**SUPPLEMENTAL MATERIAL**

Inactivation peptide production and analysis
The primary structures of the δShBPs used in this study are shown in Fig. S1. Peptides were synthesized as C-terminal amides by Cambridge Research Biochemicals Limited. The purity of the peptides was assessed to be ≥95% by reverse-phase HPLC analysis with a linear gradient water/50% acetonitrile in 0.1% trifluoroacetic acid. Amino acid analysis indicated the expected composition in all peptides. Fast atom bombardment mass spectrometry yielded the following results: δShBP, m/z 2,227.18 (calculated 2,228.56); LHBp, m/z 2,541.25 (calculated 2,542.66); MHBPI, m/z 2,256.02 (calculated 2,254.20); and MHBPII, m/z 2,256.09 (calculated 2,254.20). Lyophilized peptides were initially dissolved in a minimal amount of distilled water or a mixture of 50% DMSO/50% ethanol. All solvents used were HPLC grade.

Determination of parameters of TAA block
Parameters for block of EMD 41000–activated open RyR2 channels were determined using methods described previously (Mead and Williams, 2002). In brief, we limited our analysis to monitoring transitions within open events between the open state of the channel and the TAA-induced subconductance state. Dwell times in the open and TAA-blocked states were determined by 50% threshold analysis. Dwell times in the open and TAA-blocked states of WT and substituted ryanodine-modified RyR2 channels were determined by monitoring transitions between the modified open state and the blocked state (Tinker and Williams, 1993). Again, dwell times in the modified open and blocked states were determined by 50% threshold analysis.

Our previous investigations of large TAA block in native sheep RyR2 channels have established that, in both unmodified and ryanodine-modified channels, TBA and TPeA are concentration- and voltage-dependent open channel blockers and that dwell times in both the blocked and unblocked states within an opening can be described by single exponentials (Tinker et al., 1992; Tinker and Williams, 1993). These observations are consistent with a scheme in which individual TAA molecules interact at a single site in the open RyR2 PFR to bring about the occurrence of the blocked state. In a scheme such as this, the rate of TAA association with its binding site will be dependent on TAA concentration, whereas the rate of TAA dissociation will be uninfluenced by blocker concentration. Apparent rate constants for association (kon, per second/micromolar) and dissociation (koff, per second) are then calculated as (mean 1/TB)/(slope of 1/To). The apparent dissociation constant (Kd, micromolar) was then calculated as (mean 1/TB)/(slope of 1/Tc).

Comparison of TAA block in EMD 41000–activated and ryanodine-modified RyR2 channels
To quantify TAA and δShBP block in WT and mutant RyR2, it has been necessary to use two methods to maximize channel Popen. To compare the mechanisms of block in EMD 41000–activated and ryanodine-modified channels, we have monitored rates of TBA and TPeA association and dissociation in EMD 41000–activated and ryanodine-modified WT RyR2 channels (Fig. S3). In the EMD 41000–activated channel, the rates of association of these two TAAs are not significantly different (Fig. S3 A). Channel modification by ryanodine results in an equivalent significant reduction in the rate of association of both TAAs (Fig. S3 A). Earlier work from our group concluded that the reduced rate of TBA association seen after ryanodine modification of native sheep RyR2 resulted from a conformational change at the cytosolic entrance to the conduction pathway that reduced the capture radius for the TBA cation (Tinker and Williams, 1993).

The consequence of ryanodine modification on rates of TBA and TPeA dissociation are shown in Fig. S3 B. As highlighted elsewhere in this study, in the EMD 41000–activated WT channel, the rate of dissociation of TBA is very significantly greater than that of TPeA (Fig. S3 B). Modification of RyR2 by ryanodine results in no significant alteration in the rate of dissociation of TBA and a small increase in the equivalent parameter for TPeA. Most significantly, the overall differential between rates of dissociation of the two TAAs is maintained after ryanodine modification.

Therefore, the key finding of this comparison is that the differential effects of TBA and TPeA are essentially the same in the two forms of RyR2, an observation which indicates that these TAAs interact at the same site in EMD 41000–activated and ryanodine-modified WT RyR2 and that the ryanodine-modified RyR2 is a valid model within which to investigate the consequence of residue substitution on block.

Concentration- and voltage-dependent block of WT RyR2 by δShBPs
To characterize fully the blocking parameters of peptides of varying hydrophobicity in the WT ryanodine-modified recombinant RyR2, we have monitored both the concentration (Fig. S4) and voltage dependence (Fig. S5) of block.
REFERENCES


**Figure S1.** Distribution of hydrophobic residues in the peptide homologues. The cartoon depicts the amino acid sequences of the peptide derivatives used in this study, together with their respective net charge and a schematic representation of the hydrophobic residue distribution. Residues are color-coded as indicated in the figure.
Figure S2. Analysis of ShBP block in the ryanodine-modified RyR2 channel. (A and B) The top panels depict typical modified single RyR2 channel activity after the interaction of ryanodine (A) and block of the ryanodine-modified channel resulting from the addition of 40 µM WT ShBP (to the cytosolic side; B) at a holding potential of 50 mV in symmetrical 210 mM KCl. Lifetime distributions of channel closing and blocking events accumulated over a period of 120 s are shown in the bottom panels. In the absence of ShBP, open probability is very high and too few closing events (135) occur to allow a reliable fit of the distribution of dwell times (A, bottom). The subsequent addition of ShBP induced blocking events (2,538 events in total; B, bottom). Maximum likelihood fitting in TAC Fit established that the blocking events could be described by a minimum of four exponential components with the following parameters: No. 1 (area = 35%, time constant = 0.000331 s), No. 2 (area = 39.9%, time constant = 0.00263 s), No. 3 (area = 17.6%, time constant = 0.022909 s), and No. 4 (area = 7.5%, time constant = 0.186209 s).

Figure S3. TBA and TPeA blocking parameters in WT RyR2 after modification by ryanodine. (A and B) Rates were monitored at 60 mV in the presence of either 200 µM TBA or 100 µM TPeA in channels either activated by 20 µM EMD 41000 or after modification by 1 µM ryanodine. Rates of association (k_on) are shown in A, and rates of dissociation (k_off) are shown in B. Data are plotted as mean values (±SEM; TBA, n = 4; TPeA, n = 5–8; ***, P < 0.001).
Figure S4. Parameters for concentration-dependent block of ryanodine-modified RyR2 by ShBPs. (A–D) The dependence of the probability of block \(1 - P_o\); a) and pooled rates of peptide association (determined as the reciprocal of the mean duration of open events \(1/T_o\)) and dissociation (determined as the reciprocal of the mean duration of blocked events \(1/T_b\); b) were monitored. The dependence of these parameters on peptide concentration was monitored at a holding potential of 50 mV with ShBP (A), LHBP (B), MHBPI (C), and MHBPII (D). Each data point is the mean ± SEM of between 3 and 14 channels (A), between 3 and 11 channels (B and D), and between 3 and 8 channels (C). In the left panels (a), each set of data points are plotted together with a solid line that is the best-fit rectangular hyperbola obtained by nonlinear regression. Peptide concentrations producing 50% block \(K_m\) are as follows: ShBP, 32 ± 2 µM; LHBP, 51 ± 11 µM; MHBPI, 14 ± 2 µM; and MHBPII, 22 ± 3 µM. Variations in pooled rates of peptide association and dissociation (right; b) are plotted together with straight lines that are the best-fits obtained by linear regression. Variation in rates of peptide association and dissociation are plotted in Fig. 3 B of the main text.
Figure S5. Parameters for voltage-dependent block of ryanodine-modified RyR2 by ShBPs. (A–D) The dependence of the open probability in the presence of blocker (a) and pooled rates of peptide association (determined as the reciprocal of the mean duration of open events [1/To]) and dissociation (determined as the reciprocal of the mean duration of blocked events [1/TB]; b) were monitored. These parameters were determined in the presence of 20 μM ShBP (A), 85 μM LHBP (B), 20 μM MHBPI (C), and 20 μM MHBPII (D) at potentials between 20 and 80 mV. Each data point is the mean ± SEM of between 3 and 14 channels (A), between 3 and 11 channels (B and D), and between 3 and 8 channels (C). Variations in open probability with changing holding potential (left; a) are, in all cases, plotted together with a solid line that is the best-fit nonlinear regression line to the single site block model described by Woodhull (1973). Variations in pooled rates of peptide association and dissociation (right; b) are plotted together with straight lines that are the best-fits obtained by linear regression. The mechanisms underlying alterations in occurrence of block with holding potential are revealed by analysis of the changes in peptide 1/To and 1/TB with holding potential. As, in all cases, the relationships of natural logarithm of 1/To and 1/TB against voltage are linear with slopes $z_{on}F/RT$ and $-z_{off}F/RT$, the voltage dependence of the individual association ($z_{on}$) and dissociation ($z_{off}$) reactions can be calculated from the slopes of these plots (Tinker et al., 1992). For ShBP, $z_{on} = 0.88$ and $z_{off} = -0.61$; for LHBP, $z_{on} = 0.53$ and $z_{off} = -0.93$; for MHBPI, $z_{on} = 0.63$ and $z_{off} = -0.83$; and for MHBPII, $z_{on} = 1.18$ and $z_{off} = -1.51$. 