Methanethiosulfonate Derivatives Inhibit Current through the Ryanodine Receptor/Channel

Kerry E. Quinn and Barbara E. Ehrlich

From the Department of Physiology and Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030

ABSTRACT To identify regions of the ryanodine receptor (RyR) important for ion conduction we modified the channel with sulfhydryl-reacting compounds. After addition of methanethiosulfonate (MTS) compounds channel conductance was decreased while other channel properties, including channel regulation by ATP, caffeine, or Ca, were unaffected. The site of action was accessible to the MTS compounds from the cytoplasmic, but not the luminal, side of the channel. In addition, the hydrophilic MTS compounds were only effective when the channel was open, suggesting that the compounds covalently modify the channel from within the water-filled ion conducting pathway. The decrease in channel current amplitude occurred in a step-wise fashion and was irreversible and cumulative over time, eventually leading to the complete block of channel current. However, the time required for each consecutive modification during continuous exposure to the MTS compounds increased, suggesting that successive modification by the MTS compounds is not independent. These results are consistent with the hypothesis that the channel forms a wide vestibule on the cytoplasmic side and contains a much smaller opening on the luminal side. Furthermore, our results indicate that the MTS compounds can serve as functional markers for specific residues of the RyR to be identified in molecular studies.

KEY WORDS: Ca-release channel • sulfhydryl modification • channel conductance • conduction pathway

INTRODUCTION

The ryanodine receptor (RyR)[1] of the sarcoplasmic reticulum is the primary intracellular Ca-release channel of cardiac and skeletal muscle and is essential for Ca signalling in a number of other cell types. Through the tight regulation of RyR gating of Ca release, these muscle cells contract and relax in response to physiological demands. Similarly, the RyR in nonmuscle cells releases Ca from intracellular stores in response to a variety of cellular signals.

The RyR is the largest transmembrane channel identified to date. From the primary sequence many regulatory sites have been proposed, and putative transmembrane regions have been identified (Takeshima et al., 1989; Zorzato et al., 1990). Models of the structure of the RyR have been constructed from hydropathy plots (Takeshima et al., 1989; Zorzato et al., 1990), surface accessibility of cleavable sites (Marks et al., 1990; Callaway et al., 1994), biophysical studies (Lindsay et al., 1994; Tinker and Williams, 1995), and from electron microscopic images (Radermacher et al., 1994; Serysheva et al., 1995*a*, *b*). Nonetheless, the location of these regulatory components in the RyR are largely unknown. In addition, it is unknown how the individual subunits fold in cellular membranes to form the pore(s) of the channel, nor is it known how many pores a RyR has. Two simple possibilities exist; one is that the four identical subunits come together to form a channel containing a single pore, which is divided into quadrants and is influenced by the conformation of each of the subunits. The second possibility is that each subunit forms a pore, which contributes one-fourth to the unitary conductance of the channel. Single channel analysis has shown four subconductance states of the RyR each being one quarter of the total conductance of the channel (Smith et al., 1988). Although this would suggest that there are four equal pores that work together to produce a unified current flow through the channel, it is also possible that a single pore exists and the substates reflect shifts in the conformation of the subunits which surround the pore (Dani and Fox, 1991).

We used the methanethiosulfonate (MTS) compounds methanethiosulfonate ethylammonium ($MTSEA^+$) and methylmethane thiosulfonate (MMTS) as tools to probe the conduction pathway of the RyR. These compounds were chosen because they are highly specific sulfhydryl-reacting compounds (Akabas et al., 1992). The hydrophilic nature of the MTS compounds and the specificity for attacking cysteine residues have been used to identify and study the water-filled conduction pathway of a number of channel types (Akabas et al., 1992; Xu and Akabas, 1993; Kurz et al., 1995; Yang and Horn, 1995; Huynh et

Address correspondence to Kerry E. Quinn, Ph.D., Department of Physiology MC #3505, University of Connecticut Health Center, Farmington, CT 06030-3505. Fax: 860-679-3346; E-mail: kquinn@neuron.uchc.edu

[1]*Abbreviations used in this paper:* MMTS, methylmethane thiosulfonate; MTS, methanethiosulfonate; $MTSEA^+$, methanethiosulfonate ethylammonium; RyR, ryanodine receptor.

al., 1996). We hypothesized that covalent interactions of the MTS compounds with the RyR would alter channel properties and that the type and degree of effects would vary depending on the charge of the MTS compound used.

This paper reports our initial characterization of the interaction between the MTS compounds and the RyR. Our results show that these compounds will be useful reagents for mapping the pore-lining regions of the RyR. We found that addition of $MTSEA^+$ decreased the current in four increments, and the time increments between successive steps were unequal, indicating that the step-wise channel modifications were not occurring independently. We also found that ryanodine modification of the RyR decreased the ability of $MTSEA^+$ to modify channel conductance. In sum, the nature of the changes in channel conductance in the presence of the MTS compounds support models previously described (Lindsay et al., 1994) in which the RyR makes a single funnel-shaped pore with the widest region facing the cytoplasmic domain.

METHODS

Membrane Vesicle Preparation

Microsomes from rabbit skeletal muscle were prepared as described previously (Kim et al., 1983), except that the homogenization buffer contained 20 mM TRIS, pH 7.0. Briefly, rabbit skeletal muscle was homogenized and microsomes were separated from the rest of the cell by differential centrifugation. Samples were frozen in liquid nitrogen and stored at −80°C.

Bilayer Technique

The RyR was incorporated into planar lipid bilayers as described previously (Ehrlich and Watras, 1988). Bilayers were formed by painting a lipid mixture of phosphatidylethanolamine: phosphatidyl-choline (Avanti Polar Lipids, Alabaster, AL) in decane, across a 100-μm hole in a teflon membrane which separated two halves of a lucite chamber. The *cis* solution was a HEPES/Tris buffer lacking monovalent alkali metal and halide ions (250 mM HEPES, 125 mM Tris, pH 7.3). Ca on the *trans* side (50 mM $Ca(OH)_2$, 250 mM HEPES) served as the only permeant ion in the system. SR vesicles were added to the *cis* side and fusion with the lipid bilayer was induced by making the *cis* side hyperosmotic by the addition of 400–500 mM KCl. After the appearance of potassium and chloride channels, the *cis* side was perfused with the HEPES/Tris buffer. The free Ca concentration on the *cis* side was adjusted to 5 μM using a Ca-EGTA–buffered solution. Channel currents were amplified using a bilayer clamp amplifier (Warner Instruments, New Haven, CT) and recorded on VHS tapes. Data were filtered to 1 kHz, digitized at 5 kHz, then transferred to a personal computer and analyzed with pClamp 6.0 (Axon Instruments, Foster City, CA).

To determine single channel amplitudes, openings shorter than 2 ms were excluded from the analysis. For all other analyses, events shorter than 0.5 ms were excluded. Open probabilities were calculated from a minimum of 3 min of continuous recording. Channel openings are shown as upward deflections from the zero current line (solid line). Dashed lines represent the current amplitude of channel openings for the untreated RyR. Openings which appear above the dashed lines represent a second channel. Differences in the open probability among control recordings reflect normal channel to channel variability. Each channel serves as its own control for any noted differences in channel activity recorded after a treatment protocol. Values reported are means ± SE.

Sulfhydryl Compound Treatment

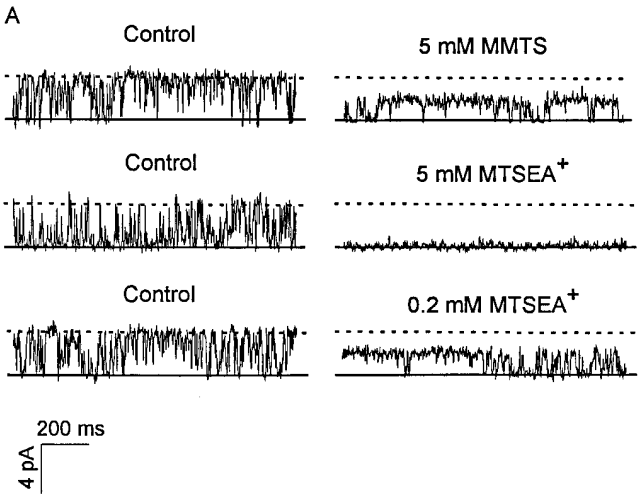
The methanethiosulfonate compound $MTSEA^+$ was initially obtained as a gift from Myles Akabas (Columbia University, NY), and subsequent samples were obtained from Toronto Research Chemicals Inc. (Ontario, Canada). MMTS was purchased from Sigma Chemical Co. (St. Louis, MO). After the incorporation of RyR channels in the bilayer, $MTSEA^+$ at 0.05, 0.1, 0.2, 0.5, or 5 mM, or MMTS at 5 mM were added to the *cis* side of the bilayer. Control segments are current recordings made before the addition of the test compounds. In one series of experiments the compound was added to the *trans* side of the bilayer. A treated channel refers to a channel that has been treated with the MTS compound and the compound has been removed. Removal is achieved by perfusion of 10 vol of compound-free solution.

RESULTS

Modification of the Current Amplitude of the RyR after MTS Treatment

The ability of the MTS compounds to modify the characteristics of the RyR was tested on native skeletal RyR incorporated into planar bilayers. Untreated control channels (Fig. 1 *A*) appeared as described previously by many investigators (Smith et al., 1988; Bezprozvanny et al., 1991; Tinker and Williams, 1992). Addition of MTS compounds to the *cis* side of the RyR caused a decrease in the current amplitude. Upon addition of the neutral MMTS (5 mM) channel current was reduced by half (Fig. 1 *A*, *top right trace*). If a positively charged MTS compound ($MTSEA^+$, 5 mM) was added to the *cis* side of the RyR, the channel was completely blocked (Fig. 1 *A*, *middle right trace*). This effect was observed at all voltages tested (Fig. 1 *B*). The slope conductance of the MMTS-modified channel was approximately half of the untreated channel (48 ± 6 vs. 108 ± 5 pS, $n = 3$).

When lower concentrations of $MTSEA^+$ were applied (i.e., 0.2 mM, Fig. 1 *A*, *bottom right trace*), full block did not occur and intermediate conducting states were seen. The $MTSEA^+$- induced reduction in current amplitude by half occurred more rapidly when higher concentrations of $MTSEA^+$ were applied (Fig. 2); 2.2 ± 0.4 min ($n = 3$) at 0.5 mM $MTSEA^+$, 6.7 ± 1.2 min ($n = 3$) at 0.05 mM $MTSEA^+$, and >20 min at 0.01 mM $MTSEA^+$ ($n = 1$). At concentrations ≥0.1 mM $MTSEA^+$, extended incubation times eventually resulted in a full block of channel current. In every experiment, change in channel conductance remained after removal of the MTS compounds. In one case, an extensive washout of $MTSEA^+$ (>30 chamber vol) still did not return channel currents to control levels. Modification of channel conductance from either MMTS or $MTSEA^+$ treatment



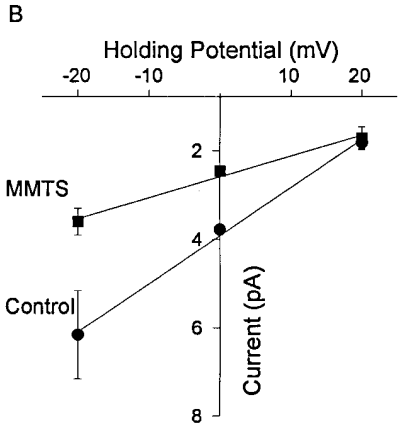


FIGURE 1. Effect of MTS compounds on current amplitude of the RyR. Single channel recordings are shown for RyR in lipid bilayers. (*A*) MMTS (5 mM, *top trace*) and $MTSEA^+$ (5 mM, *middle trace*, and 0.2 mM, *lower trace*) were added to the *cis* side of the bilayer for 5 min and was subsequently removed. The traces on the left show the current before treatment and the traces on the right show the current after treatment. The solid line is zero current. Voltage was held at 0 mV. One of three (MMTS) or four ($MTSEA^+$) similar experiments is shown. (*B*) MMTS altered channel current at all voltages tested. Slope conductance was 108 ± 5 pS for untreated and 48 ± 6 pS for treated channels ($n = 3$).

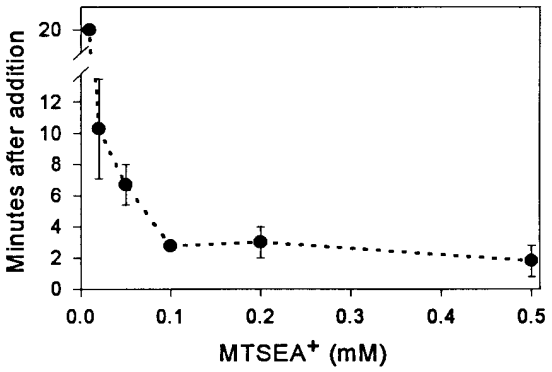


FIGURE 2. Time-dependent effect of $MTSEA^+$ on current amplitude and channel activation of the RyR. $MTSEA^+$ was added at concentrations shown on the x-axis. Channel currents were continually monitored before and after the addition of the compound and during the entire incubation period, up to 30 min. After addition of the compound, the time required to achieve a drop in channel conductance to half of the control levels was monitored. Except for the value for 0.01 mM $MTSEA^+$ which is from one experiment, each value represents the mean ± SEM of at least three similar experiments.

persisted as long as the channel remained active in the bilayer, sometimes as long as 2 h.

The maximum effect of MMTS was reduction of the current amplitude to half of the control value (see Fig. 1), even when concentrations above 5 mM MMTS were applied to the channel. If a channel was modified first with MMTS to the half conducting state, subsequent additions of $MTSEA^+$ did not change the current amplitude. Similarly, if the channel was treated first with $MTSEA^+$ then MMTS, the channel openings were inhibited as in experiments where only $MTSEA^+$ was added.

Many properties of the channel were unchanged by MTS treatment. Although the MTS compounds caused activation of the channel, the effect disappeared upon removal of the compounds. The only irreversible effect caused by the MTS compounds was the decrease in channel conductance. The mean open and closed times and the open probabilities were unaffected by $MTSEA^+$ compared to the untreated channels: open times ($\tau_o = 11 \pm 5$ vs. 12 ± 2 ms, $n = 3$; Fig. 3), closed times ($\tau_c = 13 \pm 8$ vs. 16 ± 4 ms, $n = 3$), open probability ($P_o = 0.43 \pm 0.19$ vs. 0.39 ± 0.05, $n = 3$). Compounds traditionally used to activate the RyR (e.g., ATP, Ca, and caffeine) also activated the MTS-treated channel and did not reverse any of the MTS compound's effects on channel current amplitude. Addition of *cis* ATP induced channel activation in both the untreated (Fig. 4 *A*, *left*) and $MTSEA^+$-treated RyR (Fig. 4 *A*, *right*) without altering the current amplitude in the control or $MTSEA^+$-modified channel. The concentration of ATP required for half-maximal activation of the channel was 0.5 mM for both untreated and treated RyRs (Fig. 4 *B*). Similarly, a RyR modified by $MTSEA^+$ still responded to *cis* Ca, where half-maximal activation by Ca occurred at pCa 6 as in our control experiments and in Ca release experiments (Meissner et al., 1986). Also, caffeine activated the $MTSEA^+$-modified channel as in untreated channels (not shown).

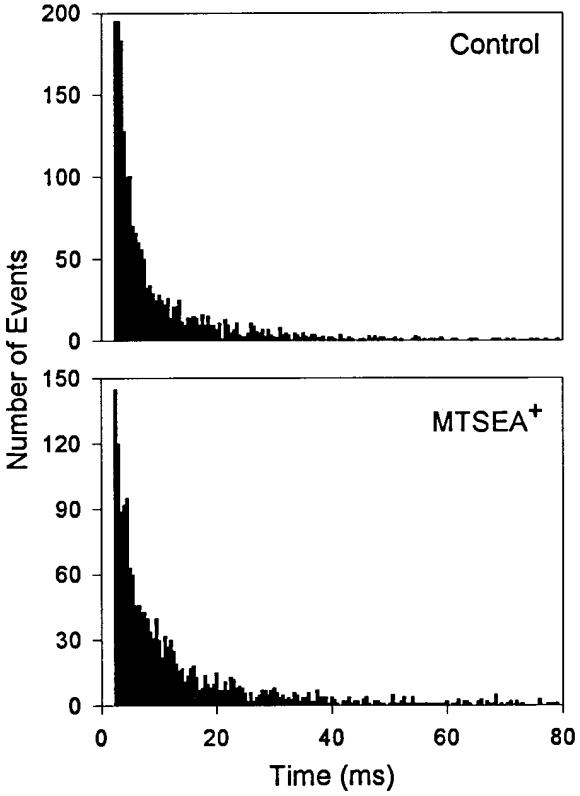


FIGURE 3. Mean open time of the RyR before and after $MTSEA^+$ treatment. The upper histogram shows the distribution of open times for the untreated channel. The lower histogram shows the same data for the channel following treatment with 0.1 mM $MTSEA^+$ (10 min, *cis* addition). One of three similar experiments is shown.

Location of the Sites for Covalent Modification by $MTSEA^+$

To localize the effect of $MTSEA^+$ and to test for nonspecific effects on the lipid bilayer, responses to application of $MTSEA^+$ to either the *cis* or *trans* side of the channel were compared. We found that addition of $MTSEA^+$ to the *trans* side of the channel had no effect on the current amplitude (Fig. 5). Channel behavior was observed for at least 15 min, in the presence of either 0.25 mM $MTSEA^+$ (Fig. 5, *left traces*) or 5 mM $MTSEA^+$ (Fig. 5, *right traces*). When $MTSEA^+$ was added to the *cis* compartment, the current amplitude was reduced to half within 3 min at 0.25 mM (Table I) and was completely inhibited within 30 s in the presence of 5 mM $MTSEA^+$. Thus, no effect of *trans* addition of $MTSEA^+$ was seen even after waiting 30 times the duration required to see an effect from the *cis* side. Experiments were also done where $MTSEA^+$ was added first to the *trans* side with no effect, and subsequently added to the *cis* side, resulting in a reduction of the current amplitude as when $MTSEA^+$ was added *cis* only.

Next, the ability of $MTSEA^+$ to modify the channel, maintained in either the closed or actively opening

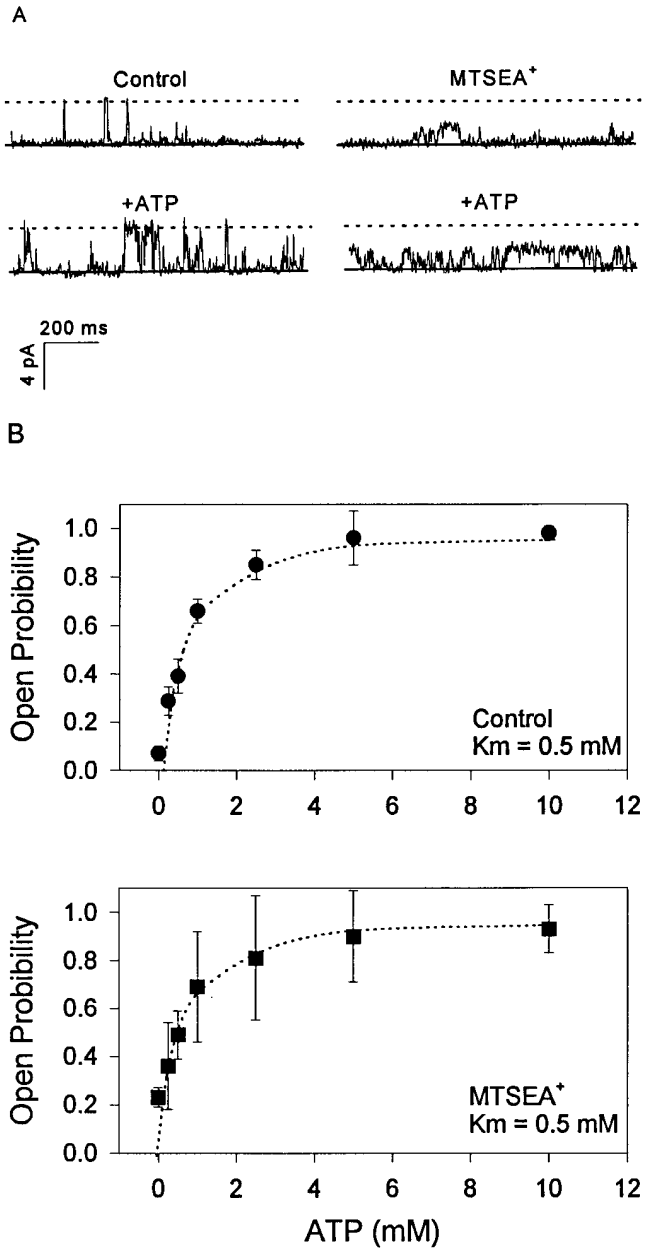


FIGURE 4. Effect of ATP on channel activation in untreated controls and $MTSEA^+$-treated RyRs. (*A*) The traces on the left show an untreated channel. The traces on the right show a channel after treatment with 0.1 mM $MTSEA^+$ (5 min, *cis* addition). The bottom traces show the respective channels after treatment with 0.5 mM ATP (*cis* addition). These results show that ATP activates the RyR in both the absence and presence of $MTSEA^+$ treatment. One of three similar experiments is shown. (*B*) The graph on the top shows the ATP-response curve of an untreated RyR ($n = 7$). The values shown are the channel's open probability at corresponding concentrations of ATP. The graph on the bottom shows the ATP-response curve of a RyR after treatment with 0.2 mM $MTSEA^+$ (5 min, *cis* addition, $n = 3$). The data points of both graphs were fit by the Michaelis Menten equation (*dashed lines*). The K_m for ATP in both the untreated and the treated channels was 0.5 mM.

TABLE I

Time Required for MTSEA$^+$-induced Transitions in the Current Amplitude

Concentration of MTSEA$^+$	Transition from 3/4 to 1/2 of the control amplitude	Transition from 1/2 to 1/4 of the control amplitude
0.05 mM	2.8 ± 0.23 min, $n = 3$	13.8 ± 3.6 min, $n = 3$
0.2 mM	1.5 (0.5, 2.5) min, $n = 2$	5.5 ± 2.5 min, $n = 4$

Channels, incorported into planar lipid bilayers, were treated with either 0.05 or 0.2 mM MTSEA$^+$ (*cis* addition). Channel currents were monitored and the time (in minutes) required for a change in the current amplitude is shown for each concentration. Values are mean ± SEM. For $n = 2$ the value is mean (range).

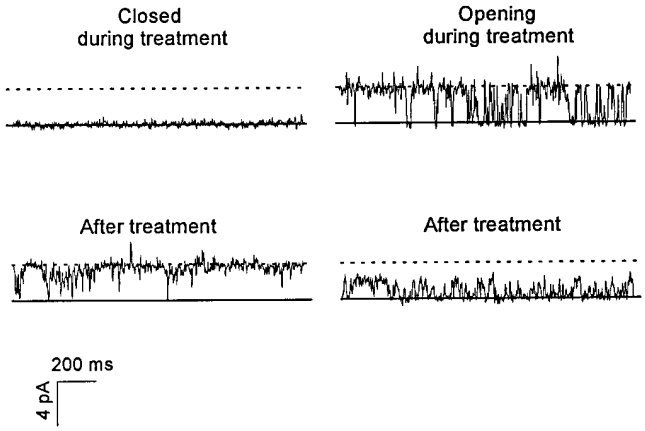


FIGURE 6. Effect of adding MTSEA$^+$ to an open or closed RyR. MTSEA$^+$ was added at 0.1 mM (10 min) to the RyR maintained in either the closed state (by the addition of 1 mM $CaCl_2$ to the *cis* buffer) or stimulated to open (by adjusting the *cis* buffer to contain 5 μM free Ca). MTSEA$^+$ treatment had no effect when added to the closed channel (*left traces*, one of three similar experiments is shown), but in the presence of an actively opening channel, decreased the current amplitude (*right traces*, one of four similar experiments is shown).

state, was assessed. The channel was either maintained closed during treatment by the addition of 1 mM *cis* $CaCl_2$ (Fig. 6, *upper left*), (Meissner and Henderson, 1987; Bezprozvanny et al., 1991), or the channel was maintained in the actively opening state by the presence of 5 μM *cis* Ca (Fig. 6, *upper right*). Both channels were incubated with 0.1 mM MTSEA$^+$ (*cis* side) for 5 min, the MTSEA$^+$ was removed, the channels were reactivated, and channel currents were observed (Fig. 6, *bottom*). If the channel was closed during exposure to MTSEA$^+$, the current amplitude was unaltered (Fig. 6, *bottom left*). However, when MTSEA$^+$ was added to the actively opening channel, current amplitude was reduced (Fig. 6, *bottom right*). Similar experiments were conducted at a very high concentration of MTSEA$^+$ (5 mM, $n = 3$); no effect of MTSEA$^+$ occurred if the channel was maintained in a closed state for as long as 5 min. A high Ca concentration alone does not inhibit MTSEA$^+$ reacting with the RyR because in four experiments, where 1 mM Ca was unable to maintain the channel in a closed state, MTSEA$^+$ decreased the current amplitude.

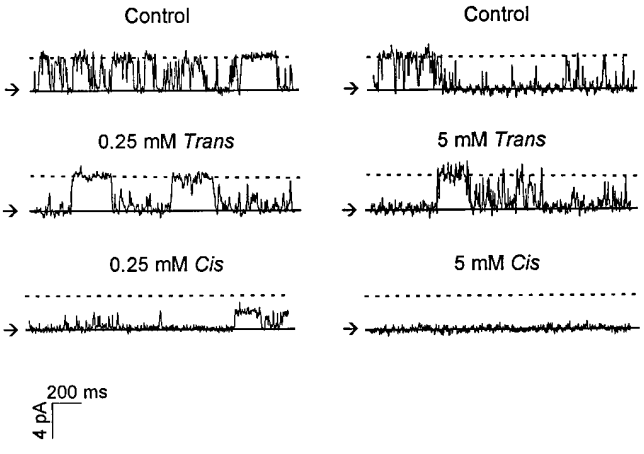


FIGURE 5. Effect of adding MTSEA$^+$ to the *trans* or *cis* side of the RyR. MTSEA$^+$ was added at 0.25 mM (*left*, one of three similar experiments is shown) or 5 mM (*right*, one of six similar experiments is shown) to the *trans* side (*middle traces*) or *cis* side (*bottom traces*) of the channel. The current recording shows that MTSEA$^+$ treatment had no effect when added to the *trans* side for up to 15 min, at either concentration, but affected the current amplitude when added to the *cis* side in 3.1 ± 0.8 min at 0.25 mM, and <30 s at 5 mM.

Step-wise Reduction Over Time

When a lower concentration (0.05 mM MTSEA$^+$) was used, the current decreased in a step-wise fashion, and the steps did not reverse (Fig. 7 *A*). 4 min after the addition of 0.05 mM MTSEA$^+$, channel openings reflecting approximately three-quarters of the control current amplitude appeared (Fig. 7 *A*, *second trace*). Because there is a period of time required for mixing and equilibrating compounds in the *cis* chamber, the actual time between the equilibration of the MTSEA$^+$ in solution and its covalent modification of the channel could actually be much less than 4 min. As the incubation continued, channel openings to approximately half of the control value appeared at 7-min (Fig. 7 *A*, *third trace*) and at 20-min openings to one-quarter of the control value appeared (Fig. 7 *A*, *bottom trace*). Transitions between substates in one experiment are shown after treatment with 0.05 mM MTSEA$^+$ (Fig. 7 *B*). It was possible to capture a record of all three transitions in a single experiment only when very low concentrations of MTSEA$^+$ were used. At higher concentrations initial transitions occurred rapidly, usually while stirring. The exact moment of a transition was often difficult to determine when the open time of the channel was short.

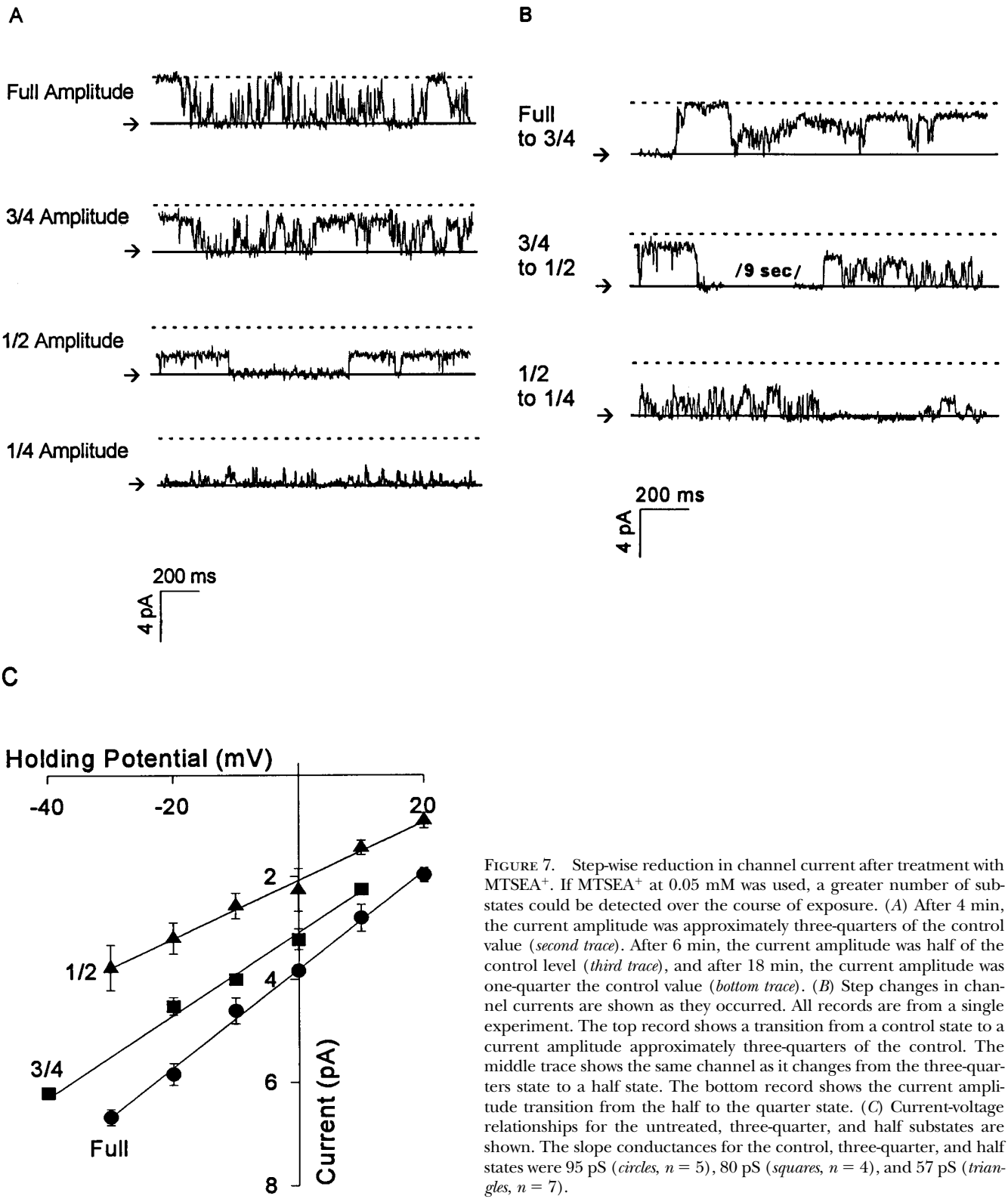


FIGURE 7. Step-wise reduction in channel current after treatment with $MTSEA^+$. If $MTSEA^+$ at 0.05 mM was used, a greater number of substates could be detected over the course of exposure. (*A*) After 4 min, the current amplitude was approximately three-quarters of the control value (*second trace*). After 6 min, the current amplitude was half of the control level (*third trace*), and after 18 min, the current amplitude was one-quarter the control value (*bottom trace*). (*B*) Step changes in channel currents are shown as they occurred. All records are from a single experiment. The top record shows a transition from a control state to a current amplitude approximately three-quarters of the control. The middle trace shows the same channel as it changes from the three-quarters state to a half state. The bottom record shows the current amplitude transition from the half to the quarter state. (*C*) Current-voltage relationships for the untreated, three-quarter, and half substates are shown. The slope conductances for the control, three-quarter, and half states were 95 pS (*circles*, $n = 5$), 80 pS (*squares*, $n = 4$), and 57 pS (*triangles*, $n = 7$).

Channel openings at the full, three-quarters and half substates had slope conductances of 95 pS ($n = 5$), 80 pS ($n = 4$), and 57 pS ($n = 7$; Fig. 7 *C*). The one-quarter substate is not included on Fig. 7 *C* as channel amplitudes were small relative to the noise, making analysis less reliable.

During continuous exposure to $MTSEA^+$ it took longer to reduce the current from the half state to the

one-quarter state than to go from the three-quarter state to the half state (Table I). When experiments using two concentrations of $MTSEA^+$ were compared, the same relationship held, but the absolute times were shorter at higher $MTSEA^+$ concentrations (Table I). Assuming independent sites and a time of 2.8 min to go from the three-quarter to the half state (Table I), it should take the same time to then go to the one-quarter state. In contrast, this transition took 13.8 min, a result inconsistent with independent sites for modification.

Ryanodine Restricts Access of MTS Compounds to Their Reaction Sites in the Channel

The order of addition of $MTSEA^+$ and ryanodine influenced the results. After a 5-min incubation with 0.2 mM $MTSEA^+$ (*cis* addition) and removal of excess compound, the amplitude of the current was half of the control (Fig. 8, *top trace*). Addition of 10 μM ryanodine to the $MTSEA^+$-modified channel locked the channel into a sub-conducting state between the half conducting state and ground (Fig. 8, *middle trace*). This remaining current was immediately inhibited by 500 μM ruthenium red (Fig. 8, *bottom trace*).

If the ryanodine and $MTSEA^+$ were added in the reverse order, the observed effect was different (Fig. 9). In a ryanodine-modified channel $MTSEA^+$ (0.05–0.5 mM) had no effect on channel conductance when observed for up to 30 min. However, the addition of high $MTSEA^+$ (5 mM) altered the current amplitude only after 20–30 min (Fig. 9, *middle trace*).

DISCUSSION

In this paper we investigated the effects of sulfhydryl-reactive compounds on the RyR. Ca current through the RyR was altered without affecting the other channel properties, including activation by ATP, Ca, and caffeine, suggesting that the sites of MTS modulation are different from other regulatory sites. We also found that the channel current was modified in four steps, but the time between successive modifications by these compounds increased with each step. This result, combined with the effects of ryanodine on the modified channel, suggests that the four sites are not independent and that the $MTSEA^+$ molecules are interacting at four sites, very close together, within the conduction pathway.

The MTS compounds were chosen because they are highly specific sulfhydryl-reacting compounds which react with thiol-containing amino acid residues (i.e., cysteines). $MTSEA^+$ has been used to block ion conductance because it reacts with cysteine residues thought to be in the water-filled conduction pathway. MTS compounds have been used to map out pore-lining cysteines in the acetylcholine receptor (Akabas et al., 1992), the

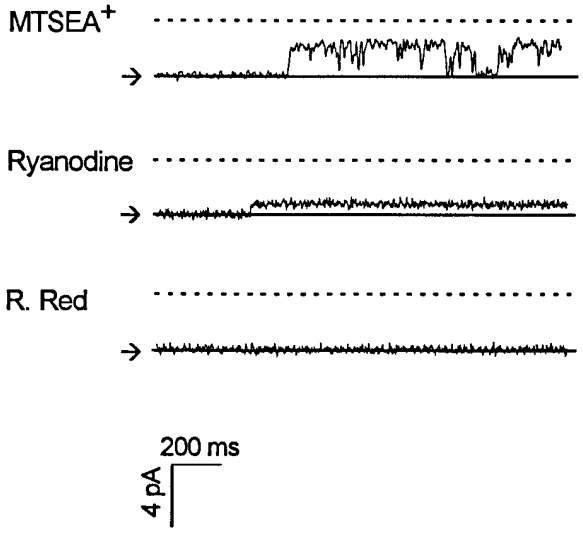


FIGURE 8. Effect of adding ryanodine to a $MTSEA^+$-modified RyR. Channels were treated first with $MTSEA^+$ (0.2 mM for 5 min). There was an irreversible decrease in the current amplitude (*top trace*) to approximately half of the untreated channels. The channels were then treated with ryanodine (10 μM, one of three similar experiments is shown). After ~10 min the current was decreased further to approximately one-quarter of the control current (*second trace*). To substantiate that the remaining current was due to the RyR, ruthenium red (500 μM) was added (*bottom trace*).

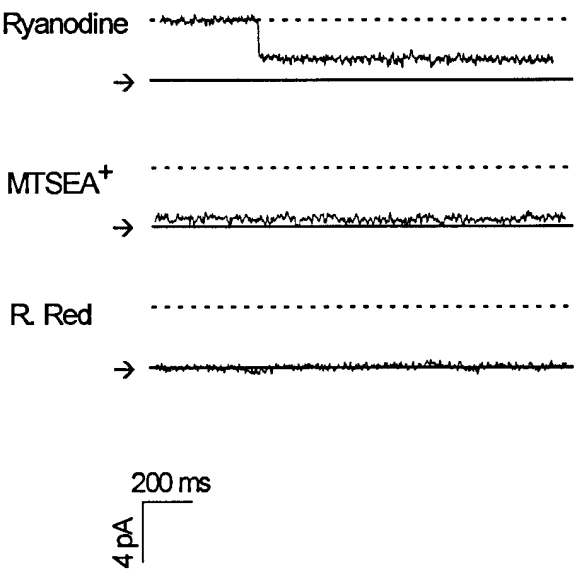


FIGURE 9. Effect of adding $MTSEA^+$ to a ryanodine-modified channel. Channels were treated first with ryanodine (10 μM). Ryanodine modified the untreated channel by locking it into a substate (*top trace*). The channels were then treated with a relatively high concentration of $MTSEA^+$ (5 mM, 25 min, *cis* addition, one of 3 similar experiments is shown). There was a single-step drop in the amplitude of the current to baseline levels (*middle trace*). Ruthenium red (500 μM) was added to show that no additional channel current remained (*bottom trace*).

$GABA_A$ receptor (Xu and Akabas, 1993), a voltage-gated K^+ channel (Kurz et al., 1995), and the channel made by diphtheria toxin (Huynh et al., 1996). Modification by the MTS compounds creates both stearic and electrostatic barriers to ion movement through the conduction pathway (Akabas et al., 1992).

Effects of MTS Compounds on the RyR

In our experiments with the RyR the MTS compounds irreversibly altered channel conductance. Although the MTS compounds initially activated the RyR, the increase in open probability did not persist after removal of the MTS compound suggesting that this effect is not related to sulfhydryl modification of the channel protein.

The positively charged compound ($MTSEA^+$) was far more effective at blocking channel current when compared to the neutral compound (MMTS). These findings support a permeation model (Tinker and Williams, 1992) where the lining of the mouth of the RyR contains fixed negative charges to concentrate cations within the channel pore in order to increase cation conduction. If surface charges in the conduction pathway of the RyR were made more positive (via the insertion of $MTSEA^+$), the concentration of cations within the channel should be reduced, resulting in a decrease in channel current. The data presented in this paper are consistent with this model.

The MTS compounds were only effective when added to the *cis* side and not to the *trans* side of the bilayer. This suggests that the structure of the channel near the *cis* side contains a wide vestibule providing little resistance against the entry of MTS molecules to their sites of action, whereas the opening of the channel on the *trans* side may be small creating a high resistance to the entry of the MTS compounds. A funnel-like structure has been proposed previously for K^+ channels (Kurz et al., 1995) and the RyR (Lindsay et al., 1994; Tinker and Williams, 1995) using different experimental paradigms. It was deduced that the length of the RyR pore was 10.4 Å from the start of the voltage drop on the *cis* side to the selectivity filter on the *trans* side and the diameter of the pore on the *trans* side was 7 Å (Lindsay et al., 1994). Most MTS derivatives, including $MTSEA^+$, fit into cylindrical spaces 6 Å in diameter by 10 Å in length (Akabas et al., 1992). Therefore, the selectivity filter proximal to the *trans* side of the RyR pore is barely wide enough to fit an MTS molecule, such that entry at the *trans* side would be possible but less likely. In contrast, regions within the membrane-spanning domain proximal to the *cis* side of the pore may be as wide as 25 Å, as suggested by electron cryomicroscopy (Radermacher et al., 1994; Serysheva et al., 1995*b*), and may provide little resistance against MTS molecules entering and modifying exposed cysteines. Even if the MTS molecules are able to pass the selectivity filter on the *trans* side, the resulting local concentration of compound near to the reactive site(s) may be too low for a reaction to take place.

Location of the Interaction between $MTSEA^+$ and the RyR

The most likely sites where covalent modification is occurring is within the conduction pathway of the RyR. Although it is possible for the MTS compounds to interact with exposed cysteines in the large foot region of the RyR, such interactions are not expected to lead to changes in channel conductance seen in this study for two reasons. First, we found that the compounds were only effective at reducing channel conductance when the channel was actively opening and were ineffective when the channel gate was closed. This result suggests that potential MTS binding sites on the large cytoplasmic domain of the channel would have to be available only when the channel was in the open configuration. Second, after ryanodine modification, the ability of the MTS compounds to decrease current was greatly reduced, suggesting that not all open conformations of the channel expose the reactive sites. The simplest explanation for these results is that the sites are within the conduction pathway.

These results also support the hypothesis that the $MTSEA^+$ molecules are occupying the conduction pathway sequentially and in close proximity with one another, creating a step-wise decrease in current flow through the channel. The evidence that the $MTSEA^+$ molecules are modifying the RyR at sites close to one another comes partially from the observation that the time required for successive modifications is longer with the progression of each substate. If the molecules were interacting with independent sites outside the range for stearic or electrostatic interactions between the molecules, then subsequent modifications would be spaced equally in time. Instead, successive modifications took longer to achieve than would be expected for independent sites. Although it is possible for the interaction of a $MTSEA^+$ molecule with one subunit to induce a conformational change in adjacent subunits, there was not any change in channel function to support this claim.

When the channel was first modified with $MTSEA^+$ to a half substate and then treated with ryanodine, an interesting effect occurred. The channel became locked in a quarter state and not the half state that typically occurs in the presence of ryanodine. Therefore, ryanodine is still able to elicit an effect on channel conductance, although ryanodine modification of the $MTSEA^+$- treated channel differs from its effects on the untreated channel. If the channel was modified first with ryanodine and then treated with $MTSEA^+$, another effect was seen. It took 10 times the normal concentration of $MTSEA^+$ and 20 times the normal incubation time to elicit any effect, suggesting that ryanodine hindered access of $MTSEA^+$

to its reactive site(s) within the channel's conducting pathway. Previous experiments showed (Tinker and Williams, 1993) that ryanodine modification reduced the capture radius at the *cis* side of the channel such that access to the conduction pathway for impermeant cations was now restricted. The diminished ability of $MTSEA^+$ to affect the ryanodine modified channel also supports the hypothesis that ryanodine reduces channel conductance by shifting the orientation of the subunits.

Putative Location of the Cysteine Residues

The evidence presented thus far suggests that $MTSEA^+$ is covalently modifying cysteine residues in the conducting pathway. One of the putative membrane spanning regions (M2 [Takeshima et al., 1989] or M6 [Zorzato et al., 1990]) contains three cysteine residues, making it the most likely candidate for the MTS-reactive sites. Other putative membrane spanning regions contain one or zero cysteines. An α helix structure of the M2 region would vertically align the cysteines on the hydrophilic face of the helix. Using Takeshima's model, cysteines No. 4663 and No. 4657 could be located approximately 1 Å and 10 Å, respectively, from the cytoplasmic side, putting two residues per subunit near the *cis* entrance within reach of the reactive end of $MTSEA^+$. In addition, an MTS molecule has a diameter of 6 Å (Akabas et al., 1992), which is approximately one-quarter the diameter of the 25 Å vestibule (Radermacher et al., 1994; Serysheva et al., 1995*b*). Therefore, it would be possible for four MTS molecules to react with four analogous sites within the pore. Although the presence of four MTS molecules would not block the pore area, the additional electrostatic hindrance caused by the charges on the $MTSEA^+$ could be responsible for the complete block of channel current.

The Structure of the RyR

The findings presented here suggest that the RyR contains a conducting pathway that is occupied by the MTS compounds in a step-wise manner. Although it is possible that these molecules could be reacting with independent sites on four separate conducting vestibules, the data presented here supports the single-pore model for the RyR. In a single-pore model each of the four subunits participates in the formation of a central pore. Computer simulations have demonstrated that a single-pore model could still yield long-lived subconductance states induced by slight alterations in the structure of the pore or in the electrostatic properties of the pore/mouth of the channel (Dani and Fox, 1991). Long-lived changes in the conformation of subunits could occur by modulation by phosphorylation, or by the addition of compounds such as ryanodine or $MTSEA^+$. Independent evidence in support of a single pore model comes from the observation that the RyR contains a single binding site for cations (Tinker and Williams, 1992; Lindsay et al., 1994), and that three-dimensional images of the RyR show a single central pore formed by the joining of the four subunits (Serysheva et al., 1995*b*).

Summary

In conclusion, the MTS compounds were able to alter RyR channel Ca current without affecting other aspects of channel function. This initial study demonstrates that MTS compounds are useful tools which interact with pore lining regions of the RyR which leads to distinct changes in the functional properties identified at the single channel level. The MTS compounds only worked from the *cis* side and when the channel was in the open state, suggesting that the site of action was within the pore of the channel and was only accessible through the wide, funnel-shaped vestibule on the cytoplasmic-facing side of the channel. The MTS-induced changes in channel conductance suggests that the RyR consists of a single conduction pathway. Further tests of the RyR structure will come from microscopic and molecular studies.

We thank Drs. Arnold Katz, Alan Finkelstein, Catherine Berlot, and Larry Cohen for comments on the manuscript. We also thank Dr. Myles Akabas for the gift of the $MTSEA^+$.

This work was supported by National Institutes of Health grants HL 33026 and a Grant-in-Aid from the Patrick and Catherine Weldon Donaghue Medical Research Foundation. K. Quinn was supported in part by a Grass Foundation Fellowship.

Original version received 29 August 1996 and accepted version received 15 November 1996.

REFERENCES

Akabas, M.H., D.A. Stauffer, M. Xu, and A. Karlin. 1992. Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Science (Wash. DC).* 258:307–310.

Bezprozvanny, I., J. Watras, and B.E. Ehrlich. 1991. Bell-shaped calcium-response curves of $Ins(1,4,5)P_3$- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature (Lond.).* 351:751–754.

Callaway, C., A. Seryshev, J.P. Wang, K.J. Slavik, D.H. Needleman, C. Cantu, Y. Wu, T. Jayaraman, A.R. Marks, and S.L. Hamilton. 1994. Localization of the high and low affinity [^{3}H]ryanodine

binding sites on the skeletal muscle Ca^{2+} release channel. *J. Biol. Chem.* 269:15876–15884.

Dani, J.A., and J.A. Fox. 1991. Examination of subconductance levels arising from a single ion channel. *J. Theor. Biol.* 153:401–423.

Ehrlich, B.E., and J. Watras. 1988. Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature (Lond.).* 336:583–586.

Huynh, P.D., H. Zhan, J.A. Mindell, R.J. Collier, and A. Finkelstein. 1996. Probing the structure of the diphtheria toxin channel with methanethiosulfonate reagents. *Biophys. J.* 70:A140.

Kim, D.H., S.T. Ohnishi, and N. Ikemoto. 1983. Kinetic studies of calcium release from sarcoplasmic reticulum in vitro. *J. Biol. Chem.* 258:9662–9668.

Kurz, L.L., R.D. Zuhlke, H.J. Zang, and R.H. Joho. 1995. Side-chain accessibilities in the pore of a K^+ channel probed by sulfhydryl-specific reagents after cysteine-scanning mutagenesis. *Biophys. J.* 68:900–905.

Lindsay, A.R.G., A. Tinker, and A.J. Williams. 1994. How does ryanodine modify ion handling in the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel? *J. Gen. Physiol.* 104:425–447.

Marks, A.R., S. Fleischer, and P. Tempst. 1990. Surface topography analysis of the ryanodine receptor/junctional channel complex based on proteolysis sensitivity mapping. *J. Biol. Chem.* 265: 13143–13149.

Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+} and adenine nucleotides. *Biochemistry.* 25:236–244.

Meissner, G., and J.S. Henderson. 1987. Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotides and calmodulin. *J. Biol. Chem.* 262:3065–3073.

Radermacher, M., V. Rao, R. Grassucci, J. Frank, A.P. Timerman, S. Fleischer, and T. Wagenknecht. 1994. Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. *J. Cell Biol.* 127:411–423.

Serysheva, I.I., E.V. Orlova, W. Chiu, M.B. Sherman, S.L. Hamilton, and M. van Heel. 1995*a*. Electron cryomicroscopy and angular reconstruction used to visualize the skeletal muscle calcium release channel. *Nat. Struct. Biol.* 2:18–24.

Serysheva, I.I., E.V. Orlova, M.B. Sherman, M. van Heel, W. Chiu, and S.L. Hamilton. 1995*b*. 3D structure of the skeletal muscle Ca^{2+} release channel in its open and closed states by electron cryomicroscopy and angular reconstitution. *Biophys. J.* 68:A128.

Smith, J.S., T. Imagawa, J. Ma, M. Fill, K.P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium release-channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92:1–26.

Takeshima, H., S. Nishimura, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Matsuo, M. Ueda, M. Hanaoka, T. Hirose, and S. Numa. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature (Lond.).* 339:439–445.

Tinker, A., and A.J. Williams. 1992. Divalent cation conduction in the ryanodine receptor of sheep cardiac muscle sarcoplasmic reticulum. *J. Gen. Physiol.* 100:479–493.

Tinker, A., and A.J. Williams. 1993. Using large organic cations to probe the nature of ryanodine modification in the sheep cardiac sarcoplasmic reticulum calcium release channel. *Biophys. J.* 65: 1678–1683.

Tinker, A., and A.J. Williams. 1995. Measuring the length of the pore of the sheep cardiac sarcoplasmic reticulum calcium-release channel using related triethylammonium ions as molecular calipers. *Biophys. J.* 68:111–120.

Xu, M., and M.H. Akabas. 1993. Amino acids lining the channel of the gamma-aminobutyric acid type A receptor identified by cysteine substitution. *J. Biol. Chem.* 268:21505–21508.

Yang, N., and R. Horn. 1995. Evidence for voltage-dependent S4 movement in sodium channels. *Neuron.* 15:213–318.

Zorzato, F., J. Fujii, K. Otsu, M. Phillips, N.M. Green, F.A. Lai, G. Meissner, and D.H. MacLennan. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265:2244–2256.