Interaction between gallopamil and cardiac ryanodine receptors

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1 In a sarcoplasmic reticulum fraction obtained from rat hearts, the analysis of equilibrium [³H]-ryanodine binding showed high and low affinity sites ($K_D = 1.3$ nM and 2.8 μ M, $B_{max} = 2.2$ pmol mg⁻¹ and 27.8 pmol mg⁻¹). The dissociation rate constant increased at 1 μ M vs 4 nM [³H]-ryanodine concentration, and micromolar ryanodine slowed the dissociation of nanomolar ryanodine.

2 The binding of 4 nM [³H]-ryanodine was not affected by gallopamil, while the binding of 100 nM to 18 μ M [³H]-ryanodine was partly displaced. Data analysis suggested that gallopamil inhibited low affinity [³H]-ryanodine binding, with IC_{50} in the micromolar range.

3 Gallopamil decreased the dissociation rate constant of 1 μ M [³H]-ryanodine. While gallopamil alone did not affect the dissociation of 4 nM [³H]-ryanodine, gallopamil and micromolar ryanodine slowed it to a greater extent than micromolar ryanodine alone.

4 Our results are consistent with the hypothesis that the ryanodine receptor is a negatively cooperative oligomer, which undergoes a sequential alteration after ryanodine binding. Gallopamil has complex actions: it inhibits ryanodine binding to its low affinity site(s), and probably modulates the cooperativity of ryanodine binding and/or the transition to a receptor state characterized by slow ryanodine dissociation. These molecular actions could account for the previously reported effect of gallopamil on the sarcoplasmic reticulum calcium release channel.

Keywords: Gallopamil; calcium antagonists; heart; sarcoplasmic reticulum; ryanodine; calcium channels

Introduction

Calcium (Ca) antagonists are a group of drugs which are currently used in the treatment of angina pectoris, arterial hypertension and arrhythmias. These compounds are able to inhibit the slow inward Ca current in cardiac and smooth muscle cells, by acting on sarcolemmal L-type voltage channels (Nayler, 1988). However, intracellular targets of Ca antagonists have been described (Zernig, 1990). In particular, it has been suggested that some Ca antagonists may interfere with the sarcoplasmic reticulum (SR) Ca release channel, and reduce the probability of channel opening and/or channel conductivity (Hirata *et al.*, 1983; Fernandez-Belda & Gomez-Fernandez, 1987; Valdivia *et al.*, 1990; Ohkusa *et al.*, 1991; Zucchi *et al.*, 1992).

The SR Ca channel binds the alkaloid ryanodine (Ry) and has been identified as the Ry receptor (Inui *et al.*, 1987; Imagawa *et al.*, 1987). In the absence of Ry, the channel undergoes spontaneous openings and closures. Ry has a biphasic effect: at low concentrations it locks the channel in a low-conductivity configuration; at higher concentrations it determines channel blockade (Meissner, 1986). This biphasic action has been related to the presence of multiple Ry binding sites. Interaction with the high affinity site induces the formation of the low-conductivity state; interaction with the low affinity site(s) leads to channel inactivation (McGrew *et al.*, 1989; Pessah & Zimanyi, 1991). Comparative analysis of Ry binding and SR channel function after different interventions [changes in electrolyte and nucleotide concentrations, addition of anthraquinone drugs, phosphorylation by different kinases (Pessah *et al.*, 1985; Meissner & Henderson, 1987; Michalak *et al.*, 1988; Abramson *et al.*, 1988; Holmberg & Williams, 1990; Chu *et al.*, 1990; Takasago *et al.*, 1991; Mack *et al.*, 1992)], has shown that interventions which affect SR Ca efflux also affect Ry binding.

In this paper we investigated the effect of gallopamil (also

known as methoxyverapamil or D-600), a phenylalkylamine Ca antagonist, on Ry binding in rat cardiac SR.

Methods

Preparation of cardiac SR

Sprague-Dawley rats, fed with standard diet (275–300 g body weight), were anaesthetized with a mixture of ether and air. After injection of 1000 iu sodium heparin in the femoral vein, the heart was quickly excised and perfused retrogradely through the aorta at a perfusion pressure of 80 mmHg. The perfusion buffer was equilibrated with a mixture of oxygen (95%) and carbon dioxide (5%), and contained (mM): NaCl 118, NaHCO₃ 25, KCl 4.5, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, glucose 11. The temperature was kept between 37°C and 37.4°C, and the pH was 7.4.

After 10 min of perfusion, in order to allow complete washout of blood from the coronary vessels and stabilization of the contractile activity, the hearts were quickly removed from the perfusion apparatus and washed in isotonic saline. The ventricles were separated from the atria and great vessels, finely minced, and homogenized in five volumes of 300 mM sucrose and 10 mM imidazole (pH 7.0 at 4°C), by 15 + 15 passes in a Potter-Elvehjem homogenizer set at 800 r.p.m. and kept in a cold room at 4°C. The homogenates obtained from 3–4 hearts were pooled and used to prepare a microsomal fraction enriched in SR, according to the procedure described by Harigaya & Schwartz (1969), with minor modifications. The homogenate was centrifuged at 800 g for 10 min, in order to remove unfractionated cells and nuclei. The supernatant was then centrifuged at 8700 g for 20 min. The resulting supernatant was re-centrifuged at 8700 g for 20 min, yielding another supernatant fraction which was centrifuged at 40000 g for 30 min. The precipitate was resuspended in a glass homogenizer with 5 volumes of 600 mM KCl and 10 mM imidazole (pH 7.0 at 4°C). This suspension

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was centrifuged at 40000 g for 25 min to remove solubilized actomyosin, and the precipitate, representing the microsomal fraction enriched in SR, was resuspended in homogenization buffer.

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin used as standard.

Assay of [^3H]-Ry equilibrium binding

High affinity Ry binding was determined as described by Holmberg & Williams (1990), with minor changes. Unless otherwise specified, membranes (protein concentration $0.1\text{--}0.3\text{ mg ml}^{-1}$) were incubated at 37°C for 120 min in a buffered medium (final volume 0.5 ml) containing 25 mM imidazole (pH 7.4 at 37°C), 1 M KCl, $0.2\text{--}50\text{ nM}$ [^3H]-Ry (6 Ci mmol^{-1}), 0.950 mM EGTA, 1.013 mM CaCl_2 , and different concentrations of gallopamil where appropriate. Free Ca concentration was $20\text{ }\mu\text{M}$. In some experiments, the CaCl_2 concentration was adjusted in order to obtain free Ca concentrations ranging from $0.1\text{ }\mu\text{M}$ to 1 mM .

In pilot experiments we observed that [^3H]-Ry binding was not modified by the inclusion of protease inhibitors in the binding buffer ($5\text{ }\mu\text{M}$ leupeptin, $2\text{ }\mu\text{M}$ pepstatin, $0.25\text{ }\mu\text{M}$ aprotinin, $200\text{ }\mu\text{M}$ phenylmethyl sulphonyl fluoride, 2 mM iodoacetamide, and 2 mM benzamide). Therefore, in subsequent assays such inhibitors were usually omitted.

The binding reaction was terminated by filtration through cellulose nitrate filters with pores of $0.45\text{ }\mu\text{M}$ (Sartorius, Göttingen, Germany) presoaked in 25 mM imidazole (pH 7.4) and 1 M KCl (washing buffer). The filters were washed with $2 \times 5\text{ ml}$ aliquots of washing buffer, and shaken overnight in 8 ml of scintillation fluid (Optiphase II, LKB). Radioactivity was then measured at 50% efficiency in a LKB Wallac 1214 scintillation counter. Each vial was counted for 10 min. Incubations were performed in duplicate, and non-specific binding was determined in the presence of $10\text{ }\mu\text{M}$ Ry. The difference between the counts of duplicate samples was $<10\%$ in all cases.

In order to assess low affinity Ry binding, [^3H]-Ry was used at concentration ranging from $0.4\text{ }\mu\text{M}$ to $20\text{ }\mu\text{M}$ (120 Ci mol^{-1}), and non-specific binding was determined in the presence of 2 mM Ry. Filters were washed with $2 \times 5\text{ ml}$ aliquots of washing buffer and 5 ml of 10% (W/V) ethanol. Careful analysis of filter binding in the absence of added membranes was performed at each concentration of [^3H]-Ry. Since we observed that filter bound [^3H]-Ry was partly displaced by unlabelled Ry, specific binding was calculated as: ([^3H]-Ry bound in the presence of membranes – [^3H]-Ry bound in the presence of membranes and 2 mM unlabelled Ry) – ([^3H]-Ry bound in the absence of membranes – [^3H]-Ry bound in the absence of membranes and in the presence of 2 mM unlabelled Ry).

Measurement of association and dissociation kinetics

[^3H]-Ry association was determined by incubating rat SR vesicles (0.15 mg ml^{-1}) at 37°C with 4 nM or $1\text{ }\mu\text{M}$ [^3H]-Ry. The binding buffer was the same as described above. After appropriate incubation times ($1\text{--}360\text{ min}$), aliquots ($50\text{ }\mu\text{l}$) of reaction medium were filtered, washed, and counted as described above. The measurements were performed in the presence or absence of 2000 fold excess of unlabelled Ry, to measure non-specific binding, and/or different concentrations of gallopamil.

In dissociation studies, rat SR vesicles (1 mg ml^{-1}) were preincubated at 37°C for 120 min with 4 nM or $1\text{ }\mu\text{M}$ [^3H]-Ry in binding buffer. At the end of this period, $20\text{ }\mu\text{l}$ of the medium were filtered and counted to measure equilibrium binding. Dissociation was then initiated by adding 100 fold excess of Ry-free binding buffer. Aliquots (1 ml) were filtered at different time intervals (30 s to 360 min), and counted to assess residual binding. Where appropriate, unlabelled Ry

and/or gallopamil were included in the buffer used to induce dissociation. Correction for non-specific binding was performed by repeating each experiment after the inclusion of 2000 fold excess unlabelled Ry during the 120 min of preincubation.

Association and dissociation experiments were repeated in the absence of SR vesicles to correct for filter binding. Such correction was performed both for total and for non-specific binding, i.e. both in the absence and in the presence of unlabelled Ry.

Chemicals and radionuclides

[9,21- ^3H (N)]-ryanodine (60 Ci mmol^{-1}) was obtained from New England Nuclear-Du Pont (Milan, Italy), and diluted to its final specific activity (see below) with unlabelled Ry. Unlabelled Ry was purchased from Calbiochem (San Diego, CA, U.S.A.), as a mixture of Ry and didehydroryanodine, and its concentration was checked on the basis of the absorbance at 268 nm , using an extinction coefficient of $1.45 \times 10^4\text{ M}^{-1}$. Such extinction coefficient was calculated by the manufacturer for the specific lot of Ry used in these experiments. EGTA was obtained from Sigma (St. Louis, MO, U.S.A.) and its alleged purity was 97%. Gallopamil hydrochloride was obtained from Knoll AG (Ludwigshafen, Germany). Gallopamil was initially dissolved in ethanol at 50 mM concentration. Control experiments showed that the amount of ethanol so introduced (final concentration: 2% with 1 mM gallopamil, $<0.2\%$ in all other cases) did not affect [^3H]-Ry binding. All other reagents were of analytical grade.

Free Ca concentration was calculated according to Fabiato & Fabiato (1979), using a computer programme which included an empirical correction for the ionic strength of the buffer.

Data analysis

Equilibrium binding data were analyzed through the SCAFIT (LIGAND) programme (Munson, 1983). The analysis of kinetic experiments was performed as described by Weiland & Molinoff (1981). The observed association rate constant (K_{obs}) was calculated as the slope of the pseudo-first order plot of $\ln[B_e/(B_e - B)]$ vs time, where B_e is the amount of radioligand bound at equilibrium. The pseudo-first order association rate constant (K_1) was calculated from the equation $K_{\text{obs}} = K_1(L \times B_{\text{max}}/B_e)$, where L is the concentration of free radioligand and B_{max} is the concentration of binding sites, derived from equilibrium studies. The dissociation rate constant (K_{-1}) was calculated as the slope of the plot of $\ln(B/B_0)$ vs time, where B_0 is the amount of radioligand bound at time zero. Regression analysis and the comparison of regression coefficients were performed as described by Armitage (1971).

Dissociation curves were also interpolated by a double exponential (two site model), i.e. by the equation $B/B_0 = \alpha e^{-Kt} + (1-\alpha) e^{-K't}$, where K and K' are the dissociation rate constants for the two sites, while α and $(1-\alpha)$ are their fractional densities. To assess whether the double exponential model provided a significantly better fit than the single exponential model, we used the 'extra sum of squares' principle (Draper & Smith, 1981; Munson, 1983).

Unless otherwise specified, when experiments were performed in duplicate, the difference between individual measurements was $<10\%$.

Results

Equilibrium studies: characterization of Ry binding

The Scatchard plots derived from [^3H]-Ry saturation binding curves in the high ($0.2\text{--}50\text{ nM}$) and low ($0.4\text{--}20\text{ }\mu\text{M}$) affinity

Figure 1 Scatchard plots of [^3H]-ryanodine (^3H -Ry) saturation binding determined at $\text{pCa} = 4.7$ (free $\text{Ca} = 20\ \mu\text{M}$). Data represent specific binding. [^3H]-Ry was used at concentrations ranging from 0.2 nM to $20\ \mu\text{M}$. See Methods for further details. (a) Overall view; (b) the nanomolar, (^3H -Ry = 0.2–50 nM) and (c) the micromolar (^3H -Ry = 0.4– $20\ \mu\text{M}$) portions of the plot are reproduced on different scales. Data analysis gave the following estimates of the binding parameters: $K_{D1} = 1.3\ \text{nM}$, $K_{D2} = 2.3\ \mu\text{M}$, $B_{\text{max}1} = 2.3\ \text{pmol mg}^{-1}$, $B_{\text{max}2} = 32.4\ \text{pmol mg}^{-1}$. Note that in this experiment the incubation time was 60 min, and on the basis of the results of our association experiments (see below) binding may have been underestimated by 5–10%.

range are shown in Figure 1. Displacement curves of 4 nM (Figure 2a) and $1\ \mu\text{M}$ [^3H]-Ry (Figure 2b) by unlabelled Ry are shown. In the nanomolar range, specific binding was >85% of total binding; about two thirds of non-specific binding were due to filter binding, and filter-bound radioactivity was not displaced by unlabelled Ry (displacement was <2%). In the micromolar range, specific binding was <50% of total binding (it was as low as 20–25% of total

Figure 2 Displacement of 4 nM (a) and $1\ \mu\text{M}$ (b) [^3H]-ryanodine (^3H -Ry) by unlabelled Ry. Binding represents specific binding and is expressed as percentage of equilibrium binding. Data are the mean of two different experiments. Data analysis by the SCAFIT (LIGAND) programme identified two sites for [^3H]-Ry, characterized by $K_{D1} = 2.8\ \text{nM}$, $K_{D2} = 3.0\ \mu\text{M}$, $B_{\text{max}1} = 1.7\ \text{pmol mg}^{-1}$, $B_{\text{max}2} = 28.0\ \text{pmol mg}^{-1}$. The Hill numbers for the two sites were 0.96 and 0.36 respectively.

binding in the high micromolar range), and filter binding accounted for up to 40% of total binding. Both saturation and displacement experiments were best fitted by a two site model ($P < 0.001$ vs the single site model). The combined analysis of equilibrium and displacement data by the SCAFIT (LIGAND) programme gave the following estimates: $K_{D1} = 1.3 \pm 0.1\ \text{nM}$, $K_{D2} = 2.8 \pm 0.5\ \mu\text{M}$, $B_{\text{max}1} = 2.2 \pm 0.1\ \text{pmol mg}^{-1}$, $B_{\text{max}2} = 27.8 \pm 3.2\ \text{pmol mg}^{-1}$. Models including more than 2 binding sites provided no further improvement in data fitting. The Hill number of high affinity binding was close to 1 (0.96 ± 0.05), while the Hill number of low affinity binding was significantly < 1 (0.35 ± 0.02).

High affinity [^3H]-Ry binding was Ca-dependent: it was negligible at $\text{pCa} \geq 7$, reached a maximum at $\text{pCa} = 4.7$ and decreased slightly at $3 \leq \text{pCa} \leq 4$. No Ca-dependence was observed for low affinity binding. The latter was not affected by changes in Mg concentration (0.5 to 50 mM) or by the non-hydrolysable ATP analogue, β - γ methylene ATP in the range of 0 to 10 mM (data not shown).

Equilibrium studies: effect of gallopamil

Gallopamil had no major effect on 4 nM [^3H]-Ry binding (Figure 3, triangles). This result was obtained at two different free Ca concentrations ($\text{pCa} = 6$ and 4.7). On the contrary, the binding of 100 nM to $3\ \mu\text{M}$ [^3H]-Ry was displaced by gallopamil. The maximum displacement ranged from 40 to 65% (Figure 3a). The residual binding decreased at increas-

Figure 3 Effect of gallopamil on $[^3\text{H}]$ -ryanodine ($[^3\text{H}]$ -Ry) binding. The experiments were performed with $p\text{Ca} = 4.7$ at the following $[^3\text{H}]$ -Ry concentrations: 4 nM (▲), 100 nM (●), 500 nM (■), 1 μM (○), 3 μM (□), 18 μM (◆). The effect of gallopamil on 4 nM $[^3\text{H}]$ -Ry binding was also determined at $p\text{Ca} = 6$, and no displacement was produced (data not shown). Each curve represents the mean of two different experiments, while the point obtained with 18 μM $[^3\text{H}]$ -Ry was derived from a single experiment: (a) represents total specific binding, and (b) represents low affinity binding, i.e. residual binding after subtraction of the high affinity component, as estimated on the basis of saturation binding experiments. IC_{50} values were: 1.4 μM, 15.9 μM, 5.0 μM, and 4.3 μM for the displacement of 100 nM, 500 nM, 1 μM, and 3 μM $[^3\text{H}]$ -Ry respectively. The corresponding Hill number values were: 0.67, 0.27, 0.35, and 0.29 respectively.

ing $[^3\text{H}]$ -Ry concentrations and is likely to represent, at least in part, the high affinity component of $[^3\text{H}]$ -Ry binding. This interpretation is supported by the observation that, in an additional experiment, 100 μM gallopamil produced 82% displacement of 18 μM $[^3\text{H}]$ -Ry. In Figure 3b, displacement data were plotted after subtraction of high affinity $[^3\text{H}]$ -Ry binding. Data analysis yielded a Hill number < 1 , and an IC_{50} in the low micromolar range. There was no clear linear relationship between IC_{50} and $[^3\text{H}]$ -Ry concentration, so that no $K_{0.5}$ value could be calculated (Weiland & Molinoff, 1981).

Kinetic studies

The association and dissociation of 4 nM $[^3\text{H}]$ -Ry are shown in Figure 4 and 5. The analysis of association data (Figure 4, ●) yielded $K_{\text{obs}} = 0.0393 \text{ min}^{-1}$ and $K_1 = 0.00716 \text{ min}^{-1} \text{ nM}^{-1}$, while the K_{-1} derived from the analysis of Ry dissociation (Figure 5, ●) averaged 0.00978 min^{-1} . The kinetic K_D , calculated as K_{-1}/K_1 , averaged 1.36 nM, in good agreement with the equilibrium K_D obtained from the Scatchard analysis. Gallopamil (100 μM) did not affect either the

Figure 4 Association of 4 nM $[^3\text{H}]$ -ryanodine ($[^3\text{H}]$ -Ry): pseudo-first order plot. (●) Control conditions; (○) results obtained after the addition of 100 μM gallopamil to the association buffer. The data were best fitted by single exponential, and the average values of the K_{obs} and K_1 were $0.0393 \pm 0.0019 \text{ min}^{-1}$ and $0.00716 \pm 0.0004 \text{ min}^{-1} \text{ nM}^{-1}$ respectively, without significant changes in the presence of gallopamil.

association or the dissociation of 4 nM $[^3\text{H}]$ -Ry (see Figures 4 and 5, ○).

As previously reported (McGrew *et al.*, 1989; Chu *et al.*, 1990; Pessah & Zimanyi, 1991), in the presence of 10 μM unlabelled Ry the dissociation of 4 nM $[^3\text{H}]$ -Ry became biphasic, and a slow component ($K_{-1} = 0.00164 \text{ min}^{-1}$) appeared after a lag of about 60 min (Figure 5, ■). This effect of micromolar unlabelled Ry persisted in the presence of gallopamil, and the K_{-1} of the slow component was further reduced in the presence of gallopamil (Figure 5, ▲), although dose-effect studies showed (Figure 6) that a significant action required high gallopamil concentration (100 μM).

The association and dissociation of 1 μM $[^3\text{H}]$ -Ry are shown in Figure 7. Association data were best interpolated by a single exponential (Figure 7a), with $K_{\text{obs}} = 0.0297 \text{ min}^{-1}$. Owing to the existence of binding cooperativity and of two-step ligand-receptor interactions (see below), it was impossible to obtain a reliable estimate of the K_1 (Weiland & Molinoff, 1981).

Dissociation was not complete, and residual binding averaging 20–25% of total binding persisted even after 4 h. Such residual binding represented high affinity Ry binding, since the 100 fold dilution used to induce dissociation left a residual $[^3\text{H}]$ -Ry concentration of 10 nM. Single-exponential analysis of the data after subtraction of the residual binding yielded a K_{-1} of 0.155 min^{-1} . If the dissociation was followed with high temporal resolution in the first few minutes (up to 8 experimental points within the first 3 min) a very fast component could be identified (Figure 7b, ●), since a significantly better fit ($P < 0.01$) was obtained with a double exponential model, whose dissociation rate constants averaged 2.267 min^{-1} and 0.132 min^{-1} . The calculated site ratio was close to 1 (1:1.38).

In the presence of gallopamil, the dissociation of 1 μM $[^3\text{H}]$ -Ry was delayed, although the residual binding was virtually unchanged. A plot of single-exponential K_{-1} versus gallopamil concentration is shown in Figure 8. The K_{-1} was significantly reduced at gallopamil concentration $\geq 1 \text{ μM}$, and the effect was clearly dose-dependent. In an additional experiment (Figure 7b, ○), the dissociation of 1 μM $[^3\text{H}]$ -Ry was followed at high temporal resolution in the presence of a saturating concentration of gallopamil (100 μM). Two components could still be observed in the dissociation curve ($P < 0.01$ for the double-exponential versus the single-expo-

Figure 5 (a) Dissociation of 4 nM [^3H]-ryanodine ([^3H]-Ry): first order plot. Each point is the mean of measurements performed in two different preparations: (●) control conditions, i.e. dissociation was induced by 100 fold dilution with Ry-free binding buffer; (○) data obtained after the addition of 100 μM gallopamil to the dissociation buffer; (■) dissociation was induced by 100 fold dilution with binding buffer containing 10 μM unlabelled Ry; (▲) dissociation was induced by 100 fold dilution with binding buffer containing 10 μM unlabelled Ry and 100 μM gallopamil. The calculated values of the K_{-1} were $0.00978 \pm 0.00055 \text{ min}^{-1}$ in control conditions, and $0.00954 \pm 0.00039 \text{ min}^{-1}$ in the presence of 100 μM gallopamil. In the presence of 10 μM Ry a slow component appeared ($K_{-1} = 0.00164 \pm 0.00011 \text{ min}^{-1}$), and when 100 μM gallopamil was added to 10 μM Ry the K_{-1} of this component decreased to $0.000898 \pm 0.00012 \text{ min}^{-1}$. Part of the figure is reproduced on a different scale in (b), in order to show the different slope observed with 10 μM Ry vs 10 μM Ry and 100 μM gallopamil.

nential fit), and both dissociation constants were reduced by gallopamil (1.660 min^{-1} and 0.047 min^{-1}).

Discussion

Several lines of evidence suggest that phenylalkylamine Ca antagonists may interfere with SR Ca channels. In skeletal muscle, gallopamil inhibited excitation-contraction coupling after conditioning K^+ contracture, and this action was independent from sarcolemmal Ca channel blockade (Eisenberg *et al.*, 1983; Berwe *et al.*, 1987). Inhibition of SR Ca release by verapamil was observed in skeletal muscle SR (Fernandez-Belda & Gomez-Fernandez, 1987; Valdivia *et al.*, 1990). In heart muscle, we observed that gallopamil increased SR Ca uptake (Zucchi *et al.*, 1992); this action was not additive with the stimulation produced by 300 μM Ry, and

Figure 6 Effect of gallopamil on the slow component of ryanodine (Ry) dissociation from its high affinity site ([^3H]-Ry concentration = 4 nM). The K_{-1} of the slow component of the dissociation (see Figure 5) is plotted vs gallopamil concentration: (●) control condition, i.e. dissociation performed in the presence of 10 μM unlabelled Ry; (○) dissociation performed in the presence of 10 μM unlabelled and 1 to 100 μM gallopamil. S.e.mean was calculated by regression analysis (Armitage, 1971). * $P < 0.01$ vs control.

Figure 7 (a) Pseudo-first order plot of 1 μM [^3H]-ryanodine ([^3H]-Ry) association. The K_{obs} averaged $0.0297 \pm 0.0016 \text{ min}^{-1}$. (b) First order plot of 1 μM [^3H]-Ry dissociation. Each point is the mean of measurements performed in two different preparations: (●) control conditions; (○) data obtained after the addition of 100 μM gallopamil to the dissociation buffer. Single exponential analysis yielded $K_{-1} = 0.155 \text{ min}^{-1}$ in control conditions, and $K_{-1} = 0.057 \text{ min}^{-1}$ in the presence of gallopamil. A significantly better fit was obtained through a double exponential model, in which dissociation rate constants averaged: 2.267 min^{-1} and 0.132 min^{-1} in control conditions; 1.660 min^{-1} and 0.047 min^{-1} in the presence of gallopamil.

Figure 8 Effect of gallopamil on the dissociation of ryanodine (Ry) from its low affinity site. The K_{-1} of $1\mu\text{M}$ [^3H]-Ry dissociation is plotted vs gallopamil concentration: (●) control conditions; (○) dissociation performed in the presence of 0.3 to $100\mu\text{M}$ gallopamil. The K_{-1} was calculated by single exponential analysis. These experiments were not projected to identify the very fast component of $1\mu\text{M}$ [^3H]-Ry dissociation, which required high temporal resolution. High resolution data were obtained only in control conditions and in the presence of $100\mu\text{M}$ gallopamil, and are shown in Figure 7. S.e.mean was calculated by regression analysis (Armitage, 1971). * $P < 0.05$, ** $P < 0.01$ vs control.

Ca-ATPase activity was not affected, suggesting that the target of gallopamil was the Ca release channel.

The SR Ca channel corresponds to the Ry receptor (Inui *et al.*, 1987; Imagawa *et al.*, 1987), and pharmacological interventions able to modify channel function affect Ry binding (Pessah *et al.*, 1985; Meissner & Henderson, 1987; Michalak *et al.*, 1988; Abramson *et al.*, 1988; Holmberg & Williams, 1990; Chu *et al.*, 1990; Takasago *et al.*, 1991; Mack *et al.*, 1992). For these reasons we determined the effect of gallopamil on Ry binding in cardiac SR.

The standard model of the Ry receptor assumes that it includes 4 negatively cooperative oligomers, and that it undergoes a sequential alteration as a consequence of Ry binding. From the functional point of view, it is thought that Ry initially binds to the open state of the channel, and that the sequential alteration corresponds to channel blockade (Pessah & Zimanyi, 1991; Buck *et al.*, 1992; Zimanyi *et al.*, 1992).

In the equilibrium studies, we obtained a curvilinear Scatchard plot, and identified two apparent binding sites, with K_D in the order of 1 nM and $2\mu\text{M}$ respectively. The Hill number calculated from displacement studies was close to 1 for the high affinity site, and <1 for the low affinity site. These findings are in agreement with previous reports (Inui *et al.*, 1987; 1988; Lai *et al.*, 1989; McGrew *et al.*, 1989; Pessah & Zimanyi, 1991), and are consistent with negative allosteric coupling between identical binding sites. As already reported by Lai *et al.* (1989) in the skeletal muscle, Ca^{2+} concentration appeared to affect high but not low affinity binding.

Pessah & Zimanyi (1991) were able to detect 4 different binding sites. In our experiments no improvement in data fitting was obtained with models including more than two binding sites. However, the existence of additional sites with intermediate affinity cannot be excluded, since we could determine only a limited number of binding points, which were selected to identify the highest and lowest affinity sites. It has been argued that up to 50 binding points may be necessary for a reliable comparison of two and three site models (Molino *et al.*, 1981). The biexponential character of $1\mu\text{M}$ Ry dissociation and the low Hill number calculated

from $1\mu\text{M}$ Ry displacement actually suggest the existence of at least two low affinity sites.

Although the tetramer model of the Ry receptor predicts a value of 3 for the ratio of low to high affinity sites (Lai *et al.*, 1988; 1989), values in the range of 10 to 50 were obtained in this study as well as by most other investigators, both in heart (Inui *et al.*, 1987; 1988; McGrew *et al.*, 1989; Pessah & Zimanyi, 1991) and in skeletal muscle (McGrew *et al.*, 1989; Pessah & Zimanyi, 1991; Buck *et al.*, 1992). It must be stressed that the accuracy of any estimate of low affinity site density is rather low, owing to the technical problems related to the binding assay.

Negative cooperativity and/or the existence of a two-step reaction can account for the increase in K_{-1} and the decrease in K_{obs} observed with micromolar vs nanomolar Ry. In particular, negative cooperativity is strongly suggested by the biexponential nature of the dissociation of $1\mu\text{M}$ Ry, both dissociation constants being higher than the K_{-1} obtained with 4 nM Ry. On the other side, a two-step reaction mechanism is necessary to explain the appearance of a slow component in the dissociation of 4 nM Ry when micromolar Ry was included in the dissociation buffer (Pessah & Zimanyi, 1991; Zimanyi *et al.*, 1992).

The kinetic constants which we obtained with 4 nM Ry are in close agreement with those reported in heart tissue by McGrew *et al.* (1989) and by Pessah & Zimanyi (1991), who used 9 nM and 1 nM Ry respectively. The K_{-1} and K_{obs} measured with $1\mu\text{M}$ Ry are much different (respectively 55 times higher and 20 times lower) from the values determined by Pessah & Zimanyi in the skeletal muscle, although values of K_{obs} close to ours were obtained by Buck *et al.* (1992). This discrepancy might be due to differences in the sequential alteration of the Ry receptor between skeletal muscle and cardiac tissue. In particular, Pessah & Zimanyi obtained a lower K_{-1} with $1\mu\text{M}$ vs 50 nM Ry, contrary to the predictions of their negative cooperative model, and speculated that under their experimental conditions (which included 3 h of incubation) sequential alteration of the Ry receptor might have occurred with $1\mu\text{M}$ Ry.

Gallopamil did not affect high affinity Ry binding to cardiac SR, consistent with previous results (Pessah *et al.*, 1985). Valdivia *et al.* (1990) reported that in skeletal muscle SR, verapamil was able to displace 5 nM Ry. However, significant displacement was observed only under peculiar conditions, i.e. alkaline pH (8.5) and ionic strength much lower than required for optimal binding (150 mM NaCl). The pH of the buffer was critical, and at neutral pH Ry displacement by gallopamil did not exceed 20%.

The effect of Ca antagonists on low affinity Ry binding had never been investigated. Our results showed that gallopamil displaced Ry from its low affinity site(s). The Hill number calculated from the displacement experiments was <1 , and no clear linear relationship was observed between [^3H]-Ry concentration and IC_{50} , suggesting that the action of gallopamil is complex, and cannot be accounted for by simple competitive inhibition.

Kinetic studies showed that the dissociation rate constants of $1\mu\text{M}$ Ry decreased in the presence of gallopamil, while the dissociation of 4 nM Ry was not affected. It is not easy to understand how gallopamil can displace Ry and yet slow its dissociation. A possible interpretation holds that the negative cooperativity of Ry binding may be reduced in the presence of gallopamil. However, the action of gallopamil must be more complex, since we also observed that gallopamil and $10\mu\text{M}$ Ry were more effective than $10\mu\text{M}$ Ry alone in introducing a slow component in the dissociation of 4 nM Ry, suggesting that gallopamil might also influence the transition to a receptor state characterized by slow Ry dissociation.

In summary, the interaction of gallopamil with the Ry receptor might produce multiple effects: (a) partial displacement of Ry from its low affinity site(s); (b) modulation of the cooperativity of Ry binding and of the sequential alteration of the receptor produced by Ry binding.

The precise site of action of gallopamil remains to be determined. Gallopamil might interact directly with the Ry receptor (Valdivia *et al.*, 1990), but it cannot be excluded that its effect may be mediated by proteins closely coupled to the receptor, such as the FK605 binding protein (Jayaraman *et al.*, 1992), or even the dihydropyridine receptor itself (Block *et al.*, 1988). Evidence of an interaction between Ry and the dihydropyridine receptor was reported by Valdivia & Coronado (1989), who observed that dihydropyridine binding to skeletal muscle membranes was displaced by high concentrations of Ry ($IC_{50} = 95 \mu M$).

In any case, it seems likely that this is the molecular basis for the pharmacological actions which have been observed in cardiac and skeletal muscle SR, i.e. inhibition of SR Ca release (Fernandez-Belda & Gomez-Fernandez, 1987; Valdivia *et al.*, 1990; Zucchi *et al.*, 1992). The comparison of dose-effect relationships obtained in binding and Ca-flux studies is not easy. In the present work, the half-effective gallopamil concentration, as calculated from equilibrium and dissociation experiments, was in the micromolar range. In the Ca-flux studies, phenylalkylamines were found to be effective at concentrations ranging from 10^{-7} to 10^{-5} M, which could be consistent with the binding data, but the dose-response relationship was bell-shaped. In particular, in bovine cardiac SR the maximum effect was observed with $0.1 \mu M$ gallopamil and concentrations $> 1 \mu M$ were ineffective (Zucchi *et al.*, 1992), while in frog skeletal muscle Berwe *et al.* (1987) reported that micromolar gallopamil inhibited excitation-

contraction coupling, but $250 \mu M$ gallopamil had the opposite effect.

Our results give no hint to explain such findings. It should be remembered that, in general, the pharmacological action of a channel modulator cannot be predicted on the basis of its effect on ligand binding. As a tentative interpretation of the above results, we might speculate that low concentrations of gallopamil stabilize an inactive state of the SR channel, while higher concentrations stabilize the active state as well (Berwe *et al.*, 1987). However, the evaluation of this hypothesis requires further investigation.

The mechanism of action of gallopamil is different from that of the SR channel blockers ruthenium red, neomycin or FLA 365. While all these substances inhibit high affinity Ry binding (Mack *et al.*, 1992), gallopamil displaced Ry only from its low affinity sites(s).

Since gallopamil can cross the sarcolemma (Hescheler *et al.*, 1982) and displaces Ry at concentrations which lie within the range that can be achieved in human plasma (Nayler, 1988), its action on the Ry receptor might have clinical importance. In particular, it is though that Ca release from the SR is a crucial issue in the pathogenesis of irreversible myocardial injury during ischaemia and reperfusion. Drugs able to interfere with this structure might therefore open new possibilities in the treatment of ischaemic heart disease.

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