Activation and inhibition of the calcium gate of sarcoplasmic reticulum by high-affinity ryanodine binding

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The occupancy of high-affinity ryanodine-binding sites of isolated heavy sarcoplasmic reticulum vesicles occurring in concentrated salt solutions affects ATP-dependent calcium accumulation and caffeine-induced calcium release. The initial suppression of calcium uptake is followed by a marked uptake activation resulting in a reduction of the final calcium level in the medium. Simultaneously, caffeine-induced calcium release is blocked. The dependence of inhibition of calcium uptake and caffeine-induced calcium release observed in assay media containing physiological concentrations of magnesium and ATP on the concentration of ryanodine corresponds to the drug’s effectiveness in living muscles.

Sarcoplasmic reticulum; Ryanodine; Caffeine; Ca\(^{2+}\) uptake; Ca\(^{2+}\) release

1. INTRODUCTION

Indirect evidence has been provided in numerous studies indicating that ryanodine might interfere with muscle function as a result of its effect on the muscles’ sarcoplasmic reticulum calcium transport system [1–7]. This notion has been pursued although the drug concentrations which affect sarcoplasmic reticulum calcium transport in vitro are several orders of magnitude higher than those affecting living muscles [1,2,8,9]. A further complication in comparing the drug’s action in vivo and in vitro is the complexity of its effects. In vitro calcium uptake of and/or calcium release from isolated sarcoplasmic reticulum vesicles can be enhanced or reduced by ryanodine [2,4,6]. In vivo ryanodine might cause contractures or reduce forced development depending on the animal species and/or muscle type [10–12]. Part of the apparently controversial findings might be due to the selected experimental conditions. Thus, observations made in media containing neither magnesium nor ATP are difficult to relate to in vivo observations [1,6,7]. Most recently, in a series of papers starting with the report of Pessah et al. [13], ryanodine has been used successfully as a high-affinity label for protein components in the sarcoplasmic reticulum associated with structures supposedly involved in calcium release [13–16]. As an essential modification Pessah et al. [14] introduced the application of high salt concentrations to favour high-affinity ryanodine binding. The reported concentrations at which ryanodine is bound by the isolated membranes are similar to those which are effective in vivo. Here, it will be shown that by applying high salt concentrations ryanodine affects ATP-supported calcium uptake by isolated sarcoplasmic reticulum vesicles as well as the release of calcium from these preparations at concentrations of the drug comparable to those previously reported for living muscles.

2. MATERIALS AND METHODS

Heavy sarcoplasmic reticulum vesicles were prepared from predominantly white rabbit hind leg
muscles according to Hasselbach and Makinose [17] by treating 100 g minced muscle for 4 × 30 s in 400 ml extraction fluid containing 0.1 M KCl, 5 mM potassium phosphate (pH 7.0), 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride in a mixer at 4°C. Myofibrils were removed from the suspension by centrifugation in a Sorvall GS3 rotor at 5000 rpm for 30 min. The heavy vesicles were separated from the supernatant by subsequent centrifugation in a Spinco type 19 rotor at 10000 rpm for 30 min. Contaminating contractile proteins were extracted by treating the pellet with 10 vols of 0.6 M KCl, 0.3 M sucrose, 3.0 mM ATP, 3.0 mM MgCl₂, 0.1 mM benzethonium-Cl and 0.1 mM benzamidine-HCl, 0.1 μM dithiothreitol for 20 min. Subsequently the vesicles were sedimented by centrifugation in a Spinco rotor Ti 60 at 55 000 rpm for 45 min and resuspended in 0.1 M KCl (30–50 mg/ml).

Calcium uptake and release experiments were performed as described by Su and Hasselbach [18]. The standard medium contained 50 mM potassium gluconate, 50 mM KCl, 100 mM sucrose, 50 mM imidazole sulfate (pH 7.0), 2 mM ATP, 8 mM phosphoenolpyruvate, 0.04 mg/ml phosphoenolpyruvate kinase, 3.0 mM magnesium gluconate, 20 μM ⁴⁵CaCl₂ (~20 000 cpm/ml). Calcium uptake was started by adding 0.2 mg/ml vesicles to 15 ml of the assay solution. Aliquots were taken at appropriate time intervals and filtered through a glass nitrocellulose filter combination (Schleicher & Schuell, GF 9 glass filter and BA 85 nitrocellulose filter, 0.45 μm). Calcium release was initiated by adding 10 mM caffeine or 0.1 mM quercetin, final concentrations. ⁴⁵Ca was determined by liquid scintillation counting of the filtrate. Ryanodine treatment of the vesicles was performed at a protein concentration of 10 mg/ml in 0.6 M KCl, 10 mM imidazole sulfate (pH 7.0) at room temperature (20°C).

3. RESULTS

Increasing amounts of ryanodine are added to standard calcium uptake media, before the reaction is started by the addition of ATP; the final drug concentration in the assays must exceed 100 μM in order to affect calcium uptake. As shown in fig.1a, the initial rate of calcium uptake is considerably retarded by 300 μM ryanodine as compared to that in the control. Yet after approx. 5 min calcium uptake starts to be accelerated, surpassing control uptake and terminating at a calcium level significantly lower than that reached in the controls. Likewise, ryanodine concentrations higher than 100 μM are needed to suppress caffeine-induced calcium release. This blockage of calcium release by ryanodine does not occur when the drug is added 1–5 min before the releasing agent. Calcium release induced by 0.1 mM quercetin is abolished by ryanodine under the same experimental conditions as described for caffeine (not shown).

In agreement with the reported enhancement of ryanodine binding to sarcoplasmic reticulum preparations in the presence of high salt concentrations, incubation of the preparation with the drug in high salt media greatly increases the preparation's drug sensitivity. Fig.1b illustrates the same experimental arrangement as shown in fig.1a, except following an incubation of 10 mg/ml of the vesicular preparation with 1.0 μM ryanodine in 0.6 M KCl for 30 min. After dilution of the vesicular suspension 50-fold into the reaction medium to 0.2 mg protein/ml and nominally 0.02 μM ryanodine, calcium uptake and caffeine-induced
calcium release are inhibited as observed with 300 μM ryanodine at low salt concentrations: the initial inhibition of calcium uptake is followed by a significantly improved calcium concentrating ability of the preparation and the abolition of caffeine-induced calcium release. Since calcium uptake and release experiments were performed at low salt concentration and last 30 min, time-dependent change of the high salt induced drug binding could occur. We have therefore suspended the high salt pretreated preparation in low salt uptake media and started the reaction by adding ATP after 30 min. No change in the activity pattern could be detected following this treatment, largely excluding time-dependent dissociation of the agent from its receptor in the uptake release assay. Figs 2 and 3 show that the inhibition of the initial calcium uptake and of caffeine-induced calcium release exhibit a similar concentration dependence. When the vesicles were treated with ryanodine in the high salt medium, an incubation time of 30 min is just sufficient for 1 μM ryanodine to reduce calcium release induced by 10 mM caffeine completely. Both reactions, calcium uptake and caffeine-induced calcium release, are half-maximally affected at 0.15–0.2 μM ryanodine in the incubation medium. The abolition of caffeine-induced release indicates that the release competent vesicle fraction present in our preparations is completely inhibited by ryanodine. On the other hand, the uptake rate is maximally reduced by 70%. Calcium uptake of approx. 30% of the preparation is not affected by ryanodine or is ryanodine-insensitive.

4. DISCUSSION

Our results show that ryanodine can affect ATP-supported calcium uptake of and calcium release from isolated sarcoplasmic reticulum membranes at submicromolar concentrations. Thus, the drug interferes with the functions of the sarcoplasmic reticulum in the same concentration range as it affects muscle function in vivo [8,9,11].

The increase in effectiveness of the drug under conditions comparable to those prevailing in living muscles has been accomplished by treating the preparations with the drug at high salt concentra-
tions [14–16]. The reported dissociation constants [15,16] agree well with the concentration range where the drug affects calcium uptake and caffeine-induced calcium release. As observed previously, the drug appears to produce two opposing, subsequently occurring changes in the membrane properties [6]. The initial calcium uptake inhibition is followed by an uptake activation. A modulation of the calcium pump activity by ryanodine which could cause this activity pattern can largely be excluded since the calcium transport ATPase is not affected by the drug [1,4]. Hence the drug’s effects on the calcium permeability of the membranes should be responsible for the complex pattern of calcium uptake. At first, the permeability of the membrane must be increased by the drug. Subsequently, the apparent leak must be closed, resulting in diminished passive calcium efflux. In this state caffeine and quercetin have become ineffective as calcium-releasing agents. The two opposing effects presumably originate from the same drug-protein complex. Since a corresponding transition of the properties of the membranes is observed at low and high concentrations of the drug in the incubation medium it is unlikely that minor ryanodine derivatives, present in the preparation, might give rise to this complicated action profile [21]. It should be stressed that opposing permeability changes occur in the presence of physiological magnesium and ATP concentrations. In contrast, in the absence of ATP, millimolar concentrations of magnesium suppress the action of the drug on actively and passively loaded preparations [1,6,7].

The question as to how the transition from the high- to low-permeability state might occur during ATP-supported calcium uptake is difficult to approach. Similar biphasic reaction patterns have repeatedly been described [22–25], but no generally accepted explanation has been found. Another feature of the vesicular preparation which could be related to the observed biphasic uptake pattern is its inhomogeneity. All preparations contain two subpopulations of vesicles [5,6,18], since it is rather difficult to achieve a complete separation of the two populations. Both fractions can pump calcium, but only one is equipped with the calcium-release channels. The latter is unable to store calcium when its channels are open. If the state of the calcium-release channel is closed by a process depending only on time.

REFERENCES