The homotetrameric structure of the ryanodine-sensitive intracellular calcium (Ca\(^{2+}\)) release channel (ryanodine receptor (RyR)) suggests that the four RyR subunits either combine to form a single pore or that each RyR subunit is an independently conducting pathway. Previously we showed that methanethiosulfonate ethylammonium (MTSEA\(^+\)) covalently modifies the RyR to reduce current amplitudes in a time-dependent and stepwise manner. To ascertain the number of functionally conducting pores in the RyR, two approaches were combined: modification of the receptor by MTSEA\(^+\) and the use of different sized current carriers. Previous reports (Tinker, A., and Williams, A. J. (1993) *J. Gen. Physiol.* 102, 1107–1129) have shown that the organic cations methameline, dimethylamine, ethyleneamine, and trimethylamine are permeant through the RyR but with reduced current amplitude depending upon the diameter of the respective amine. Experiments using the thiol reagent MTSEA\(^+\) to modify the channel protein showed that the current amplitudes decrease in steps leading to complete block of the channel when cesium (Cs\(^+\)) is the current carrier. MTSEA\(^+\)-modification decreased the number of channel substates as the diameter of the current carrier increased. Comparison of the degree of inhibition of MTSEA\(^+\)-modified currents allows for differentiation between the two models for channel architecture. These results demonstrate that the conduction pathway for the RyR is comprised of a single central pore.

The ryanodine receptor (RyR)\(^1\) is an intracellular calcium (Ca\(^{2+}\)) release channel found in the endoplasmic reticulum of numerous cell types and is especially concentrated in the sarcoplasmic reticulum of striated muscle. These channels play crucial roles in Ca\(^{2+}\)-mediated signaling pathways leading to T-lymphocyte activation, fertilization, excitation-contraction coupling, and many other cellular functions (2). The RyR channel is a multimeric structure comprised of four identical subunits assumed to contribute directly to currents through the RyR (3, 4). 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 are contained within a RyR. Two simple possibilities exist. One possibility is that the four identical subunits come together to form a single pore that is divided into quadrants, and the pore is influenced by the conformation of each subunit. The second possibility is that each subunit forms an independent pore that contributes one-fourth of the unitary current of the channel (5). Previous efforts to elucidate the structure of the RyR pore have produced conflicting results. Using single channel analysis of the RyR, four subconductance states have been observed where each state represents one-quarter of the full conductance of the channel (4). The equal spacing of the substates suggests that the receptor is comprised of four distinct pores. The functional association between FK-506-binding protein and the RyR also supports the suggestion that the channel complex consists of multiple pores. The dissociation of FK-506-binding protein from the RyR after the addition of the immunosuppressant agents FK-506 or rapamycin leads to the occurrence of subconductance states that have been interpreted as ion flow through individual uncoordinated RyR monomers (6–8). Evidence further supporting the four-pore theory comes from three-dimensional image reconstructions of the RyR (9). These studies routinely show four small radial openings, which branch out from a common origin. However, some views also show a large central opening, which makes interpretation of these studies complex. In contrast, there are also a number of studies that support a one-pore model. Only one cationic binding site was measured in the RyR (10), suggesting a single permeation pathway. Although an equal distribution of subconductance states has been used to support a four-pore model, a theoretical analysis demonstrating that single pore channels can also exhibit subconductance states of equal steps lends support to the single pore theory (11). Additional theoretical bases for favoring a single pore model derive from the calculation that each subunit would need a pore large enough to pass cations measuring up to 0.6 nm in diameter (10), a pore size difficult to create from the limited number of transmembrane helixes present in a single subunit (5). Further support for the single pore model of the RyR tetramer comes from co-expression of a modified RyR construct designed to produce a low conductance channel with the wild type RyR. The expressed heteromeric proteins produced channel currents that could be separated into six groups where the unitary conductance ranged from wild type to that of the homomeric mutant (8). The six conductance groups were then correlated with six possible arrangements of mutant and wild type RyR subunits. The conclusion from this study was that the conduction pathway was a single pore created by an equal contribution from each subunit (12). Finally images using negative stain electron microscopy showed that the RyR complex exists as a four-subunit structure encircling a central “hole” (3). Similarly a three-dimensional model of the RyR,
TABLE I

Diameters of the current carriers

| Current carriers | Diameter (Å) |
|------------------|-------------|
| Cs⁺              | 3.4         |
| Methylamine CH₃NH₃⁺ | 3.8        |
| Dimethylamine (CH₃)₂NH₂⁺ | 4.6       |
| Ethylamine C₂H₅NH₂⁺  | 4.4        |
| Trimethylamine (CH₃)₃NH⁺  | 5.6       |

TABLE II

Conductance of the current carriers

| Current carriers | Control | MTSEA⁺ | Percentage of maximum |
|------------------|---------|--------|-----------------------|
| Cs⁺              | 733 ± 35| 416 ± 55| 57                    |
| Methylamine      | 425 ± 43| 306 ± 31| 72                    |
| Dimethylamine    | 333 ± 9 | 145 ± 21| 43                    |
| Ethylamine       | 189 ± 79| 134 ± 30| 71                    |
| Trimethylamine   | 120 ± 10|        |                       |

Fig. 1. A comparison of the single channel currents using each of the test cations. Upward deflections are channel openings. Top trace, Cs⁺ currents obtained at a holding potential of −30 mV; second trace, methylamine (MA) currents at −30 mV; third trace, dimethylamine (DMA) currents at −30 mV; fourth trace, ethylamine (EA) currents obtained at −30 mV; bottom trace, trimethylamine (TriMA) currents obtained at −50 mV. All traces represent currents in the absence of MTSEA⁺ and are 500 ms long. To the right of each trace, the full conductance for Cs⁺ is represented as a long dashed line, the full conductance for the amine is a short dotted line, and the solid line represents the closed state.

Fig. 2. The Effect of MTSEA⁺ on Cs⁺ conductance. Currents obtained from −10 to −50 mV before treatment with MTSEA⁺ are represented by circles, and MTSEA⁺-modified currents are represented by triangles. The Cs⁺ concentration (250 mM) was the same in cis and trans chambers. Data from one of three similar experiments is shown. Error bars represent S.E.

Values for the conductance were taken from experiments shown in Figs. 2 and 3. Only one MTSEA⁺-modified state was examined over the entire voltage range to calculate a conductance. The ability of MTSEA⁺ to modify the current was unaffected by the permeating ion. Values are presented as mean ± S.E. pS, picosiemens.

EXPERIMENTAL PROCEDURES

Materials—The methanethiosulfonate compound MTSEA⁺ was obtained from Toronto Research Chemicals Inc. (Ontario, Canada). The cationic amines methylamine, dimethylamine, ethylamine, and trimethylamine were purchased from Sigma. All other chemicals were acquired from standard commercial sources.

Preparation of Sarcolemmic Reticulum Membrane Vesicles—Cardiac sarcolemmic reticulum vesicles were isolated from dog heart according to the method of Feher (18) with a few modifications. Ventricles were isolated (10–15 g) and washed in ice-cold homogenization buffer (1 M KCl, 10 mM imidazole, 10 μM leupeptin, 1 μM pepstatin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 100 μM phenylmethylsulfonyl fluoride, pH 7.2), blotted with absorbent paper, weighed, and minced. Tissues were homogenized in 5 volumes of ice-cold homogenization buffer using a Polytron (Brinkmann Instruments) in two steps. (a) The homogenate from a soft tissue grind, on ice, of three 10-s pulses at...
20,000 rpm was centrifuged for 20 min at 10,000 × g_{max} at 4 °C, and the supernatant was discarded. (b) The pellet was resuspended in ice-cold homogenization buffer to its original volume and rehomogenized in a hard tissue grind, on ice, of five 15–20-s pulses at 20,000 rpm. The resulting homogenate was centrifuged at 6,000 × g_{max} for 20 min. The supernatant was collected and further centrifuged at 24,000 × g_{max} for 25 min, and the pellet was discarded. The supernatant was Dounced, layered on a discontinuous sucrose gradient (two layers of 19 and 34% sucrose), and centrifuged at 41,000 × g_{max} for 2 h in a swinging bucket rotor. The band at the interface was collected, diluted 100% with homogenization buffer, and centrifuged at 100,000 × g_{max} for 1 h. The final pellet was resuspended in storage buffer (10% sucrose, 20 mM Tris, pH 7.0) at ~5 mg/ml protein. Aliquots were snap frozen in liquid nitrogen and stored at −80 °C until used.

**FIG. 3.** The Effect of MTSEA^− on the current-voltage relationship for each of the amines tested. Currents obtained before treatment with MTSEA^− are represented by circles, and MTSEA^−-modified currents are represented by triangles. In each panel, data from a single experiment are shown; the number of experiments used in the analysis is provided after each test compound. The current amplitude for methylamine (n = 4) (A), dimethylamine (n = 4) (B), and ethylamine (n = 4) (C) were obtained at holding potentials ranging from −20 to −70 mV, and for trimethylamine (n = 3) (D) the holding potential was between −30 and −70 mV. MTSEA^− decreased the current amplitude for all current carriers. Only one MTSEA^−-modified state was examined over the entire voltage range to calculate a conductance for this figure. Each point is the average of >100 channel openings. Error bars represent S.E. E (traces a–e), current traces of Cs^+ and trimethylamine (TriMA) under bi-ionic conditions (250 mM CsCl in the cis chamber, 250 mM trimethylamine in the trans chamber). Trace a, the fully open state; trace b, the 3/4 state; trace c, the 1/2 state; trace d, the 1/4 state; trace e, blocked state. To the right of each trace, the full conductance for Cs^+ is represented as a long dashed line, the subconductance state of both Cs^+ and trimethylamine is represented as a short dotted line, and the solid line represents the closed state. Note that trimethylamine current is abolished by MTSEA^− modification at the same time that the Cs^+ current is only attenuated.
to the channel activity being observed was ion currents through the RyR. The MTSEA and stored on a personal computer. Subsequent data analysis was performed using pClamp (Axon Instruments). The MTSEA served previously (5).

amines (250 mM amine, 10 mM HEPES, pH 7.4) were present in both the cis and trans chamber, which contained 500 mM CsCl, 200 mM CaCl₂, and 10 mM HEPES, pH 7.4, whereas the trans chamber contained 250 mM CsCl, 10 mM HEPES, pH 7.4. After fusion of vesicles, the cis chamber was rapidly perfused with 15 ml of the trans buffer with no added Ca²⁺. Single channel activity was measured at various holding potentials with respect to the trans (ground) side in the presence or absence of MTSEA.

In experiments using organic amines as the primary current carrier, sarcoplasmic reticulum vesicles were fused to the bilayer, and the cis chamber was perfused with either CsCl or an amine-containing solution. Ruthenium red (2 μM) was applied to the cis chamber to verify that the channel activity being observed was ion currents through the RyR.

Data were amplified (BC-525C, Warner Instruments, Inc., Hamden, CT), filtered at 1–3 kHz with a low pass eight-pole Bessel filter, digitized at 10 kHz (Digitata 1200, Axon Instruments, Foster City, CA), and stored on a personal computer. Subsequent data analysis was performed using pClamp (Axon Instruments). The MTSEA⁻-induced, stepwise decrease of the RyR current was irreversible and cumulative over time resulting in complete inhibition of channel currents as observed previously (5).

Sulfhydryl Compound Treatment—Modification of channel currents by MTSEA⁻ was performed in the presence of CsCl and various organic amines. After incorporation of RyR channels into the bilayer membrane, control measurements were acquired for at least 2 min followed by addition of MTSEA⁻ to the cis chamber. In all experiments, 50 μM MTSEA⁻ was used to modify the channel. At least three experiments were completed with each organic cation.

Organic Amine Experiments—Current-voltage experiments were performed under bi-ionic conditions. Organic amines (250 mM amine, 10 mM HEPES, pH 7.4) were present in the trans chamber, and the cis buffer was comprised of 250 mM CsCl, 10 mM HEPES, pH 7.4. Cs⁺ currents were measured at positive potentials, whereas the amine currents were measured at negative holding potentials. For experiments monitoring the development of subconductance levels, organic amines (250 mM amine, 10 mM HEPES, pH 7.4) were present in both the cis and trans chambers. The amine subconductance levels were measured at ~30 mV.

Analysis of Current Amplitudes—Current amplitudes for the current-voltage relationships as shown in Figs. 2 and 3 were generated using all-point histograms (pClamp, Axon Instruments) for each current carrier. Open channel current amplitudes were collected for channels in the presence and absence of 50 μM MTSEA⁻. For clarity of presentation, only one transition state is shown for each current carrier tested. Lines through the points were generated by linear regression fits to the data.

The open channel current amplitudes sequentially decreased in response to prolonged exposure to MTSEA⁻. Once a channel reached the blocked state, transitions out of the blocked state were never observed. The subconductance current values were generated using all-point histograms. All-point histograms were generated from at least 2 s of continuously recorded data. Data were filtered at 1 kHz and fit with a Gaussian curve. Current amplitudes were calculated as the difference between the Gaussian means of the open and closed states. Errors shown in the figures were derived using the standard deviations from the Gaussian fit described above. Subconductance levels were determined by following the time course of the stepwise decrease in current amplitude. The fully open channel current amplitude for a given amine was divided by 4 to estimate the expected substate current amplitudes. The estimated and measured values were similar.

All representative current traces shown in the figures were filtered at 500 Hz and are at least 500 ms long. Mean open and closed dwell times were calculated by generating events lists from filtered (700 Hz) data recordings lasting at least 2.5 min using pClamp (Axon Instruments). Histograms were drawn, and the data were fit to exponential curves using a simplex-least squares fitting method. The open and closed dwell time values were extracted from the fits to the data. In all cases error bars represent the S.E. of the exponential fit.

### RESULTS

Effect of MTSEA⁻ on RyR Cationic Conductance—The modification of RyR currents by MTSEA⁻ was used to probe the conductance of several cations through the channel. Previous work showed that MTSEA⁻ reduced the single channel conductance of the RyR in a stepwise manner when Ba²⁺ was used as the current carrier (5). A difficulty associated with using this approach is that the relatively low conductance of Ba²⁺ (~100 picosiemens) makes subconductance currents difficult to resolve. To address this issue, Cs⁺ was used as the control current carrier because the large amplitude currents carried by this ion allow greater discrimination of MTSEA⁻-induced subconductance states of the RyR. Then organic amines of increasing size (Table I) were used as test cations to probe the nature of the conducting pathway.

### TABLE III

| State | Cs⁺ | Methylamine | Dimethylamine | Ethylamine | Trimethylamine |
|-------|-----|-------------|--------------|------------|---------------|
|       | τ₀  | τ₁         | τ₂           | τ₀         | τ₁           |
| Full  | 16.12 ± 1.18 | 1.20 ± 0.12 | 12.98 ± 0.45 | 1.17 ± 0.32 | 6.15 ± 0.35 |
| 3/4   | 10.12 ± 0.60 | 4.44 ± 0.31 | 3.17 ± 0.34  | 7.95 ± 0.72 | 3.72 ± 0.61 |
| 1/2   | 1.81 ± 0.25  | 1.00 ± 0.44 | 1.02 ± 0.28  | 7.42 ± 0.35 | 4.20 ± 0.16 |
| 1/4   | 1.44 ± 0.13  | 28.85 ± 0.72 | 1.99 ± 0.18  | 52.75 ± 0.89 |

Values are presented as mean ± S.E.

### TABLE IV

| State | Cs⁺ | Methylamine | Dimethylamine | Ethylamine | Trimethylamine |
|-------|-----|-------------|--------------|------------|---------------|
|       | τ₀  | τ₁         | τ₂           | τ₀         | τ₁           |
| Full  | 0.85 ± 0.31 | 0.90 ± 0.24 | 0.73 ± 0.39  | 0.26 ± 0.39 | 0.16 ± 0.26  |
| 3/4   | 0.74 ± 0.41 | 0.14 ± 0.30 | 0.51 ± 0.47  | 0.21 ± 0.34 | —            |
| 1/2   | 0.63 ± 0.41 | 0.11 ± 0.26 | 0.45 ± 0.47  | 0.16 ± 0.34 | —            |
| 1/4   | 0.04 ± 0.17 | 0.09 ± 0.26 | —            | —          | —            |

Values are presented as mean ± S.E.

### Notes

- a, subconductance states were not observed.
- b The number of openings was too low for analysis.
The relative difference in current amplitude among the test cations (Cs⁺, methylamine, dimethylamine, and ethylamine) was measured in the unmodified state at a holding potential of −30 mV except for trimethylamine, which was measured at −50 mV (Fig. 1). Trimethylamine currents were measured at a larger holding potential to increase the amplitude of the currents. As can be seen in Fig. 1, the observed current decreases with increasing ionic diameter. Although dimethylamine has a larger diameter than ethylamine, it has a larger conductance than ethylamine (Tables I and II). This result has been previously reported, and an explanation was provided that is based upon differences in the three-dimensional shape of the amines (20). The rod-like structure of dimethylamine results in a smaller orthogonal profile for this amine.

The calculated conductances for the amines (Table II) are slightly larger than those reported previously (20) because the concentration of amine used in our experiments was larger than those in the prior report (250 versus 210 mM, respectively (20)). However, when experiments in this series were performed using the same concentration as previously reported, similar conductance values were obtained. The permeability of the amines relative to Cs⁺ is similar to those reported by Tinker and Williams (1) considering that the permeability ratio of K⁺/Cs⁺ is 0.6.

To modify the channel, 50 μM MTSEA⁺ was used in all experiments because this concentration reduced RyR currents on a time scale slow enough for substate transitions to be monitored. Current-voltage relationships using Cs⁺ as the current carrier were acquired when the channel was in the full state and a subconductance state, showing that MTSEA⁺ modified the transmembrane current at every applied potential (Fig. 2).

When transmembrane currents were measured using organic amines as the primary current carriers, MTSEA⁺ modified the currents at every applied potential (Fig. 3, A–D). The experiments using organic amines were performed under biionic conditions. In all cases, MTSEA⁺ modification of amine currents resulted in a concomitant modification of Cs⁺ currents. When the largest cation, trimethylamine, was used as current carrier, complete block of amine currents occurred before full block of Cs⁺ currents. That is, following partial modification of the RyR by MTSEA⁺, Cs⁺ was still able to pass through the channel with a reduced current amplitude, whereas trimethylamine currents could not be detected (Fig,
3E). This result indicates that the immediate loss of all trimethylamine currents was associated with partial block of the pore.

When monitoring Ba\(^{2+}\) currents through the RyR, MTSEA\(^+\) modified the amplitude of the currents but not the ability to activate the channel with Ca\(^{2+}\) or ATP. Neither were the open probability \((P_o)\) nor the mean open and closed times of the receptor affected (5). In this study, MTSEA\(^+\) also had no significant effect on the same parameters. In addition, the mean open and closed times for each tested current carrier (Table III) correlates directly with the \(P_o\) (Table IV). Dashes in Table IV indicate the absence of channel activity due to modification by MTSEA\(^+\). The only alteration observed was the appearance of subconductance levels (Figs. 4–8 and Table V).

To verify that the observed single channel behavior was specific to the RyR, organic amine currents were monitored in the presence and absence of 2\(/\text{H}9262\)M ruthenium red. Currents with each test compound were completely blocked by ruthenium red (Fig. 9).

**Effect of MTSEA\(^+\) on Subconductance Levels of the RyR—**

The next series of experiments were designed to quantify the number of subconductance states seen at a fixed voltage using either Cs\(^+\) or organic amines as the current carrier and in the presence of MTSEA\(^+\). A summary of the subconductance current amplitudes are shown in Table V. In control experiments using Cs\(^+\), MTSEA\(^+\) decreased current amplitudes in four discrete steps (Fig. 4) in a manner similar to that observed with Ba\(^{2+}\) (5). As MTSEA\(^+\) acts to modify the channel, the current of the RyR is sequentially reduced from full, to three-quarters, to one-half, to one-quarter, and finally to complete block of the full current amplitude.

Methylamine, the smallest organic cation tested, has a diameter of 3.8 Å and is 1.12 times larger than Cs\(^+\). When this cation was used as the current carrier, MTSEA\(^+\) decreased transmembrane currents in four discrete steps (Fig. 5). As the size of the current carrier was increased, the corresponding number of observed subconductance states was decreased.

Dimethylamine has a diameter of 4.6 Å and is 1.35 times larger than Cs\(^+\). When dimethylamine was used as the current carrier, channel activity was observed in the full, three-quarter, one-half, and one-quarter states in the presence of MTSEA\(^+\) (Fig. 6). With dimethylamine as the current carrier, the percentage of time the modified channel was in the one-quarter state was significantly reduced compared with those seen when using Cs\(^+\) and methylamine as current carriers. Interestingly, when ethylamine (whose diameter is 4.4 Å and is 1.29 times the diameter of Cs\(^+\)) was used as the current carrier, no channel activity was observed in the one-quarter conductance state. Only the full, three-quarter, and one-half states were observed in the presence of MTSEA\(^+\) (Fig. 7).

Trimethylamine, the largest cation tested, has a diameter of 5.6 Å and is 1.65 times the diameter of Cs\(^+\). When trimethylamine was used as a current carrier, the RyR channel activity was completely blocked after approximately 15 min of exposure.
to MTSEA\(^+\) (Fig. 8). Only the full state was observed. This phenomenon could only be due to the modification of trimethylamine currents by MTSEA\(^+\). This could not have been due to the loss of the channel from the bilayer membrane because the trimethylamine current was eliminated by MTSEA\(^+\), but the Cs\(^+\) current in the same experiment was only reduced (Fig. 3E).

All-point histograms for the subconductance state for each current carrier (Figs. 4–8) obtained in the presence of MTSEA\(^+\) reveal the sequential modification of channel currents by this compound. Numbers to the right of each trace indicate the time when each subconductance state was first observed. In the previous report describing MTSEA\(^+\) modification of the RyR, the time between successive modifications increased (5) suggesting that there was a size limitation in the region of modification that became more limiting as additional modifications to the RyR occurred. In this series of experiments, the time between successive modifications for each current carrier showed a similar trend (Figs. 4–8). The shift in the histogram peaks reinforces the time-dependent and sequential decrease in current amplitudes reported above.

**DISCUSSION**

In this study, we explored the functional configuration of the RyR pore. The ability of MTSEA\(^+\) to sequentially modify the RyR, and consequently channel conductance, in the presence of a series of organic cations of increasing diameter was used to address this question. Four organic cations (methylamine, dimethylamine, ethylamine, and trimethylamine) were selected that spanned a range of molecular sizes and consequently a range of conductances through the RyR (1). We found that MTSEA\(^+\) modification decreased the number of channel substates as the diameter of the current carrier increased. From these results we conclude that the conduction pathway for the RyR is comprised of a single central pore.

MTSEA\(^+\) modifies the RyR current by covalently modifying cysteine residues. Because the modification only occurs when the channel is open it is assumed that the modification occurs within the conduction pathway of the receptor (5). The molecular mechanism by which this process occurs in the RyR is yet to be elucidated. It is estimated that the RyR contains 80–100 cysteine residues/subunit of which \(\sim 25\%\) are free for covalent modification and the remainder are unavailable because they do not reside on the surface of the protein or they form intraprotein disulfide bonds (21). A truncated version of the RyR, which has only a small portion of the cytoplasmic domain but the entire transmembrane domain (22), can still be modified by MTSEA\(^+\),\(^2\) further limiting the possible targets for modification by MTSEA\(^+\). Three cysteine residues that have been proposed as targets for MTSEA\(^+\) reside in the second (M2) and sixth (M6) membrane-spanning region of the RyR (23, 24). The two residues on the M2 \(\alpha\) helix are \(\sim 1\) and 10 Å from the cytoplasmic side of the membrane (23), a distance that should be within the channel vestibule on the cytoplasmic side and accessible to the MTSEA\(^+\) molecule.

\[^2\]B. E. Ehrlich, unpublished observations.
MTSEA is believed to interact with four specific sites within the cytoplasmic vestibule of the RyR pore (5). Taken by itself, the observed sequential alteration in channel current due to MTSEA modification could be due to the complete block of each of four individual conducting pathways or from steric hindrance by MTSEA within a single, larger conduction pathway. These two mechanisms cannot be uniquely separated on the basis of MTSEA modification alone. Therefore, MTSEA in conjunction with organic amines of increasing sizes was used to dissect the configuration of the pore. If the RyR was comprised of four independently conducting pathways, the observed MTSEA-modified currents should have decreased in four discrete steps even in the presence of the largest conducting cation. By comparison, if the RyR was comprised of a single conducting pathway, the observed MTSEA-modified current should abruptly cease when the diameter of the permeating ion exceeds the size of the MTSEA-modified channel pore even if the channel is not yet fully blocked by MTSEA. An illustration of these two model structures is presented in Fig. 10. In the one-pore model, the four subunits each contribute to form a single pore, whereas in the four-pore structure, each subunit has its own pore within the transmembrane region of the protein (Fig 10).

Previously the size of the sheep cardiac RyR was estimated using the relative permeability of a series of organic cations (20). It was deduced that the length of the RyR was 10.4 Å from the start of the voltage drop on the cis side to the selectivity filter on the trans side (1, 25). The selectivity filter was found to have a diameter of 7 Å. These dimensions can be compared with those obtained from the three-dimensional structure deduced from single particle reconstructions (26, 27). When the solid body representation of the three-dimensional structure is viewed from the side, the RyR resembles a mushroom with a “cap” composed of the cytoplasmic domains of the component monomers and a “stalk” made up of the putative membrane-spanning domain (8). When viewed from the cytoplasm, the cap appears as a square slab (270 × 270 Å) with a depth of 110 Å (28, 8). The size of the vestibule deduced from functional studies is 25 Å wide and 10.4 Å tall, dimensions that would easily fit into the cap as drawn from three-dimensional images (8). The stalk is also square in appearance with a height through the membrane of ~65 Å and lateral dimensions of ~120 Å on a side (8). The size of the stalk is much larger than those obtained from the functional information, suggesting that the narrow region of the pore and the selectivity filter are only a small portion of the transmembrane segment of the protein. This observation is similar to that described for the KcsA channel where the selectivity filter is only 4 Å long (29). Most methanethiosulfonate derivatives, including MTSEA, fit into cylindrical spaces of 6 Å in diameter and 10 Å long (16), which is approximately one-quarter of the diameter of the 25-Å RyR vestibule, but similar to the size of the selectivity filter and the height of the vestibule (1).

Using the functional information described above, along with the dimensions of the reagents used in our experiments, coupled with the assumption that MTSEA modifies a specific
cysteine residue within the vestibule of the RyR, the diameter of the altered RyR pore can be calculated. If the binding of one MTSEA/H11001 molecule decreases the maximum diameter of the RyR vestibule from 25 to 19 Å, then the diameter will sequentially decrease to 13 Å, 7 Å, and finally 1 Å as additional MTSEA/H11001 molecules bind. These calculations suggest that all of the cations used in these experiments should be able to pass through the modified RyR pore when up to three MTSEA/H11001 molecules are bound. However, dimethylamine and ethylamine currents are blocked after only one or two MTSEA/H11001 modifications, and trimethylamine is unable to conduct after a single modification. These organic amines conduct through the RyR in a completely hydrated fashion; therefore, the individual size of these amines as they pass through the conduction pathway are increased by 2 Å due to hydration (1). In addition, these results imply that other factors play a role in determining conduction such as electrostatic interactions between the positively charged MTSEA/H11001 and the positively charged permeant ion. In this context a permeation model has been proposed where the lining of the mouth of the RyR contains fixed negative charges to concentrate cations within the channel pore to increase cation conduction (10). If surface charges in the conduction pathway of the RyR were made more positive (via the insertion of MTSEA/H11001), the concentration of cations within the channel should be reduced, resulting in a decrease in channel current. The model is supported by preliminary data using a negatively charged analog of methylmethanethiosulfonate, which generated increased Ba²⁺ currents through the RyR.² The results of the present study also agree with the permeation model.

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**FIG. 8.** MTSEA⁺-induced subconductance states of trimethylamine currents. Labeling is as described in the legend for Fig. 4. A, current traces representing channel activity before modification and at the only observed substate. Note that no subconductance states were observed; only the fully open state was observed. The holding potential was −50 mV, and 250 mM trimethylamine was present on both sides of the membrane. B, all-point histograms, representative of each observed subconductance state in A, are shown. One of four similar experiments is shown.

**TABLE V**

| Current Carriers | Holding potential | Full  mV | 3/4  mV | 1/2  mV | 1/4  mV |
|------------------|-------------------|---------|--------|--------|--------|
| Cs⁺              | −30               | 20 ± 3.54| 15 ± 2.03| 10 ± 2.24| 5 ± 1.85|
| Methylamine      | −30               | 15 ± 2.38| 13 ± 2.54| 9 ± 2.56| 5 ± 1.48|
| Dimethylamine    | −30               | 12 ± 2.28| 10 ± 2.43| 7 ± 2.01| 4 ± 2.25|
| Ethylamine       | −30               | 7 ± 1.91 | 5 ± 1.87 | 3 ± 0.92| —       |
| Trimethylamine   | −50               | 4 ± 1.37 | —       | —       | —       |

—, subconductance states were not observed.
A similarity in the pore stoichiometry of other channels in the intracellular calcium channel family such as the inositol trisphosphate channel and the polycystin-2 channel is likely. The RyR and the inositol trisphosphate channel share a high sequence homology and have the same quaternary structure (30). These two receptors also share a strong similarity to the K⁺ channels as they are tetrameric complexes, an α helix lines the pore, and a short sequence defines the selectivity filter (29, 31, 32). It is anticipated that the intracellular calcium channels have pores that are related architecturally to the K⁺ channels (33, 34). At the present time, the subunit configuration of the polycystin-2 channel is undefined, but the pore structure can be approached with the same experimental procedures used in this study. The above analysis of the pore of the RyR will allow for further understanding of the mechanism of ion conduction, channel regulation, and the mechanism of cross-talk among subunits.

Introduction of disulfide bonds and modification by MTSEA⁺ can also be used to determine whether the subunits of an ion channel gate independently or as a unit. The recent experiments investigating the pore formation of the MscL, a bacteria mechanosensitive channel (35), showed that cysteine substitutions and oxidizing agents that interact with the cysteine residues of the M1 and M2 domains of the channel did not impede channel activity. This result applied that the M1 and M2 domains do not change their relative position during gating conformation, but rather they tilt as a unit. This experimental approach can be used to study the gating of the RyR subunits and to determine whether all four subunits “tilt” together. Previous work showed that modification of the RyR by methylmethanethiosulfonate or MTSEA⁺ did not alter the gating kinetics or calcium dependence of channel activation (5). Similarly, by generating cysteine mutations on the S6 tail of the Shaker potassium channel, putative residues lining the inner cavity of the pore region were identified based on their ability to reduce the single channel conductance in addition to preventing pore blockage in the presence of tetrabutylammonium (36). In comparison, it was possible to modify the channel only when it was in its open state regardless of which side of the channel the methanethiosulfonate reagent was added (5). These reports show that manipulation of the interactions among the subunits will lead to further insights into the configuration of the pore pathway.

In this study, we have shown that MTSEA⁺ modifies the current of the RyR in the presence of organic amines of increasing diameter. If the RyR was comprised of four independent pores, then it would have been possible to observe all four conductance levels with each amine used after the addition of MTSEA⁺. Instead we saw a size-dependent decrease in the number of states observed with each amine. The ability to completely inhibit permeation by trimethylenamine when Cs⁺ was still able to pass current showed that MTSEA⁺ was able to modify the parameters of the permeation pathway in a very specific manner. The size-dependent block of amine currents shown here provides strong support for the one-pore model of the permeation pathway of the RyR.

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