Electrophysiological Effects of Ryanodine Derivatives on the Sheep Cardiac Sarcoplasmic Reticulum Calcium-Release Channel

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ABSTRACT We have examined the effects of a number of derivatives of ryanodine on K^+ conduction in the Ca^{2+} release channel purified from sheep cardiac sarcoplasmic reticulum (SR). In a fashion comparable to that of ryanodine, the addition of nanomolar to micromolar quantities to the cytoplasmic face (the exact amount depending on the derivative) causes the channel to enter a state of reduced conductance that has a high open probability. However, the amplitude of that reduced conductance state varies between the different derivatives. In symmetrical 210 mM K^+, ryanodine leads to a conductance state with an amplitude of 56.8 ± 0.5% of control, ryanodol leads to a level of 69.4 ± 0.6%, ester A ryanodine modifies to one of 61.5 ± 1.4%, 9,21-dehydroryanodine to one of 58.3 ± 0.3%, 9β,21β-epoxyryanodine to one of 56.8 ± 0.8%, 9-hydroxy-21-azidoryanodine to one of 56.3 ± 0.4%, 10-pyrroleryanodol to one of 52.2 ± 1.0%, 3-epiryanodine to one of 42.9 ± 0.7%, CBZ glycyl ryanodine to one of 29.4 ± 1.0%, 21-*p*-nitrobenzoyl-amino-9-hydroxyryanodine to one of 26.1 ± 0.5%, β-alanyl ryanodine to one of 14.3 ± 0.5%, and guanidino-propionyl ryanodine to one of 5.8 ± 0.1% (chord conductance at +60 mV, ± SEM). For the majority of the derivatives the effect is irreversible within the lifetime of a single-channel experiment (up to 1 h). However, for four of the derivatives, typified by ryanodol, the effect is reversible, with dwell times in the substate lasting tens of seconds to minutes. The effect caused by ryanodol is dependent on transmembrane voltage, with modification more likely to occur and lasting longer at +60 than at −60 mV holding potential. The addition of concentrations of ryanodol insufficient to cause modification does not lead to an increase in single-channel open probability, such as has been reported for ryanodine. At concentrations of ≥500 μM, ryanodine after initial rapid modification of the channel leads to irreversible closure, generally within a minute. In contrast, comparable concentrations of β-alanyl ryanodine do not cause such a phenomenon after modification, even after prolonged periods of recording (>5 min). The implications of these results for the site(s) of interaction with the channel protein and mechanism of the action of ryanodine are discussed. Changes in the structure of ryanodine can lead to specific changes in the electrophysiological consequences of the interaction of the alkaloid with the sheep cardiac SR Ca^{2+} release channel.

INTRODUCTION

The identification of ryanodine as a specific ligand for the sarcoplasmic reticulum (SR) Ca^{2+} release channel has advanced significantly the fields of excitation-contraction coupling in both skeletal and cardiac muscle; ryanodine is often used in electrophysiological settings to specifically isolate or block the SR. Much information about the function of this intracellular network has been gained from such studies (Marban and Wier, 1985; Beuckelmann and Wier, 1988; Bers and Bridge, 1989; DuBell et al., 1993; Negretti et al., 1993). These observations are paralleled by observations from SR vesicle studies in which ryanodine leads to an increase in Ca^{2+} efflux at nanomolar to low micromolar concentrations, whereas in high μM to mM quantities it leads to an inhibition. The availability of a tritiated form of ryanodine has led to detailed binding studies being performed on SR membranes. These experiments have supported the existence of one, two, or more binding sites (Pessah and Zimanyi, 1991; Wang et al., 1993). In addition, $[^3H]$ryanodine has allowed us and others to carry out biochemical purification of the Ca^{2+} release channel (Williams, 1992).

It has now been well established that when either native SR membranes, under the appropriate ionic conditions, or the purified ryanodine receptor is reconstituted into planar phospholipid bilayers, under voltage-clamp conditions, a cation-selective ligand-regulated channel of high conductance can be observed. The channel displays many of the properties expected of the Ca^{2+} release protein (Williams, 1992; Meissner, 1994). The interaction of ryanodine at the single-channel level in concentrations above 50 nM is characterized most strikingly by a highly specific effect, namely the irreversible production of a reduced conductance state of high open probability (Rousseau et al., 1987). Furthermore, concentrations of ryanodine in the 10–50 nM range have also been observed to increase single-channel open probability without modification of conductance, and in the 100 μM to 2 mM concentration range ryanodine has been reported to lead to irreversible channel closure (Lai et al., 1989; Buck et al., 1992).

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The structural features of some ryanodine derivatives are shown in Scheme 1. Ryanodine (1) is isolated from the stems of *Ryania speciosa* Vahl and consists principally of two active constituents, ryanodine (1) and 9,21-dehydroryanodine (not shown). Recently, a series of derivatives of ryanodine have been synthesized. Competition binding assays with [^{3}H]ryanodine indicate that some interact with the channel with higher affinity than ryanodine, whereas others are of much lower affinity (Humerickhouse et al., 1993; Gerzon et al., 1993). The experimental behavior of these derivatives has been examined in binding assays and in experiments in which $^{45}Ca^{2+}$ efflux from SR vesicles has been determined. However, the effect of these derivatives has not been characterized at the single-channel level. In this paper we present an overview of the electrophysiological effects of a series of selected ryanodine derivatives on the purified Ca^{2+} release channel of sheep cardiac muscle SR reconstituted into planar phospholipid bilayers.

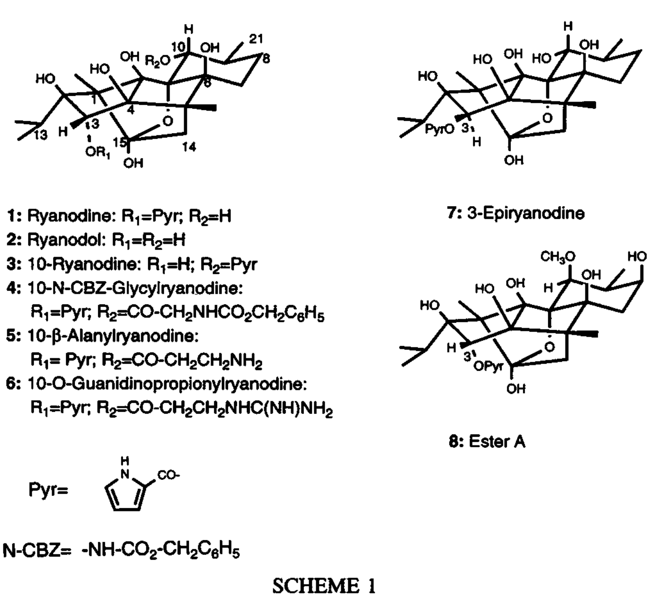
MATERIALS AND METHODS

Materials

Phosphatidylethanolamine was purchased from Avanti Polar Lipids (Birmingham, AL), and phosphatidylcholine was from Sigma (Poole, Dorset, England). [^{3}H]Ryanodine was obtained from New England Nuclear (Boston, MA). Aqueous counting scintillant was purchased from Packard (Groningen, The Netherlands). Other chemicals were obtained as the best available grade from BDH (Dagenham, Essex, England), Aldrich Chemical Company (Gillingham, Dorset, England), or Sigma.

Synthesis of the ryanodine derivatives

The compounds studied in this communication are loosely termed ryanodine derivatives; in strict chemical terms this is not correct for all those studied. Ryanoids or ryanodine congeners are naturally occurring compounds isolated from the *Ryania speciosa* Vahl bark and include ryanodine (1), dehydroryanodine (not shown), and ester A ryanodine (8). The O_{10eq}-ryanodine esters, including CBZ glycyl ryanodine (4), β-alanyl ryanodine (5), and guanidino-propionyl ryanodine (6), are true derivatives, as they are obtained by derivatization of ryanodine, retain some biological activity of the parent alkaloid, and can regenerate the parent alkaloid by chemical or enzymatic means. Ryanodol (2) is a degradation product in which the pyrrole carboxylic acid fragment is lost. The others are probably best considered as modification products in which the skeleton structure has been altered—changes which under physiological conditions cannot be altered. However, despite these chemical considerations, for ease of reference we refer to them all as derivatives. The methods of synthesis are as given previously (Humerickhouse et al., 1993; Gerzon et al., 1993).


1: Ryanodine: R_1=Pyr; R_2=H
2: Ryanodol: R_1=R_2=H
3: 10-Ryanodine: R_1=H; R_2=Pyr
4: 10-N-CBZ-Glycylryanodine: R_1=Pyr; R_2=$CO\text{-}CH_2NHCO_2CH_2C_6H_5$
5: 10-β-Alanylryanodine: R_1= Pyr; R_2=$CO\text{-}CH_2CH_2NH_2$
6: 10-O-Guanidinopropionylryanodine: R_1=Pyr; R_2=$CO\text{-}CH_2CH_2NHC(NH)NH_2$
7: 3-Epiryanodine
8: Ester A
Pyr=
N-CBZ= $\text{-}NH\text{-}CO_2\text{-}CH_2C_6H_5$

SCHEME 1

Small aliquots of the derivatives were added to the cytoplasmic face of the channel from concentrated stock solutions. A shorthand terminology is adopted in the ensuing discussion: ester A ryanodine (8) is referred to as ester A, CBZ glycyl ryanodine (4) as CBZ glycyl, 21-*p*-nitrobenzoylamino-9-hydroxyryanodine (not shown) as 21-*p*-nitro, β-alanyl ryanodine (5) as β-alanyl, guanidino-propionyl ryanodine (6) as guanidino-propionyl, 9,21-dehydroryanodine (not shown) as 9,21 dehydro, 9β,21β-epoxyryanodine (not shown) as epoxyryanodine, 9-hydroxy-21-azidoryanodine (not shown) as azidoryanodine, 10-pyrroleryanodol (3) as 10-pyrrole, and 3-epiryanodine (7) as 3-epi.

Preparation of sheep cardiac heavy sarcoplasmic reticulum membrane vesicles

Sheep hearts were collected from a farm (Selborne Biological) in ice-cold cardioplegic solution (Tomlins et al., 1986). Junctional or heavy sarcoplasmic reticulum membrane (HSR) vesicles were isolated from the interventricular septum and left ventricular free wall as previously described (Sitsapesan and Williams, 1990). Differential centrifugation of the muscle homogenate provides a mixed membrane fraction, which when fractionated further on a discontinuous sucrose gradient yields a HSR fraction at its 30/40% (w/v) interface. The HSR fraction was resuspended in 0.4 M KCl before sedimentation at 36,000 rpm (100,000 × g_{av}) for 1 h in a Sorvall A641 rotor. The resulting pellet was resuspended in a solution containing 0.4 M sucrose, 5 mM 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, subsequent separation, and reconstitution into proteoliposomes were performed as described previously (Lindsay and Williams, 1991).

Planar lipid bilayer methods

Lipid bilayers, formed from suspensions of phosphatidylethanolamine in *n*-decane (35 mg/ml), were painted across a 200-μm-diameter hole in a polystyrene copolymer partition that separated two chambers, referred to as the *cis* (volume 0.5 ml) and *trans* (volume 1.5 ml) chambers. The *trans* chamber was held at virtual ground, while the *cis* chamber could be clamped at various holding potentials relative to ground. Current flow across the bilayer was measured with an operational amplifier as a current-voltage converter as described by Miller (1982). 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 210 mM K^+. Solutions contained 10 μM free Ca^{2+} (8–12 μM as measured using an Orion Ca^{2+}-selective electrode) as contaminant, which was generally sufficient for channel activation. Occasionally it was necessary to raise the Ca^{2+} in the *cis* chamber to 50–100 μM to obtain sufficient activation. Experiments were carried out at room temperature (21 ± 2°C).

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

Single-channel data acquisition

Single-channel current fluctuations were displayed on an oscilloscope and stored on videotape. For analysis, data were replayed, filtered with an 8-pole Bessel filter, and digitized on an AT based computer system (Satori; Intracel, Cambridge, England). Data were filtered at 1 kHz and digitized at 4 kHz. Single-channel current amplitudes and single-channel open probability were determined from digitized data.

The ryanodine derivatives produce substates of varying amplitude in symmetrical 210 mM K^+. With some derivatives the amplitude of the substate was small, closures from this level were rare, and sometimes these occurred at levels of conductance intermediate between the substate and closed conductance. The combination of these factors meant that current-voltage relationships were difficult to determine for all of the derivatives. As a result, the change in single-channel current amplitude between the modified and unmodified channels was determined at the point of modification at a holding potential of +60 mV (effectively the ratio of the chord conductances). The result was expressed as a ratio (the fractional conductance in percentage terms) of the currents before and after modification. We have adopted this method previously in our work on ryanodine modification of channel function (Lindsay et al., 1994). Channel open probability was determined by using 50% threshold criteria (Colquhoun and Sigworth, 1983). Single-channel P_o was obtained from at least 150 s of continuous recording. In those experiments with ryanodol where a simple comparison of dwell times was necessary, the data were played out on a very slow time base on a pen recorder. The relevant dwell times were compared by direct measurement with a ruler.

The representative traces shown in the figures were obtained from digitized data acquired with Satori V3.2 transferred as an HPGL graphics file to a graphics software package (CorelDraw; Corel Systems Corporation, Ottawa, Canada) for annotation and printing.

RESULTS

The derivatives lead to modification

The most striking action of ryanodine at the single-channel level is a profound modification of gating and conduction such that after a variable period of exposure of the cytosolic face of the channel to concentrations greater than 100 nM, the channel suddenly enters a state of reduced conductance that has a high open probability. Do the ryanodine derivatives cause a similar effect? The addition of these derivatives to the cytosolic face of the receptor channel also leads to the production of a substate with very high open probability (Fig. 1). However, one striking difference is that the amplitude of the current of the reduced conductance state is different for each of the derivatives. We have quantified this information in terms of the fractional conductance (see Materials and Methods), and the data are summarized in Table 1. It can be seen from Fig. 1 that closures from the modified state are rare, and this is even more pronounced at negative holding potentials. Transitions do occasionally occur to states of conductance intermediate between the modified and the closed conductance levels, with all of the derivatives as with ryanodine (not shown). At the concentrations indicated in Table 1, modification generally occurred within 5 min of addition.

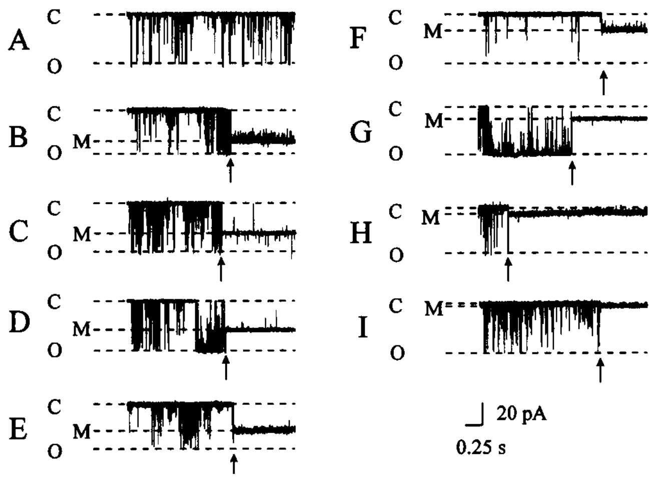


FIGURE 1 Representative single-channel traces at a holding potential of 60 mV in symmetrical 210 mM K^+, showing the various values of fractional conductance occurring upon modification by the various derivatives. The records were filtered at 1 kHz and digitized at 4 kHz. The control trace is shown in *A*, modification by 40 μM ryanodol in *B*, by 2 μM ester A in *C*, by 500 nM 9,21-dehydroryanodine in *D*, by 200 nM ryanodine in *E*, by 5 μM CBZ ryanodine in *F*, by 500 nM 21-p-nitro ryanodine in *G*, by 500 μM β-alanyl ryanodine in *H*, and by 100 nM guanidino propionyl ryanodine in *I*. The point of modification is marked with an arrow below the trace. A second unmodified channel is present in record *C*, and occasional openings of this channel can be seen on top of the modified channel. The dotted lines indicate the levels of the closed (C), modified (M), and open (O) states.

On two occasions the 21-*p*-nitro derivative caused modification to levels (approximately 13% and 50%) other than that indicated in Table 1. It is possible that these events occurred as the result of the interaction of other ryanodine derivatives, present at low concentrations as impurities generated during the synthesis of this derivative, with the channel.

Little difference was seen in the current amplitudes after modification at −60 and +60 mV for all but one of the derivatives. For example, fractional conductances determined at −60 mV for ryanodol were 67.9 ± 0.5% (±SEM $n = 8$), a value not much different from that in Table 1. There was one exception to this trend. In the case of the guanidino propionyl derivative, currents at −60 mV were obviously larger, and a figure of 12.1 ± 1.2% (±SD, $n = 3$) was obtained for fractional conductance at this holding potential.

Some derivatives are reversible

In Table 1 we classify the derivatives as either reversible or irreversible. The majority of these compounds behave in a

TABLE 1 Fractional conductance resulting immediately after modification with the series of ryanodine derivatives

Derivative	Fractional conductance	N	Effective concentration	Reversibility
Ryanodine	56.8 ± 0.5%	12	≥100 nM	Irreversible
9,21-Dehydro	58.3 ± 0.3%	6	≥500 nM	Irreversible
Ryanodol	69.4 ± 0.6%	12	≥20 μM	Reversible
Ester A	61.5 ± 1.4%	8	≥1 μM	Irreversible
$9\beta,21\beta$-Epoxy	56.8 ± 0.8%	4	≥8 μM	Irreversible
9-Hydroxy-21-azido	56.3 ± 0.4%	9	≥10 μM	Reversible
10-Pyrrole	52.2 ± 1.0%	7	≥4 μM	Mixed
3-Epi	42.9 ± 0.7%	8	≥8 μM	Reversible
CBZ-glycyl	29.4 ± 1.0%	6	≥2 μM	Irreversible
21-*p*-Nitro	26.1 ± 0.5%	11	≥500 nM	Irreversible
β-Alanyl	14.3 ± 0.5%	5	≥50 nM	Irreversible
Guanidino-propionyl	5.8 ± 0.1%	11	≥40 nM	Irreversible

The fractional conductance is the ratio of the chord conductance at +60 mV before and after modification. The statistics are calculated as ±SEM, where N refers to the number of measured modifications. The effective concentration is the concentration that led, in most cases, to modification within 5 min. It does not exclude the possibility that modification could occur at lower concentrations. The implications of the terms "reversible/irreversible" and "mixed" are indicated in the text.

fashion comparable to that of ryanodine in that the modification is essentially irreversible within the lifetime of the experiment. Perfusion of the derivative from the cytosolic face of the channel leaves this property unaltered. However, four of the compounds tested led to modification that spontaneously reversed during recording. These compounds, generally with lower binding affinities (ryanodol, azido, 10-pyrrole, and 3-epi; Welch et al., manuscript submitted for publication), led to modification that was spontaneously reversible in the continued presence of the derivative. Fig. 2 illustrates modification at positive holding potentials and its reversal at negative holding potentials with 10-pyrrole and 3-epi as examples. Fig. 3 *A* shows an example of spontaneous modification with ryanodol at +60 mV (indicated by arrow 1 in the diagram) and reversal of that modification (indicated by arrow 2 in the diagram).

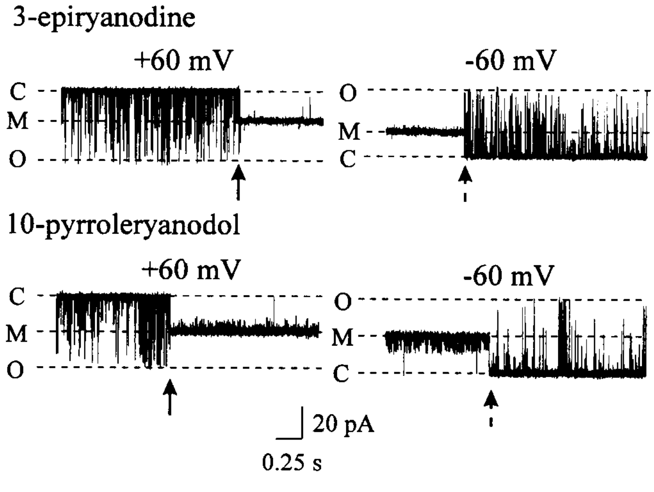


FIGURE 2 Representative single-channel traces at holding potentials as indicated on the figure in symmetrical 210 mM K^+. The records were filtered at 1 kHz and digitized at 4 kHz. The diagram shows modification at the positive holding potentials (marked by the *solid arrow*) and its reversal at the negative holding potential (marked by the *dashed arrow*) by 8 μM 3-epiryanodine and by 4 μM 10 pyrrole ryanodol. The dotted lines indicate the levels of the closed (C), modified (M), and open (O) states.

Dwell times in the modified state varied for the reversible derivatives. For 10-pyrrole, 3-epi, and azido dwell times were very long at +60 mV holding potential, with reversal of modification occurring only occasionally over a 3-min recording period. For ryanodol, dwell times were shorter, generally lasting tens of seconds to minutes at a holding potential of +60 mV. Perfusion of the reversible derivative from the cytoplasmic face of the channel led to channel behavior identical to that of control. A simple interpretation of these phenomena is that the binding and unbinding of the reversible derivatives correspond to "modification" and "reversal of modification" observed at the single-channel level. The probability of these derivatives causing modification seemed to be quite steeply concentration dependent. For example, with ryanodol, during 5-min observation periods, modification never occurred at 10 μM at a +60 mV holding potential, only occasionally with 20 μM, but frequently at 40 μM. It was apparent with all of the reversible derivatives that modification was more likely to occur and lasted longer at positive as compared to negative holding potentials. Dwell times were much shorter at negative holding potentials for these derivatives, with reversal of modification generally occurring within a minute.

In addition, the 10-pyrrole derivative showed a further property. After a variable period of time (~10 min) the effect would become irreversible. In other words, the modification would not reverse even holding for over 5 min at −60 mV, and the modified state would be present for the lifetime of the experiment after perfusion of 10-pyrrole ryanodine from the cytoplasmic face of the channel. This tended to occur more quickly as higher concentrations were used.

Voltage dependence of modification

As outlined above, the likelihood of modification and the length of dwell time in the modified state were dependent on holding potential with all the reversible derivatives. It was also apparent with the irreversible basic derivatives (β-alanyl and guanidino propionyl) that modification occurred more quickly at +60 mV and only occasionally and at higher concentrations at −60 mV holding potential.

This interesting behavior was investigated further with the reversible derivative ryanodol. Although the length of the dwell times made it very difficult to obtain quantitative estimates of the voltage dependence, the reversibility of the ryanodol effect made it possible to make a qualitative statistical demonstration of the effect. Two types of experiment

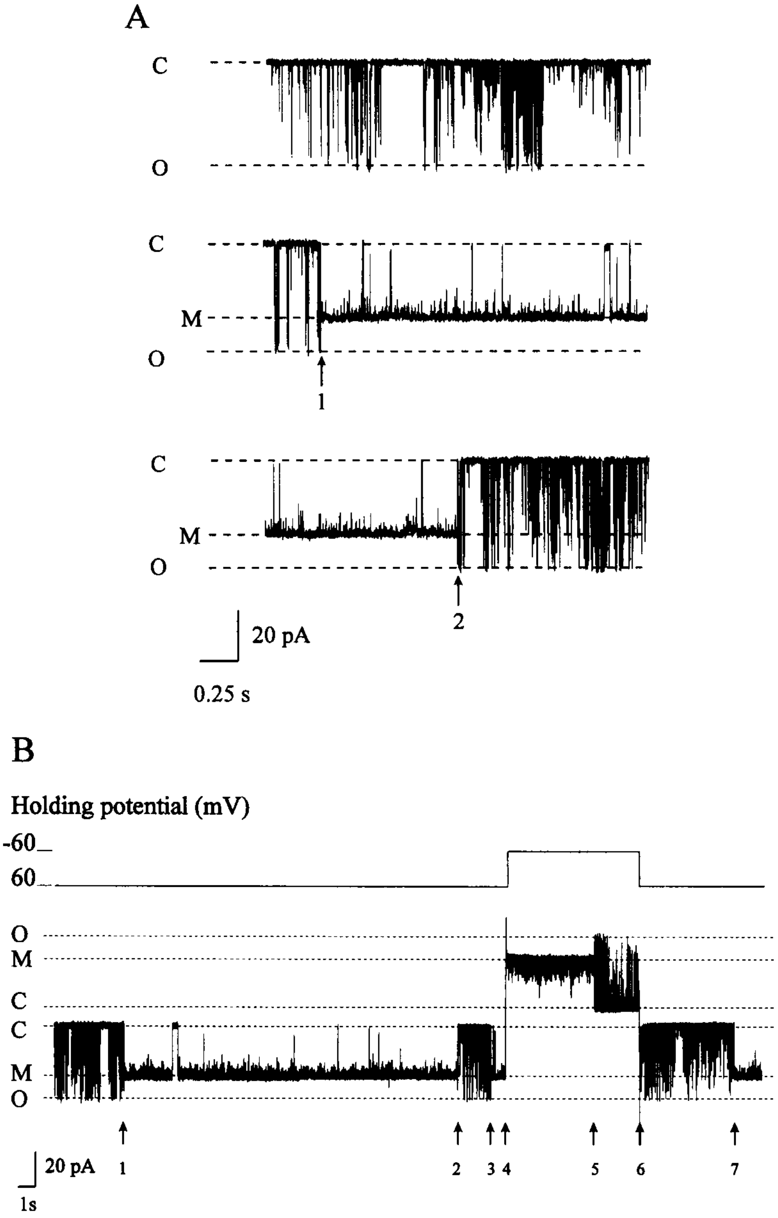


FIGURE 3 The effects of ryanodol. (*A*) All traces recorded in symmetrical 210 mM K^+ at 60 mV holding potential filtered at 1 kHz and digitized at 4 kHz. (*Upper trace*) Control trace in the absence of ryanodol. (*Middle trace*) Onset of modification in the presence of 20 μM (marked by *arrow 1*). *Lower trace*) Point of reversal of modification (marked by *arrow 2*). (*B*) A continuous period of 40 s of recording showing the interaction of 40 μM ryanodol with the receptor channel at holding potentials as illustrated in the diagram in symmetrical 210 mM K^+. The trace was filtered at 500 Hz and digitized at 2 kHz. The protocol shown is that adopted for the qualitative examination of the voltage dependence of ryanodol dwell times. The dotted lines indicate the levels of the closed (C), modified (M), and open (O) states. At +60 mV channel openings are indicated by an upward deflection; at −60 mV channel openings are indicated by a downward deflection of the trace. An unmodified channel is held at +60 mV before the onset of modification (marked by *arrow 1*). There follows a dwell period in the modified state before the spontaneous reversal of modification (marked by *arrow 2*). The period between arrows 1 and 2 is equivalent to t_1. A second modification occurs (marked by *arrow 3*), and the holding potential is switched to −60 mV (marked by *arrow 4*; capacitance spike reduced in size for display). At the point marked by arrow 5, the modification once again reverses. The period $t_2 + t_3$ is equivalent to the length of time between arrows 3 and 5. In this piece of the trace it can be seen that $t_1 > t_2 + t_3$. The holding potential is once again switched to +60 mV (marked by *arrow 6*; capacitance spike reduced in size for display). Modification once again occurs at +60 mV (marked by *arrow 7*).

were performed with 40 μM ryanodol at ± 60 mV holding potential.

In the first series of experiments an attempt was made to demonstrate the qualitative dependence of the on rate of ryanodol on holding potential. The channel was held at +60 mV and the "trial" series begun at the end of the first modification. After this event, holding potential was held at +60 mV for 30 s and at the end of this period was switched to a holding potential of −60 mV for 30 s. If the channel modified at +60 but not −60 mV, this was scored as 1; if it modified at −60 but not +60 mV, this was scored as −1; and no score was awarded if modification occurred at both potentials or did not occur at either. Quite commonly the channel rapidly modified and did not "unmodify" at +60 mV during the 30-s period. In these circumstances switching to a negative holding potential led to "unmodification" within approximately 10 s, and the 30-s recording period was begun at this moment. If modification was equally likely to occur at both the positive and negative membrane potentials, then the net score was zero, with an equal number of "modifications at +60 but not at −60 mV" as "modifications at −60 but not at +60 mV." In fact, in experiments performed with the above protocol, the score was +48, with no trials having the result of "modification at −60 but not +60 mV" (results obtained from $n = 4$ bilayers; full results in Table 2). If the results "modification at +60 but not at −60" and "modification at −60 but not at +60 mV" were equally likely, then the probability of obtaining this result by chance would be $(0.5)^{48}$. More formally, it is possible to estimate confidence intervals for the binomial distribution using Student's *t*-distribution and given that the variance is $np(1 - p)$ (where n is the number

TABLE 2 **Dependence of the on rate of ryanodol on holding potential**

Trial	No. of such results
Modification at +60 but not −60 mV	48
Modification at −60 but not +60 mV	0
Modification at both +60 and −60 mV	3
No modification at either potential	10

of trials and p is the probability of the event occurrence) (Clarke and Cooke, 1983). The null hypothesis that modification is equally likely to occur at +60 as at −60 mV ($p = 0.5$) holding potential is rejected at the 0.1% level (one- and two-tailed). In fact, the null hypothesis is near rejection, with $p \approx 0.9$ at the 1% level (in other words modification is nine times more likely to occur at +60 mV). By use of the Boltzmann relationship it is possible to estimate that the valency of the on reaction must be at least on the order of 0.6–0.7. This voltage dependence of the ryanodol modification was independent of P_o, as activation with sulmazole analogs (McGarry and Williams, 1994) did not affect the observation, and voltage dependence was independent of whether ryanodol was bound or unbound at the point of switching to a negative holding potential.

Experiments similar in principle were used to determine whether the off rate for ryanodol was voltage dependent, i.e., if once modified, were the channel dwell times in the modified state dependent on holding potential? The following protocol was used. A channel was held at +60 mV and the dwell time in a modification at +60 mV (t_1) was obtained. The holding potential was held at +60 mV until a second modification occurred and then switched to −60 mV. The time spent at +60 mV was added to the time spent at −60 mV until "unmodification" ($t_2 + t_3$) occurred. An example of one such trial is shown in Fig. 3 *B*; t_1, t_2, and t_3 are further explained in the legend. If dwell times are independent of voltage, then there ought to be as many trials with $t_1 > t_2 + t_3$ as there are with $t_1 < t_2 + t_3$. The results from a series of four bilayers are given in Table 3.

The results lead to the rejection of the null hypothesis (at the 0.2% level using a two tailed Student's t-test) that ryanodol dwell times are unaffected by holding potential and suggest that, as is the case with the on rate, the off rate of ryanodol is dependent on voltage.

Other comparisons with ryanodine

In addition to the modification reaction described above, two other unrelated effects have been suggested to occur with ryanodine. At low concentrations (10–50 nM) an increase in single-channel P_0 has been noted to occur (Bull et al., 1989; Buck et al., 1992). We have examined one derivative to see whether it causes a similar phenomenon. The ryanodol derivative was used at concentrations of 10–20 μM, where modification occurs rarely, to see whether single-channel P_o increases with this derivative. In symmetrical 210 mM K^+ with contaminant Ca^{2+} (~10 μM) at the cytoplasmic face of the channel, there was no convincing increase in single-channel P_o at ±60 mV holding potential. The results are shown in Table 4. Whether this pattern extends to all of the other derivatives remains to be established, but it does imply that derivatives need not necessarily possess this additional property.

TABLE 3 **Dependence of the off rate of ryanodol on holding potential**

Trial	No. of such results
$t_1 > t_2 + t_3$	25
$t_1 < t_2 + t_3$	6

The second effect of ryanodine has been less well documented at the single-channel level. We have noted previously that there is a tendency for modified channels to close irreversibly during long experiments with continued exposure of the cytosolic face of the channel to micromolar quantities of ryanodine. This tendency can be overcome by the removal of ryanodine from the cytosolic face of the channel after modification.

Channel closure is more readily observed at higher concentrations of ryanodine. In our hands, when ≥500 μM ryanodine is added to the cytoplasmic face of the channel, an initial rapid modification of all channels to the substate occurs, subsequently followed by closure, i.e., cessation of channel activity, within 1 min ($n = 7$). However, in a similar series of experiments in which we used 100 μM ryanodine and followed channel activity for 3 min, only one channel closed during this period. The subsequent elevation of the concentration of ryanodine to 1 mM led to rapid closure of all channels within 1 min ($n = 9$). The effect is irreversible, as perfusion of the ryanodine from the cytosolic face of the channel after closure does not lead to a return of single-channel activity. The effect is illustrated in Fig. 4 *A*. Measurement of bilayer capacitance indicated that channel activity was not lost as the result of a ryanodine-induced, nonspecific thickening of the bilayer.

As with ryanodine, comparable concentrations (1 mM) of the basic derivative β-alanyl cause rapid modification; however, with this derivative there is no subsequent closure of the modified channel up to 3–5 min later. In a series of five bilayers (with $n = 14$ channels) only one channel closed in this period. The subsequent addition of 1 mM ryanodine to the β-alanyl modified channel also failed to produce channel closure. This was the case under conditions where the β-alanyl ryanodine was either maintained or perfused from the cytosolic face of the channel. This effect is illustrated in Fig. 4 *B*.

TABLE 4 **Influence of low concentrations of ryanodol on open probability**

Concentration	P_o at +60 mV (±SD)	P_o at −60 mV (±SD)
Control	0.015 ± 0.006 ($n = 4$)	0.003 ± 0.001 ($n = 4$)
10 μM ryanodol	0.009 ± 0.007 ($n = 3$)	0.004 ± 0.003 ($n = 3$)
20 μM ryanodol	0.012 ± 0.005 ($n = 4$)	0.003 ± 0.004 ($n = 4$)

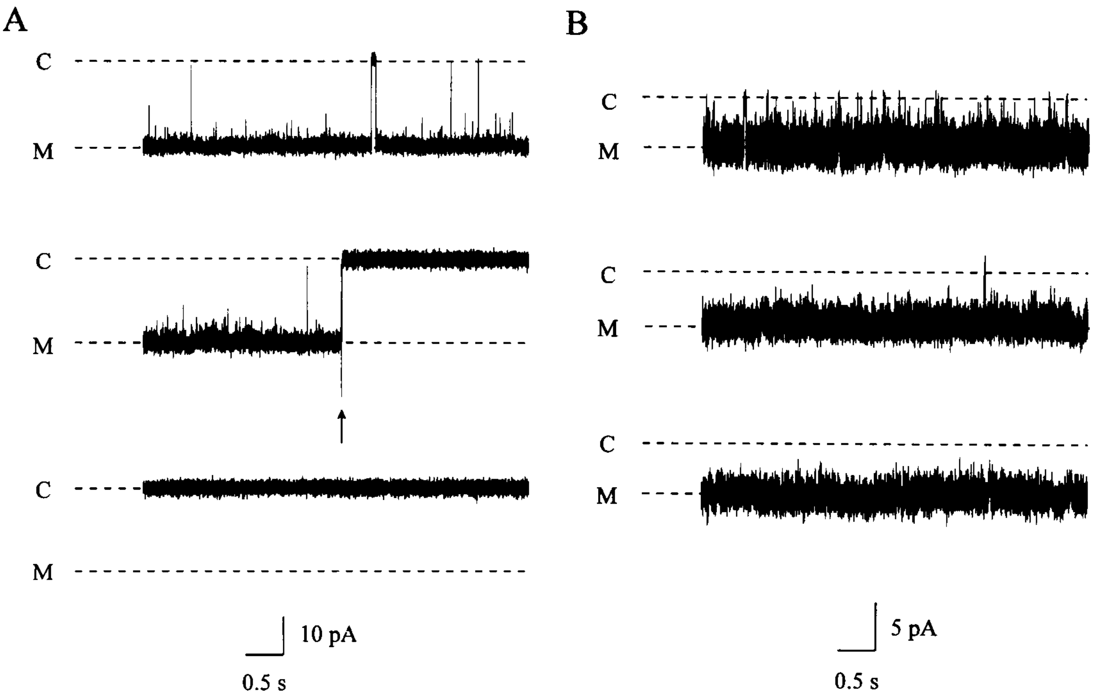


FIGURE 4 All traces recorded in symmetrical 210 mM K^+ at a holding potential of 60 mV. The records were filtered at 1 kHz and digitized at 4 kHz. Closed (C) and modified (M) levels are indicated by the dotted lines on the diagrams. (*A*) (*Upper trace*) Modified channel in the presence of 100 μM ryanodine. (*Middle trace*) Closure within 1 min after elevation of the concentration of ryanodine to 1 mM at the cytoplasmic face of the channel. In this particular record there is a very brief excursion to a higher conducting state before closure. This was seen occasionally in other experiments, but closure was by no means invariably accompanied by this phenomenon. (*Lower trace*) After perfusion of ryanodine from the cytoplasmic face, showing that this does not reverse the closure. (*B*) The upper trace shows a modified channel in the presence of 1 mM β-alanyl ryanodine at the cytoplasmic face of the channel, the middle trace shows the channel after perfusion of the ryanodine derivative away, and the lower trace shows the channel after the addition of 1 mM ryanodine at the cytoplasmic face. It is to be noted from the traces that the modified channel closes more frequently in the presence of 1 mM β-alanyl ryanodine. This was seen in all other experiments. It may represent a form of block by the positively charged derivative. It was not investigated further.

DISCUSSION

Modification induced by ryanodine and its derivatives

It seems most likely that the observation of modification with ryanodine and the derivatives corresponds to interaction with the high-affinity binding site observed in binding studies. Initially this may seem at odds with the observation of a slow on rate in binding studies and the variable time to modification observed at the single-channel level. However, the overall binding affinity is determined by both the unbinding and the binding rate constants. In the case of ryanodine the unbinding rate as determined from binding studies is very slow, and the ryanodine modification of channel gating and conduction is essentially irreversible, certainly within the lifetime of a single-channel experiment. Thus the combination of a slow binding rate constant but a much slower unbinding rate constant leads overall to a high affinity. Why is the binding rate constant so slow? If it were determined simply by diffusion it would be much faster (10^7-10^9 M^{-1} s^{-1}). This observation implies a more complex ligand-receptor interaction. Previous work from our laboratory (Williams and Holmberg, 1990) suggests that ryanodine binds to the open channel, and perhaps it is even necessary that the channel enters a specific open conformation before the binding site is sufficiently exposed. It has been suggested that interaction of ryanodine with the high-affinity binding site in fact corresponds, at the single-channel level, to the increase in P_o observed with low nanomolar concentrations of ryanodine (Bull et al., 1989; Buck et al., 1992). However, even this effect required several minutes before onset and was readily reversible with perfusion, suggesting a relatively fast unbinding rate (Buck et al., 1992).

The reversibility of modification seen with, for example, ryanodol and the relatively lower affinity of these compounds compared to ryanodine also support the contention that a major determinant of the binding equilibrium is the unbinding rate from the modified state. It has been proposed (Welch et al., 1994) that one component of importance in the binding of ryanodine is stabilized by a hydrophobic interaction at the 3 substituted position. The shorter dwell times of the more polar ryanodol and 10-pyrrole ryanodol support this proposition. However, the two other reversible derivatives identified here, 3-epi and 9-hydroxy-21-azido ryanodines, have a pyrrole substitution at the 3 position identical to that of ryanodine. Clearly, other chemical alterations in the ryanodine structure can have a significant effect on the off rate. Dwell times for all of the reversible derivatives studied so far were too long to obtain sufficient quantitative information for kinetic studies. We have previously used such studies to reveal kinetic information concerning the interaction of QX314 and the large tetraalkyl ammonium cations with the sheep cardiac Ca^{2+} release channel conduction pathway (Tinker et al., 1992b; Tinker and Williams, 1993). Such approaches can be quite powerful in revealing quantitative detail of molecule-channel interaction. The synthesis of a derivative with even lower affinity may open up these possibilities.

The observation that for 10-pyrrole ryanodol, reversible modification can progress to an irreversible state is intriguing and open to a number of interpretations. It could suggest sequential positively cooperative binding of at least two derivatives, i.e., with one bound the off rate is relatively fast, whereas with two the off rate becomes much slower and the effect becomes irreversible. Alternatively, there may be two conformations of a single 10-pyrrole in the binding site, one with a fast and the other with a slow off rate.

The nature of the conductance change induced by ryanodine and its derivatives

One of the most intriguing observations made with the derivatives is that the fractional conductance of the modified level in 210 mM K^+ shows a wide spectrum of values from approximately 70% for ryanodol to nearly 5% for guanidino propionyl. What does this say about the mechanism of substate production and how ryanodine derivative structure influences the resulting fractional conductance? There are two broad hypotheses. The first supposes that the change is induced by direct interaction within the conduction pathway, in other words a form of partial block in which the ryanoid interacts with a site within the conduction pathway and reduces, but does not completely prevent, single-channel current flow. The increased open probability is caused by the occupation of the site, making it much more difficult for the open channel to close. This is the explanation we have used to account for a number of other substate phenomena in the sheep cardiac Ca^{2+} release channel (Tinker and Williams, 1993). The alternative thesis envisages ryanodine binding at a site distant from the conduction pathway but inducing a conformational change that results in a stabilized open state with reduced conductance, i.e., an allosteric hypothesis. How does the available evidence weigh in favor of each of these possibilities?

The multitude of different fractional conductances seen with the derivatives and the rectification seen with guanidino propionyl but not the other derivatives would seem easier to rationalize in terms of partial block. Derivatives bind to and unbind from the same site located within the conduction pathway, and the nature of the interacting species determines the conductance change because of local effects within the conduction pathway. The alternative allosteric hypothesis could be formulated along the lines of the Monod-Wyman-Changeux (MWC) concerted mechanism as used by enzyme kineticists. K^+ is considered as the substrate and conductance as the enzymatic rate, and the channel consists of two conducting states. Ryanodine when bound would favor the ryanodine-modified over the unmodified conducting state (equivalent to the "tense" and "relaxed" states in the MWC mechanism). Interacting species at the binding site are considered as stabilizing one or the other of the states, reflecting the all-or-none nature of the MWC mechanism. The problem then with including all of the derivatives in such a scheme is that one would have to suppose a series of different quaternary protein structures, each corresponding to a unique conductance state and each uniquely stabilized by a particular derivative. Although it is impossible to exclude this situation, it is not especially appealing. Also, we have demonstrated that the binding and unbinding of ryanodol are influenced by membrane potential. For charged blocking or substate-inducing molecules this is generally taken as adequate, although by no means watertight evidence (Moczydlowski et al., 1984) for direct entry into the conduction pathway. Ryanodol carries no net charge, although it does have a fixed dipole. If the positively charged part of the dipole had to lie in the voltage drop for interaction, while the negative pole lay outside, the effect would show an overall voltage dependence. Alternatively, the ryanodine-binding site could be influenced by transmembrane potential such that binding is more likely to occur at positive holding potentials. The most telling evidence in favor of a partial block hypothesis would be the demonstration of permeant ion competition with the interacting species. Even ryanodol has dwell times an order of magnitude too large to quantify this effect, but it may be possible in the future to synthesize a derivative with much shorter dwell times as we obtain more information on the relationship between derivative structure and function.

Previously we performed a detailed analysis of the conduction properties of the ryanodine-modified state and compared these with those of the unmodified SR Ca^{2+} release channel (Lindsay et al., 1994). The conclusion of these studies is that ryanodine produces a profound, diffuse change in many aspects of ion handling such that it is impossible to propose one simple change to account for the changes in fractional conductance seen with different ions. This is highly suggestive of a widespread conformational change and supports an allosteric hypothesis. It seems that both hypotheses have their weaknesses.

One way of resolving these conflicting ideas is to suppose that perhaps both hypotheses are correct—in other words, that ryanodine derivatives bind in a vestibule-like region of the conduction pathway. This has direct physical consequences in terms of ion translocation, for example, by affecting the electrostatics of permeant ion to channel interaction or by simply reducing the capture radius for ion entry, and leads to changes in ion handling at sites distant from the binding site through a long-range conformational change. This reasoning is perhaps not so far-fetched. Zn^{2+}

has been observed to induce a substate in the cardiac batrachotoxin-modified sodium channel. For the Zn^{2+} effect in the cardiac batrachotoxin-modified sodium channel, kinetic arguments support an allosteric hypothesis, whereas evidence from site-directed mutagenesis in the putative vestibule of the pore supports a binding site location at this point (Schild et al., 1993; Schild and Moczydlowski, 1994). Furthermore, there is some preliminary evidence that the ryanodine binding site lies within the pore-forming transmembrane regions of the SR Ca^{2+} release channel protein (Callaway et al., 1994; Witcher et al., 1994).

Ryanodine causes specific inhibition of single-channel activity

Ca^{2+} efflux from SR vesicles is enhanced by ryanodine in concentrations from 100 nM to 100 μM. Increases of ryanodine concentration above this lead to a rapid decline in efflux. However, the basic derivative β-alanyl ryanodine does not show such behavior, and Ca^{2+} efflux plateaus at its maximum value above concentrations of 100 μM (Humerickhouse et al., 1993). The observations in this paper suggest an explanation for this finding. Ryanodine, at concentrations comparable to those used in flux experiments to reduce Ca^{2+} efflux, is able to produce a rapid, irreversible inhibition of single-channel activity, a property not shared by β-alanyl ryanodine. Interestingly, the addition of millimolar quantities of ryanodine to a channel modified in the presence of β-alanyl ryanodine does not lead to inhibition. One possible explanation for this interesting finding is that the ability to presumably bind a further ryanodine to close the channel is contingent upon the channel already having been modified with ryanodine.

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