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Using Large Organic Cations to Probe the Nature of Ryanodine Modification in the Sheep Cardiac Sarcoplasmic Reticulum Calcium Release Channel

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ABSTRACT We have reported that the large impermeant organic cations tetrabutyl ammonium (TBA^+), tetrapentyl ammonium, and the charged local anesthetic QX314 produce unique reduced conductance states in the purified sheep cardiac sarcoplasmic reticulum Ca^{2+} release channel when present at the cytoplasmic face of the channel. We have interpreted this as a form of partial occlusion by the blocking cation in wide vestibules of the conduction pathway. Following modification with ryanodine, which causes the channel to enter a reduced conductance state with long open dwell time, these cations block the receptor channel to a level that is indistinguishable from the closed state. The voltage dependence of TBA^+'s interaction with the Ca^{2+} release channel is the same before and after ryanodine modification. The concentration dependence is different, in that the ryanodine-modified channel has one-third the affinity for TBA^+, which is accounted for predominantly by changes in the TBA^+ on rate. The data are compatible with a structural change in the vestibule of the conduction pathway consequent upon ryanodine binding that reduces the capture radius for blocking ion entry.

INTRODUCTION

Ryanodine is a plant alkaloid that binds with high affinity and specificity to the sarcoplasmic reticulum Ca^{2+} release channel (Fleischer et al., 1985; Pessah et al., 1986; Lattanzio et al., 1987). In addition, it has characteristic electrophysiological effects on single Ca^{2+} release channels reconstituted into planar phospholipid bilayers. The most striking effect is the modification of the channel to a substate with a high open probability (Rousseau et al., 1987). In a general sense this action has some similarity to the effect of batrachotoxin on the voltage-dependent sodium channel: a naturally occurring toxin leads to a profound modification of channel gating and conduction (Krueger et al., 1983; Moczydlowski et al., 1984; Khodorov, 1985). The relationship between the "normal" and the "toxin-modified" channels is of considerable biophysical interest in both instances and has been explored in the batrachotoxin-modified sodium channel (Correa et al., 1991). Additionally, the use of the toxin-modified channels has allowed the properties of voltage-dependent sodium channels to be investigated in planar phospholipid bilayers. In a similar way, there may be experimental advantages in studying the ryanodine-modified Ca^{2+} release channel. The ryanodine-modified receptor channel has the attraction of long open times and a high open probability, which is ideal for the bioassay of compounds with long dwell times in the conduction pathway.

We have previously described an unusual form of block caused by large tetraalkyl ammonium cations, namely tetrabutyl ammonium (TBA^+) and tetrapentyl ammonium ($TPeA^+$) (Tinker et al., 1992a) and the charged local anaesthetic QX314 (Tinker and Williams, 1993) in the unmodified ryanodine receptor channel. It is characterized by the production of a substate (approximately 20% of the control conductance for TBA^+, 14% for $TPeA^+$, and 34% for QX314), the occurrence of which is highly voltage and concentration dependent. The effect occurs on addition of these organic cations to the cytoplasmic face of the channel but not the luminal. We have interpreted this observation as a form of partial occlusion in which several TAA^+s or QX314s bind to a vestibule region on the cytosolic face of the conduction pathway to induce a substate by electrostatic means.

In this short report we provide a qualitative description of the interaction of these large positively charged cations with the ryanodine-modified Ca^{2+} release channel and quantify the effect of TBA^+. The differences in behavior before and after ryanodine modification are interpreted in terms of structural changes in the mouth regions of the pore consequent upon ryanodine binding.

MATERIALS AND METHODS

Materials

Phosphatidylethanolamine was purchased from Avanti Polar Lipids (Birmingham, AL), and phosphatidylcholine was from Sigma, Ltd. (Poole, Dorset, England). [^{3}H]Ryanodine was obtained from New England Nuclear (Boston, MA). Aqueous counting scintillant was purchased from Packard (Groningen, the Netherlands). The bromide salts of tetrabutyl ammonium and tetrapentyl ammonium were obtained from the Aldrich Chemical Company (Gillingham, Dorset, England). The chloride salt of QX314 was a kind gift from Astra Pharmaceuticals, Ltd. (Kings Langley, Hertfordshire, England). These ions were dissolved in the standard experimental 210 mM K^+ solution to make concentrated stock solutions from which small aliquots were added to the solutions in the *cis* and *trans* chambers. Ryanodine was obtained from Agrisystems International (Windgap, PA). Other chemicals were the best available grade from BDH, Ltd. (Dagenham, Essex, England), Aldrich Chemical Company, or Sigma, Ltd.

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Preparation of sheep cardiac heavy sarcoplasmic reticulum membrane vesicles

Sheep hearts were collected from a local abattoir in ice-cold cardioplegic solution (Tomlins et al., 1986). Junctional or heavy sarcoplasmic reticulum membrane vesicles were isolated from the interventricular septum and left ventricular free wall as previously described (Sitsapesan and Williams, 1990). Differential centrifugation of the muscle homogenate provides a mixed membrane fraction, which when fractionated further on a discontinuous sucrose gradient yields a heavy sarcoplasmic reticulum (HSR) fraction at its 30/40% (w/v) interface. The HSR fraction was resuspended in 0.4 M KCl before sedimentation at 36,000 rpm (100,000 × g_{av}) for 1 h in a Sorvall A641 rotor. The resulting pellet was resuspended in a solution containing 0.4 M sucrose and 5 mM *N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid titrated to pH 7.2 with tris (hydroxymethyl)-methylamine (Tris) and then snap frozen in liquid nitrogen for storage overnight at −80°C.

Solubilization and separation of the ryanodine receptor

The solubilization of the ryanodine receptor by the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate and subsequent separation of the receptor from other SR membrane proteins was performed as described previously (Lindsay and Williams, 1991). HSR membrane vesicles were solubilized on ice for 1 h with 0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate in the presence of 2.5 mg/ml L-α-phosphatidylcholine and 1 M NaCl, 0.1 mM EGTA, 0.15 mM $CaCl_2$, and 25 mM piperazine *N,N'*-bis(2-ethanesulfonic acid)-NaOH (pH 7.4), at a protein concentration of 2 mg/ml. Following sedimentation of all unsolubilized material by centrifugation, separation of the ryanodine receptor from the other solubilized protein components was achieved by centrifugation on a linear 5–25% (w/v) sucrose gradient. Fractions were drawn from the bottom of the tube, and the fraction containing the receptor protein was identified by comparison with identical tubes containing membrane components incubated in the presence of [^{3}H]ryanodine during solubilization. The purified ryanodine receptor was then reconstituted into liposomes by dialysis overnight against a buffer (0.1 M NaCl, 0.1 mM EGTA, 0.15 mM $CaCl_2$, 25 mM piperazine *N,N'*-bis(2-ethanesulfonic acid)-NaOH, pH 7.4) with five changes of solution, for incorporation into planar phospholipid bilayers.

Planar lipid bilayer methods

Lipid bilayers, formed from suspensions of phosphatidylethanolamine in *n*-decane (35 mg/ml), were painted across a 200-μm-diameter hole in a polystyrene copolymer partition that separated two chambers referred to as the *cis* (volume 0.5 ml) and *trans* (volume 1.5 ml) chambers. The *trans* chamber was held at virtual ground, while the *cis* chamber could be clamped at various holding potentials relative to ground. Current flow across the bilayer was measured using an operational amplifier as a current-voltage converter as described by Miller (1982). Bilayers were formed in solutions of 200 mM KCl and 20 mM *N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, titrated with KOH to pH 7.4, resulting in a solution containing 210 mM K^+. An osmotic gradient was established by the addition of a small quantity (usually 50 to 100 μl) of 3 M KCl to the *cis* chamber. Proteoliposomes were added to the *cis* chamber and stirred. To induce fusion of the vesicles with the bilayer a second small aliquot (50 to 100 μl) of 3 M KCl was added to the *cis* chamber. After channel incorporation, further fusion was prevented by perfusion of the *cis* chamber with 210 mM K^+. Solutions contained 10 μM free Ca^{2+} as contaminant, which was generally sufficient for channel activation. Experiments were carried out at room temperature (21 ± 2°C). In general 100 to 200 nM ryanodine was added to the *cis* chamber to invoke modification. The chamber was subsequently perfused to remove all traces of ryanodine.

The receptor channel incorporates in a fixed orientation in the bilayer; the *cis* chamber corresponds to the cytosolic face of the channel and the *trans* to the luminal (Lindsay and Williams, 1991; Tinker et al., 1992b). In the subsequent discussion this naming convention will be adopted, and current flowing from the cytoplasm to the interior of the SR will be referred to as positive to ground (Bertl et al., 1992).

Single-channel data acquisition

Single-channel current fluctuations were displayed on an oscilloscope and stored on videotape. For analysis, data were replayed, filtered with an 8-pole Bessel filter, and digitized using an AT based computer system (Satori; Intracel, Cambridge, England). Single-channel current amplitudes and dwell time histograms were determined from digitized data. The representative traces shown in the figures were obtained from digitized data acquired from Satori V3.2 and transferred as an HPGL graphics file to a graphics software package (CorelDraw) for annotation and printing.

Methods of Analysis

Determining kinetics for block by TBA^+

The addition of TBA^+ to the ryanodine-modified channel leads to the break-up of the normal long open events into rapid fluctuations between the open level and a level indistinguishable from the closed level. The effect occurs only on addition of TBA^+ to the cytoplasmic face of the channel.

By using single channels it is possible to determine kinetic parameters from the interaction of TBA^+ with the modified channel. For such analyses the data were filtered at 4 kHz and digitized at 10 kHz. Open and closed lifetime files were compiled using a 50% amplitude detection threshold with the cursors set manually on the open level and the blocked level. Events above the midpoint were considered to be open events and those below it to be blocked events. Using this approach it was possible to obtain total times spent in each state and dwell time histograms for each corresponding state. Two measurements were made, one related to the equilibrium of the open < > blocked reaction and the other to its kinetics.

In the first of these, the length of time spent in the open or blocked state was obtained from the raw lifetime file and was used to calculate a measure of the open to blocked equilibrium. The ratio ($R_{b/o}$) of the total time spent in the blocked state to the total time spent in the open state was determined. An equivalent parameter was also calculated—the fraction (F_b) of total events occurring as the blocked state (in other words, the total time spent in the blocked state divided by the total time spent in both the open and blocked states).

In the second method, information was obtained on the kinetics of the reaction by lifetime analysis. Open and blocked state dwell-time histograms were compiled with the 50% amplitude threshold method as detailed above. Events of less than 0.18 ms could not be fully resolved with low-pass filtering at 4 kHz and were removed from the file. Lifetimes were calculated on stripped files containing at least 500 transitions and often more. We followed the methods of Colquhoun and Sigworth (1983) in fitting lifetimes to probability density functions. These lifetimes were fitted to probability density functions with one or two exponential terms optimizing the variables to minimize the negative log-likelihood using a simplex algorithm. A missed events correction was used as described by Colquhoun and Sigworth (1983). The fit to one and two exponential terms was assessed by eye and by using the log-likelihood ratio test (Blatz and Magleby, 1986); twice the difference in log-likelihood between the values for one and two exponential terms was tested against a χ^2 distribution at the 1% level with the relevant degrees of freedom. Both open and blocked lifetime histograms were found to be adequately described by probability density functions with a single exponential term.

The SEM statistic was calculated with a commercially available software package (Graphpad Inplot v 4.03; GraphPad Software, San Diego, CA) from linear and nonlinear regression.

A framework for analysis

The analysis of the interaction of TBA^+ in the modified channel is based on the simple kinetic scheme

CLOSED < > OPEN < > BLOCKED.TBA.

This is a simplification of the true situation (discussed in detail in Tinker et al., 1992a, and Tinker and Williams, 1993); however, it provides a useful basis for analysis. Characteristically the ryanodine-modified channel has a high open probability with few closures. Therefore in the presence of TBA^+, the vast majority of transitions represent open < > blocked fluctuations. If voltage dependent, the equilibrium (the ratio of total times spent in the blocked and open states) will be determined by the Boltzmann distribution,

$$R_{b/o} = \exp([z_t FV - G_i]/RT), \quad (1)$$

where z_t is the voltage dependence of the block and G_i/RT is an expression of the equilibrium of the reaction at zero holding potential. Taking the natural logarithm leads to a convenient linearization in which the slope gives the voltage dependence of the open to blocked state transitions and the equilibrium term can be derived from the intercept. In addition, if voltage dependent, K_{on} and K_{off} to and from the blocked state will be described by

$$K_{on}(V) = K_{on}(0) \cdot \exp[z_{on} \cdot (FV/RT)] \quad (2)$$

and

$$K_{off}(V) = K_{off}(0) \cdot \exp[-z_{off} \cdot (FV/RT)], \quad (3)$$

where $K(V)$ and $K(0)$ refer to the rate constant at a particular voltage and at 0 mV, respectively. z is the valence of the respective reaction. From a plot of the natural logarithm of the rate constant against holding potential it is possible to determine z from the slope and $K(0)$ from the intercept. The total voltage dependence of the reaction is given by $z_{on} + z_{off}$.

The fraction of time spent in the blocked state (F_b) will saturate with increasing TBA^+ concentration. This will be accounted for kinetically by a linear rise in K_{on} with TBA^+ concentration. K_{off} will be independent of concentration.

RESULTS

The purified sheep cardiac Ca^{2+} release channel has a high single-channel conductance of approximately 720 pS in symmetrical 210 mM K^+ (Lindsay et al., 1991). The addition of 100 nM to 1 μM ryanodine to the cytoplasmic face of the channel results in modification to a reduced conductance level (Fig. 1), with an amplitude of approximately 60% of the control conductance under these ionic conditions. Following modification the open probability is close to 1.

Large organic cations interact with the ryanodine-modified channel

TBA^+, $TPeA^+$, and QX314 have a characteristic effect on the sheep cardiac sarcoplasmic reticulum Ca^{2+} release channel before modification by ryanodine. Characteristically they lead to the generation of a reduced conductance level, the value of which is dependent on the interacting species (Fig.2 *a*). The occurrence of the reduced conductance level is dependent on the holding potential and concentration of the interacting species and occurs only on addition to the cytoplasmic face of the channel (Tinker et al., 1992a; Tinker and Williams, 1993). Those organic cations known to induce the occurrence of a reduced conductance state in the sheep cardiac Ca^{2+} release channel were examined for their effect on single-channel current fluctuations following ryanodine modification. A strikingly different behavior was found. TBA^+, $TPeA^+$, and QX314 all cause the channel to fluctuate

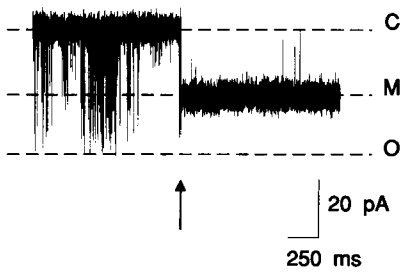

FIGURE 1 Single-channel current fluctuations in 210 mM K^+. The trace is filtered at 2 kHz and digitized at 8 kHz. Ryanodine modification (marked by the arrow) induced by the addition of 100 nM ryanodine at the cytoplasmic face of the channel at a holding potential of 60 mV. *C*, closed level; *O*, open level; *M*, modified level.

between the fully open level and a blocked level indistinguishable from the closed level. This is demonstrated for TBA^+ and QX314 in Fig. 2 *a*. The effect occurs only on addition to the cytoplasmic face of the channel. However, to avoid the development of asymmetric surface potentials, the organic cations were added to both chambers when data were analyzed quantitatively. The effect is freely reversible when the *cis* chamber is perfused with 210 mM K^+. Ryanodine modification occurs in the presence of the above-mentioned large organic cations and results in a comparable effect. The effect is dependent on both blocker concentration and holding potential, and this is illustrated for TBA^+ in Fig. 2 *b*.

Quantitative analysis of the TBA^+ interaction

We have previously obtained considerable quantitative information on the kinetics of the interaction of TBA^+ in the unmodified Ca^{2+} release channel. Consequently a quantitative kinetic analysis and comparison are made with this ion in the ryanodine-modified channel. The dwell-time histograms in the open and blocked states in the presence of TBA^+ are well described by probability density functions with a single exponential term (Fig. 3). It was thus possible to calculate K_{on} and K_{off} for the interaction of TBA^+ with the ryanodine-modified channel.

The voltage dependence of the effect was examined in four experiments in the presence of 100 μM TBA^+. It was estimated (see Materials and Methods) from a linearized form of the Boltzmann distribution (Fig. 4 *a*) and by an analysis of the voltage dependence of the blocker on rate (K_{on}) and the off rate (K_{off}) (Fig. 4 *b*). The valency calculated from the former approach yields a figure of 1.74 ± 0.04 (SEM). $\ln(K_{on})$ and $\ln(K_{off})$ against voltage yield a valency of 0.45 ± 0.05 and 1.11 ± 0.03 (SEM), giving a total valency of 1.56. This compares with figures of 1.66 ± 0.10, 0.47 ± 0.05, and 1.34 ± 0.09 obtained in the unmodified channel for the transition to and from the reduced conductance level (Tinker et al., 1992a). The comparison leads to the conclusion that there is no change in the voltage dependence of the transition following ryanodine modification.

The concentration dependence of block was examined in four experiments at a holding potential of 60 mV and does

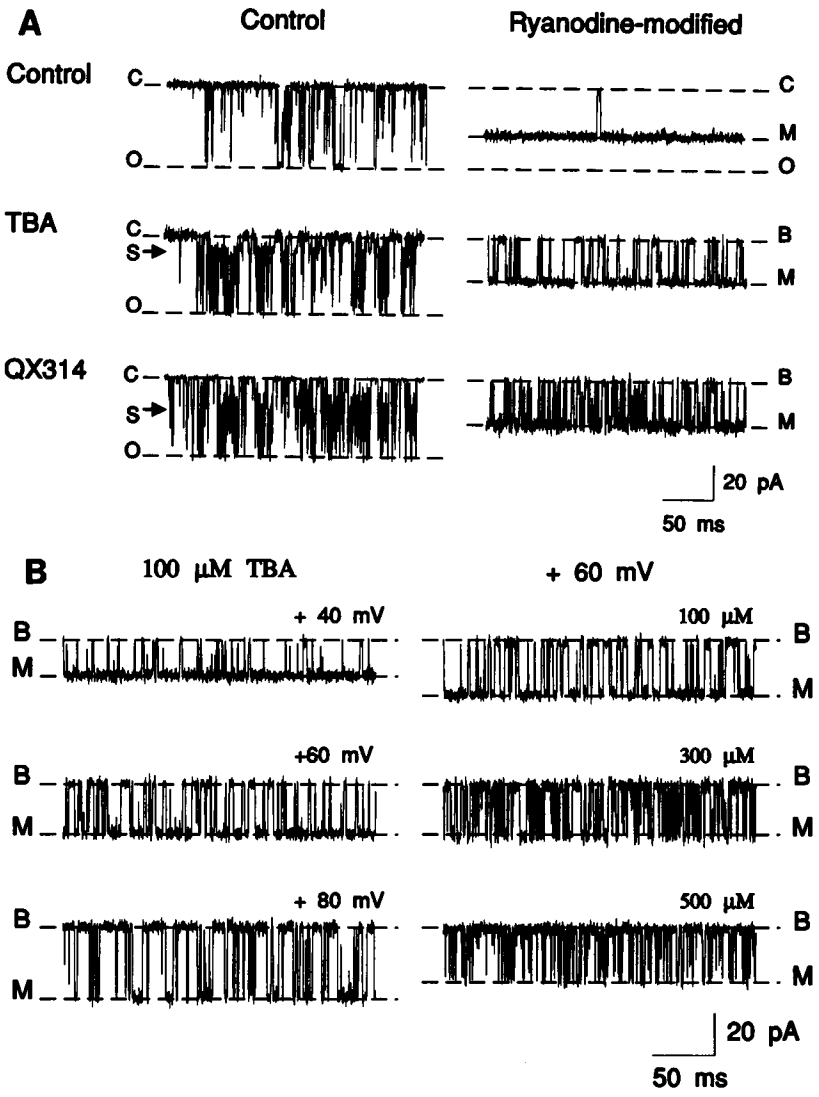

FIGURE 2 Single-channel current fluctuations in 210 mM K^+. The traces are filtered at 2 kHz and digitized at 8 kHz. *C*, closed level; O, open level; *M*, modified level; *B*, blocked level. (*a*) Different effects of 100 μM TBA^+ and 300 μM QX314 at a holding potential of 60 mV in the unmodified (*left*) and modified (*right*) channels. The top panels are representative control traces, the middle panels are traces taken following the addition of TBA^+, and the bottom panels are traces taken following the addition of QX314. The arrow in the middle left panel indicates the level of the TBA^+-related reduced conductance state (*S*) (~20% of control conductance) and the arrow in the lower left panel indicates the level of the QX314-related reduced conductance state (~34% of control conductance). (*b*) Voltage and concentration dependence of the TBA^+ effect in the ryanodine-modified channel.

reveal some differences between the normal and ryanodine-modified channel. In Fig. 5 *a* a saturation curve, of the type

$$F_b = V_{max} \cdot \frac{[TBA^+]}{[TBA^+] + K_m}, \quad (4)$$

where K_m is the affinity and V_{max} the maximal degree of block, is fitted by nonlinear regression to the variation of F_b with TBA^+ concentration. It yields half-maximal binding (K_m) of 165 ± 19 μM and saturation (V_{max}) at 1.0 ± 0.05. The slope of the Hill plot of the data is 1.0 (not shown). This compares with a K_m of 45 ± 7 μM and a V_{max} of 0.91 ± 0.02 in the unmodified channel. The reason for this decline in affinity for TBA^+ in the modified channel is illustrated in Fig. 5 *b*. The on rate is linearly dependent on TBA^+ concentration, with a slope of 2.94 ± 0.2E-3 ms^{-1} μM^{-1} (SEM, $r = 0.99$; y intercept = 0.204). This compares with a figure of 8.9 ± 1.2E-3 (SEM) in the unmodified channel (Tinker et al., 1992a). Both before and after ryanodine modification the off rates are concentration independent, giving figures of 1.14 (Tinker et al., 1992a) and 0.64 ms^{-1}, respectively.

DISCUSSION

We have interpreted the occurrence of reduced conductance states induced by organic cations in the sheep cardiac Ca^{2+} release channel, prior to ryanodine modification, as a form of partial occlusion in which several of the interacting species can bind to a vestibule-like region in the conduction pathway and cause block by electrostatic means. Quantitative attempts to model the voltage dependence of K_{on} and K_{off} for QX314 and TBA^+ suggest it is likely that a single TBA^+ or QX314 binds to the open state to produce the reduced conductance state but that it is subsequently possible to bind further organic cations to this reduced conductance state.

It seems likely that the TBA^+ binding site does not alter its location in the electric field following ryanodine modification, inasmuch as the total voltage dependence of the effect and the voltage dependence of K_{on} and K_{off} are unaltered. The flux of TBA^+ to its binding site in the electrical field, within a vestibule-like region of the conduction pathway, will be determined by the equations of electrodiffusion and the geometry of the vestibule. If the pathway is assumed

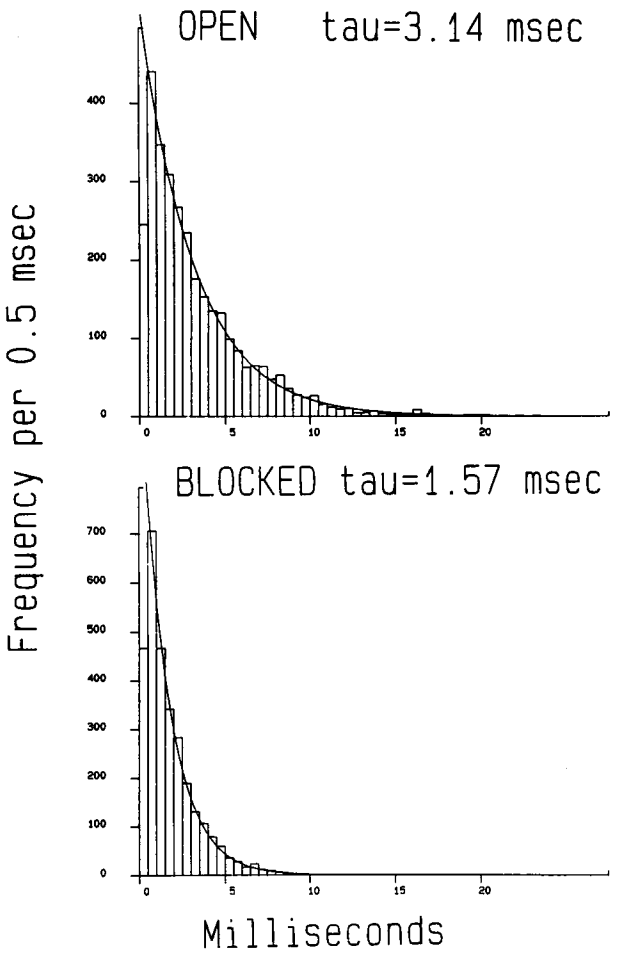

FIGURE 3 The panels show representative lifetime histograms of the ryanodine-modified channel obtained in the presence of symmetrical 100 μM TBA^+ at a holding potential of 60 mV. (*Top*) open lifetime histogram; (*bottom*) blocked state lifetime histogram together with the superimposed probability density functions (*solid lines*). The data are best described by probability density functions with a single exponential term with τ as indicated in the figure.

to be approximately cylindrical, the on rate will be proportional to the area presented (i.e., the squared capture radius) (Läuger, 1976). It should be appreciated that the capture radius is the difference between the channel vestibule radius and the interacting ion radius.

Two observations in this communication suggest a profound alteration in the vestibule of the conduction pathway upon ryanodine modification. The most striking observation is that in the modified channel the cations TBA^+, $TPeA^+$, and QX314 block to a conductance level indistinguishable from the closed state, and not to a reduced conductance level, as in the unmodified channel. In addition, there is an almost fourfold decrease in the slope of K_{on} against TBA^+ concentration following ryanodine modification. Both of these pieces of data are compatible with a reduction in the capture radius of the cytoplasmic vestibule of the sarcoplasmic reticulum Ca^{2+} release channel consequent upon ryanodine modification; the change allows complete rather than partial occlusion of the conduction pathway by the cations TBA^+, $TPeA^+$, and QX314, and K_{on} falls because the area for the electrodiffusion of TBA^+ to its binding site is reduced.

In addition, the lower affinity of TBA^+ in the modified channel and the failure of K_{off} to fall by a factor similar to the fall by K_{on}, as would be expected from diffusion considerations (see Blaustein and Finkelstein, 1990, for a thermodynamic explanation of this effect), also indicates that ryanodine induces a further conformational alteration that decreases the affinity of the binding site for this organic cation (e.g., if the hydrophobicity of the binding site were decreased by a structural rearrangement consequent upon ryanodine modification). Whether ryanodine binds directly in the conduction pathway to produce these effects or causes them by an allosteric change following binding to a receptor site distant to the pathway is unknown. How these observations relate to the reduced single-channel conductance found with both monovalent and divalent cations following ryanodine modification is also unclear, as the rate limiting step may not be the same for blocker and permeant ion.

In conclusion, we have used large organic cations as probes for possible changes in structure occurring upon ryanodine modification. The study of single-channel block may give additional clues as to its nature. In addition, the ryanodine-modified channel may represent a useful experimental system in which to investigate the interaction of

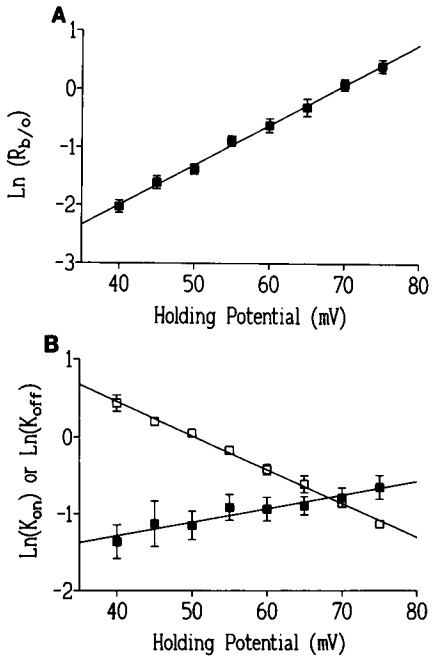

FIGURE 4 (*a*) The variation of $\ln(R_{b/o})$ with holding potential with 100 μM TBA^+ present at both faces of the ryanodine-modified channel. The points indicated by the squares are the mean of four observations from four experiments. The SEM on the points is indicated by the error bars. The solid line was obtained by linear regression with parameters as indicated in the text. (*b*) Variation of $\ln(K_{on})$ (■) and $\ln(K_{off})$ (□) with holding potential in the ryanodine-modified channel. The data were obtained from a series of four experiments, and the data points are the means of at least four observations. The SEM on the points is indicated by the error bars; where it is not indicated the SEM is included within the symbol. The solid linear regression lines are calculated and have parameters as described in the text.

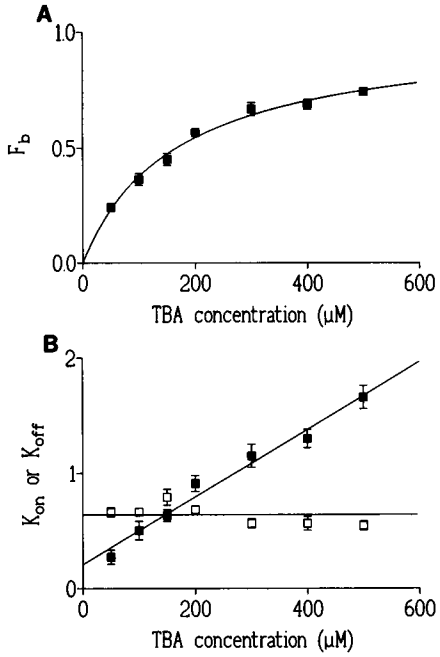

FIGURE 5 (*a*) The variation of F_b at a holding potential of 60 mV with increasing TBA^+ concentration in the ryanodine-modified channel. The data points indicated by the squares are the mean of four observations from four experiments. The SEM is indicated by the error bars (if not indicated it is included within the symbol). The solid line is the best-fit rectangular hyperbola to the data with parameters as indicated in the text. (*b*) Concentration dependence of K_{on} (■) and K_{off} (□) in the ryanodine-modified channel for TBA^+ at a holding potential of 60 mV. The points are the mean of four observations from four experiments. The SEM is indicated by the error bars (if not indicated it is included within the symbol). The solid lines through the points have theoretical significance, and the parameters are as indicated in the text.

blockers with the conduction pathway of the SR Ca^{2+} release channel. However, the extrapolation of certain quantitative details to the unmodified channel should be made with caution. Modification of the voltage-dependent Na^+ channel by batrachotoxin has been shown to result in altered properties of ion translocation (Correa et al., 1991). The details of the ion-transporting properties of the ryanodine-modified SR Ca^{2+} release channel remain to be characterized.

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