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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1. Propranolol, a β -blocker, inhibited or stimulated ryanodine binding to both the membrane-bound and purified ryanodine receptor (RyR) depending 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.

pranolol 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.

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–contraction coupling is the junctional Ca^{2+} release channel which binds the plant alkaloid ryanodine with nanomolar affinity [2].

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. Pro-

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 [3H]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. [3H]Ryanodine (60 Ci/mmol) was purchased from New England Nuclear, and unlabelled ryanodine from Calbiochem. Lidocaine was dissolved in methanol and then 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_4 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 $\mu\text{g}/\text{ml}$) 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 nM [^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, pro-

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 $\mu\text{g}/\text{ml}$) (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.

pranolol 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 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 two different experiments.

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

^3H 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 ^3H 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_{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.

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_{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- (μM) and high- (nM) affinity ryanodine-binding sites [15]. The 20% increase in B_{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, $k_{-1} = 0.01 \text{ min}^{-1}$). 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 than 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

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.

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 effects, according to the receptor conformation. To study the relationship between the RyR conformational state and the effects of propranolol, we examined the effect of propranolol on ryanodine binding in the presence of different pH values and ligands such as Ca^{2+} and ATP that are known to alter the conformational states of the protein [7].

Ryanodine binding to its receptor is strongly dependent on the incubation pH [7,16]. The effect of propranolol on ryanodine binding as a function of incubation pH at 0.2 M and 0.75 M NaCl is presented in Figure 4. The pH value for maximal ryanodine binding is strongly dependent on NaCl concentration; optimal pH values of 8.2 and 7.4 were obtained at 0.2 and at 0.75 M NaCl respectively. At 0.2 M NaCl, increasing the assay pH from 6.5 to 8.0 increased the binding by 4-fold. However, in the presence of propranolol the binding was constant between pH 7.0 and 8.5, while ryanodine-binding inhibition by propranolol was increased between pH 7.0 and 8.0 (Figure 4A). At

Figure 5 The effects of inhibiting and stimulating Ca^{2+} concentrations on ryanodine binding in the presence of propranolol

SR membranes (0.5 mg/ml) were assayed for [^3H]ryanodine binding at 0.2 M NaCl, in the absence (●) and in the presence (○) of 1.6 mM propranolol as described in the Materials and methods section, except that the free Ca^{2+} concentration was varied. Free Ca^{2+} concentration was controlled by EGTA and was calculated using a special computer program as described previously [26]. This is one of three similar experiments.

Table 1 Effect of nucleotides on the inhibition of ryanodine binding by propranolol

[^3H]Ryanodine binding was assayed as described in the Materials and methods section in the absence or presence of propranolol (1.2 mM) and the absence or presence of the indicated nucleotides (2 mM). Free calcium concentration was 50 μM in the absence and presence of the different nucleotides and was calculated as described previously [26]. The numbers in parentheses indicate percentages of stimulation of ryanodine binding by the nucleotides. The results are from a representative experiment of a total of three.

Nucleotide	Ryanodine bound (pmol/mg of protein)		Inhibition by propranolol (%)
	— Propranolol	+ Propranolol	
None	2.5 (100)	0.6	76.8
ATP	7.8 (312)	5.2	33.3
ADP	6.0 (240)	3.6	39.5
GTP	2.0 (80)	0.8	64.4

0.75 M NaCl, however, the same pH profile of ryanodine binding was obtained in the absence and in the presence of propranolol. Stimulation of ryanodine binding by propranolol was obtained in the pH range of 6.5 to 8.5. These results reflect the different conformations of the RyR that are stabilized by different NaCl concentrations and reflected in the pH-dependence and propranolol effects on ryanodine binding.

Ryanodine binds to its receptor in a strictly Ca^{2+} -dependent manner. Micromolar concentrations of Ca^{2+} are required for both ryanodine-binding Ca^{2+} release and channel opening, while millimolar concentrations of Ca^{2+} inhibit these activities [3,7]. The bell-shaped curve of ryanodine-binding dependence on Ca^{2+} is shown in Figure 5, with half-maximal stimulation and inhibition occurring at 0.13 μM and 622 μM respectively. In the presence of propranolol, ryanodine binding reached a constant value at about 1 μM and then was independent of the Ca^{2+} concentration used up to 3.0 mM.

These results strengthen the complexity of the effect of propranolol on the RyR at low NaCl concentrations, suggesting

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 ($36 \pm 6\%$ 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.

The opposite experiment, incubation of the RyR at a high NaCl concentration and then transfer to a low NaCl concentration, is experimentally impossible, since it is well established that ryanodine binds very tightly to the receptor [3,7].

Relationship between the local anaesthetics and propranolol-interacting sites

In a previous study we have shown a direct interaction between

Figure 6 Effect of propranolol on single Ca²⁺ release channel activity reconstituted in planar lipid bilayers

A single Ca²⁺ release channel from heavy SR vesicles was fused into a planar lipid bilayer in the presence of 250 mM CsCl, 10 mM Hepes, pH 7.4, 50 μ M CaCl₂ in the *cis* chamber and 50 mM CsCl, 10 mM Hepes, pH 7.4, 40 μ M CaCl₂ in the *trans* chamber. Single-channel reconstitution and recordings (at +60 mV holding potential) were carried out as described previously [14]. In (A), to the incorporated Ca²⁺ release channel (first trace) propranolol was added to the *cis* chamber to a final concentration of 0.2 and 0.4 mM (second and third trace), followed by the addition of 1.0 mM ATP (fourth trace). In (B), after the reconstitution of a single channel (first trace), 1.0 mM ATP was added (second trace), followed by 1.0 mM propranolol (third trace). Afterwards, the *cis* chamber was perfused for 3 min at 2.5 ml/min with identical solution with no propranolol (fourth trace), and then 1 mM propranolol was added (last trace). All the additions of heavy SR vesicles and the ATP and propranolol were to the *cis* chamber. Abbreviation: c-, indicates for each trace the closure state of the channel. (A) and (B) show representative traces of 10 to 20 similar ones and are representative results of three and two different experiments, respectively.

different local anaesthetics and the RyR/Ca²⁺ release channel [19]. The various local anaesthetics had different effects on ryanodine binding; some stimulated and others inhibited the

Figure 7 The inhibition of ryanodine binding by propranolol at 0.2 M NaCl is not recovered by increasing NaCl concentration to 0.75 M NaCl

SR membranes (0.5 mg/ml protein) were incubated with 20 nM ryanodine at the indicated NaCl concentration, and at 37 °C in the absence and presence of 1.6 mM propranolol, as described in the Materials and methods section. After 1 h of incubation, NaCl was added to a final concentration of 0.75 M NaCl, and 1 h later aliquots were assayed for bound ryanodine. This is one of three similar experiments.

binding of ryanodine to its receptor. Since propranolol exerted similar effects on ryanodine binding, we tested whether local anaesthetics and propranolol interact with the same site on the RyR. A competition experiment was performed between tetracaine (an inhibiting local anaesthetic) and propranolol at 1.0 M NaCl. Tetracaine decreased the stimulatory effect of propranolol but did not diminish it. The extent of ryanodine binding in the presence of both tetracaine and propranolol reflected partial inhibition or stimulation, and none of the drugs had a dominant effect (results not shown). Lineweaver–Burk analysis of the results was not conclusive with regard to a possible competition between the two drugs (results not shown).

The results of another competition experiment, between lidocaine (a stimulating drug) and propranolol at 0.2 M NaCl, show that propranolol also inhibits ryanodine binding in the presence of lidocaine with an IC₅₀ of 0.4 mM compared with an IC₅₀ of 1.2 mM in the absence of lidocaine (Figure 8A). It seems that, in the presence of lidocaine, propranolol is better recognized by the RyR and that the two drugs have different interacting sites. A complementary experiment in which the effect of lidocaine on ryanodine binding was tested in the presence of propranolol is presented in Figure 8(B). The results clearly show that the extent of ryanodine binding inhibition by 1.2 mM propranolol is increased with increasing lidocaine concentration from 30% inhibition in the absence of lidocaine to 90% inhibition in the presence of 1 mM lidocaine (Figure 8B, inset).

DISCUSSION

RyR interacts with many hydrophobic drugs including local anaesthetics [19,20], volatile anaesthetics [21,22], duxorobacin [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/Ca²⁺ 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-

Figure 8 Lidocaine-stimulated ryanodine binding at 0.2 M NaCl is inhibited by propranolol

SR membranes were assayed for [3 H]ryanodine binding at 0.2 M NaCl, in the presence of the indicated propranolol concentration and without (●) or with (○) 6 mM lidocaine (A), or in the presence of the indicated lidocaine concentrations and without (●) or with (○) 1.2 mM propranolol (B), as described in the Materials and methods section. This is one of four similar experiments.

fying the kinetic parameters of ryanodine binding, thereby increasing the $B_{\max}$ value. At low NaCl concentrations (< 0.3 M), propranolol inhibited the binding of ryanodine to both membrane-bound and purified RyR (Figure 1). The effect of propranolol on Ca^{2+} release from SR vesicles has been described, and was often explained by its non-specific interaction with the phospholipids [8]. In this study we clearly show that propranolol interacts specifically with the RyR and modifies its activities in both ryanodine-binding and single-channel activities. This conclusion is based on the following: (a) propranolol has similar effects on ryanodine binding to the membrane-bound and to the purified RyR, suggesting the existence of an interaction site(s) on the protein (Figure 1); (b) modulators of the RyR, such as pH, ATP, lidocaine and Ca^{2+} , alter the propranolol effects on both ryanodine-binding and single-channel activities (Figures 4–6, 8 and Table 1); (c) propranolol modifies the kinetics parameters of ryanodine binding (Figure 3); and (d) propranolol blocks the single-channel activity of RyR reconstituted into a bilayer, and ATP reactivates the channel to a subconductance state (Figure 6).

Propranolol at low NaCl concentrations inhibits both ryanodine binding and channel opening

Propranolol interaction with the RyR and the modulation of its interaction by ATP is demonstrated using single-channel activity. Propranolol blocks the channel opening completely and in a reversible manner (Figure 6A). Surprisingly, the addition of ATP either before or after adding propranolol (Table 1) reactivates the propranolol-modified channel to a subconductance state. This is in accord with the effect of ATP on ryanodine binding. ATP decreases, but does not completely prevent, the inhibition of ryanodine binding by propranolol (Table 1). It seems that ATP stabilizes the RyR in a conformation that is modified differently by propranolol. Ryanodine has been shown to lock the channel in a subconductance state [3,7]. Interestingly, propranolol stabilizes the RyR channel in a subconductance state only in the presence of ATP. These observations suggest that, in the presence of ATP, the interaction of propranolol with the RyR is reflected differently in channel activity and ryanodine-binding activities. These results strengthen the suggestion that propranolol effects are strongly dependent on the protein conformation.

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 and local anaesthetics have different interacting sites

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 pro-

pranolol has both a stimulatory and an inhibitory effect on ryanodine binding, we asked whether propranolol interacts with binding site of the local anaesthetic. The competition experiments performed between propranolol (under conditions in which it stimulates ryanodine binding) and the inhibitory tetracaine yielded no conclusive results. 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 IC₅₀ 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.

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