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Block of the Sheep Cardiac Sarcoplasmic Reticulum Ca^{2+}-Release Channel by Tetra-alkyl Ammonium Cations

Andrew Tinker, Allan R.G. Lindsay, and Alan J. Williams
Department of Cardiac Medicine, National Heart and Lung Institute, University of London, London SW3 6LY, United Kingdom

Summary. The purified ryanodine receptor channel of the sheep cardiac muscle sarcoplasmic reticulum (SR) membrane functions as a calcium-activated cation-selective channel under voltage-clamp conditions following reconstitution into planar phospholipid bilayers. We have investigated the effects of the tetra-alkyl ammonium (TAA) cations, $(C_nH_{2n+1})_4N^+$ and the trimethyl ammonium cations, ethyltrimethyl ammonium and propyltrimethyl ammonium, on potassium conductance through the receptor channel. Small TAA cations (n = 1–3) and the trimethyl ammonium derivatives act as asymmetric, voltage-dependent blockers of potassium current. Quantitative analysis of the voltage dependence of block indicates that the conduction pathway of the sheep cardiac SR ryanodine receptor channel contains two distinct sites for the interaction of these small organic cations. Sites are located at approximately 50% for tetramethyl ammonium (TMA^+) and 90% for tetraethyl ammonium (TEA^+) and tetrapropyl ammonium ($TPrA^+$) of the voltage drop across the channel from the cytosolic face of the protein. The chemical substitution of an ethyl or propyl group for one of the methyl groups in TMA^+ increases the voltage dependence of block to a level similar to that of TEA^+ and $TPrA^+$. The zero-voltage dissociation constant ($K_{b(0)}$) falls with the increasing number of methyl and methylene groups for those blockers acting 90% of the way across the voltage drop. This is interpreted as suggesting a hydrophobic binding site at this point in the conduction pathway. The degree of block increases as the concentration of small TAA cations is raised. The concentration dependence of tetraethyl ammonium block indicates that the cation interacts with a single site within the conduction pathway with a K_m of 9.8 ± 1.7 mM (mean ± SD) at 40 mV. Larger TAA cations (n = 4–5) do not induce voltage-dependent block of potassium current of the form seen with the smaller TAA cations. These data support the contention that the sheep cardiac SR ryanodine receptor channel may be occupied by at most one ion at a time and suggest that a large proportion of the voltage drop falls over a relatively wide region of the conduction pathway.

Key Words sarcoplasmic reticulum · ryanodine receptor · heart · channel blockade

Introduction

Calcium release from the sarcoplasmic reticulum (SR) intracellular membrane stores of striated muscle cells occurs via a Ca^{2+}-regulated ion channel located in specialized regions of the membrane system found in close apposition to the sarcolemma [27]. Characteristics of gating and conduction of both the skeletal [28] and cardiac [4, 25] forms of this channel have been monitored following the incorporation of isolated membrane vesicles into planar phospholipid bilayers. Identification of the SR Ca^{2+}-release channel as a high molecular weight ryanodine binding protein and the subsequent purification of this oligomeric complex from both skeletal [10, 11, 13, 15, 19, 29, 33] and cardiac [1, 12, 14, 18, 24] muscle has provided additional functional and structural information.

The purified ryanodine receptor functions as a ligand-regulated ion channel under voltage-clamp conditions following its reconstitution into planar phospholipid bilayers. In addition to divalent cations such as Ca^{2+}, Ba^{2+} and Mg^{2+}, the ryanodine receptor channel is permeable to a range of monovalent cations and displays extremely high single-channel conductance with the group Ia monovalent cations [17, 29].

We have provided preliminary evidence demonstrating that K^+ current through the purified sheep cardiac muscle SR ryanodine receptor channel may be inhibited by a commonly used K^+ channel blocking cation, tetraethyl ammonium (TEA^+) [17]. In this report we extend our initial observations by examining the actions of a range of tetra-alkyl ammonium (TAA) and trimethyl ammonium cations on K^+ conductance through the sheep cardiac SR ryanodine receptor channel. The short-chain TAA and trimethyl ammonium cations act as asymmetric, voltage-dependent blockers of K^+ conductance. Quantitative analysis of block has identified two sites within the voltage drop of the receptor channel with which these cations interact. The results support our earlier suggestion that the sheep cardiac ryanodine receptor channel functions as a single-ion

channel [17] and provide information on the dimensions of regions of the receptor-channel conduction pathway.

Materials and Methods

Materials

Phosphatidylethanolamine was purchased from Avanti Polar Lipids and phosphatidylcholine from Sigma. [^{3}H]-ryanodine was obtained from New England Nuclear. Aqueous counting scintillant was purchased from Amersham International. All quaternary ammonium compounds were from Aldrich. The bromide salts of tetramethyl ammonium (TMA^+), tetrapropyl ammonium ($TPrA^+$), tetrabutyl ammonium (TBA^+) and tetrapentyl ammonium ($TPeA^+$) and the chloride salt of TEA^+ were used. In general these ions were dissolved in deionized water to make concentrated stock solutions from which small aliquots were added to the solutions in the *cis* and *trans* chambers. For conduction experiments, solutions of TMA^+ and TEA^+ were buffered with 20 mM HEPES and titrated to pH 7.4 with the appropriate TAA hydroxide. All other chemicals were the best available grade from BDH or Sigma.

The trimethyl ammonium derivatives, ethyltrimethyl ammonium ($U2^+$) and propyltrimethyl ammonium ($U3^+$), were synthesized by reacting the appropriate *n*-bromoalkane with a concentrated stock solution of trimethylamine in acetonitrile. The reaction was carried out in two- to threefold excess of trimethylamine and refluxed for 4 hr. The product was washed with ether and dried under suction with an anhydrous calcium chloride trap. The solids were stored under vacuum over phosphorus pentoxide. NMR spectroscopy was used to check the identity of the compounds, and purity was assessed as at least 95%.

Preparation of Sheep Cardiac Heavy Sarcoplasmic Reticulum (HSR) Membrane Vesicles

Sheep hearts were collected from a local abattoir in ice-cold cardioplegic solution [31]. Junctional or HSR membrane vesicles were isolated from the interventricular septum and left ventricular free wall as previously described [26]. Differential centrifugation of the muscle homogenate provides a mixed membrane fraction, which when fractionated further on a discontinuous sucrose gradient yields a HSR fraction at its 30/40% (wt/vol) interface. The HSR fraction was resuspended in 0.4 M KCl before sedimentation at 36,000 rpm (100,000 $\times$ g_{av}) for 1 hr in a Sorvall A641 rotor. The resulting pellet was resuspended in a solution containing 0.4 M sucrose, 5 mM N-'2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 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 sulphonate (CHAPS) and subsequent separation of the receptor from other SR membrane proteins was performed as described previously [18]. HSR membrane vesicles were solubilized, on ice for 1 hr, with 0.5% (wt/vol) CHAPS in the presence of 2.5 mg/ml L-α-phosphatidylcholine (PC) and 1 M NaCl, 0.1 mM ethyleneglycolbis(aminoethyl ether)tetra-acetic acid (EGTA), 0.15 mM $CaCl_2$, 25 mM piperazine *N,N'*-bis(2-ethanesulphonic acid) (PIPES)-NaOH (pH 7.4), at a protein concentration of 1.5–2 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% (wt/vol) 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, as described previously [18], for incorporation into planar phospholipid bilayers.

Planar Lipid Bilayer Methods

Lipid bilayers, formed from suspensions of phosphatidylethanolamine in decane (35 mg/ml), were painted across a 200 μm diameter hole in a polystyrene partition which 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 [22]. Bilayers were formed in solutions of 200 mM KCl, 20 mM HEPES, 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 200 mM KCl, 20 mM HEPES, titrated with KOH to pH 7.4. Solutions contained 10 μM contaminant free calcium which was sufficient for channel activation. Experiments were carried out at room temperature (21 $\pm$ 2°C).

Single-Channel Data Acquisition and Analysis

Single-channel current fluctuations were displayed on an oscilloscope and stored on videotape. For analysis, data were replayed, filtered using an 8-pole Bessel filter (Frequency Devices 902) and digitized using an AT-based computer system (Satori, Intracel, Cambridge, UK). Single-channel current amplitudes were determined from digitized data using manually controlled cursors. Current fluctuations monitored in the presence of TMA^+ or TEA^+ and the trimethyl ammonium derivatives were filtered at 1.0 kHz and digitized at 4.0 kHz, while data obtained in the presence of $TPrA^+$ were filtered at 600 Hz and digitized at 2 kHz. The representative traces shown in the figures were displayed on a Hewlett Packard 7475A plotter following digitization using a PDP 11/73-based system (Indec, Sunnyvale, CA).

Calculation of Blocking Parameters

The blocking parameters given in the Table were calculated from linear regression of plots based on the linearized form of the Woodhull equation (Eq. (2) in Results). The blocking effect with TEA^+, $TPrA^-$, $U2^+$ and $U3^+$ was apparent only on addition of the cation to the *cis* chamber. Addition of these blockers to the *trans* chamber had no effect. Despite this asymmetry of effect these blockers were added symmetrically to both *cis* and *trans* chambers to avoid the possibility of asymmetric surface potentials resulting from the binding of the TAA cations to the bilayer [21]. In the case of TMA^+ the addition of high concentrations to the *trans* chamber (≥400 mM) led to a very small, but resolvable, reduction in single-channel current amplitude. The *cis* blocking effect of TMA^+ occurred at much lower concentrations. A comparison of the current-voltage relationship obtained after *cis* addition and that obtained after symmetrical addition of 200 mM TMA^+ revealed a very small difference at high negative holding potentials (Fig. 3*b*). In view of the possible slight complication arising from interactions of TMA^+ from the *trans* side of the membrane, the blocking parameters quoted in the Table were calculated from experiments in which TMA^+ was added only to the *cis* chamber. The values determined following symmetric addition ($z\delta = 0.52 \pm 0.02$, $K_{b(0)} = 265 \pm 34$; $n = 6$, ±SEM) differed little from the values quoted in the Table despite a possible small deviation from the Woodhull model at high negative holding potentials.

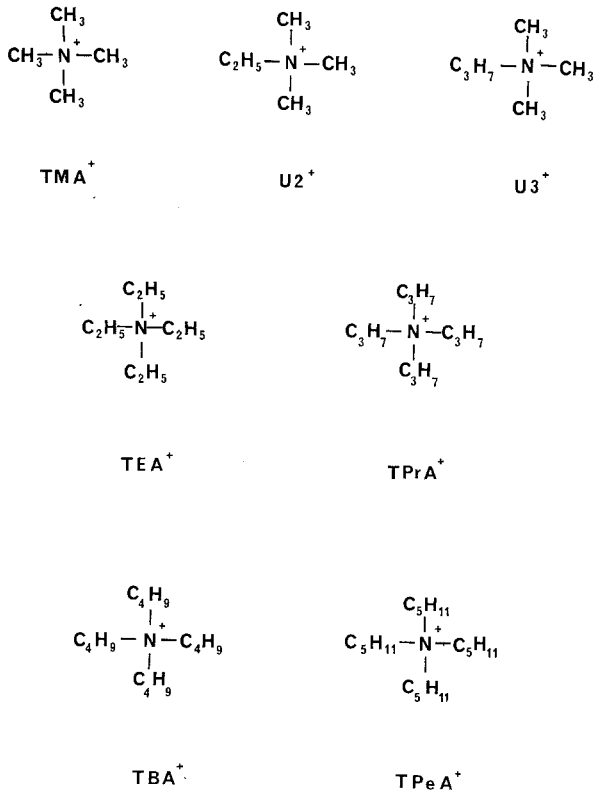


Fig. 1. The chemical structures and abbreviations of the TAA cations and their trimethyl ammonium derivatives used in these experiments.

Results

The sheep cardiac muscle SR ryanodine receptor functions as a ligand-regulated ion channel when reconstituted into planar phospholipid bilayers [17, 18]. The channel is ideally selective for cations over anions but allows current to be carried by both divalent and monovalent cations [18]. In symmetrical 210 mM K^+ the current-voltage relationship of the receptor channel is linear over the voltage range of ±80 mV, with a conductance of 723 pS [17]. The separation of the ryanodine receptor channel from the K^+ channel of the native SR [20, 32] enables monovalent-cation conduction and block to be studied in detail. We have investigated the effects of TAA cations and their trimethyl ammonium derivatives on K^+ conductance. The chemical structures of the compounds are shown in Fig. 1.

TAA Cations are Impermeant in the Sheep Cardiac Ryanodine Receptor Channel

Experiments with TEA^+ under bi-ionic conditions, i.e., with 210 mM K^+ on the *trans* side of the membrane and 210 mM TEA^+ on the *cis*, indicate that this cation is impermeant [17]. Similar experiments using the smaller TMA^+ indicate that this cation is also unable to act as a charge carrier in the receptor channel. Under these conditions, it is not possible to determine a reversal potential with TMA^+; no net TAA^+ current was measurable at any holding potential up to and including 80 mV (Fig. 2). Similarly, no current fluctuations were detectable in the presence of symmetrical solutions of 210 mM TMA^+. In all cases the existence of very small current fluctuations was excluded by inspection of data at high gain and with low frequency filtering (*data not shown*).

TMA^+, TEA^+ and $TPrA^+$ are Voltage-Dependent Blockers of K^+ Current

Although the small TAA cations are unable to carry charge through the sheep cardiac SR ryanodine receptor channel, they are capable of influencing the conduction of permeant monovalent cations through the channel. With K^+ as the permeant ion, the addition of millimolar concentrations of TEA^+ [17] and TMA^+ to solutions bathing the channel protein produced a reduction in single-channel current amplitude. Despite symmetric addition of these ions to both sides of the bilayer, single-channel current re-

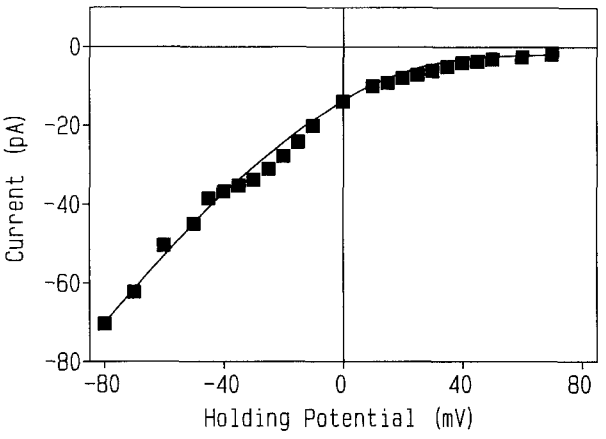


Fig. 2. Single-channel current-voltage relationship obtained under bi-ionic conditions. The *trans* chamber contained 210 mM K^+, and the *cis* chamber contained 210 mM TMA^+. The solid line was drawn by eye and is of no theoretical significance.

duction was more prominent at positive voltages (Figs. 3 and 4). This behavior is consistent with these impermeant TAA cations entering the conduction pathway of the ryanodine receptor channel and blocking K^+ conductance. The dwell time of the blocking cations in the conductance pathway is so short that individual blocking reactions are not resolved and block appears as a time-averaged reduction in single-channel current amplitude.

$TPrA^+$ produces a comparable voltage-dependent reduction in single-channel current amplitude. However, the reduced open-state current levels seen in the presence of this TAA cation appear to be considerably more noisy than those seen with either TMA^+ or TEA^+. Open-state noise was reduced when the data were filtered at 600 Hz rather than 1.0 kHz (Fig. 4). This implies that the dwell time of $TPrA^+$ in the conduction pathway of the receptor channel was considerably longer than those of TMA^+ or TEA^+. The three short-chain TAA cations act as asymmetric blockers of K^+ current in the ryanodine receptor channel. Block of K^+ conductance by the short-chain TAA cations was freely reversed by perfusion of the cation from the *cis* chamber.

It is apparent from Fig. 3*a* that the addition of TMA^+ produced an increase in receptor-channel open probability (P_o). This effect was not observed with the other TAA cations investigated in this study, and the mechanisms involved in TMA^+'s elevation of P_o remain to be established.

TAA^+ Block Fits a Simple Scheme

The reduction in single-channel current amplitude seen with the TAA^+ blockers at any particular voltage can be fitted by a model first suggested by Woodhull [34]. In its simplest form it assumes that there is a single site lying a fraction δ across the voltage drop and that the site is only accessible to the blocker from one side of the channel. The expression for the relative conductance, i.e., in these experiments the ratio of the conductance with and without the blocker at a particular holding potential, is

$$g/g_o = 1/[1 + [B]/K_{b(0)} \cdot \exp(z\delta FV/RT)] \quad (1)$$

and in a linearized form

$$\mathrm{Ln}(g_o/g - 1) = \mathrm{Ln}([B]/K_{b(0)}) + z\delta \cdot FV/RT \quad (2)$$

where $[B]$ is the blocker concentration; $K_{b(0)}$ is the

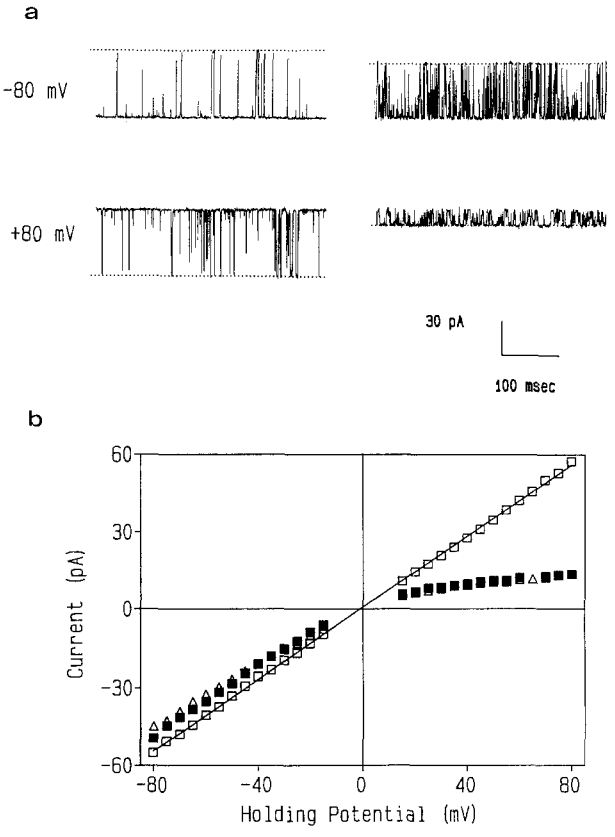


Fig. 3. (*a*) Single-channel current fluctuations are shown with a control trace in symmetrical 210 mM K^+ at holding potentials of ±80 mV (left panel) and following the symmetrical addition of 200 mM TMA^+ (right panel). Open-channel current levels are indicated by the dotted lines. (*b*) Single-channel current-voltage relationship is shown demonstrating the reduced current observed following the addition of 200 mM TMA^+ to the *cis* chamber and symmetrically (□, control; ■, plus 200 mM TMA^+ *cis*; △, 200 mM TMA^+ symmetrically).

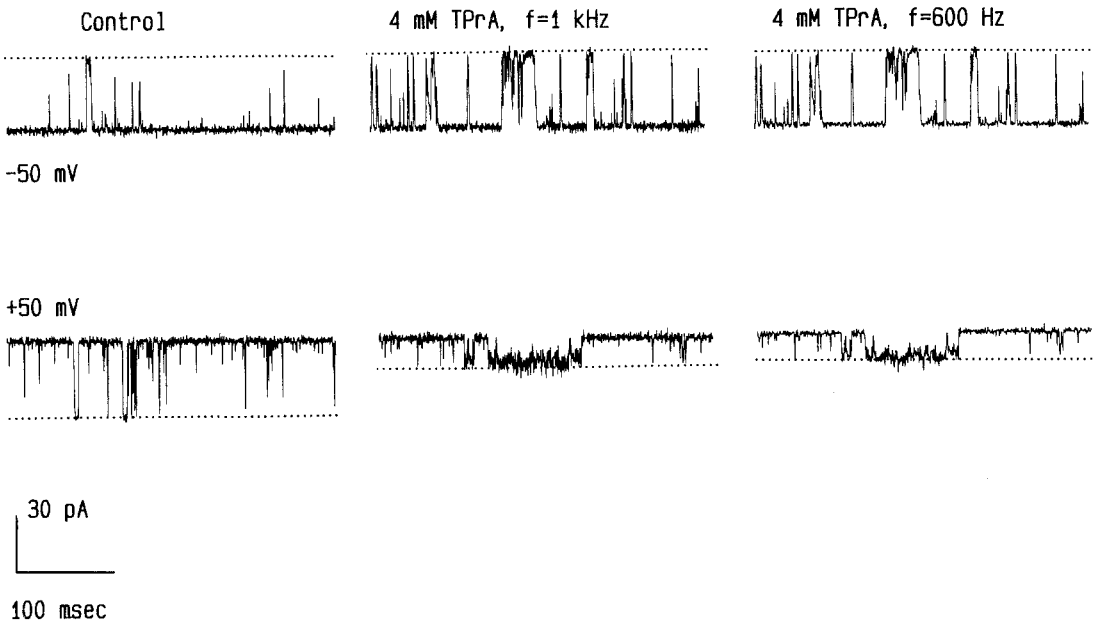


Fig. 4. Representative traces of single-channel current fluctuations at ±50 mV. The control trace in 210 mM K^+ is shown in the left panels, with the effects of symmetrical addition of 4 mM $TPrA^+$ shown in the middle and right panels. In the middle panels the records were filtered at 1 kHz, with the right panels showing the same data filtered at 600 Hz. Open-channel current levels are indicated by the dotted lines.

dissociation constant at zero holding potential; F, R and T have their usual meanings and $z\delta$ is conventionally referred to as the effective valence of the blocker. RT/F is 25.2 mV at 20°C.

The K^+ conductance data obtained in the presence of the three short-chain TAA cations correlate well with this model (Fig. 5), and the values of $z\delta$ and $K_{b(0)}$ for each blocker, obtained from linear-regression analysis of plots using Eq. (2), are shown in the Table. As would be expected for a single-ion channel, the $z\delta$ values determined for the three TAA cations were not significantly altered by up to a threefold variation of blocker concentration (*data not shown*).

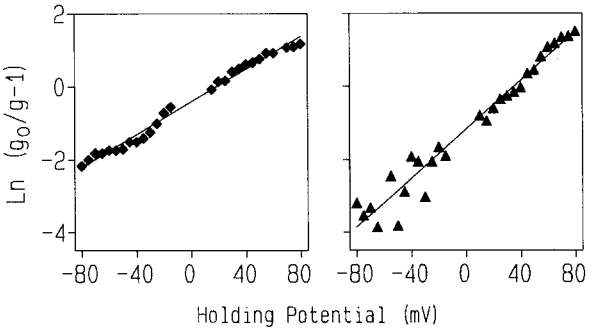


Fig. 5. The voltage-dependent block of K^+ conduction in the sheep cardiac SR ryanodine receptor channel with 200 mM TMA^+ (r = 0.99, left panel) and 4 mM $TPrA^+$ (r = 0.98, right panel). Mean parameters are quoted in the Table.

The Voltage Dependence of Block of the Trimethyl Ammonium Derivatives is Similar to that of TEA^+ and $TPrA^+$

To examine the effect of blocker structure on the voltage dependence of block two short-chain trimethyl ammonium derivatives were synthesized (*see* Materials and Methods). These organic cations also acted as voltage-dependent blockers of K^+ current, and again block was well described by the simple form of the Woodhull model (Fig. 6). A series of such experiments gave the blocking parameters for $U2^+$ and $U3^+$ given in the Table. It appears that the substitution of an ethyl or propyl group for one of the methyl groups in TMA^+ dramatically increases the voltage dependence of block to a level that is similar to that of TEA^+ and $TPrA^+$.

TEA^+ Block Occurs at a Single Site

The effect of varying TEA^+ concentration on relative K^+ conductance was measured in symmetrical solutions containing 200 mM K^+. TEA^+ was chosen

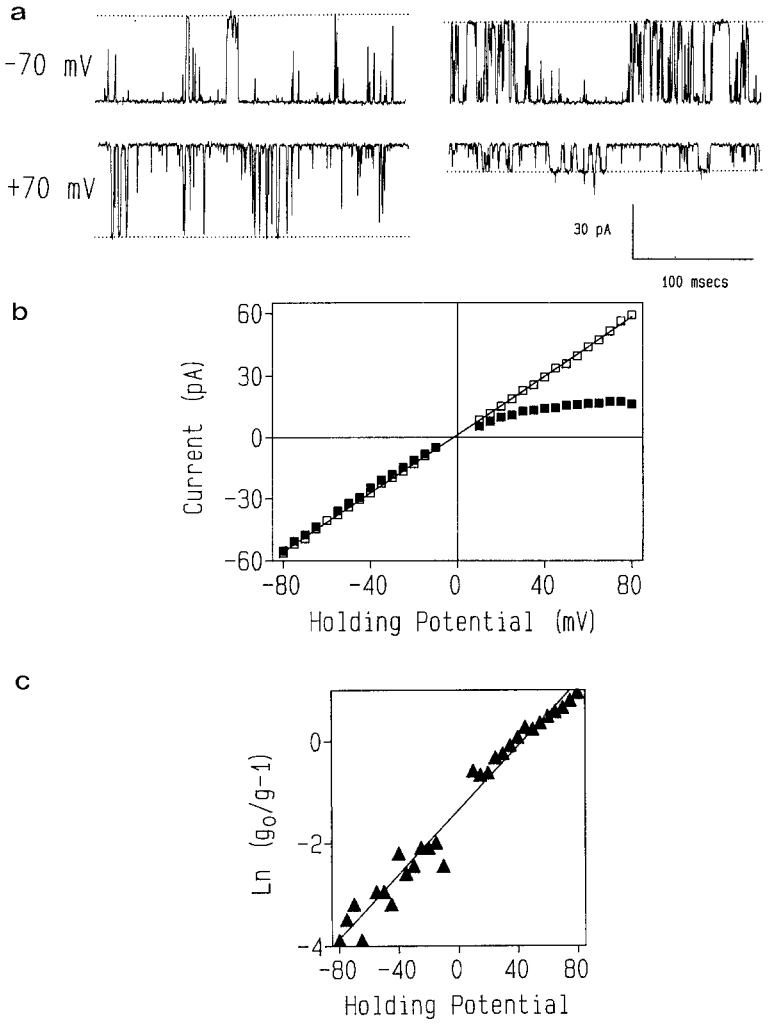


Fig. 6. (*a*) Representative traces of single-channel current fluctuations at ±70 mV from a bilayer containing at least two channels. Control fluctuations are shown in the left panel and fluctuations following the symmetrical addition of 120 mM U2$^+$ are shown in the right panel. Open-current levels for a single channel are indicated by the dotted lines, and the upper traces are at the negative and the lower ones at the positive holding potential. Downward deflections from the dotted line in the bottom right trace are openings of a second channel. (*b*) Single-channel current-voltage relationship demonstrating the reduced current seen following the symmetrical addition of 90 mM U2$^+$ (□, control; ■, plus 90 mM U2$^+$). (*c*) The linearized plot of the Woodhull model (Eq. (2)) for 90 mM U2$^+$ (r = 0.99). Mean parameters are quoted in the Table.

Table. Blocking parameters

Cation	n	zd	$K_{b(0)}$
TMA	5	0.54 ± 0.01	273 ± 18
TEA	4	0.91 ± 0.02	50.4 ± 3.4
TPrA	7	0.91 ± 0.02	15.2 ± 2.6
U2	4	0.85 ± 0.02	418 ± 21
U3	4	0.89 ± 0.02	201 ± 9

The table shows the effective valence of block ($z\delta$) (±SEM) and $K_{b(0)}$ (mM, ±SEM) for the TAA cations and their trimethyl ammonium derivatives.

because of its smooth block and its effect at relatively low concentrations. The degree of block was measured at 40 mV and is given by $(1 - g/g_o)$. The increasing degree of block seen with rising TEA$^+$ concentrations is consistent with Michaelis-Menten saturation kinetics. An example of an Eadie-Hoftsee plot of the data is shown in Fig. 7. The mean K_m (the concentration at which single-channel conductance is reduced by 50%) is 9.8 ± 1.7 mM (±SD, n = 5), and the normalized V_{max} is 0.91, close to the expected value of 1.0. Correlation of conductance blocking data with this type of kinetic scheme implies that TEA$^+$ is acting at a single site.

The Effect of Larger TAA Cations Is Not Classical Voltage-Dependent Block

Tetrabutyl ammonium (TBA$^+$) and tetrapentyl ammonium (TPeA$^+$) do not cause voltage-dependent block of the form reported here for the three shorter-chain TAA cations and the two trimethyl ammonium derivatives. However, they do modify K$^+$ conductance, and this effect is illustrated for TBA$^+$ in Fig.

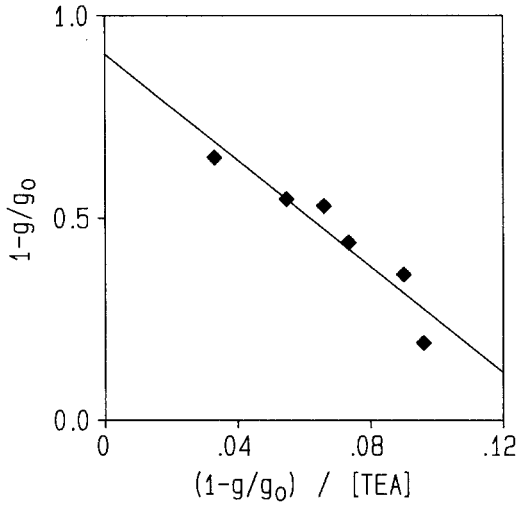


Fig. 7. An Eadie-Hoftsee plot showing the concentration dependence of TEA^+ block at 40 mV ($r = -0.94$). Mean parameters are quoted in the text.

8*a*. $TPeA^+$ causes a qualitatively similar but quantitatively different effect. In contrast to the smaller TAA^+ cations the relative conductance of the "blocked" state (the level marked by the arrow in the lower right trace in Fig. 8*a*) is constant over the range of voltages at which the effect is apparent (Fig. 8*b*) and over a range of TBA^+ concentrations. In other words in the presence of TBA^+ and $TPeA^+$, in addition to normal full-opening events the receptor channel appears to enter a reduced conductance state. The occurrence of the reduced conductance state is concentration and voltage dependent, being evident only at relatively high positive holding potentials. The interactions of the longer-chain TAA cations with the conduction pathway are complex and are clearly different from those reported here for the short-chain TAA cations. A quantitative description of the effects of TBA^+ and $TPeA^+$ is given in a subsequent paper [30].

Discussion

Tetraalkyl ammonium ions block cation conduction in a wide variety of K^+ channels, including the delayed rectifier of the squid giant axon [2, 6], channels of amphibian nervous tissues [3], the high conductance calcium-activated K^+ channel [16] and the K^+ channel of the SR [5, 21]. Block in such channels may be voltage independent, for example, the external TEA^+ site of the delayed rectifier of the frog node of Ranvier [8], or have a variable degree of voltage dependence. In this report we have demonstrated that smaller members of this group of organic cations can produce voltage-dependent block of K^+ current in the purified sheep cardiac muscle SR ryanodine receptor channel.

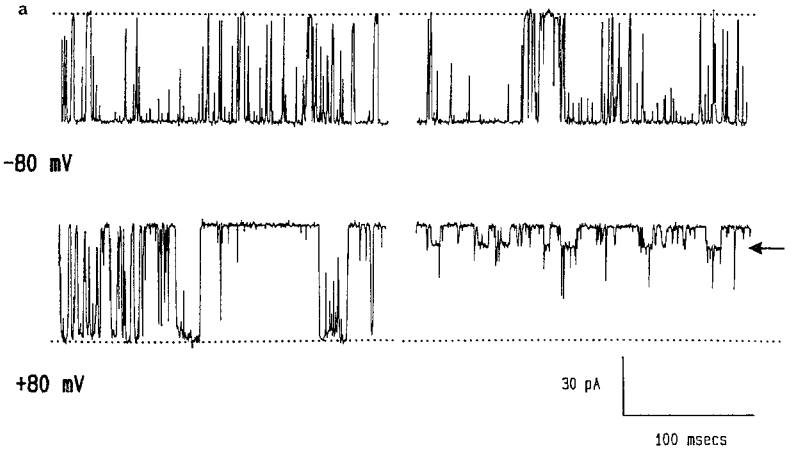
These Blocking Experiments Support Single-Ion Behavior for the Sheep Cardiac SR Ryanodine Receptor Channel

Previous work from this laboratory has indicated that a variety of conductance and permeability characteristics of the purified sheep cardiac SR ryanodine receptor channel are consistent with single-ion occupancy of the channel when monovalent cations are the permeant species [17]. The single-channel conductance-activity relationships for K^+, Na^+, and Li^+ display saturation consistent with Michaelis-Menten kinetics at ionic activities up to 10-fold greater than K_D. Further evidence for single-ion behavior comes from experiments in which single-channel conductance was monitored in the presence of mixtures of permeant group 1a cations. Under these conditions, single-channel conductance varied monotonically as the proportions of K^+/Na^+ or K^+/Li^+ were altered, with no apparent anomalous behavior.

Our proposal that the sheep cardiac SR ryanodine receptor channel functions as a single-ion channel has certain implications for the interpretation of the effects of blocking ions on channel conductance. In such a system, monovalent blocking ions must have an effective valence of less than 1.0 [9], and it is apparent from our data that this is so for the three short-chain TAA cations and the two trimethyl ammonium derivatives examined. It is also implied that a given blocking ion should interact with a single site in the conduction pathway, with no evidence of cooperativity. The concentration dependence of TEA^+ block of the receptor channel is compatible with this prediction. Multi-ion occupancy is not suggested by any experimental evidence presented here or in previous reports from our laboratory [17]. Therefore, we believe it is valid to adopt the simplest available scheme and to regard the sheep cardiac SR ryanodine receptor channel as a single-ion channel.

There Are Two Binding Sites for Small TAA Cations in the Sheep Cardiac SR Ryanodine Receptor Channel

Analysis of TAA^+, $U2^+$ and $U3^+$ block of K^+ conductance points to the presence of two distinct values of effective valence, indicating different locations for the sites of interaction of these ions with



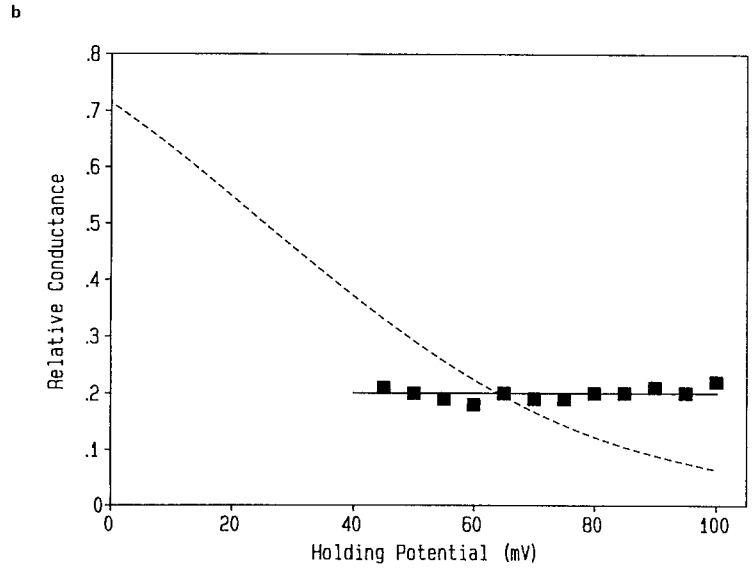


Fig. 8. (*a*) Representative traces of single-channel current fluctuations at ±80 mV. The left panel shows control traces and the right panel shows fluctuations following the symmetrical addition of 500 μM TBA^+. The upper traces are at the negative and the lower ones at the positive holding potentials. The dotted lines in all traces indicate the full open-channel current level. The arrow in the bottom right trace indicates the reduced conductance state induced by TBA^+. Opening events from this level are attenuated events to the full open level. The traces are filtered at 1 kHz. (*b*) The behavior of the relative conductance, as measured above, with 500 μM TBA^+ added symmetrically. The straight line indicates the constant value of relative conductance with voltage at holding potentials where the effect is apparent. The dashed line indicates the behavior of a voltage-dependent blocker with an effective valence of 0.9 for comparison.

the conduction pathway of the receptor channel. The smallest ion, TMA^+, would appear to bind at a site approximately halfway into the voltage drop, while the larger TEA, TPrA, U2 and U3 cations all interact with a site located approximately 90% across the voltage drop of the conduction pathway from the cytosolic face of the channel.

It is interesting to note that TEA^+ and $TPrA^+$ are able to penetrate further into the conduction pathway than TMA^+. This contrasts with the findings of French and Shoukimas [7] who demonstrated that the effective valence of block of organic cations in the squid axon K^+ channel decreased with increasing size. One possible explanation for our observations is that TEA^+ and $TPrA^+$ are sterically excluded from the TMA^+ binding site. This suggestion is supported by the observation that the voltage dependence of block is so sensitive to small changes in blocker structure. The substitution of an ethyl group in $U2^+$ and a propyl group in $U3^+$ for one of the methyl side chains in TMA^+ leads to a dramatic increase in voltage dependence to a level comparable to that of TEA^+ and $TPrA^+$. This is compatible with the TMA^+ binding site being a relatively small rigid structure into which larger blockers are unable to enter because of steric restriction.

It has been consistently observed in a number of studies on other channels that with increasing organic side chain length of blocking cations the $K_{b(0)}$ falls. The explanation given is that within the predominantly hydrophilic conduction pathway there is a hydrophobic "pocket" where such blockers bind [6, 21]. The general trend of $K_{b(0)}$ with the four blockers that act at the site 90% of the way across the voltage drop, i.e., TEA^+, $TPrA^+$, $U2^+$ and $U3^+$, is consistent with the presence of such a site in the ryanodine receptor channel. It is possible to estimate the change in affinity for each methyl group added [21] by plotting the natural logarithm of the mean $K_{b(0)}$ against the number of methyl groups in the blocker (Fig. 9). The proviso in our case is that the addition of a methyl group to a lengthening

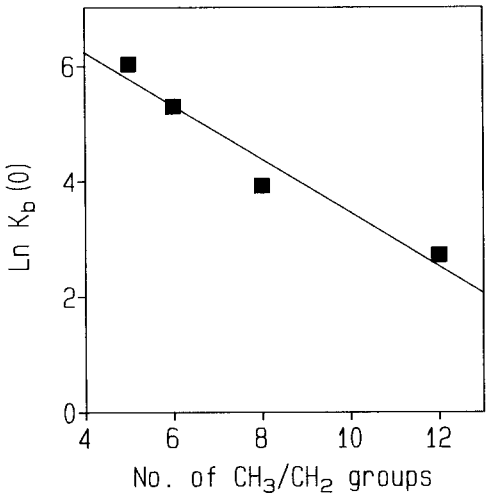


Fig. 9. A plot of natural logarithm $K_{b(0)}$ against the number of methyl and methylene groups in the organic cation for those blockers with an effective valence of approximately 0.9 (*see* Table).

chain is probably different in thermodynamic terms from the successive addition to a series of groups. However, this difference is likely to be small, and the plot is fitted reasonably by a straight line. For the addition of each methyl group the $K_{b(0)}$ decreases by a fraction 0.629, i.e., a 1.5- to 2-fold decrease. If TMA^+ were to interact at this site the predicted $K_{b(0)}$ would be 513 mM. The observed $K_{b(0)}$ for TMA^+ is almost half this value and is once again suggestive that the site of interaction of TMA^+ is different from that of the other blockers studied. It is not possible to be specific about the nature of the interaction of TMA^+ with its site.

One outstanding question is why TMA^+ does not interact at the $TEA^+/TPrA^+$ site when added to the cytosolic face of the receptor channel? The observed $K_{b(0)}$ is approximately half that predicted for interaction at the more voltage-dependent site, reflecting a higher affinity of TMA^+ for the central as opposed to the peripheral site. It may be that although TMA^+ interacts at two sites, the overall effect is dominated by its interaction at the site located 50% across the voltage drop. Alternatively, for some reason unrelated to size, TMA^+ may be unable to permeate further into the channel. TMA^+, unlike the other TAA^+ cations and trimethylammonium derivatives, has a very slight effect from the *trans* chamber. The data obtained after symmetrical addition of 200 mM TMA^+ are compatible with TMA^+ binding to the $TEA^+/TPrA^+$ site from the *trans* chamber with low affinity. It is unable to gain access to the central site from this side of the membrane.

Conduction Pathway Structure and Mechanisms of Ion Discrimination within the Sheep Cardiac SR Ryanodine Receptor Channel

The observation that the short-chain TAA cations act as voltage-dependent blockers of K^+ conductance in the sheep cardiac SR ryanodine receptor channel allows us to draw a number of preliminary conclusions regarding the architecture of part of the receptor-channel conduction pathway. The designation of the $TEA^+/TPrA^+$ interaction site at approximately 90% across the electrical distance from the cytosolic face of the channel suggests that access to this site must be via a pathway with a minimum aperture diameter of 0.7 nm to allow for entry of the fully dehydrated TPrA cation. However, it should be stressed that, although we have demonstrated that TEA^+ and $TPrA^+$ can traverse the greater part of the voltage drop across the channel, we have no information on the corresponding physical distances or locations of these sites relative to the dimensions of the purified ryanodine receptor protein obtained from ultrastructural studies [15, 33].

The cross-sectional dimensions of the conduction pathway revealed by TAA^+ block are consistent with our previous observation that another organic cation, $Tris^+$, which is of a similar size to TEA^+, is permeant in the receptor channel [17]. The different locations of the TMA^+ and $TEA^+/TPrA^+$ sites of interaction and the different permeability properties of TEA^+ and $Tris^+$ give a clear indication that factors other than the physical dimensions of a cation determine its movement within and across the conduction pathway of the receptor channel.

The Effect of the Larger TAA Cations

The effect of the larger TAA cations is clearly different from the form of block discussed above, in which the entry of a blocking cation prohibits the channel from carrying current. In studies on single channels such entry and exit may be visible as a rapid flickering between a fully open level and a blocked level which is indistinguishable from the normal closed level [21, 23]. Alternatively the rates of entry and exit may be so fast that a time-averaged reduction in current is apparent. This kind of behavior, so-called smooth block, is observed with TMA^+, TEA^+, $U2^+$ and $U3^+$. $TPrA^+$ is intermediate in behavior with a noisy fully open current level. Is it possible that TBA^+ and $TPeA^+$ act in a similar manner to $TPrA^+$? With TBA^+ and $TPeA^+$, the apparent relative conductance, as defined by the current amplitude between baseline and the level indicated by

the arrow in the lower right trace in Fig. 8*a*, is constant over a range of positive voltages. This contrasts with classical voltage-dependent block as exemplified by the small TAA cations. The difference is emphasized by the dashed line in Fig. 8*b* which illustrates the relative conductance of an archetypal voltage-dependent blocker with an effective valence of 0.9, equivalent to that of $TPrA^+$. The inability of TBA^+ and $TPeA^+$ to produce a voltage-dependent inhibition of K^+ current, of the form reported for the shorter-chain TAA cations, may result simply because these ions are too large to reach appropriate sites within the conduction pathway or may reflect an incompatibility of these cations with the TMA^+ and $TEA^+/TPrA^+$ binding sites. The effects of TBA^+ and $TPeA^+$ will be analyzed and discussed in greater detail in a subsequent paper [30].

In conclusion, we have demonstrated that the short-chain TAA cations and trimethyl ammonium derivatives act as asymmetric, voltage-dependent blockers of K^+ conductance in the purified sheep cardiac SR ryanodine receptor channel. These experiments support the view that the receptor channel functions as a single-ion channel and suggest that a major proportion of the voltage drop across the channel extends into a wide conduction pathway. Quantitative analysis supports the existence of two binding sites within the conduction pathway for these cations.

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