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Evidence for Negative Charge in the Conduction Pathway of the Cardiac Ryanodine Receptor Channel Provided by the Interaction of K^+ Channel N-type Inactivation Peptides

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Abstract. We have investigated the interaction of two peptides (*Sh*B — net charge +3 and *Sh*B:E12KD13K — net charge +7) derived from the NH_2-terminal domain of the *Shaker* K^+ channel with purified, ryanodine-modified, cardiac Ca^{2+}-release channels (RyR). Both peptides produced well resolved blocking events from the cytosolic face of the channel. At a holding potential of +60 mV the relationship between the probability of block and peptide concentration was described by a single-site binding scheme with 50% saturation occurring at 5.92 ± 1.06 μM for *Sh*B and 0.59 ± 0.14 nM for *Sh*B:E12KD13K. The association rates of both peptides varied with concentration (4.0 ± 0.4 sec^{-1} μM^{-1} for *Sh*B and 2000 ± 200 sec^{-1} μM^{-1} for *Sh*B:E12KD13K); dissociation rates were independent of concentration. The interaction of both peptides was influenced by applied potential with the bulk of the voltage-dependence residing in K_{off}. The effectiveness of the inactivation peptides as blockers of RyR is enhanced by an increase in net positive charge. As is the case with inactivation and block of K^+ channels, this is mediated by a large increase in K_{on}. These observations are consistent with the proposal that the conduction pathway of RyR contains negatively charged sites which will contribute to the ion handling properties of this channel.

Key words: Ryanodine receptor — Sarcoplasmic reticulum — Calcium channel — Inactivation peptide

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Introduction

Ryanodine receptors are ligand-regulated, cation-selective, ion channels located in intracellular membrane systems. The physiological role of these channels is to provide a pathway for the release of stored Ca^{2+} to initiate cellular processes such as muscle contraction and fertilization. The mechanisms underlying ion handling in RyR are markedly different from those governing conduction and selectivity in other well characterized systems such as voltage-dependent Na^+ and K^+ channels. This is not surprising since the task performed by the RyR channel differs considerably from those of the voltage-activated plasmalemmal channels. For example in striated muscle, on excitation, RyR must provide a pathway for the rapid translocation of Ca^{2+} from an intracellular store to the cytosol under conditions where it is probable that Ca^{2+} is the only cation with an electrochemical driving force. Consistent with such a role, each RyR channel has an extraordinarily high single channel conductance for Ca^{2+} (Tinker & Williams, 1992; Tinker, Lindsay & Williams, 1993). Investigations of the ion-handling properties of single cardiac ryanodine receptor channels have established the basic characteristics of conduction and selectivity in this channel and have provided some indications as to how these rates of ion translocation are achieved (Lindsay, Manning & Williams, 1991; Tinker & Williams, 1992; Tinker et al., 1992*a;* Williams, 1992; Tinker & Williams, 1993*a*). The channel is relatively nonselective, being permeable to both divalent and monovalent group 1a cations. The alkaline earth divalents are essentially equally permeant as are the group 1a monovalents. However, RyR does discriminate between these classes of cation; broadly $pX^{++}/pY^{+} = 6$. Within each of these groups of cation there is considerable variation in single channel conductance. A simple,

single-ion, Eyring rate theory model qualitatively and quantitatively reproduces these characteristics (Tinker, Lindsay & Williams, 1992*a*).

In the model, selection between divalent and monovalent cations arises from differential binding at a high affinity site and a process that allows preferential translocation of divalent cations between binding sites in the conduction pathway. In theory, a physical basis for this latter mechanism could be provided by a high density of negatively charged sites within the conduction pathway (Tinker et al., 1992*a*).

In this communication we describe studies in which we have used K^+ channel N-type inactivation peptides as probes to investigate the possible occurrence of negatively charged sites within the conduction pathway of RyR. The mechanisms underlying the inactivation or block by these peptides of *Shaker* and Ca^{2+}-activated K^+ channels have been investigated by monitoring the modes of action of peptides in which net charge or hydrophobicity had been altered by amino acid substitution (Murrell-Lagnado & Aldrich, 1993; Toro et al., 1994). Increasing the net positive charge on the peptide increases its affinity for the channels by an enhancement of the rate of association of the peptide. The rate of dissociation of the peptide is effectively independent of peptide net charge. Increasing the overall hydrophobicity of the peptide stabilizes its interaction with the channel, primarily by decreasing its dissociation rate.

A generally accepted model of the peptide 'receptor' in K^+ channels has emerged. A channel needs certain structural features to act as a receptor for an inactivation peptide, including a vestibule that contains negatively charged sites to attract and orientate the peptide and hydrophobic sites that stabilize the bound peptide. These requirements might be considered as nonspecific, i.e., there is no absolute requirement for particular residues in a classical, specific, binding site as long as the channel provides an appropriate environment of charge and hydrophobicity. The resulting, relatively low affinity, interaction is ideally suited for the functional role of channel inactivation (Murrell-Lagnado & Aldrich, 1993).

If, as we have postulated, the conduction pathway of RyR contains a high density of negative charge it would be likely that (i) the inactivation peptides will interact with the RyR channel and (ii) that the rate of association of the peptides with the channel will be influenced by the net positive charge of the peptide. We have tested this hypothesis by monitoring the interaction of two peptides (*Sh*B — net charge +3 and the *Sh*B:E12KD13K — net charge +7) with the conduction pathway of the ryanodine-modified RyR channel.

Materials and Methods

Preparation of SR Membrane Vesicles

SR membrane vesicles were prepared as described previously (Sitsapesan & Williams, 1990). Sheep hearts were collected from a local farm and transported to the laboratory in ice-cold cardioplegic solution (Tomlins et al., 1986). All subsequent procedures were carried out at 4°C. Left ventricle and septum were homogenized in a solution containing 300 mM sucrose and 20 mM potassium piperazine-N′N′-bis-ethane-sulphonic acid (PIPES), pH 7.4 supplemented with 1 mM phenylmethanesulphonyl fluoride (PMSF). The homogenate was centrifuged at 7000 × *g* for 20 min. The supernatant was centrifuged at 100,000 × *g* for 45 min and the resulting pellet was resuspended and homogenized vigorously in (mM): 400 KCl, 0.5 $MgCl_2$, 0.5 $CaCl_2$, 0.5 1,2-di(2-aminoethoxy)ethane-N,N,N′,N′-tetraacetic acid (EGTA), 25 mM PIPES, and 10% sucrose w/v, pH 7.4. The same salt solution was used to make 20, 30 and 40% sucrose solutions for a discontinuous sucrose density gradient. The mixed membrane suspension was layered onto the top of the gradient prior to centrifugation for 120 min at 100,000 × *g*. Heavy SR (HSR) membrane vesicles were collected from the 30–40% interface, resuspended in an excess of 400 mM KCl and centrifuged for 45 min at 100,000 × *g*. The resulting pellet was resuspended in 400 mM sucrose and 5 mM N′-2-hydroxyethylpiperazine-N′-2-sulphonic acid (HEPES), pH 7.4 (Tris) and aliquots snap frozen in liquid nitrogen prior to storage at −80°C.

Solubilization of the Ryanodine Receptor

The ryanodine receptor was purified as previously described (Lindsay & Williams, 1991), following solubilization with 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS). HSR membrane vesicles were suspended in a solution containing 1 M NaCl, 0.15 mM $CaCl_2$, 0.1 mM EGTA and 25 mM Na PIPES, pH 7.4 (NaOH) with 0.4% (w/v) CHAPS and 2 mg/ml L-∝-phosphatidylcholine (PC), at a protein concentration of 2 mg/ml. This was incubated for 1 hr on ice prior to sedimentation of the unsolubilized material at 100,000 × *g* for 45 min. Two samples of HSR membrane vesicles were prepared. During solubilization, one sample was incubated with 5 nM [^{3}H] ryanodine to enable detection of the solubilized ryanodine receptor during subsequent procedures.

The ryanodine receptor was isolated from the other solubilized material by centrifugation on a 5–15% linear sucrose gradient containing the same concentrations of CHAPS and PC used during the solubilization, with a 40% sucrose cushion. This was carried out overnight (16 hr) at 100,000 × *g*. 2 ml fractions were collected and the fraction containing the ryanodine receptor located by [^{3}H] ryanodine binding. The purified ryanodine receptor was then reconstituted into liposomes by a rapid dialysis technique against a dialysis buffer containing 0.1 M NaCl, 0.15 mM $CaCl_2$, 0.1 mM EGTA and 25 mM Na PIPES, pH 7.4 (NaOH), and 2 mM DTT.

Planar Lipid Bilayer Methods

Planar phospholipid bilayers containing phosphatidylethanolamine in *n*-decane (35 mg/ml) were painted across a 200 μm diameter hole in a polystyrene partition separating two fluid filled chambers referred to as the *cis* (0.5 ml) and the *trans* (1.0 ml) chambers. The *trans* chamber was held at ground, and the *cis* chamber was held at various holding potentials relative to ground. Current flow was measured using an operational amplifier as a current-voltage converter (Miller, 1982). Bilayers were formed in a solution of 210 mM KCl and 20 mM HEPES, pH 7.4 (KOH). Proteoliposomes were added to the *cis* chamber, and the K^+ concentration was increased to encourage vesicle fusion with the bilayer. After incorporation, the *cis* chamber was perfused with 210 mM K^+ to prevent further vesicle fusion. The channel incorporates into the bilayer in a fixed orientation so that the *cis* chamber corresponds to the cytosolic face of the channel, and the *trans* chamber, the luminal

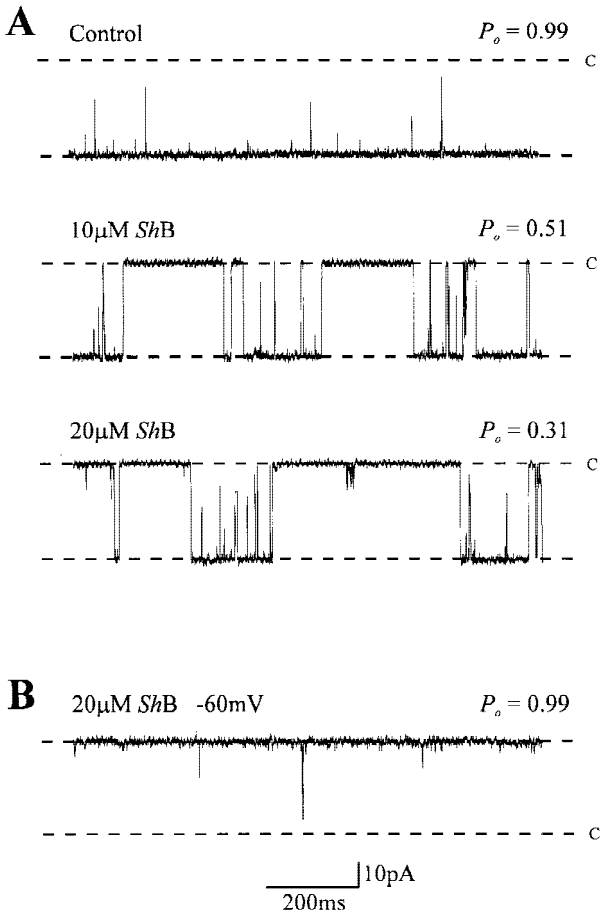

Fig. 1. Effect of the *Shaker* B inactivation peptide on the unmodified sheep cardiac SR Ca^{2+}-release channel. Single-channel current fluctuations of a channel activated with 1 mM cytosolic EMD 41000 and 1 mM luminal calcium at a holding potential of +60 mV (Fig. 1*a*) and −60 mV (Fig. 1*b*). Under these conditions, the channel resides predominantly in the normal open state. In Fig. 1*a*, channel openings correspond to downward deflections (C–closed). The top panel was recorded in the absence of peptide, and the subsequent panels show the block induced by addition of *Sh*B peptide to the cytosolic face of the channel. In Fig. 1*b*, 20 μM peptide (cytosolic) did not block the channel when the holding potential was adjusted to −60 mV (channel openings are upward deflections, C–closed). Open probability (P_o) was calculated from 1 min recordings of this channel.

face. The free Ca^{2+} concentration of the solutions used was 10 μM. The experiments were performed at room temperature.

The basic observation reported in this communication is that inactivation peptides derived from *Shaker* B interact with RyR and reduce open probability (P_o). To provide a quantitative description of this interaction it is important to minimize the likelihood of the occurrence of closing events not induced by the peptides. Therefore, the channels used in this study should have a P_o of 1.

Initially, we attempted to investigate the action of the inactivation peptides on RyR channels in the absence of ryanodine. Combinations of luminal calcium plus an activator of the cytosolic caffeine site are likely to produce maximal P_o (Sitsapesan & Williams, 1994). Using these ligands we occasionally saw channels that maintained a P_o of approximately 1, as shown in Fig. 1. Under these conditions, the addition of micromolar concentrations of *Sh*B to the solution at the cytosolic face of the channel produced well resolved blocking events. Unfortunately, the probability of producing channels with these very high P_os was too low to allow us to acquire sufficient data for quantitative analysis.

As an alternative approach, all subsequent experiments were conducted under conditions in which the P_o of channels was held at approximately 1 following the interaction of ryanodine (Lindsay et al., 1994). Using this approach we were able to minimize the likelihood of normal closing events and hence quantify blocking events arising from the interaction of the inactivation peptides with the channel. Irreversible ryanodine modification of the channel was accomplished by the addition of 100–200 nM ryanodine to the *cis* chamber. Following modification, unbound ryanodine was removed from the *cis* chamber by perfusion. Channels remained in the ryanodine-modified state for the duration of the experiment. The functional consequences of channel modification, in relation to the interaction of the inactivation peptides, are considered in the discussion section of this communication.

Data Acquisition and Analysis

Single channel current fluctuations were displayed on an oscilloscope and recorded on Digital Audio Tape (DAT). For analysis, data were replayed, low-pass filtered with an 8-pole Bessel filter at 1kHz and digitized at 4kHz using an AT based computer system (Satori, Intracel, Cambridge, UK). P_o and dwell times were determined from 1 min steady-state recordings by 50% threshold analysis, with cursors set manually on the ryanodine-modified open and fully blocked levels (Tinker & Williams, 1993*b*).

Materials

Ryanodine was obtained from Agrisystems International (Wind Gap, PA) and phosphatidylethanolamine was obtained from Avanti Polar Lipids (Alabaster, AL).

The wild type *Sh*B inactivation peptide (MAAVAGLYGLGEDRQHRKKQ, net charge +3) and the *Sh*B:E12KD13K peptide (MAAVAGLYGLGKKRQHRKKQ, net charge +7) were synthesized at commercial facilities, either the Brandeis University Peptide Facility (Waltham, MA) or Bio-Synthesis (Lewisville, TX). They are amidated at the C-terminus. The peptides were purified using reverse phase HPLC with a C18 Vyadac column. The purified peptides were resuspended in 20 mM Hepes, 100 mM NaCl, pH 7.4 for use.

Results

General Observations

To maximize the possibility of resolving the interaction of the inactivation peptides, ryanodine was used to increase the open probability (P_o) of the cardiac SR Ca^{2+}-release channels (Rousseau, Smith & Meissner, 1987; Lindsay, Tinker & Williams, 1994). When ryanodine interacts with the channel, conductance is reduced to a level approximately 60% of that seen in the absence of ryanodine and P_o increases to approximately 1.

Addition of μM quantities of *Sh*B peptide to the solution bathing the cytosolic face of the ryanodine-modified channel resulted in the occurrence of well defined blocking events (Fig. 2). The majority of events

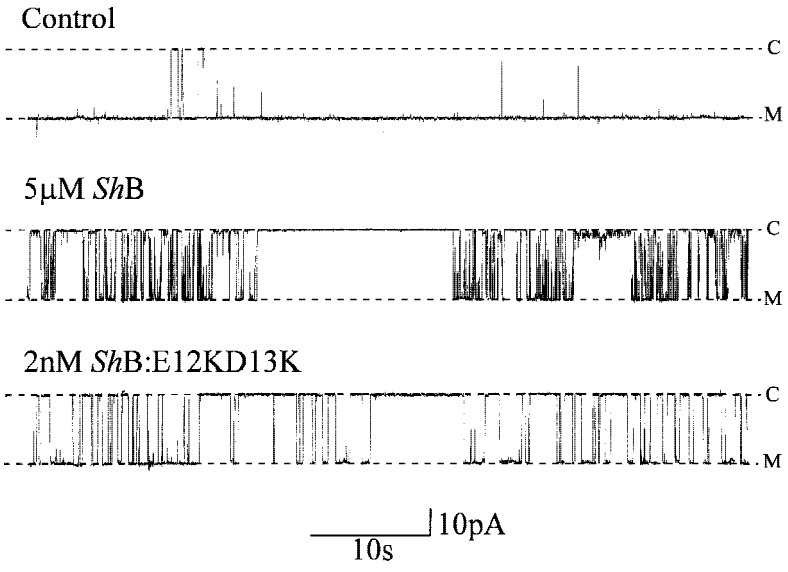

Fig. 2. Block of the ryanodine-modified sheep cardiac SR Ca^{2+}-release channel by *Shaker* inactivation peptides. Representative single-channel current fluctuations of ryanodine-modified channels at a holding potential of +60 mV. In all cases channel openings correspond to downward deflections (C–closed, M–Modified conductance level). The top panel was recorded in the absence of peptide. Under these conditions the channel resides predominantly in the modified conductance state with only occasional closing events. Addition of inactivation peptides (*Sh*B–middle panel or *Sh*B:E12KD13K–lower panel) to the cytosolic face of the channel resulted in the occurrence of well resolved blocking events (*see* text for details).

occurred to a conductance level that was indistinguishable from the closed level, however a small proportion of blocking events were to levels at 10–20% of the normal modified conductance. *Sh*B induced two distinct forms of block. The majority of blocking events seen were of short duration (<500 msec) but a few events were of much longer duration. The occurrence of these long blocking events was not dependent on peptide concentration or voltage (*data not shown*), so for the purposes of analysis, no discrimination was made between blocking events of long or short duration.

Qualitatively similar results were found when a modified peptide *Sh*B:E12KD13K was added to the solution bathing the cytosolic face of the ryanodine-modified channel (Fig. 2). In this case, nM concentrations of the peptide produced well defined blocking events. Unlike the situation observed with the *Sh*B peptide, the blocking events produced by *Sh*B:E12KD13K did not fall clearly into two populations of short and long duration. As with *Sh*B, the majority of events occurred to a conductance level indistinguishable from the normal closed state of the channel, with a small proportion occurring as subconductance events of 10–20% of the normal current amplitude.

With both *Sh*B and *Sh*B:E12KD13K, block was only observed when the peptide was present in the solution bathing the cytosolic face of the channel. No blocking events were seen when the peptides were added only to the luminal face of the channel. In addition, block by both peptides was reversible on washout.

By analogy with previous studies in which peptides derived from the NH_2-terminal sequence of the *Shaker* potassium channel have been shown to block voltage-dependent and Ca^{2+}-activated potassium channels, we have assumed that the block of the RyR-channel observed here results from the interaction of one peptide molecule with a site located in the cytosolic facing vestibule of the channel. Under these conditions we would expect the blocking reaction to be described by the following simple bimolecular scheme (A):

$$OPEN_{\text{mod}} + PEPTIDE \rightleftharpoons BLOCKED$$

We have characterized the interaction of the *Sh*B and *Sh*B:E12KD13K peptides by monitoring the consequence of varying the concentration of the peptide and the influence of voltage on the interaction of the peptides with the channel.

The Concentration-dependence of Block by *Sh*B and *Sh*B:E12KD13K

We investigated the influence of increasing concentrations of *Sh*B and *Sh*B:E12KD13K peptides with 210 K^+ as the permeant species at a holding potential of +60 mV. Under these conditions, in the absence of the peptides, the ryanodine-modified Ca^{2+}-release channel is predominantly open (Fig. 3*a* and *b*). *Sh*B and *Sh*B:E12KD13K added to the solution bathing the cytosolic face of the channel induce blocking events and the likelihood of occurrence of block increases as the concentration of the peptides is raised (Fig. 3*a* and *b*). In both cases the increase in block can be described by a simple saturation curve of the type

$$1 - P_o = B_{\text{limit}} \cdot \frac{[Pep]}{K_D + [Pep]} \quad (1)$$

where the probability of block is expressed as $1\text{-}P_o$, K_D is the concentration at which half maximal block occurs, B_{limit} is the maximal degree of block and [*Pep*] is the concentration of the peptide. The relationship between *Sh*B and *Sh*B:E12KD13K concentration and $1\text{-}P_o$ for

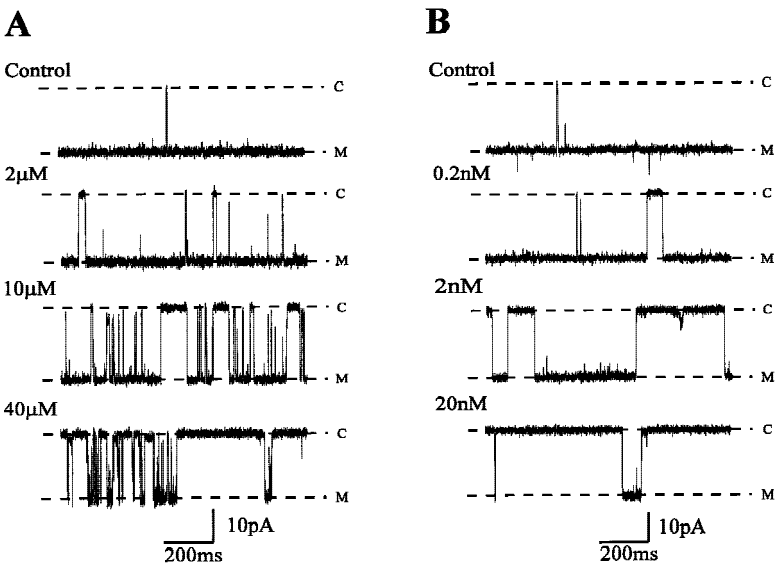

Fig. 3. The influence of inactivation peptide concentration on the probability of block. Representative single-channel current fluctuations of ryanodine-modified channels at a holding potential of +60 mV. Increasing the concentration of the inactivation peptide in the solution at the cytosolic face of the channel (*Sh*B 0 to 40 μM, Fig. 3*a*: *Sh*B:E12KD13K 0 to 20 nM, Fig. 3*b*) results in an increase in the probability of block. The majority of events are to conductance levels indistinguishable from the normal closed level of the channel, however some occur as incomplete blocking events to reduced conductance levels.

several experiments is shown in Fig. 4*a* and *b*. The solid lines were drawn using the best-fit parameters for K_D and B_{limit} obtained using nonlinear regression (Table 1). *Sh*B is an effective blocker of K^+ conductance in the sheep cardiac ryanodine receptor channel at μM concentrations; *Sh*B:E12KD13K produces a similar degree of block at nM concentrations.

In scheme (A) the rate of association of the blocking peptide with its receptor on the channel should vary with concentration while the rate of dissociation should be independent of concentration. We have determined the association rates (K_{on}) for *Sh*B and *Sh*B:E12KD13K as the reciprocal of the mean time in the unblocked states and the dissociation rates (K_{off}) of the peptides as the reciprocal of the mean time in the blocked states. The relationships between these parameters and *Sh*B concentration are shown in Fig. 5*a*. K_{on} is linearly dependent upon *Sh*B concentration, with a slope of 4.0 ± 0.4 sec^{-1} μM^{-1} while K_{off} is essentially independent of *Sh*B concentration with a mean value of 23 ± 1.0 sec^{-1}. The relationships between these parameters and *Sh*B:E12KD13K concentration are shown in Fig. 5*b*. Again, K_{on} is linearly dependent upon peptide concentration, with a slope of 2000 ± 200 sec^{-1} μM^{-1} and K_{off} is essentially independent of *Sh*B:E12KD13K concentration with a mean value of 2.4 ± 0.4 sec^{-1}. Therefore the difference in efficiency of the two peptides as blockers of the channel resides predominantly in the difference in association rates (*Sh*B:E12KD13K ~500 times faster) although the difference in dissociation rates also makes a contribution (*Sh*B:E12KD13K ~10 times slower). The apparent dissociation constants (K_D) derived from these rate constants for the two peptides (*Sh*B, 5.75 μM; *Sh*B:E12KD13K, 1.2 nM) are in good agreement with the values monitored directly from the concentration dependence of block (Fig. 4*a* and *b*).

The Voltage-dependence of Block by *Sh*B and *Sh*B:E12KD13K

In the absence of blocking peptides the P_o of the ryanodine-modified sheep cardiac SR Ca^{2+}-release channel is approximately 1.0 over the range of holding potentials used in this investigation (+20 to +80 mV). At a fixed concentration, the degree of block observed with both *Sh*B and *Sh*B:E12KD13K varies with the applied potential. In both cases the probability of block increases as the applied potential is made more positive. Figure 6*a* shows current fluctuations of a single ryanodine-modified channel in the presence of 20 μM *Sh*B. Figure 6*b* shows data obtained with another ryanodine-modified channel in the presence of 2 nM *Sh*B:E12KD13K. For both peptides, the relationship between the probability of the channel being in the blocked state and applied potential can be described by the Woodhull model in which it is assumed that there is a single site of interaction of the peptide lying a fraction δ across the voltage drop and that this site is only accessible to the blocking peptide from one side of the channel (Woodhull, 1973). The relationship between block, expressed here as relative P_o, and applied potential is then given by

$$P_{o_{Rel}} = \left[1 + \frac{[Pep]}{K_b(0)} \exp\left(z\delta \frac{FV}{RT} \right) \right]^{-1} \qquad (2)$$

where [*Pep*] is the peptide concentration, *V* is the applied potential, $K_b(0)$ is the dissociation constant at 0 mV, zδ is the effective valence and *F*, *R* and *T* have their usual meanings. The change in relative P_o was monitored in 10-mV steps from +20 to +80 mV with either 20 μM *Sh*B or 2 nM *Sh*B:E12KD13K in the solution at the cytosolic face of the channel. Equation 2 was fitted by nonlinear regression to the mean relative P_o values for the 2 pep-

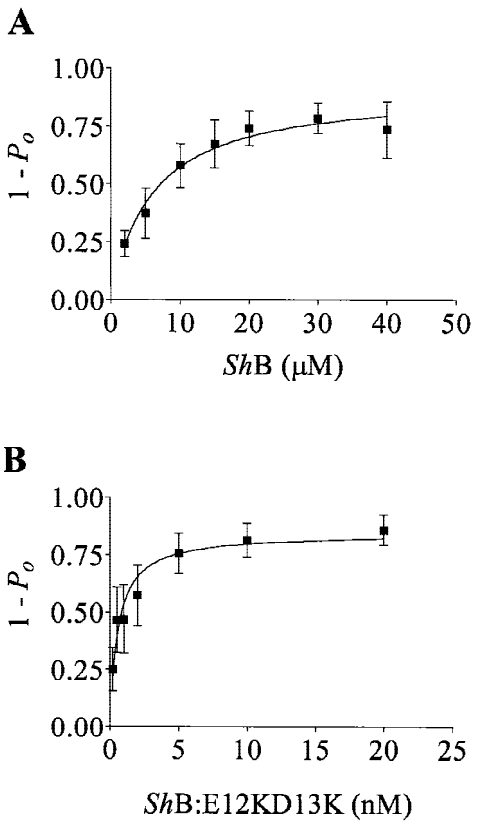

Fig. 4. The relationship between inactivation peptide concentration and the probability of block. The probability of block (1-P_o) of ryanodine-modified channels was determined as described in Materials and Methods at a holding potential of +60 mV in the presence of increasing concentrations of either *Sh*B (Fig. 4*a*) or *Sh*B:E12KD13K (Fig. 4*b*). Each point represents the mean ± SEM for at least 4 experiments. The solid lines are best-fit rectangular hyperbolas drawn with the parameters given in Table 1 obtained by nonlinear regression.

tides (Fig. 7*a* and *b*). The parameters derived from these fits are shown in Table 2. Therefore the zero voltage dissociation constant of *Sh*B:E12KD13K is approximately 1,000 times lower than that of *Sh*B. The effective valence, i.e., the product of the valence of the blocking peptide and the fraction across the voltage drop at which the binding site is located, is approximately equivalent for the two peptides.

If the association and dissociation rates of the peptides are described by the Boltzmann relationship the rate constants at a given voltage will be described by

$$K_{on}(V) \quad K_{on}(0) \cdot \exp^{z_{on}} \cdot (FV/RT) \tag{3}$$

$$K_{off}(V) \quad K_{off}(0) \cdot \exp^{-z_{off}} \cdot (FV/RT) \tag{4}$$

where $K(0)$ is the rate constant at 0 mV and z is the valence of the reaction. z may then be determined as the slope of a plot of ln of the rate constant against applied potential. Plots of this form are shown for the association and dissociation constants of *Sh*B in Fig. 8*a* and *Sh*B:E12KD13K in Fig. 8*b*. In both cases it is clear that

Table 1. Parameters for concentration-dependent block

	K_D ± SEM	B_{limit} ± SEM
*Sh*B	5.92 ± 1.06 μM	0.91 ± 0.05
*Sh*B:E12KD13K	0.59 ± 0.14 nM	0.84 ± 0.04

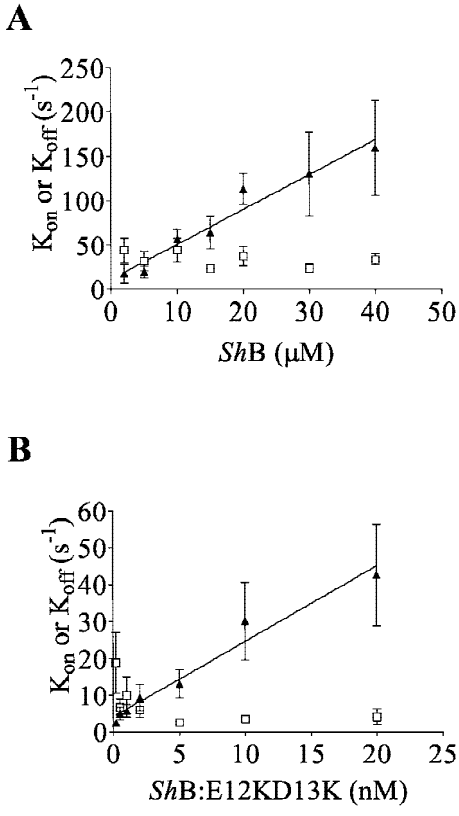

Fig. 5. The relationship between inactivation peptide concentration and rates of association (K_{on}–▲) and dissociation (K_{off}–□). *Sh*B (Fig. 5*a*) and *Sh*B:E12KD13K (Fig. 5*b*). Rates were determined for the experiments shown in Fig. 4 at a holding potential of +60 mV. In both cases the solid line drawn through the K_{on} data was obtained by linear regression.

the bulk of the voltage dependence of the interaction of the peptide is derived from the dissociation of the peptide from the channel. K_{off} decreases as applied potential is made more positive. The valences of the dissociation reactions are 1.27 for *Sh*B and 1.76 for *Sh*B:E12KD13K, these values are comparable with those derived from the Woodhull model (*above*).

Discussion

The aim of this investigation was to use synthetic peptides derived from the N-type inactivation domain of the *Shaker* K^+ channel to probe putative negatively charged sites in the conduction pathway of RyR. One peptide (*Sh*B) is equivalent to the 20 amino acid NH_2-terminal

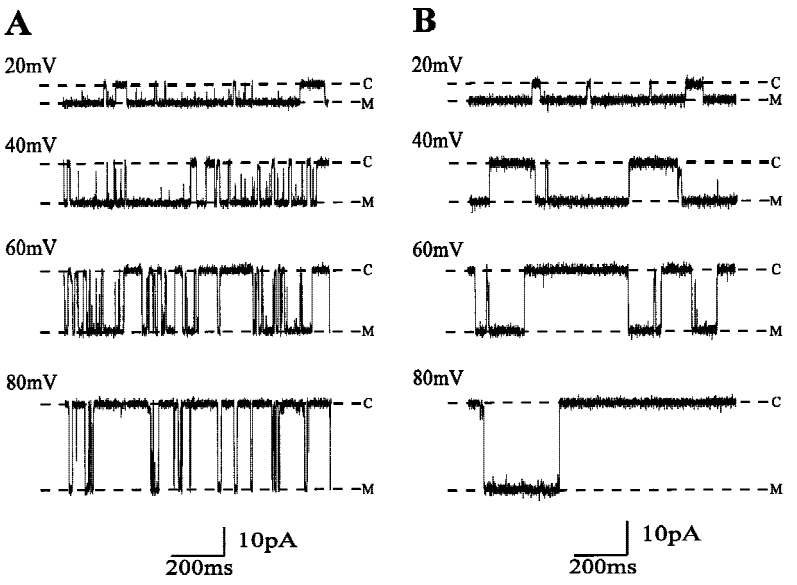

Fig. 6. The influence of holding potential on the probability of block. Representative single-channel current fluctuations of ryanodine-modified channels in the presence of 20 μM *Sh*B (Fig. 6*a*) or 2 nM *Sh*B:E12KD13K (Fig. 6*b*) at various holding potentials (Peptides were added to the solution at the cytosolic face of the channel. C–closed, M–Modified conductance level). In both cases, the probability of observing block increases as the holding potential is made more positive. As in Fig. 3, the majority of blocking events occur to conductance levels indistinguishable from the normal closed level, however some events do occur to intermediate levels of conductance.

sequence of the *Shaker* K^+. The other (*Sh*B:E12KD13K) has the indicated amino acid substitutions in the COOH-terminal half of the peptide which result in an increase in the net charge of the peptide of +4 in comparison with *Sh*B.

The first novel finding of this investigation is that these peptides do interact with RyR. When applied to the cytosolic face of the channel, both peptides induce well resolved blocking events in the ryanodine-modified sheep cardiac SR Ca^{2+}-release channel. Block is freely reversible on washout of the peptides and no blocking activity was seen when the peptides were added to the luminal face of the channel.

What are the Mechanisms Underlying Block and are They Consistent with the Presence of Negative Charge in the Conduction Pathway?

We have analyzed the interaction of the inactivation peptides with the cardiac SR Ca^{2+}-release channel in terms of a simple, bimolecular scheme (Scheme A). In keeping with this scheme, the relationship between the concentration of both peptides and the probability of block can be described by a simple single-site binding scheme. In addition the rates of association of both peptides are dependent upon peptide concentration, while the rates of dissociation are essentially independent of the concentration of the peptide. The probability of either peptide inducing block is dependent upon the applied potential. In both cases the probability of block increases with increasing positive potential and the bulk of this voltage-dependence resides in the dissociation reaction. Therefore, at a qualitative level, the mechanisms underlying the interaction of *Sh*B and *Sh*B:E12KD13K with the channel to cause block appear to be identical. In addition, these mechanisms would also describe the interaction of inactivation peptides with the voltage-dependent and Ca^{2+}-activated K^+ channels.

Inactivation or block of these K^+ channels involves the interaction of a single peptide molecule with the cytosolic vestibule of the channel. To be effective, the inactivation peptide must contain both positively charged and hydrophobic residues. Alterations to the net charge of the peptide modify the rate of association of the peptide with the channel, increasing the net positive charge increases K_{on}, decreasing net positive charge reduces K_{on} (Murrell-Lagnado & Aldrich, 1993; Toro et al., 1994; Kukuljan, Labarca & Latorre, 1995). Changing the net charge of the peptide produces only small changes in the dissociation rate of the peptide from the channel (Murrell-Lagnado & Aldrich, 1993). The identity of the charged amino acids is not important, substitutions which alter the primary structure of the peptide but maintain the overall net charge have little effect on either rates of association or dissociation (Murrell-Lagnado & Aldrich, 1993). It is envisaged that the inactivation peptide acts as a positively charged ball and that long range electrostatic interactions with negatively charged residues in the conduction pathway of the channel tend to concentrate and possibly orientate the peptide in the vicinity of its binding site on the channel (Murrell-Lagnado & Aldrich, 1993; Toro et al., 1994). The interaction of the peptides with the channel is voltage-dependent, however, as is the case for the experiments reported here, the net charge on the peptide has little effect on the voltage-dependence of its interaction with the Ca^{2+}-activated K^+ channel (Toro et al., 1994). Once the peptide makes contact with its receptor, hydrophobic residues play an important role in stabilizing the interaction. Substituting polar for hydrophobic residues increases the dissociation rate with only small effects on

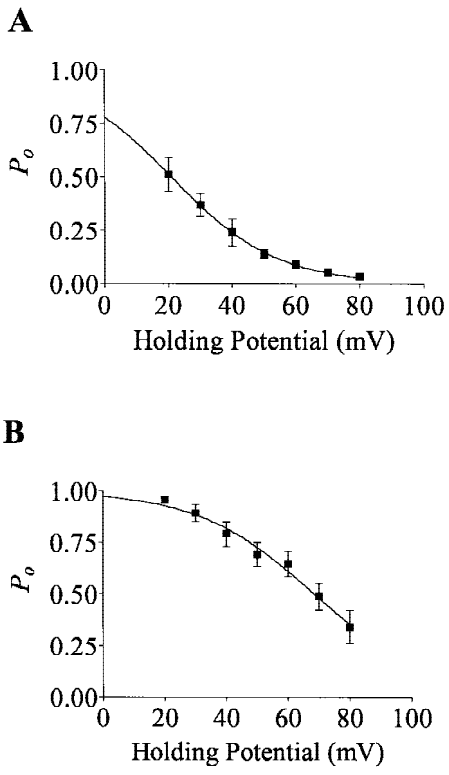

Fig. 7. The relationship between holding potential and the open probability (P_o) of ryanodine-modified channels in the presence of 20 μM *Sh*B (Fig. 7*a*) or 2 nM *Sh*B:E12KD13K (Fig. 7*b*). P_o decreases, i.e., the probability of block increases, as the holding potential is made more positive. Each point represents the mean ± SEM for at least 4 experiments. The solid lines are best-fits to the Woodhull equation (Equation 2) obtained by non-linear regression with the parameters quoted in Table 2.

Table 2. Parameters for voltage-dependent block

	$K_b(0)$ ± SEM	$z\delta$ ± SEM
*Sh*B	70.76 ± 2.84 μM	1.52 ± 0.02
*Sh*B:E12KD13K	74.35 ± 18.61 nM	1.32 ± 0.09

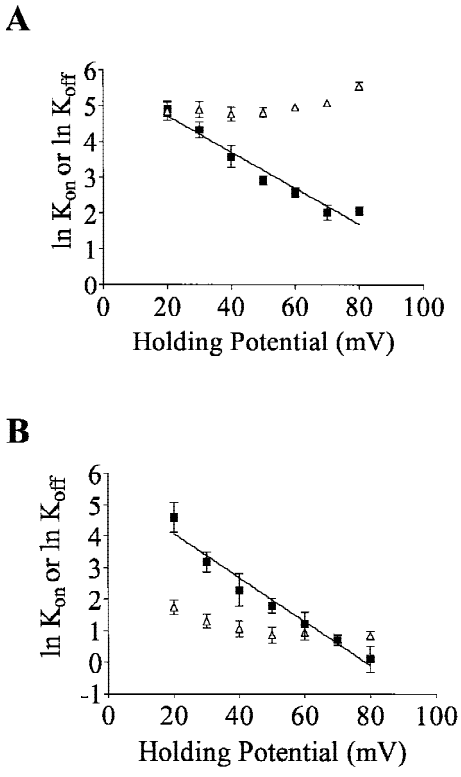

Fig. 8. Variation in LnK_{on} (△) and LnK_{off} (■) with holding potential with 20 μM *Sh*B (Fig. 8*a*) or 2 nM *Sh*B:E12KD13K (Fig. 8*b*). Each point represents the mean ± SEM for at least 4 experiments. The solid lines were obtained by linear regression and have the parameters quoted in the text.

the association rate (Murrell-Lagnado & Aldrich, 1993; Toro et al., 1994).

Clearly, negatively charged sites in the conduction pathways of the voltage- and Ca^{2+}-activated K^+ channels play a significant role in the interaction of the inactivation peptides with these channels. This is emphasized by the quantitative differences seen when the net charge of the peptides is altered. Similarly, in the present study we have observed quantitative differences in the blocking behavior of the two peptides, *Sh*B and *Sh*B:E12KD13K.

The most obvious difference in the action of *Sh*B and *Sh*B:E12KD13K on the ryanodine-modified cardiac SR Ca^{2+}-release channel is the effective concentration range. Block is seen with μM concentrations of *Sh*B while an equivalent degree of block is seen with nM concentrations of *Sh*B:E12KD13K. An explanation for this disparity can be found by considering the rates of association and dissociation of the two peptides. Most striking is the difference in K_{on} which is approximately 500-fold greater for *Sh*B:E12KD13K than *Sh*B (Fig. 5*a* and *b*). In addition, K_{off} is approximately 10-fold lower for *Sh*B:E12KD13K than *Sh*B (Fig. 5*a* and *b*). Therefore, as is the case with the interaction of the inactivation peptides with K^+ channels, an increase in the net positive charge of the peptide results in an increase in the affinity of its interaction with the SR Ca^{2+}-release channel. Again, as is the case with the interaction of the inactivation peptides with K^+ channels, this increase in affinity results from a dramatic increase in the rate of association of the peptide with the channel, with a much smaller effect on the dissociation rate of the peptide.

While the primary structure of the cytosolic vestibule of the SR Ca^{2+}-release channel is likely to be very different from that of the voltage-dependent and Ca^{2+}-activated K^+ channels it would appear that they share a functional homology by providing suitable environments for the interaction of the inactivation peptides. The qualitative and quantitative similarities between the consequences of modifying the net charge of the inactivation peptides on their interaction with K^+ channels and RyR suggest that, as with the K^+ channels, negative charge in

the conduction pathway of RyR plays an important role in concentrating and possibly orientating the peptides in the pathway. It is interesting to note that the relative difference in K_{on} between *Sh*B and *Sh*B:E12KD13K is considerably greater for RyR than for either the voltage-dependent or Ca^{2+}-activated K^+ channels investigated in previous studies (Murrell-Lagnado & Aldrich, 1993; Toro et al., 1994). This might indicate that the density of negatively charged sites in the peptide ''receptor'' is greater in RyR than in these other species of channel.

In previous studies we have suggested that interactions with hydrophobic sites within the cytosolic vestibule of RyR stabilize the interaction of the large tetra alkyl ammonium blocking cations (Tinker et al., 1992*b*). The interaction of inactivation peptides with this region of the SR Ca^{2+}-release channel described here adds support to the suggestion that this region of the channel contains hydrophobic sites.

Differences in Amplitude of Blocked State and Kinetics of Block

Both *Sh*B and *Sh*B:E12KD13K produce a variety of blocked states of RyR. While the majority of blocking events result in complete block, i.e., to conductance levels indistinguishable from the closed channel, other, incomplete, blocking events are seen. The occurrence of these events suggests that interaction of the peptides with the cytosolic vestibule of RyR does not always result in complete occlusion of the conduction pathway. This implies that the peptides do not always interact with exactly the same site within the vestibule or do not always interact in exactly the same orientation. This would be consistent with a relatively nonspecific interaction of the peptides with the channel. Long-range electrostatic interactions lure the peptide into the vestibule and hydrophobic interactions anchor it, however the exact orientation of the bound peptide may vary in its loosely fitting 'receptor'.

Such a mechanism might also explain the occurrence of two clearly distinguishable durations of blocking event by *Sh*B in RyR. Some orientations of the peptide might permit more extensive hydrophobic interactions with the channel than others and such interactions would result in longer duration blocking events. An alternative explanation of this phenomenon was put forward by Toro et al. (1994) who observed differing blocked states in a Ca^{2+}-activated K^+ channel and suggested that these might arise from the interaction of peptide molecules adopting different conformations in solution (Lee, Aldrich & Gierasch, 1992).

Functional Consequences of Ryanodine Modification in Relation to the Interaction of the Inactivation Peptides

The use of ryanodine-modified channels in this study allowed us to make quantitative determinations of block induced by the inactivation peptides. As outlined above, similar blocking events were seen in a limited number of unmodified channels in which P_o could be driven to approximately 1 and we are confident that our basic observations apply to both unmodified and modified channels. In previous investigations we have characterized ion handling in both forms of RyR and have noted that the modification of function seen following the interaction of ryanodine with the channel arises as the result of a number of subtle changes in the properties of the conduction pathway (Lindsay et al., 1994). Ryanodine-modification results in a decrease in the relative permeability of Ba^{2+} with respect to the group 1a monovalent cations and we have suggested that this could occur as a consequence of a structural re-organization of the channel which produced a lowering of the density of putative negative charge within the conduction pathway (Lindsay et al., 1994). If this is the case, we might predict that the conduction pathway of the unmodified ryanodine receptor channel would display an affinity for the inactivation peptides even higher than that reported here.

Conclusion

The demonstration that K^+ channel N-type inactivation peptides interact with RyR to produce block, and that their effectiveness as blockers is determined by the net charge of the peptides is entirely consistent with our earlier proposal of the presence of negative charge within the conduction pathway of this channel. These studies also reinforce the proposal that the interaction of inactivation peptides with their 'receptors' is governed not by the presence of specific residues in a binding site but rather by more general features such as charge and hydrophobicity.

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