any coupling of the carbamate isomerization to protein conformational changes would alter the kinetics of isomerization.

On the basis of these arguments, we have now designed and synthesized a thiol reagent designated 2-[(methylsulfonyl)thio]ethyl-*N*-[(*N*,*N*-dimethylamino)ethyl]carbamate [MTSAC (**1**)] (Figure 1). To test the properties of this reagent, and to compare it to the commonly used MTSEA reagent (Figure 1), we have investigated its effect on single-channel currents in a structurally well-defined channel, gramicidin (Andersen & Koeppe, 1996; Woolley & Wallace, 1992). We synthesized two different derivatives of the gramicidin channel, bearing thiol groups at different distances from the C-terminal ends. An analysis of single-channel recordings obtained with these derivatives after reaction with MTSAC indicates that this reagent can serve as a qualitative indicator of the proximity to the pore.

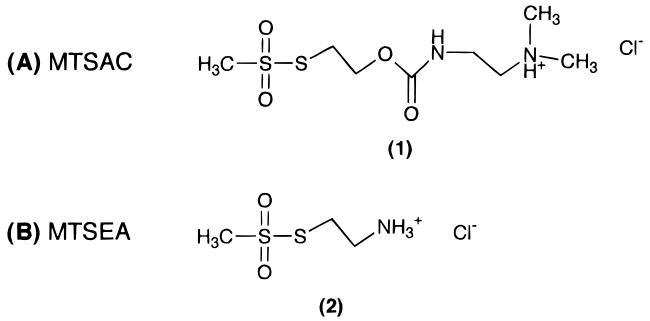


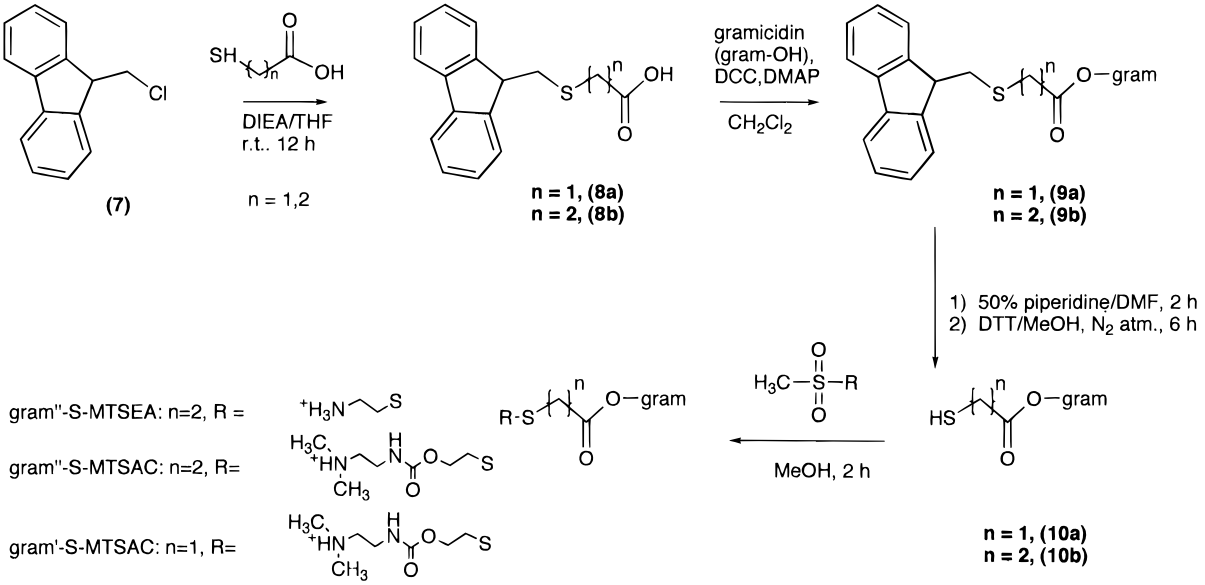
FIGURE 1: Chemical structures of the thiol reagents: (A) 2-[(methylsulfonyl)thio]ethyl-*N*-[(*N*,*N*-dimethylamino)ethyl]carbamate (MTSAC) and (B) 2-[(methylsulfonyl)thio]ethylamine (MTSEA).

EXPERIMENTAL PROCEDURES

Gramicidin was obtained from Sigma (St. Louis, MO); lipids used for single-channel recording were obtained from Nu-Chek-Prep, Inc. (Elysian, MO), and MTSEA was obtained from Toronto Research Chemicals (Toronto, ON). All other chemicals were obtained from Aldrich (Milwaukee, WI).

(I) Synthesis of 2-[(Methylsulfonyl)thio]ethyl N-[(N,N-Dimethylamino)ethyl] carbamate (MTSAC). The synthesis of MTSAC is outlined in Scheme 1. Sodium sulfide nonahydrate (24 g, 0.2 mol) was dissolved in absolute ethanol (60 mL) and cooled to −15 °C, and methanesulfonyl chloride (**3**) (12.5 g or 0.11 mol in 20 mL of absolute EtOH) was added dropwise. The sodium chloride was filtered off and the sodium methylthiosulfonate (**4**) isolated and recrystallized from absolute ethanol to obtain 3.8 g (0.03 mol) of pure product [NMR: δ 3.3 (s, 3H)]. 2-Bromoethanol (2.5 g, 20 mmol) was added dropwise to sodium methylthiosulfonate (**4**) (1.34 g or 10 mmol in 15 mL of absolute EtOH) and the mixture refluxed for 8 h. Sodium bromide formed and was filtered off, and the filtrate was concentrated and extracted with $CHCl_3/H_2O$. The water phase was concentrated and then suspended in 30 mL of THF to precipitate remaining NaBr. Concentration of the filtrate gave 2-[(methylsulfonyl)-thio]ethanol (**5**) (0.86 g, 5.5 mmol) [NMR: δ 3.35 (t, 2H), 3.5 (s, 3H), 3.85 (t, 2H)]. Next, *p*-nitrophenyl chloroformate (0.2 g, 0.5 mmol) in 1 mL of THF was cooled to −10 °C. To this was added TEA (150 mL) over 10 min, and a suspension of 2-[(methylsulfonyl)thio]ethanol (**5**) (95 mg, 0.6 mmol) in THF (1 mL) was added within 15 min while maintaining the temperature. The reaction mixture was stirred overnight at 4 °C. The resultant yellow suspension

Scheme 2



was filtered to isolate a yellow solid which was dissolved in $CHCl_3$ and washed with H_2O. Concentration of the chloroform phase yielded 2-[(methylsulfonyl)thio]ethoxycarbonyl *p*-nitrophenolate (**6**) (70 mg, 0.2 mmol) [TLC (65:25:4 $CHCl_3$/MeOH/H_2O): $R_f = 0.9$. NMR: δ 3.43 (s, 3H), 3.55 (t, 2H), 4.55 (t, 2H), 7.4 (m, 2H), 8.3 (m, 2H)]. Next, this carbonate compound (**6**) (119 mg or 0.37 mol in 2 mL of absolute THF) was cooled to −15 °C. To this was added dropwise *N*,*N*-dimethylethylenediamine (29.4 mg or 0.33 mmol in 2 mL of THF). After addition, the temperature was maintained at 0 °C for 10 min followed by stirring for an additional 10 min at room temperature. The reaction mixture was then cooled to 0 °C again, and HCl (1.8 mL of 1.0 M Et_2O) was added dropwise. Stirring was continued for a further 30 min and the oily solid produced isolated and washed with THF. Dissolution in H_2O followed by freeze-drying yielded the final compound, MTSAC (**1**) (77 mg, 0.3 mmol). NMR: δ 2.85 (s, 6H), 3.25 (t, 2H), 3.45 (t, 2H), 3.45 (t, 2H), 3.5 (s, 3H), 4.35 (t, 2H). High-resolution MS-EI: 270.071 793 for $M^+ - HCl$ ($C_{16}H_{14}O_2S$; calculated, 270.073 137).

(II) Synthesis of Gram-(OCO)-CH_2SH and Gram-(OCO)-CH_2CH_2SH. The C terminus of gramicidin was derivatized to obtain a terminal thiol group, as outlined in Scheme 2. 9-Fluorenylmethyl chloride (**7**) was prepared as detailed by Wawzonek (1956). This compound (**7**) (600 mg, 2.8 mmol) was then reacted with 3-mercaptopropionic acid or 2-mercaptoacetic acid (3.0 mmol) in diisopropylethylamine (1.5 mL) and THF (10 mL) at room temperature, overnight. The solvent was removed under vacuum and resuspended in aqueous Na_2CO_3 at pH 8−9. The mixture was then extracted with $CHCl_3$. The aqueous layer was acidified and extracted with EtOAc. The EtOAc layer was dried and the solvent removed to give the product (**8a,b**). The following data are for (9-fluorenylmethyl)-3-mercaptopropionic acid (**8b**). NMR: δ 2.6 (t, 2H), 2.75 (m, 2H), 3.1 (d, 2H), 4.1 (t, 1H), 7.35 (m, 4H), 7.7 (m, 4H). MS-EI: 284 for M^+ ($C_{17}H_{16}O_2S$; calculated, 284). The following data are for (9-fluorenylmethyl)-2-mercaptoacetic acid (**8a**). NMR: δ 3.7 (s, 2H), 4.2 (t, 1H), 7.35 (m, 4H), 7.7 (m, 4H). MS-EI: 270 for M^+ ($C_{16}H_{14}O_2S$; calculated, 270).

Gramicidin (40 mg, 21.3 mmol) was dissolved in 6 mL of dichloromethane with DCC (175 mg, 850 mmol) and DMAP (26 mg, 213 mmol). (9-Fluorenylmethyl)-3-mercaptopropionic acid (**8b**) (18 mg, 63.7 mmol) was added to the gramicidin/DCC/DMAP solution and stirred overnight. The product (**9b**) was purified by gel-filtration chromatography using lipophilic Sephadex (LH-20) with methanol as the mobile phase. The protected gramicidin derivative (**9b**) (19 mmol, 40 mg) was deprotected by dissolution in 50% piperidine in DMF (1.2 mL) followed by concentration to half-volume by evaporation under nitrogen. To this was added methanol (1 mL), and the solution was passed through the LH-20 column again to give a disulfide-linked gramicidin dimer. To reduce the disulfide bond, 2 mL of the gramicidin dimer solution in MeOH was added dropwise to a N_2-saturated DTT solution (18 mg in 1.0 mL of MeOH) under a N_2 atmosphere. The desired product, gram-(OCO)-CH_2-CH_2SH (**10b**) (gram″-SH), was obtained after 6 h of stirring at room temperature and purification by passage through the LH-20 column. TLC (65:25:4 $CHCl_3$/MeOH/H_2O): R_f = 0.8. MS-FAB: 1970 for M^+ ($C_{102}H_{146}N_{20}O_{18}S$; calculated, 1971).

To obtain gram-(OCO)-CH_2SH (**10a**) (gram′-SH), the same procedure was followed except that (9-fluorenylmethyl)-2-mercaptoacetic acid (**8a**) was substituted for (9-fluorenylmethyl)-3-mercaptopropionic acid (**8b**).

(III) Reaction of Thiol-Derivatized Gramicidin with the Thiol Reagents. The thiol derivatives of gramicidin, gram′-SH (**10a**) and gram″-SH (**10b**), were linked to the two thiol reagents, MTSAC (**1**) and MTSEA (**2**) (3-fold excess to gram^n-SH), by reaction in methanol for 2 h at room temperature (Scheme 2). The following data are for gram″-S-MTSEA. MS-FAB: 2046 for MH^+ ($C_{104}H_{150}N_{21}O_{18}S_2$; calculated, 2045). The following data are for gram″-S-MTSAC. MS-ES: 2160 for M^+ ($C_{109}H_{159}N_{22}O_{20}S_2$; calculated, 2160).

Due to gram′-SH's instability during the deprotection step, an alternative synthesis was also employed where MTSAC (**1**) (60 mL) was coupled with 2-mercaptoacetic acid (3.0 mL) in MeOH (0.4 mL) by stirring for 1 h at room temperature to give the disulfide acetic acid. The disulfide

(300 mL) was coupled to gramicidin (14 mg) in DCC (87 mg)/DMAP (14 mg)/CH_2Cl_2 (4.5 mL) to give gram′-S-MTSAC. The following data are for gram′-S-MTSAC. MS-ES: 2146 for MH^+ ($C_{108}H_{156}N_{22}O_{20}S_2$; calculated, 2145).

Purification of the gramicidin derivatives for single-channel measurements was accomplished by HPLC using a reverse phase Rx-C8 column (Zorbax, Rockland Technologies Inc.). A mobile phase consisting of 80% MeOH in H_2O at pH 3 (0.1% trifluoroacetic acid, adjusted with TEA) was employed. Retention times were as follows: gram″-S-MTSEA, 12.0 min; gram″-S-MTSAC, 9.2 min; and gram′-S-MTSAC, 8.8 min.

(IV) Single-Channel Measurements. Currents through a glycerol monooleate lipid bilayer containing the gramicidin derivative were recorded and controlled using an Axopatch 1D patch clamp amplifier (Axon Instruments). The lipid bilayer was formed over the opening of a pipette tip as described by Busath and Szabo (1988). The recordings were obtained at room temperature (23 ± 1 °C) with a 200 mV holding potential and filtered at 200 Hz. The aqueous solution used was 1 M CsCl and 5 mM BES at pH 6.3 (adjusted with CsOH). Current recordings were stored and analyzed using Synapse software (Synergistic Research Systems). Current amplitudes and step sizes were measured directly using cursors available in the Synapse software. Errors given reflect standard deviations in the calculated values (number of samples as indicated). For a more detailed analysis of the kinetics of carbamate isomerization observed via single-channel recordings, see Jaikaran and Woolley (1995).

Heterodimers of the MTSAC derivatives and unmodified gramicidin were obtained by first adding pure gram^n-S-MTSAC to obtain homodimers, followed by addition of unmodified gramicidin to one compartment only. Heterodimers and homodimers were distinguished by reversing the polarity of the applied voltage during the recording of an open channel. Homodimer currents were of the same magnitude with either voltage, whereas heterodimer currents were of different magnitudes.

RESULTS AND DISCUSSION

(I) Synthesis of a Novel Thiol Reagent, MTSAC. The structure of MTSAC incorporates three functional groups: a (methylsulfonyl)thio (MTS) group at one end of the molecule, an amino group at the other end, and an internal carbamate bond. The MTS group is known to react with thiol groups with high specificity (Kenyon & Bruice, 1977). The terminal charged amino group will affect cation currents electrostatically, and the cis−trans isomerization of the internal carbamate bond provides the unique "current signature" of the reagent. The synthesis of MTSAC was adapted from Bruice and Kenyon (1982) and the synthesis of gramicidin/ethylenediamine (Woolley et al., 1995); the synthetic route is outlined in Scheme 1. Methanesulfonyl chloride (**3**) was converted to the thiosulfonate salt (**4**) by reaction with sodium sulfide. Reaction with 2-bromoethanol afforded 2-[(methylsulfonyl)thio]ethanol (**5**). This was then reacted with *p*-nitrophenyl chloroformate to give 2-[(methylsulfonyl)thio]ethoxycarbonyl *p*-nitrophenolate (**6**). The *p*-nitrophenolate moiety was then displaced by *N*,*N*-dimethylethylenediamine to give the thiol reagent, MTSAC (**1**).

(II) Reaction of Thiol Reagents with Thiol Derivatives of Gramicidin. To test MTSAC, and compare its action with that of MTSEA (a reagent without a carbamate bond), we required a model system. Gramicidin was chosen; it is structurally well characterized and is popular as a system for studying structure−function relationships in ion channels (Koeppe & Andersen, 1996). Gramicidin forms cation-selective channels in a variety of membranes; channels form through N terminus to N terminus dimerization of peptide monomers. The C termini of the peptides form the entrance and exit of the channel. A variety of C-terminal derivatives of gramicidin have been described; the structure of the channel does not seem to be sensitive to modification at this site (Vogt et al., 1991; Woolley & Wallace, 1992).

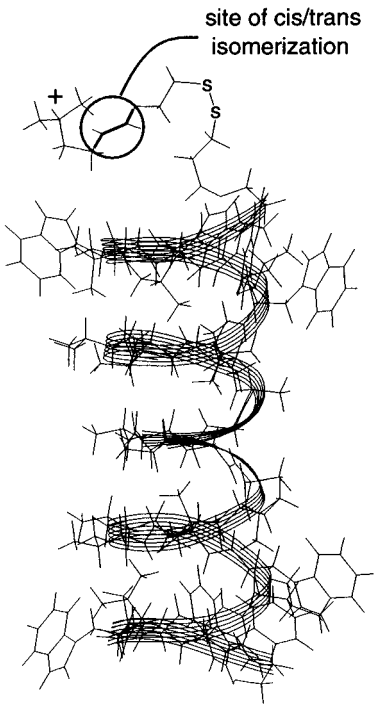


FIGURE 2: Structure of a gramicidin channel modified at one end with the MTSAC reagent (*i.e.* a heterodimer channel). The carbamate bond that isomerizes is circled. The channel structure is that reported by Arseniev et al. (1985). The modified C terminus is flexible, and only one of many possible conformations is shown.

We derivatized the C terminus of gramicidin by forming esters with two different thiol-carboxylic acids. In this way, a thiol group is positioned at the channel entrance and exit, close to the ion pathway. Reaction of this thiol group with a thiol reagent would thus place the reagent near the mouth of the channel as shown in Figure 2. The average distance of the thiol group from the channel mouth was varied by using either 2-mercaptoacetic acid or 3-mercaptopropionic acid. We predicted that the closer the charged amino group of the reagent was to the mouth of the channel (*i.e.* the shorter the derivative), the greater the effect on the ion flux.

(III) Single-Channel Recordings. Figure 3 shows single-channel current recordings of unmodified gramicidin (A), gram″-S-MTSEA (B), and gram″-S-MTSAC (C). Currents through each modified channel were smaller than for gramicidin under the same experimental conditions. Thus, the presence of the positively charged thiol reagents at the entrance (and exit) of the channel reduces cation flow through the channel, consistent with previous observations (Mindell et al., 1994; Akabas et al., 1992). Importantly, gram″-S-MTSAC exhibited a flicker in current, as predicted (Figure 3C). This flicker is not simply noise since it is not present

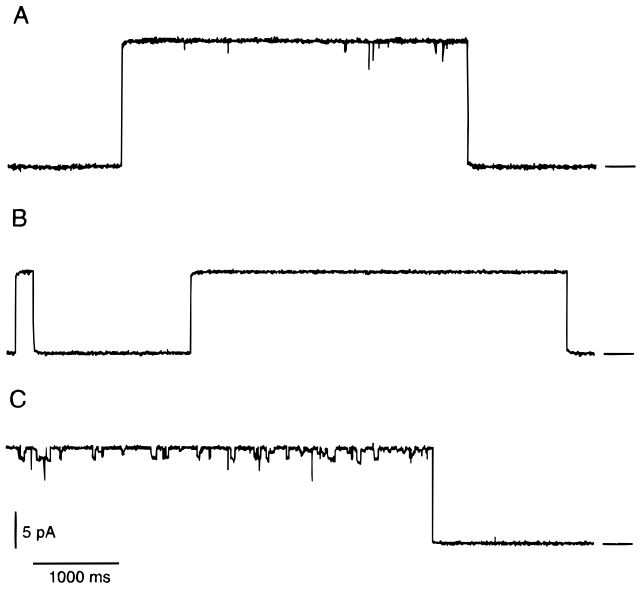


FIGURE 3: Single-channel recordings of gramicidin and derivatives. The baselines (no channel open) are indicated by the horizontal bars: (A) unmodified gramicidin (15.3 ± 0.9 pA, $n = 59$), (B) gram″-S-MTSEA (10.7 ± 0.4 pA, $n = 176$), and (C) gram″-S-MTSAC (topmost level 13.5 ± 0.5 pA, $n = 133$).

in the baseline current (Figure 3C, horizontal bar). Gram″-S-MTSEA, which contains a disulfide bond and a positively charged amino group, but not the carbamate group, also does not show current flickers. The disulfide bond does have two preferred rotomers, but because the barrier to interconversion is relatively low [7 kcal/mol (Creighton, 1993)], disulfide rotation is too rapid to be detected in these recordings. Thus, the current steps in the gram″-S-MTSAC recording can be attributed to thermal cis−trans isomerization of the carbamate bond in the MTSAC reagent.

Previous studies have shown that the trans state of these types of carbamate groups is more stable than the cis state (Kessler & Molter, 1976; Jaikaran & Woolley, 1995). Thus, the longer-lived state (the upper current level in Figure 3C) presumably reflects MTSAC reagents in trans conformations. Average lifetimes for the states (38 ms for cis and 266 ms for trans) estimated from recordings of channels in which only one MTSAC reagent was present (see below) are consistent with previous measurements (Jaikaran & Woolley, 1995).

Since each molecule of MTSAC can exist in two states, cis and trans, the number of current levels can in principle increase as 2^N, where N is the number of thiol reagents present. Figure 4 shows magnified traces of currents flowing through an open channel with one (panel B) or two (C) MTSAC groups. The current observed when two channels are open (each with two MTSAC groups) is shown in panel D. A heterodimer of unmodified gramicidin and gram″-S-MTSAC, which has only one MTSAC group, exhibits two levels of current (Figure 4B). The size of this step is dependent on the direction of the current flow. For one orientation of the applied voltage, the single-channel current is 12.9 ± 0.8 pA ($n = 31$) (the upper conductance state), whereas for the other orientation, this current is 16 ± 1 pA ($n = 30$) [which is comparable to that for unmodified gramicidin (15.3 ± 0.9 pA, $n = 59$)] and the step size is 0.4 pA. The homodimer of gram″-S-MTSAC shows at least three levels of a possible four. MTSAC's effect on the

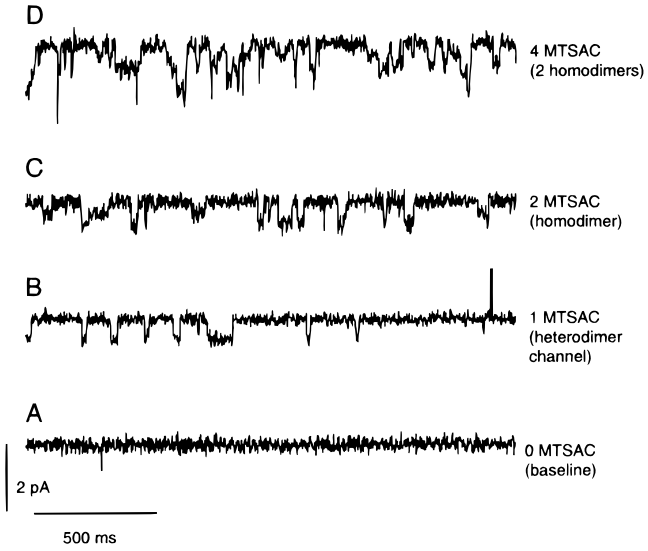


FIGURE 4: Effects of different numbers of MTSAC reagents on the open channel current. The number of channels open is known from the number of large (~14 pA) steps observed above the baseline. For clarity, the baselines are not shown in B−D and current through the open channel(s) is shown on an expanded scale: (A) baseline current, (B) current through an open gram″-S-MTSAC/gramicidin heterodimer showing two current levels (one step), (C) current through an open gram″-S-MTSAC homodimer (at least three different current levels can be identified), and (D) current observed when two gram″-S-MTSAC homodimers are open.

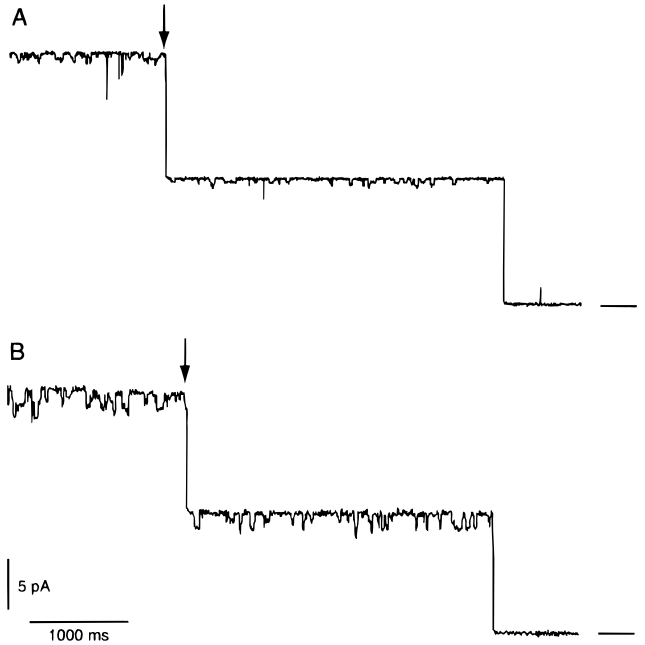


FIGURE 5: Comparison of single-channel records for gram″-S-MTSAC (A) and gram′-S-MTSAC (B) homodimers. Two channels are open at the start of each record. One channel closes at the point marked with an arrow. The baselines are indicated with horizontal bars. The steps in current are more pronounced with the shorter derivative (B).

current becomes more marked if two (or more) channels are open simultaneously (panel D).

The effect of varying proximity of the MTSAC group to the channel mouth was tested by comparing single-channel recordings of gram″-S-MTSAC and gram′-S-MTSAC (Figure 5). Gram′-S-MTSAC has one less methylene unit in the linkage to the terminal amino group than gram″-S-MTSAC so that the charged group should (on average) be

closer to the channel mouth. It was expected that gram′-S-MTSAC would show more pronounced current flickers since changes in "shape" of the MTSAC group would more directly influence ion flux. In Figure 5, two channels are open at the beginning of each record; at the arrow, one channel closes, then this channel closes, and the baseline is recorded (shown by the horizontal bar). The steps in gram′-S-MTSAC current (B) are significantly more pronounced than with gram″-S-MTSAC (A). The effect is particularly obvious when two channels are open. In the single-channel recording of gram′-S-MTSAC, there are four current levels apparent, as expected for the homodimer, whereas the fourth level was not clearly apparent in gram″-S-MTSAC. Also, the difference between the topmost and bottommost levels is 2 pA for gram′-S-MTSAC, as compared to 1 pA for gram″-S-MTSAC. The maximal current was reduced slightly from 13.5 ± 0.5 pA for gram″-S-MTSAC to 12.2 ± 0.4 pA for gram′-S-MTSAC, also indicating that the MTSAC group was having a greater effect on the current. Thus, the effect of MTSAC does indeed appear to be a function of its distance from the pore.

MTSAC behaves essentially as predicted and may prove useful as a new probe of ion channel structure. If it were added to a cysteine-substituted channel of unknown three-dimensional structure, and current steps appeared with lifetimes similar to those shown in Figures 3−5, one could conclude that the reagent was interacting directly with the ionic flux. If the reagent instead induced conformational switching in the protein, it is unlikely that the activation barrier for such switching would be the same as that for carbamate isomerization. An increase of even 1 kcal/mol in the barrier would lead to more than a 5-fold increase in state lifetimes. With further development, better reagents may be obtained. For example, placement of an electron-withdrawing group adjacent to the carbamate nitrogen would be expected to increase the rate of isomerization. This would be desirable for use in a channel with a shorter open lifetime. The charge of the reagent could also be altered to maximize effects on ion flow. Smaller reagents may be useful if site accessibility proves to be a problem. Such designed thiol reagents should expand the range of techniques available to study the structures of ion channel proteins.

REFERENCES

Akabas, M. H., & Karlin, A. (1995) *Biochemistry 34*, 12496−12500.

Akabas, M. H., Stauffer, D. A., Xu, M., & Karlin, A. (1992) *Science 258*, 307−310.

Akabas, M. H., Kaufmann, C., Archdeacon, P., & Karlin, A. (1994) *Neuron 13*, 919−927.

Arseniev, A. S., Barsukov, I. L., Bystrov, V. F., Lomize, A. L., & Ovchinnikov, Y. A. (1985) *FEBS Lett. 186*, 168−174.

Backx, P. H., Yue, D. T., Lawrence, J. H., Marban, E., & Tomaselli, G. F. (1992) *Science 257*, 248−251.

Bodansky, M., & Bednare, M. A. (1982) *Int. J. Pept. Protein Res. 20*, 437.

Bruice, T. W., & Kenyon, G. L. (1982) *J. Protein Chem. 1*, 47−58.

Busath, D., & Szabo, G. (1988) *Biophys. J. 53*, 689−695.

Cheung, M., & Akabas, M. H. (1996) *Biophys. J. 70*, 2688−2695.

Creighton, T. E. (1993) *Proteins: Structure and Molecular Properties*, 2nd ed, W. H. Freeman & Co., New York.

Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd ed., Sinauer Associates Inc., Sunderland.

Jaikaran, D., & Woolley, G. A. (1995) *J. Phys. Chem. 99*, 13352−13354.

Karlin, A., & Akabas, M. H. (1996) *Neuron 15*, 1231−1244.

Kenyon, G. L., & Bruice, T. W. (1977) *Methods Enzymol. 47*, 407−430.

Kessler, H., & Molter, M. (1976) *J. Am. Chem. Soc. 98*, 5969−5973.

Koeppe, R. E., II, & Andersen, O. S. (1996) *Annu. Rev. Biophys. Biomol. Struct. 25*, 231−258.

Kürz, L. L., Zühlke, R. D., Zhang, H.-J., & Joho, R. H. (1995) *Biophys. J. 68*, 900−905.

Lu, Q., & Miller, C. (1995) *Science 268*, 304−307.

MacKinnon, R. (1995) *Neuron 14*, 889−892.

Mindell, J. A., Zhan, H., Huynh, P. D., Collier, R. J., & Finkelstein, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5272−5276.

Sakmann, B., & Neher, E., Eds. (1983) *Single-Channel Recording*, Plenum Press, New York.

Stauffer, D. A., & Karlin, A. (1994) *Biochemistry 33*, 6840−6849.

Sun, Z. P., Akabas, M. H., Goulding, E. H., Karlin, A., & Siegelbaum, S. A. (1996) *Neuron 16*, 141−149.

Tomaselli, G. F., Chiamvimonvat, N., Nuss, H. B., Balser, J. R., Perez-Garcia, M. T., Xu, R. H., Orias, D. W., Backx, P. H., & Marban, E. (1995) *Biophys. J. 68*, 1814−1827.

Vogt, T. C. B., Killian, J. A., Demel, R. A., & De Kruijff, B. (1991) *Biochim. Biophys. Acta 1069*, 157−164.

Wawzonek, S., & Dufek, E. (1956) *J. Am. Chem. Soc. 78*, 3530−3533.

Woolley, G. A., & Wallace, B. A. (1992) *J. Membr. Biol. 129*, 109−136.

Woolley, G. A., Jaikaran, A. S. I., Zhang, Z., & Peng, S. (1995) *J. Am. Chem. Soc. 117*, 4448−4454.

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