STRUCTURAL ASPECTS OF THE SARCOPLASMIC RETICULUM K^+ CHANNEL REVEALED BY GALLAMINE BLOCK

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ABSTRACT We have studied single-channel conductance fluctuations of K^+ channels present in the sarcoplasmic reticulum (SR) membrane systems of rabbit cardiac and skeletal muscle. K^+ conductance through the channels is reversibly blocked by gallamine. Conductance block occurs only from the *trans* side of the channel and is resolved as a smooth reduction in the open state conductance. At a fixed K^+ concentration, conduction decreases with increasing gallamine concentration and the data can be fitted to a single-site inhibition scheme. The degree of block seen at a constant gallamine concentration decreases as K^+ concentration is increased, indicating competition between gallamine and K^+. Gallamine block is voltage dependent, the degree of block increasing with increasing negative holding potential. Quantitative analysis of block yields a zero voltage dissociation constant of 55.3 ± 16 μM and an effective valence of block of 0.93 ± 0.12. We conclude that gallamine blocks by interacting with a site or sites located at an electrical distance 30–35% into the voltage drop from the *trans* side of the channel. This site must have a cross-sectional area of at least 1.2 nm^2. The results of this study have been used to modify and extend our view of the structure of the channel's conduction pathway.

INTRODUCTION

Large organic cations have been used as structural probes of a number of monovalent cation selective channels (Hille, 1971, 1973, 1975; Armstrong, 1975; Huang et al., 1978; Kirsch et al., 1980; French and Shoukimas, 1981; Swenson, 1981). More specifically, Miller and his colleagues have used a range of monovalent and divalent organic cations as probes of the monovalent cation selective channel of mammalian skeletal muscle sarcoplasmic reticulum membranes. By correlating single channel conductance with the cross-sectional area of a range of monovalent organic cations, Coronado and Miller (1982) were able to determine that the cross-sectional area of the narrowest region of the channel is ~0.2 nm^2. Further information was obtained using a series of impermeant cations that block K^+ conduction through the channel. Nineteen monovalent organic cations blocked K^+ conduction from the *trans* side of the channel. Block was voltage dependent and all blockers appeared to interact with a site located at ~65% of the voltage drop from the *trans* side of the channel. These experiments demonstrated that this 65% site was accessible to cations with cross-sectional areas of up to 0.5 nm^2 (Coronado and Miller, 1982).

The length of the voltage drop from the 65% site to the *trans* face of the channel was determined by studying the interaction of a series of bis-quaternary ammonium ion blockers (Miller, 1982*a*). By using these ions as potential sensors Miller was able to demonstrate that the voltage drop extends 0.6–0.7 nm from the 65% binding site towards the *trans* side of the membrane, and by implication, that the entire voltage drop occurred over ~1 nm rather than over the entire 5 nm membrane. Combining the various forms of evidence outlined above, Miller has described a model of the conductance pathway of the channel (Miller, 1982*b*). The important structural features of this model are that the conductance pathway contains a selectivity region with a cross-sectional area of ~0.2 nm^2, and that the conductance pathway opens from this site towards the *trans* face of the membrane into a tunnel region with a cross-sectional area of at least 0.5 nm^2. The voltage drop across the channel extends 0.6–0.7 nm from the selectivity filter and is assumed to occur within the 0.5 nm^2 tunnel region. It is envisaged that the tunnel region then opens into a wider mouth or vestibule. Little or no information is available concerning the dimensions of the conductance pathway extending from the selectivity filter to the *cis* face of the membrane.

Here we describe the interaction of a large, trivalent, organic cation, gallamine (1,2,3 Tris [2-triethyl-ammonium ethoxy] benzene^{3+}), with the functionally identical monovalent cation selective channels of mammalian cardiac and

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skeletal muscle SR membranes. Gallamine blocks K^+ conductance through the channels from the *trans* side of the membrane. Block is voltage dependent and high affinity. Analysis of gallamine block provides additional information which extends our understanding of the structure of the *trans*- facing opening of the channel protein.

MATERIALS AND METHODS

Isolation of Rabbit Cardiac and Skeletal SR Membranes

SR membranes were isolated from cardiac ventricular tissue of male New Zealand white rabbits using a method based on that of Jones and Cala (1981) as previously described (Tomlins et al., 1984). The membrane vesicles used in the experiments described here were those which actively accumulated calcium oxalate (fraction III).

Skeletal muscle SR membranes were isolated from back and hind-leg muscles of male New Zealand white rabbits using the method described by Garcia and Miller (1984).

Measurement of Single-channel Activity

Single monovalent cation selective channel activity of both cardiac and skeletal muscle SR membranes was monitored under voltage clamp conditions after the fusion of membrane vesicles into preformed planar phospholipid bilayers (Miller, 1978; Tomlins et al., 1984; Gray et al., 1985). Bilayers were formed from 100% phosphatidyl ethanolamine (PE) (Avanti Polar Lipids, Inc., Birmingham, AL) in decane. The bilayer separated two aqueous chambers, one of which, designated *trans*, was held at virtual ground, while the other, designated *cis*, could be clamped at a range of holding potentials relative to ground. Current flow through the bilayer was monitored using an operational amplifier as a current-voltage convertor (Miller, 1982*d*). The output of the amplifier was displayed on an oscilloscope and stored on FM tape for later analysis.

SR membrane vesicles were incorporated into bilayers in the presence of an osmotic gradient (Gray et al., 1985). After a fusion event unfused vesicles were perfused out of the chamber. Experiments were performed at room temperature (20–22°C). Unless otherwise stated the experiments reported here were carried out with symmetrical 150 mM K^+ solutions (as sulphate salt), and 5 mM Hepes pH 7.2 with Tris or KOH, in the *cis* and *trans* chambers.

When gallamine was used it was added symmetrically to the *cis* and *trans* chambers as the iodide salt.

The cross-sectional area of gallamine was determined from silhouette drawings of CPK models (Koltun, 1965) of the cation. Drawings were made from photographs of the model taken head on and showing effectively, the cross-sectional area of the three triethylammonium groups of the cation.

Gallamine triethiodide was purchased from Sigma Chemical Co., Poole, U.K.

RESULTS

Cardiac SR Channel Activity

The fusion of isolated vesicles of rabbit cardiac SR membranes with planar phospholipid bilayers leads to the incorporation of a single species of monovalent cation selective channel (Montgomery et al., 1983) (Fig. 1). The channel has two conducting states designated α and β (Tomlins et al., 1984). The conductance of both states is independent of voltage over the holding potential range -70 to $+70$ mV (Gray et al., 1985).

Channels become incorporated into the bilayer with a fixed orientation so that one side of the channel may be designated *cis* and the other *trans* (Tomlins et al., 1984).

Block of K^+ Conductance by Gallamine

The inclusion of μM concentrations of gallamine in the chamber solutions leads to an inhibition of K^+ conductance (Fig. 1 *b*). K^+ conductance block is resolved as a reduction in single-channel current flow at negative holding potentials. K^+ conductance block by gallamine is reversible. Fig. 1 *c* demonstrates the return of single-channel current flow to control levels after the removal of gallamine by perfusion. The observed channel behavior in the presence of gallamine is consistent with a scheme in which the trivalent gallamine cation is driven into the channel's conduction pathway from the *trans* side of the membrane at negative holding potentials. When the channel is occupied by gallamine, K^+ conductance is zero and the channel is blocked. The individual blocking reactions are too short to be resolved and consequently we observe a smooth, time-averaged, reduction in the conductance of the open state.

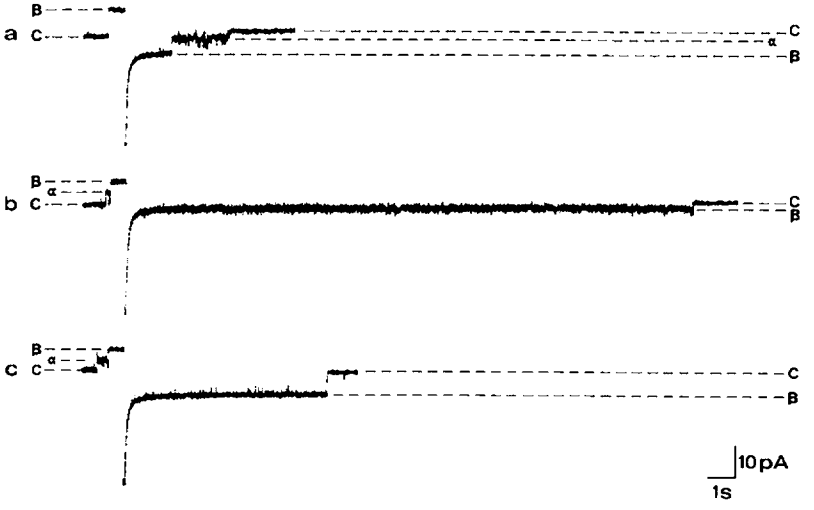

FIGURE 1 Single-channel current fluctuations of rabbit cardiac muscle SR K^+ channel incorporated into a 100% PE planar bilayer. The solution in both the *cis* and *trans* chambers was 75 mM K_2SO_4, 5 mM Hepes, pH 7.2 with Tris. In all cases the membrane is initially held at +40 mV and then switched to −40 mV. At positive holding potentials channel opening is seen as an upwards deflection and at negative holding potentials channel opening is seen as a downwards deflection. (*a*) Channel fluctuations in control conditions. Note the two conducting states of the channel designated α and β. (*b*) Channel fluctuations in the presence of symmetrical 30 μM gallamine. Single-channel current is markedly reduced at −40 mV but is unaffected at +40 mV. (*c*) Channel fluctuations after the removal of gallamine by perfusion. Channel current returns to control levels.

In fact, although individual blocking reactions cannot be resolved, the blocked conductance state is considerably noisier than the normal β open state.

Gallamine block is voltage dependent (Fig. 2). The degree of block observed at a fixed concentration of gallamine increases as negative holding potential increases. This behavior is reminiscent of that observed previously with a number of other organic cation blockers of the SR K^+ channel and may be explained in terms of a model in which gallamine can block only the open channel. In this case the time-averaged conductance in the presence of gallamine is given as:

$$g = g_o [1 + [G]/Kb(0) \exp (Fz\delta V/RT)]^{-1}, \quad (1)$$

where [G] is the concentration and z the valence of gallamine, $Kb(0)$ is the zero voltage dissociation constant of gallamine, g_o is the conductance in the absence of gallamine and δ is an electrical distance equivalent to the fraction of the total voltage drop across the channel, V, experienced at the site of gallamine interaction. The quantity $z\delta$ is the effective valence of the blocking reaction and is related to the voltage dependence of the block (Woodhull, 1973; Coronado and Miller, 1979; Miller, 1982*a*). The blocking parameters $Kb(0)$ and $z\delta$ obtained from the linearised plot of Eq. 1 shown in Fig. 2 *b* are 62 μM and 1.0 respectively. Therefore, gallamine is a high-affinity blocker of the channel. The value of the effective valence would suggest that gallamine interacts with a site, or class of sites, located at ~30% of the voltage drop from the *trans* side of the channel. This is, of course, based on the assumption that $z = 3$, that is that all three charged groups of the cation are interacting at a site approximately the same distance into the voltage drop. This point is dealt with in more detail in the discussion section of this report.

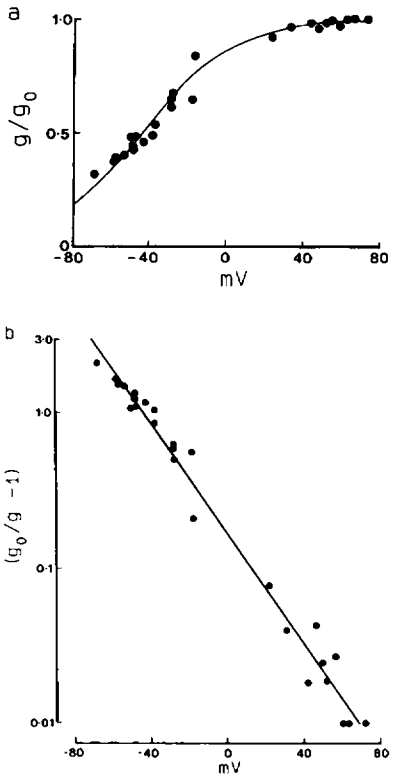

FIGURE 2. Voltage dependence of gallamine block. Single-channel β state conductance was measured using PE bilayers in symmetrical 75 mM K_2SO_4, 5mM Hepes pH 7.2 with Tris, containing 10 μM gallamine. g = β state conductance in the presence of the blocker, g_o = β state conductance measured in the absence of the blocker. Data from four separate experiments are combined in these plots. The solid line in part *a* of the figure is drawn according to Eq. 1 with values for Kb (0) = 62 μM and $z\delta$ = 1.0 obtained from the linearised plot of the data shown in *b*.

As has been demonstrated previously with several organic cations in the SR K^+ channel (Coronado and Miller, 1982; Miller, 1982*a*; Gray et al., 1985), gallamine block is consistent with a single-site blocking scheme. Fig. 3 demonstrates that, at a fixed K^+ concentration, time-averaged conductance decreases with increasing gallamine concentration. The data obtained for the cardiac SR channel can be fitted to a single-site inhibition curve with an apparent dissociation constant of 13.6 μM measured at −40 mV. The values of effective valence ($z\delta$) and zero voltage dissociation constant ($Kb(0)$) are independent of gallamine concentration at a fixed K^+ concentration (Fig. 4), as would be predicted for a single-site, single-ion channel. The mean values obtained at gallamine concentrations ranging between 5 and 50 μM are $z\delta = 0.93 \pm 0.12$ ($n = 22$) and $Kb(0) = 55.3 \pm 16.0$ μM ($n = 22$).

The degree of conductance block observed with a fixed concentration of gallamine decreases as K^+ concentration is increased. The zero voltage dissociation constant

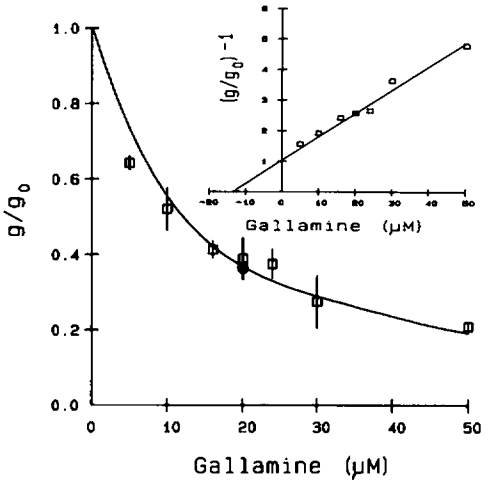

FIGURE 3 Influence of gallamine concentration on channel conductance. Single-channel conductance was monitored at a holding potential of −40 mV in the presence of symmetrical solutions of 75 mM K_2SO_4, 5 mM Hepes pH 7.2 with Tris, containing the appropriate concentration of gallamine. g = β state conductance in the presence of gallamine, g_o = β state conductance in the absence of gallamine. (*Open square*) Cardiac SR channel; (*solid circle*) skeletal SR channel. Each point represents the mean (±S.D.) of at least three experiments. The solid curve was drawn according to $g/g_o = [1 + [G]/Kb(V)]^{-1}$, with $Kb(V)$ = 13.6 μM, obtained from the linearised plot shown in the inset of the figure.

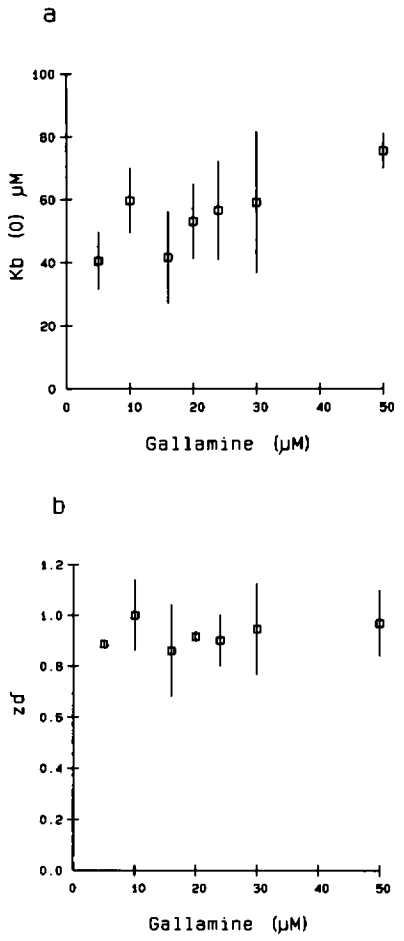

FIGURE 4 Influence of gallamine concentration on channel blocking parameters. The zero voltage dissociation constant ($Kb(0)$) and the effective valence of the blocker ($z\delta$) were determined as described in the legend to Fig. 2, at a range of gallamine concentrations. Each point represents the mean (±S.D.) of at least three experiments. No significant variation was observed for either parameter. Mean values for the entire range of gallamine concentrations are $Kb(0)$ 55.3 ± 16 μM and $z\delta$ 0.93 ± 0.12 (±S.D., n = 22).

($Kb(0)$) of block varies linearly with K^+ concentration as is shown in Fig. 5, demonstrating competition between gallamine and K^+.

All of the data detailed above were obtained in experiments carried out using neutral (100% PE) bilayers. In another series of experiments, data were obtained using bilayers with a net negative charge (30% phosphatidyl serine, 70% phosphatidyl ethanolamine). None of the parameters measured in these experiments were significantly different from those observed in neutral bilayers.

Skeletal SR Channel Activity

Our previous studies have shown many similarities between the monovalent cation selective channels found in rabbit cardiac and skeletal muscle SR membranes. In terms of conduction, gating characteristics and blocking parameters observed with bis-quaternary ammonium compounds, the two species of channel are virtually identical (Tomlins et al., 1984; Gray et al., 1985). In an attempt to determine whether K^+ conductance through the skeletal muscle SR channel is blocked by

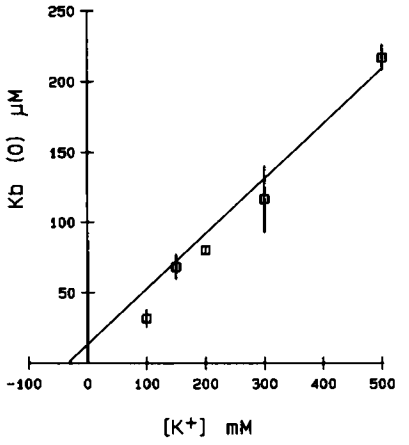

FIGURE 5 Variation in $Kb(0)$ with K^+ concentration. Values of $Kb(0)$ were obtained as described in the legend to Fig. 2 in symmetrical solutions of 50 to 250 mM K_2SO_4, 5 mM Hepes pH 7.2 with Tris, containing gallamine in the concentration range 10–30 μM. Each point represents the mean (±S.D.) of at least three experiments.

gallamine, we carried out a series of experiments using SR membrane vesicles isolated as described by Garcia and Miller (1984) and incorporated into neutral (100% PE) bilayers. In the presence of 20 μM gallamine, conductance through the skeletal SR channel was found to be blocked in an identical fashion to that seen for cardiac SR. Block occurred as a smooth reduction in conductance, was voltage dependent and occurred exclusively from the *trans* side. The blocking parameters obtained from three experiments were as follows; zero voltage dissociation constant ($Kb(0)$) = 59.1 μM, the effective valence of block ($z\delta$) = 1.0. The normalised conductance at −40 mV = 0.38 (see Fig. 3). Therefore, the characteristics of gallamine block of the skeletal SR channel are in good qualitative and quantitative agreement with those obtained with the cardiac SR channel.

DISCUSSION

The data presented in this report demonstrate that gallamine is an asymmetric, high-affinity, voltage-dependent blocker of K^+ conductance through the monovalent cation selective channels of both cardiac and skeletal SR membranes. From the fact that block is voltage dependent, one can infer that the site of interaction between gallamine and the channel protein must occur within the voltage drop across the channel and as such can yield information regarding the dimensions of this region of the channel protein.

The existing model of the *trans*-facing portion of the conduction pathway of the SR K^+ channel has been outlined in the introduction to this report and is summarized in Fig. 6 *a*.

What does gallamine block add to this model? Gallamine (Fig. 7 *a*) is a large, trivalent cation with a cross-sectional area of ~1.2 nm^2, as estimated from measurements of space-filling models (see Methods section). Our data indicate that, in the presence of 75 mM K_2SO4, gallamine interacts with a site at which ~30% of the voltage drop from the *trans* face of the channel is sensed. This value is based on the assumption that all three positively-charged groups of the cation are present in the

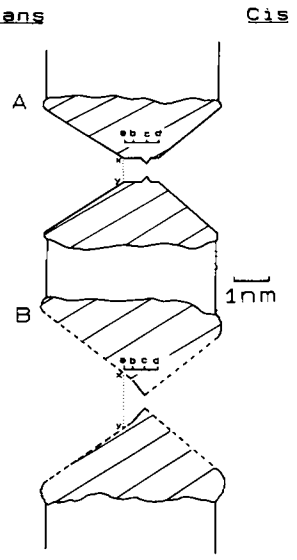

FIGURE 6 Cartoons of the SR K^+ channel. (*A*) Proposed structure of the conduction pathway based on conduction and block by a range of monovalent cations and block by a series of divalent organic cations (after Miller, 1982*b*). The line *a–d* represents the length of the voltage drop as sensed with bis-quaternary ammonium ions (Miller, 1982*a*). The voltage drop is assumed to occur in a 0.5 nm^2 tunnel region. *x–y* represents the diameter of the conduction pathway at the limit of the voltage drop. c is the 65% binding site proposed for the location of the selectivity filter. *b* represents the site of interaction of gallamine with the channel protein. (*B*) The structure of the conduction pathway proposed in the light of data presented in this report. The line *a–d* represents the range of the voltage drop across the channel in the presence of 75 mM K_2SO_4, as used by Miller in his bis-quaternary ammonium voltage sensing experiments (Miller, 1982*a*, (*A*) above) and in the majority of the experiments presented here. *c* represents the 65% binding site assumed to be the selectivity filter. The major development of this model is the expansion of the region of the channel protein in which gallamine interaction occurs, *b*. As a consequence, the voltage drop extends out into a region of large cross-sectional area, providing the channel with a large capture radius for K^+ ions. The *cis* opening of the channel is assumed to have similar dimensions to the *trans* opening (see Discussion).

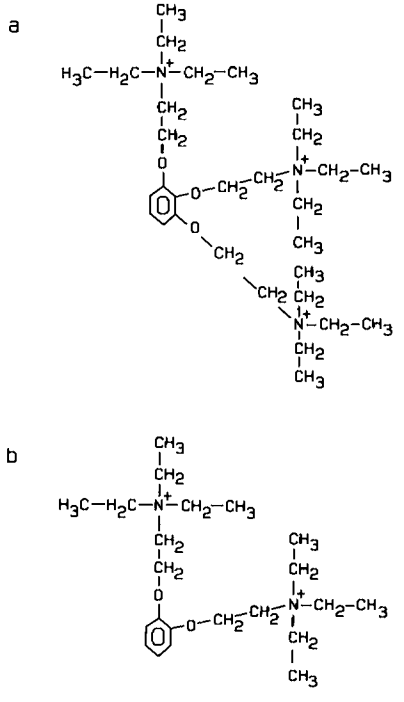

FIGURE 7 Structural formulae of *a* 1,2,3, Tris (2-triethylammonium ethoxy) benzene^{3+}, gallamine and *b* 1,2 bis (2-triethylammonium ethoxy) benzene^{2+}. Space filling models of these structures were used to determine cross-sectional areas as described in the Methods section.

voltage drop at approximately the same distance; this is a reasonable assumption when steric limitations of the ion are considered. Further support for this view has been provided by experiments in which we have studied the interaction of a related cation, 1,2 bis (2-Tris ethyl ammonium ethoxy) benzene^{2+} (Fig. 7 *b*) with the cardiac SR channel. This cation produces two clearly distinguishable and separate voltage dependent, asymmetric forms of K^+ conductance block. In addition to a smooth reduction in K^+ current, analogous to gallamine block, this cation produces well defined flickering block (data not shown). Quantitative analysis of smooth block yields a zero voltage dissociation constant ($Kb(0)$) of 268 ± 74 μM and an effective valence of block ($z\delta$) of 0.7 ± 0.1 (mean ± SD, $n = 6$ at concentrations of 50 and 100 μM for both parameters). With $z = 2$ for this cation δ will then be equal to 0.35, a value which is in good agreement with that obtained with gallamine. Therefore, the site of interaction of both cations would be 0.35–0.4 nm from the selectivity filter and must have a cross-sectional area of at least 1.2 nm^2. To accommodate these findings the tunnel region of the existing model must be considerably expanded (Fig. 6 *b*). As a consequence, the selectivity filter opens abruptly into a relatively large pathway and the voltage drop extends out into this region.

Gallamine does not block K^+ conductance from the *cis* side of the channel. This lack of effect may be explained if either gallamine is too large to enter the region of the protein over which the voltage drop occurs on the *cis* side of the channel, or the *cis* portion of the channel does not have a suitable site or sites for interaction. Little information is available concerning the dimensions of the voltage drop on the *cis* side of the channel. However, we do know that blockers such as decamethonium and succinyl choline interact with sites within the voltage drop on the *cis* side of the channel (Tomlins et al., 1984; Gray et al., 1985). It is not possible to assign a physical location to these sites, as no studies such as those carried out by Miller (1982*a*) for the *trans* opening of the channel have been undertaken for the *cis* portion of the voltage drop. Nevertheless, block by decamethonium and succinyl choline does establish that the voltage drop on the *cis* side of the channel extends into a region with a cross-sectional area of at least 0.5 nm^2.

Our picture of the SR K^+ channel is that of a *trans*-membrane protein with a reasonably symmetrical structure. The channel makes contact with the bulk solutions on either side of the membrane via large mouth regions which narrow to a constriction responsible for ionic selectivity. On both sides of the channel the voltage drop extends out into regions of large cross-sectional area. This is wholly consistent with the findings of Jordan (1982, 1984*a*, *b*, 1986), who describes an electrostatic treatment of a stylised pore through a membrane of low dielectric constant. The treatment demonstrates that for such a system, part of the voltage applied across the channel will extend out into the aqueous region at the entrance to the pore. This effect

becomes more pronounced as the ratio of the pore's length to its radius decreases. Translating this argument to our image of the SR K^+ channel we can consider the short, constricted region of the channel (Fig. 6 *b*) to be the pore of Jordan's model, with the applied potential extending out into the relatively large mouth regions.

A consequence of Jordan's model is that the voltage drop should not occur over a fixed distance but rather should vary with changing external factors. In particular, he suggests that alterations in the ionic strength of the bulk solutions should influence the range of voltage drop (Jordan, 1986). This would be reflected in a decrease in the effective valence ($z\delta$), determined with ions that interact with sites within the voltage drop, as the ionic strength of the bulk solution is raised. Our data are consistent with this prediction (Fig. 8). The effective valence of gallamine decreases with increasing ionic strength, reflecting a decrease in the range of the voltage drop.

The SR K^+ channel has been defined as a maxi K^+ channel (Latorre and Miller, 1983). This group of channels is characterized as having high single-channel conductance, while maintaining a high degree of selectivity. Superficially, the level of conductance achieved by these channels appears to be greater than the maximal levels allowed by free diffusion. In an attempt to resolve this paradoxical behavior Latorre and Miller (1983) described factors limiting ion flow through a single-ion channel such as the SR K^+ channel. The rate of ion flow will be influenced by both the rate of ion entry into the channel, which will be limited by diffusion of ions up to the channel opening, and the rate of exit of ions from the channel, which describes the maximal conductance of the channel.

Latorre and Miller (1983) suggest that the rate of ion exit from the channel can be optimized, while maintaining a high degree of selectivity, if the channel is short, or, in other words, if the region of the channel protein through which restricted diffusion occurs does not span the entire 5 nm membrane, but rather occurs between two large mouths filled with the bulk solution. Indeed, evidence from streaming potential determinations suggests that the constricted region of the SR K^+ channel, that is the region of the channel in which ions must flow in single file, cannot be >0.5 nm in length (Miller, 1982*b*, *c*).

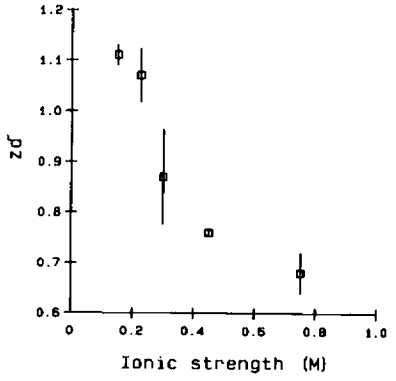

FIGURE 8 The effect of alterations in ionic strength on the effective valence of gallamine. Effective valence ($z\delta$) was determined as described in the legend to Fig. 2, in the presence of symmetrical solutions of 50 to 250 mM K_2SO_4, 5 mM Hepes pH 7.2 with Tris, containing gallamine in the range 10–30 μM. Each point represents the mean (±SD) of at least three experiments.

The rate of ion entry into the channel will be increased by providing the channel with a large capture radius, that is the area available for a freely diffusing ion to come into contact with the opening of the channel (Läuger, 1976). As Latorre and Miller (1983) emphasize, the existence of large mouth regions at either end of the channel does not, in itself, provide a large capture radius. These regions are continuous with the bulk solution and therefore ions within the mouths will be able to diffuse freely. Ions will enter the channel proper only when they come under the influence of the potential drop.

The data provided in this report strongly support and extend the image of the SR K^+ channel as set out by Latorre and Miller (1983). For a large ion, such as gallamine, to interact with the channel protein at a significant distance into the voltage drop across the channel, the constricted region, in which the selectivity filter is located, must be short.

Most significantly, gallamine block demonstrates that the voltage drop at the *trans* side of the channel extends into a region with a cross-sectional area of at least 1.2 nm^2 (Fig. 6 *b*), a value considerably greater than previously estimated (Miller, 1982*b*). This will provide the channel with a large cross-sectional area at the limits of the voltage drop and hence a large capture radius, so enhancing rates of K^+ entry into the channel.

A final point worthy of discussion is that the expansion of the region of the channel protein in which the voltage drop occurs helps to clarify a somewhat puzzling aspect of previously observed channel block by long-chain bis-quaternary ammonium ions (Miller, 1982*a*; Tomlins et al., 1984; Gray et al., 1985). The blocking parameters obtained with these cations indicate that both positively-charged residues of the ion interact with sites located at the same distance into the voltage drop across the channel. That is, the ion must adopt a horseshoe configuration with the two charged groups side by side. It is difficult to envisage how such a structure could be attained in a tunnel region with cross-sectional area of 0.5 nm^2, which is barely larger than the blocking ion.

However, in the light of the evidence provided here, it seems reasonable to speculate that the initial contact of the long-chain bis-quaternary ammonium cations is the binding of one positively charged group, followed by the flipping over of the second group, now unhindered by a 0.5 nm^2 tunnel.

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