Ryanodine receptor/calcium release channel conformations as reflected in the different effects of propranolol on its ryanodine binding and channel activity

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INTRODUCTION

Muscle cell contraction is initiated by a depolarization of the transverse tubular membranes that is coupled to a rapid Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) into the myoplasm [1]. It is accepted that a key protein involved in excitation–release from the sarcoplasmic reticulum (SR) into the myoplasm is the transverse tubular membranes that are coupled to a rapid Ca\(^{2+}\) release channel [3,6]. The purified Ca\(^{2+}\) release channel/ryanodine receptor (RyR) has been found to consist of high-molecular-mass polypeptides which are assembled into a homotetrameric complex of apparent sedimentation coefficient 30 S [3]. The cDNA of the RyR from skeletal muscle has been cloned and sequenced, and a molecular mass of 565 kDa was determined [4,5]. When incorporated into a planar lipid bilayer, the purified protein exhibits a Ca\(^{2+}\) mass of 565 kDa was determined [4,5]. When incorporated into skeletal muscle has been cloned and sequenced, and a molecular sedimentation coefficient 30 S [3]. The cDNA of the RyR from which are assembled into a homotetrameric complex of apparent has been found to consist of high-molecular-mass polypeptides.

The purified Ca\(^{2+}\) release channel/ryanodine receptor (RyR) depends on the assay conditions. At high NaCl concentrations, propranolol increased the number of ryanodine-binding sites (B_max) with no effect on the binding affinity. In the presence of 0.2 M NaCl, ryanodine binding was inhibited by propranolol. Half-maximal inhibition was obtained at 1.2 mM and complete inhibition at 2 mM propranolol. The inhibitory effect of propranolol obtained at low NaCl concentration was not restored by increasing the NaCl concentration to 1 M. 2. Modulators of the RyR that are known to alter its conformational states, such as adenine nucleotides, Ca\(^{2+}\) concentration and pH, modified the effect of propranolol on ryanodine binding. In the presence of propranolol and at low NaCl concentrations, ryanodine binding was inhibited and showed no Ca\(^{2+}\)-, pH- or time-dependence. 3. Propranolol immediately and completely blocked the channel opening of RyR reconstituted into a planar lipid bilayer. Propranolol-modified non-active channel was reactivated to a subconductive state (about 40% of the control conductance) by ATP. 4. Competition experiments between lidocaine (a stimulatory drug) or tetracaine (an inhibitory drug) and propranolol at 0.2 or 1.0 M NaCl, respectively, suggest the existence of different interaction sites for local anaesthetics and propranolol. 5. These results suggest that propranolol interacts directly with the RyR and modifies its ryanodine binding and single-channel activities. Propranolol effects are altered by the RyR conformational state, suggesting its possible use as a conformational probe for RyR.

INTRODUCTION

Muscle cell contraction is initiated by a depolarization of the transverse tubular membranes that is coupled to a rapid Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) into the myoplasm [1]. It is accepted that a key protein involved in excitation–release from the sarcoplasmic reticulum (SR) into the myoplasm is the transverse tubular membranes that are coupled to a rapid Ca\(^{2+}\) release channel [3,6]. The purified Ca\(^{2+}\) release channel/ryanodine receptor (RyR) has been found to consist of high-molecular-mass polypeptides which are assembled into a homotetrameric complex of apparent sedimentation coefficient 30 S [3]. The cDNA of the RyR from skeletal muscle has been cloned and sequenced, and a molecular mass of 565 kDa was determined [4,5]. When incorporated into a planar lipid bilayer, the purified protein exhibits a Ca\(^{2+}\) conductance with pharmacological properties of the native SR Ca\(^{2+}\) release channel [3,6].

Ligands such as micromolar Ca\(^{2+}\), caffeine or millimolar ATP, which are known to stimulate Ca\(^{2+}\) release, ryanodine binding and channel opening, stimulate ryanodine binding to a high-affinity site. Ligands such as micromolar Ruthenium Red and millimolar Mg\(^{2+}\) or Ca\(^{2+}\) that close the channel and inhibit Ca\(^{2+}\) release, also inhibit ryanodine binding. Thus, it became apparent that ryanodine preferentially binds to the open state of the channel. This is an important finding, since it means that ryanodine binding can be used as a conformational probe to indicate the gating state of the Ca\(^{2+}\) release channel [7].

Propranolol is a blocker of the β-adrenergic receptor. Propranolol was shown to inhibit Ca\(^{2+}\) accumulation by the SR [8], and this effect can be partially prevented by Ca\(^{2+}\), but not by isoprenaline (β-adrenergic-receptor agonist) [9], suggesting that its effect is mediated by interaction with a system distinct from the β-adrenergic receptor. The ATP-dependent interaction of propranolol with the SR has been demonstrated [10]. Propranolol apparently inhibited Ca\(^{2+}\) accumulation and stimulated ATPase activity by more than 2-fold due to activation of Ca\(^{2+}\) efflux. This ATP-dependent propranolol effect has been observed also in non-junctional SR membranes.

In this study we demonstrate the interaction of propranolol with the RyR and modification of its ryanodine binding and Ca\(^{2+}\) release channel activities. Propranolol, according to the assay conditions, has either an inhibitory or stimulatory effect on the binding of [H]ryanodine to the receptor, suggesting different interactions of propranolol with the RyR according to its conformational state. Thus, propranolol effects on ryanodine binding or single-channel activity can be used as a ‘tool’ to study different conformational states of the RyR/Ca\(^{2+}\) release channel.

MATERIALS AND METHODS

Materials

ATP, EGTA, Tris, CsCl, Hepes, Tricine, Mops, propranolol, tetracaine and lidocaine were obtained from Sigma. [H]-Ryanodine (60 Ci/mm) was purchased from New England Nuclear, and unlabelled ryanodine from Calbiochem. Lidocaine was dissolved in methanol and than diluted 1:4 (v/v) with water.

Abbreviations used: SR, sarcoplasmic reticulum; CHAPS; 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulphonic acid; RyR, ryanodine receptor.

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The final methanol concentration in control and lidocaine-containing samples never exceeded 2%.

Membrane preparation

Junctional SR membranes were prepared from rabbit fast-twitch skeletal muscle as described by Saito et al. [11]. In most of the experiments the fraction R$_{i}$ was used. The membranes were suspended to a final concentration of about 25 mg of protein/ml in a buffer containing 0.25 M sucrose and 10 mM Tricine, pH 8.0, and stored at -70 °C. Protein concentration was determined by the method of Lowry et al. [12].

Purification of the RyR

RyR was purified by the spermine–agarose method [13]. The purified protein (30–70 µg/ml) was assayed for [3H]ryanodine binding (in 0.1 ml) as described below for the membranes, except that RyR was first incubated for 5 min with soybean lecithin (5 mg/ml), and then diluted 5-fold in the assay medium. After 2 h at 30 °C, the bound ryanodine was assayed by polyethylene glycol 600 (PEG) precipitation in the presence of carrier protein (1.4 mg/ml BSA), followed by filtration through Whatman GF/B filters and three washes of 4 ml each with 10% PEG solution [13].

[3H]Ryanodine binding

Unless otherwise specified, junctional SR membranes (final concentration of 0.5 mg/ml) were incubated for 2 h at 37 °C with 20 mM [3H]ryanodine (30 Ci/nmol), in a standard binding solution containing either 0.2 or 1.0 M NaCl, 20 mM Mops, pH 7.4, and 50 µM CaCl$_2$. Unbound ryanodine was separated from protein-bound ryanodine by filtration of protein aliquots (50 µg) through Whatman GF/C filters, followed by washing three times with 5 ml each of ice-cold buffer containing 0.2 M NaCl, 10 mM Mops, pH 7.4, and 50 µM CaCl$_2$. The filters were dried and the retained radioactivity was determined by standard liquid scintillation counting techniques. Specific binding of [3H]ryanodine is defined as the difference between the binding in the absence and in the presence of 20 µM unlabelled ryanodine.

Single-channel reconstitution and analysis

Reconstitution and analysis of single-channel experiments were carried out as described previously [14]. Planar lipid bilayers were made with a 5:3 mixture of phosphatidylethanolamine and phosphatidylserine (Avanti Polar Lipids) at 50 mg/ml in decane. SR vesicles (1–3 µg) suspended in 0.3 M sucrose and 10 mM Hepes, pH 7.4, were added to the chamber defined as the cis side of the planar lipid bilayer setup. The cis chamber contained 250 mM CsCl, 20–50 µM CaCl$_2$, and 10 mM Hepes, pH 7.4, whereas the trans chamber contained 50 mM CsCl, 20–50 µM CaCl$_2$, and 10 mM Hepes, pH 7.4. Following a single step-like fusion event, the cis chamber was immediately perfused with an identical buffer to stop further fusion. All current recordings were measured at +60 mV holding potential with respect to the trans (ground) side. In a typical experiment, reagent was added to the cis or trans chamber and, after 1–2 min, single-channel fluctuations were recorded for at least 2 min.

RESULTS

The effects of propranolol on ryanodine binding by junctional SR membranes and purified RyR at 1 M NaCl and 0.2 M NaCl are illustrated in Figure 1. At low NaCl concentrations, propranolol inhibits ryanodine binding to both membrane-bound and purified RyR, with IC$_{50}$ values of 1.2 and 1.12 mM, respectively, and complete inhibition with about 2 mM propranolol. However, at high NaCl concentrations, propranolol stimulates ryanodine binding (140%) to the membrane-bound RyR and has no effect on the binding to the purified RyR. Similar results were obtained with KCl (results not shown). Figure 2 shows the different effects of 1.6 mM propranolol on ryanodine binding as a function of NaCl concentration. The propranolol inhibition of ryanodine binding, at low NaCl concentrations, is diminished and becomes a stimulation at above 0.4 M NaCl. These results suggest that propranolol interacts directly with the RyR, and its inhibitory or stimulatory effects on ryanodine binding are dependent on the ionic strength which stabilizes different conformations of the RyR.

The effects of propranolol on ryanodine binding affinity at high and low ionic strength

The two different effects of propranolol on ryanodine binding observed at different NaCl concentrations were characterized in

![Figure 1](image1.png)

**Figure 1** Effects of propranolol on ryanodine binding to the membrane-bound and purified RyR

Junctional SR membranes (0.5 mg/ml) (A) or purified RyR (38 µg/ml) (B) were assayed for [3H]ryanodine binding in 0.2 M NaCl (closed symbols) or 1.0 M NaCl (open symbols) in the absence and presence of propranolol as described in the Materials and methods section. Control activities (100%) were: 2.5 and 52.6, and 10 and 61.6 pmol/mg of protein in 0.2 or 1.0 M NaCl for the membrane-bound and purified RyR respectively. (A) The different symbols represent different experiments. (B) This is a representative experiment from a total of four.

![Figure 2](image2.png)

**Figure 2** Propranolol inhibition of ryanodine binding is modified by high NaCl concentrations

(A) [3H]Ryanodine binding was assayed in the absence (●) and in the presence (○) of propranolol as described in Figure 1, except that NaCl concentration was varied as indicated. (B) Ryanodine binding in the presence of propranolol is presented as a percentage of the binding in the absence of propranolol (at the same NaCl concentration). Open and closed symbols represent different experiments.
the following experiments. Figure 3(A) shows stimulation by propranolol of ryanodine binding to SR membranes at 1.0 M NaCl at all the ryanodine concentrations tested. Scatchard plot analysis of these results (Figure 3A, inset) indicates that stimulation of ryanodine binding by propranolol at 1.0 M NaCl results from an increase in the total number of ryanodine high-affinity binding sites ($B_{\text{max}}$) from 15.8 to 20.6 pmol/mg of protein, with no effect on the apparent ryanodine-binding affinity. It is well established that the RyR has low- ($\mu$M) and high- (nM) affinity ryanodine-binding sites [15]. The 20% increase in $B_{\text{max}}$ value could result from conversion of low-affinity sites into high-affinity sites. Kinetic experiments of ryanodine binding at high NaCl concentration, in the absence and in the presence of propranolol, show that propranolol has no effect on either the ryanodine association- or dissociation-rate constants, and thus no effect on the ryanodine-binding affinity (results not shown).

The effect of propranolol at 0.2 M NaCl on ryanodine binding as a function of its concentration is shown in Figure 3(B). The results indicate that in the presence of propranolol a constant ryanodine binding value is obtained with no dependence on ryanodine concentration. These data have been analysed according to Scatchard and yielded no linear correlation (results not shown).

The association of ryanodine with its binding site, at low NaCl concentrations, is altered in the presence of propranolol; within 20 min the binding reached a constant value that did not change during 100 min, although the binding in its absence was increased over 4-fold over the same period of time. On the other hand, dissociation of bound ryanodine at equilibrium with propranolol present only during the dissociation assay was the same as in its absence (dissociation rate constant, $k_{\text{diss}} = 0.01 \text{ min}^{-1}$). It seems that at 0.2 M NaCl, propranolol associates with the RyR and stabilizes it in a conformation that does not recognize ryanodine thereafter. The results also suggest that propranolol interacts with the RyR faster then ryanodine and, therefore, its effect is dominant.

**Alteration of propranolol interaction with the RyR by specific modulators**

The changes observed in propranolol effects on ryanodine binding under different assay conditions may suggest that different interaction sites for propranolol become accessible at different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR. Alternatively, the interaction of propranolol with the same binding site results in different conformational states of the RyR.

**Figure 3** Effect of propranolol at high- and low-NaCl concentrations on the ryanodine-binding sites

$[^{3}H]$Ryanodine binding was assayed in the presence of 1.0 M NaCl (A) or 0.2 M NaCl (B) and in the absence (●) and presence (○) of 1.2 mM propranolol as described in Figure 1, except that $[^{3}H]$ryanodine concentration was varied as indicated. In (A), the $K_{d}$ values calculated from the Scatchard plots are 8.1 nM both in the absence and in the presence of propranolol. The calculated $B_{\text{max}}$ values are 15.8 and 20.6 pmol/mg of protein in the absence and presence of propranolol respectively. This is a representative experiment from a total of four.

**Figure 4** pH-dependence of ryanodine binding in the absence and presence of propranolol, at 0.2 and 0.75 M NaCl

SR membranes (0.5 mg/ml) were assayed for ryanodine binding in the absence (●) and in the presence (○) of propranolol, and in the presence of 0.2 M NaCl (A) or 1.0 M NaCl (B) as described in the Materials and methods section, except that the pH was varied as indicated. The buffers used were: 20 mM Mops for the pH values: 6.5, 7.0, 7.5, and 20 mM Tricine for the pH values: 7.5, 7.8, 8.0, 8.2, 8.5. This is a representative experiment from a total of three.
that propranolol stabilizes the protein in a conformation that does not recognize ryanodine and is no longer responding to pH or to Ca$^{2+}$ concentration. Adenine nucleotides are known to stimulate Ca$^{2+}$ release, single-channel activity and the binding of ryanodine to its receptor [7]. They exert their effect on the RyR through a specific interaction with a nucleotide interaction site(s) located on the protein [17]. ATP was shown to increase the ryanodine-binding affinity by 5-fold [17] and to stabilize the receptor in an open conformational state [18]. The results presented in Table 1 show that in the presence of 2 mM ATP the inhibition of ryanodine binding by propranolol is decreased from 77% to 33%. A similar result is obtained in the presence of 2 mM ADP, but not with GTP. The lack of effect by GTP is not surprising since the nucleotide-binding site on the RyR has high affinity for adenine nucleotides and less for other nucleotides [17]. It seems that ATP and ADP stabilize a certain conformational state of the RyR that is less sensitive to propranolol.

### Single-channel inactivation by propranolol

The RyR has the intrinsic activity of a ryanodine-sensitive Ca$^{2+}$ release channel. The effect of propranolol on the single-channel activity of RyR reconstituted into planar lipid bilayer is shown in Figure 6. Propranolol, added to the cis chamber at low concentrations (200 µM) blocks channel opening completely within 20 s (five experiments). Figure 6 also shows the effect of 1 mM ATP on the channel activity, either after (Figure 6A) or before (Figure 6B) the addition of propranolol. Propranolol-modified non-active channel was reactivated by the addition of ATP, but to a low conductive state (36±6% of the control conductance). Perfusion of cis solution containing propranolol and ATP reactivates the low-conductive channel to a high-conductance state. Addition of propranolol to the ATP-activated channel results in a conversion of the active high-conducting channel into a less active channel with low conductance (Figure 6B).

### Transformation between RyR conformations in the absence and in the presence of propranolol

Figure 7 addresses the question of whether the RyR conformation stabilized by low NaCl concentrations in the absence and in the presence of propranolol could be altered by increasing the NaCl concentration. The results show that, in the absence of propranolol, raising the NaCl concentration from 0.2 to 0.75 M increases the binding of ryanodine to the level obtained in the presence of 0.75 M. However, if the incubation at low NaCl concentration is performed in the presence of propranolol, increasing the salt concentration to 0.75 M where propranolol stimulates the binding did not restore the inhibition by propranolol. It seems that the RyR conformation stabilized by propranolol at 0.2 M NaCl is a stable one that cannot be converted, by increasing the NaCl concentration, into the more active conformation in which propranolol stimulates ryanodine binding.

### Relationship between the local anaesthetics and propranolol-interacting sites

In a previous study we have shown a direct interaction between
Propranolol interacts with the ryanodine receptor/Ca\(^{2+}\) release channel

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**DISCUSSION**

RyR interacts with many hydrophobic drugs including local anaesthetics [19,20], volatile anaesthetics [21,22], duxorubicin [23,24] and more (see [7]). These agents affect the RyR either by stimulating or inhibiting its ryanodine-binding activity and single-channel activities. In this study we demonstrate the interaction of propranolol with RyR. Propranolol has dual effects: stimulation or inhibition of RyR activities, depending on the assay conditions.

Propranolol interacts with the RyR/Ca\(^{2+}\) release channel

Propranolol has stimulatory or inhibitory effects on ryanodine binding and single-channel activities of the membrane-bound and purified RyR. At high NaCl concentrations ( > 0.4 M), propranolol increases ryanodine binding up to 140\% by modi-
The effects of different local anaesthetics on ryanodine binding to its receptor have been presented in a previous study [19]. Some of the local anaesthetics completely inhibited ryanodine binding, and others increased the binding by up to 4-fold. We proposed that the inhibitory and the stimulatory local anaesthetics interact with the same site on the RyR, and the different effects are due to the interaction with the subsites which result from differences in the structure of the local anaesthetic molecules. Since propranolol stabilizes the RyR channel in a subconductance state [3,7]. Interestingly, propranolol blocks the channel opening completely and in a reversible manner (Figure 6A). Surprisingly, the addition of ATP reactivates the channel to a subconductance state (Figure 6). 

Different conformations of the RyR as reflected by the inhibitory or stimulatory effects of propranolol

We found that propranolol exerts different effects at different conformational states of the RyR, as stabilized by NaCl or ATP concentrations. Propranolol inhibits ryanodine binding at low NaCl concentrations and increases the binding at high NaCl concentrations (Figure 1). However, in the presence of propranolol, other RyR modulators such as ryanodine, Ca$^{2+}$ and pH were found to have no effect on ryanodine binding. The RyR conformation stabilized by propranolol at low NaCl concentrations showed virtually no Ca$^{2+}$ (Figure 5) or ryanodine (Figure 3B) concentration-dependence, and also no pH- (Fig. 4) or time-dependence. Thus, the results suggest that propranolol at low NaCl concentration stabilizes an inactive conformation that cannot be reactivated by conditions and ligands which are well characterized effectors of Ca$^{2+}$ release and ryanodine binding [7]. Furthermore, the propranolol-stabilized conformation at low NaCl concentration was not reactivated by elevating the NaCl concentration (Figure 7). Adenine nucleotides, however, are the only modulators that reactivate the propranolol-modified protein as reflected in increasing ryanodine binding (Table 1) and reactivating of single-channel activity to a subconductance state (Figure 6). It seems that the complex [RyR–propranolol], stabilized at low ionic strength, no longer binds ryanodine, and therefore does not respond to changes in Ca$^{2+}$ and ryanodine concentrations or to incubation-pH or time. The different effects of propranolol at low- and high-NaCl concentrations, and the fact that the inhibitory effect at low NaCl concentrations could not be converted into the stimulatory effect by elevating the NaCl concentration, suggest that at high- and low-NaCl concentrations propranolol binds at two different sites on the protein.
Propranolol has both a stimulatory and an inhibitory effect on ryanodine binding. We asked whether propranolol interacts with the binding site of the local anaesthetic. A similar experiment between the inhibitor propranolol and stimulator lidocaine (at 0.2 M NaCl) showed that lidocaine did not disturb the ryanodine-binding inhibition by propranolol; on the contrary, it decreased the IC\textsubscript{so} value by 3-fold (Figure 8). It seems that lidocaine stabilizes a conformational state of the RyR that is more sensitive to propranolol. These results suggest that propranolol and local anaesthetics have different interacting sites on the RyR.

In conclusion, the different propranolol effects on ryanodine binding reflect different conformational states of the RyR. Propranolol interaction with the RyR, under low ionic strength, stabilizes a protein conformation that is no longer responsive to modulators of RyR activity. It was expected that in the presence of propranolol the general effects of NaCl concentration and pH on this highly negatively charged protein would be exerted. The results suggest that the major effect of propranolol is on the ryanodine-binding site where, although modulation of RyR conformation by the various effectors occurs, this is not reflected in RyR activity. Thus, propranolol could be used as a probe of RyR conformations.

REFERENCES


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