Volume 244, number 2, 333–337 FEB 06836 February 1989

Inhibition of dihydropyridine-sensitive calcium channels by the plant alkaloid ryanodine

Hector H. Valdivia and Roberto Coronado

Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030, USA

Received 14 December 1988

At micromolar concentrations, ryanodine interacts with the dihydropyridine receptor of rabbit skeletal muscle transverse tubules. Ryanodine displaces specifically bound [^{3}H]PN200-110 with an apparent inhibition constant of approx. 95 μM and inhibits dihydropyridine-sensitive calcium channels in the same preparation with an IC_{50} of approx. 45 μM. These concentrations of ryanodine are approximately three orders of magnitude higher than those required to saturate binding of the alkaloid to the ryanodine receptor of sarcoplasmic reticulum and to open the calcium release channel of sarcoplasmic reticulum (i.e. 20 nM (1988) J. Gen. Physiol. 92, 1–26). Thus at sufficiently high dose, ryanodine may affect SR as well as plasma membrane Ca permeabilities.

Dihydropyridine receptor; Ca^{2+} channel inhibition; Ryanodine

1. INTRODUCTION

The muscle-paralysing alkaloid ryanodine [1] binds with nanomolar affinity to a 450 kDa protein of the sarcoplasmic reticulum (SR) membrane of skeletal muscle, i.e. the ryanodine receptor [2]. In the SR membrane, the ryanodine receptor forms a channel that is thought to control the Ca permeability of the SR membrane during excitation-contraction coupling [2]. Ryanodine in the nanomolar range (20 nM), opens the Ca release channel of SR [3] and this effect explains the increase in ^{45}Ca efflux rate reported in vesicular preparations of SR [4,5] treated with the alkaloid. However in many studies in intact and fragmented preparations, micromolar instead of nanomolar levels of ryanodine are used, mainly to reduce the time of onset of the physiological effect [6–8]. Evidently, the association rate of ryanodine to its receptor is exceedingly slow, on the time scale of several hours at concentrations commensurable with the radioligand binding affinity [9]. We show that ryanodine at micromolar concentrations interferes with dihydropyridine (DHP) receptors of the transverse tubular membrane of rabbit skeletal muscle. The alkaloid displaces the dihydropyridine [^{3}H]PN200-110 from the DHP receptor and inhibits opening of DHP-sensitive calcium channels activated by the DHP agonist BAY K 8644 [10]. At sufficiently high concentration, ryanodine may thus produce two opposite effects. It would decrease the calcium permeability of the plasma membrane and it would increase the calcium permeability of the SR membrane.

Correspondence address: R. Coronado, Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030, USA

2. MATERIALS AND METHODS

2.1. *Transverse tubules and planar bilayer recording*

T-tubule vesicles were prepared from rabbit back and leg white muscle as described [10]. Light muscle microsomes sedimenting at the 10/20% sucrose interface were used in all experiments. T-tubules were resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Na-Pipes, pH 6.8, and frozen in liquid N_2 until use. Lipid bilayers were cast from an equimolar mixture of phosphatidylethanolamine and phosphatidylserine dissolved in decane at a concentration of 20 mg lipid/ml. Lipid solution was spread across a 300 μm diameter polystyrene aperture separating two aqueous chambers designated *cis* and *trans*. The volume of each chamber was 3.0 ml and 3.5 ml, respectively. T-tubule vesicles (10–50 μg) were added to the *cis* solution as

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

described in detail elsewhere [10]. *Cis* and *trans* solutions were: *cis* (internal), 100 mM $BaCl_2$, 50 mM NaCl, 10 mM Hepes-Tris, pH 7.0; *trans* (external), 50 mM NaCl, 10 mM Hepes-Tris, pH 7.0.

2.2. *Radioligand-binding assays*

Samples of 15–40 μg protein/ml were incubated 15 min at room temperature and 60 min at 36°C in 1 ml of 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, and the required concentration of (+)-[*methyl*-^{3}H]PN200-110 (0.05–7 nM). Specific binding was defined as the amount of radiolabel that could be displaced competitively by 1 μM cold PN200-110 or nitrendipine; [^{3}H]PN200-110 was the last reagent added. For determination of the total amount of [^{3}H]PN200-110, a small aliquot (20 μl) was removed before filtration. Binding was terminated by rapid filtration on Whatman GF/B or GF/F glass fiber filters. Filters were washed twice with 5 ml of an ice-cold solution containing 20 mM Tris-HCl, pH 7.2, and 200 mM choline chloride. Nonspecific binding to filters was negligible and independent of the presence of unlabeled ligand in the incubation medium. Radioactivity was measured in 6 ml of a Beckman HP/b scintillant on a Beckman LS 3801 scintillation counter. All experiments were performed under dim light to avoid photolysis. Inhibition of [^{3}H]PN200-110 binding by ryanodine was carried out using 0.2 nM [^{3}H]PN200-110 and 20 μg/ml t-tubule protein. Receptor occupancy by the radiolabel was 20–30%. Protein concentration was determined by the Lowry method using bovine serum albumin as standard.

2.3. *Chemicals*

Phosphatidylethanolamine and phosphatidylserine were from Avanti Polar Lipids (Birmingham, AL). *n*-Decane was purchased from Aldrich (Milwaukee, WI). (+)-[*methyl*-^{3}H]PN200-110 (71 Ci/mmol) was from New England Nuclear (Boston, MA). BAY K 8644 was a gift from Dr Scriabine at Miles Institute, New Haven, CT. Ryanodine was purchased from Calbiochem (La Jolla, CA).

3. RESULTS AND DISCUSSION

T-tubules purified from rabbit skeletal muscle are an abundant source of dihydropyridine receptors for electrophysiological [10–13], biochemical [14,15], and molecular [16,17] studies. In this membrane preparation we have implemented single-channel recording of Ca channels using the planar bilayer technique [13]. Fig.1 (control) shows dihydropyridine-sensitive Ca channels of rabbit t-tubules activated by the DHP agonist BAY K 8644 (1 μM). The current carrier is 100 mM Ba^{2+} and the membrane potential is 0 mV. We have previously shown that once activated by agonist, three types of Ca antagonists namely dihydropyridines, phenylalkylamines, and benzothiazepines, inhibit channel activity at doses similar to those reported in vivo [12]. At the concentrations indicated in each set of traces in fig.1, ryanodine

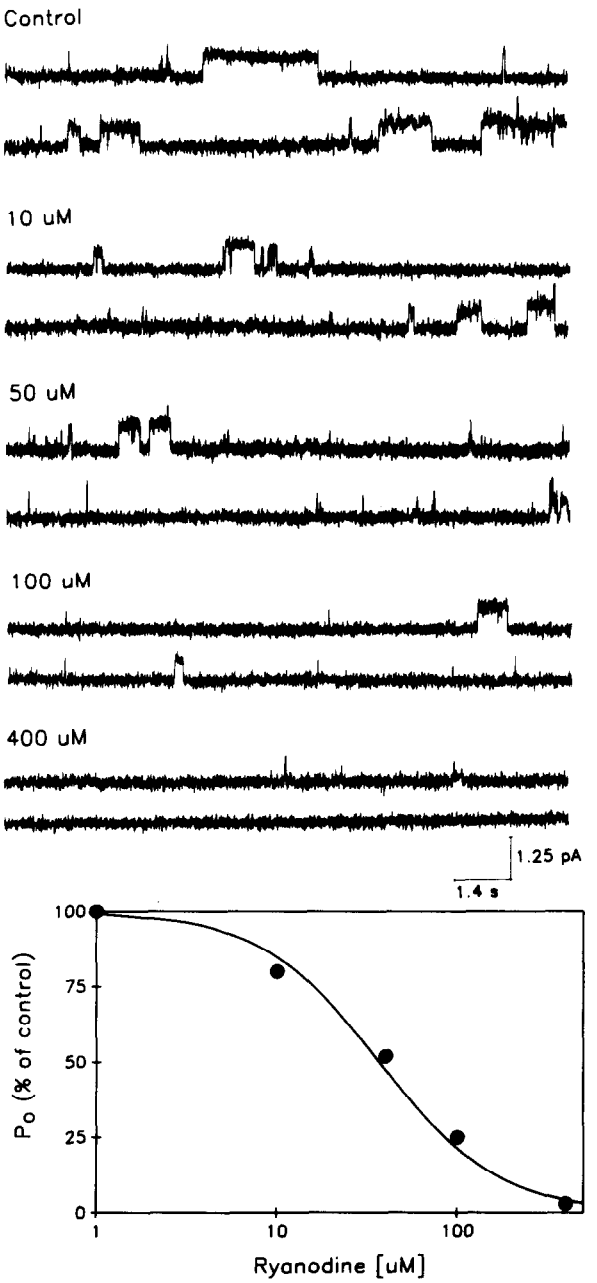

Fig.1. Ryanodine blockade of dihydropyridine-sensitive calcium channels of skeletal muscle. Channels in 'control' were activated by 1 μM BAY K 8644 to the *cis* compartment [10]. Traces below control are representative of 140 s of channel recording at the indicated concentration of ryanodine added to the *trans* compartment. Dose-response curve was constructed normalizing P_o (0.16 in this particular case) to 100%. Data points in the presence of ryanodine were calculated accordingly. Solid line is a fit using Hill equation assuming a single binding site with a Hill coefficient $n_H = 0.92$ and $K_i = 45$ μM.

progressively decreases the number of channels opening per trace and the mean duration of single events. A dose-response curve at the bottom of fig.1 was constructed by plotting the fraction of time in which one or more channels were open, relative to control, at the four concentrations tested. The apparent K_i of the inhibition was approx. 45 μM and the Hill coefficient was n_H = 0.92. Left panel of fig.2 shows the inhibition by ryanodine (filled circles) of binding of [^{3}H]PN200-110 to the DHP receptor (open circles). Scatchard analysis of the same data in the right panel of fig.2 shows that PN200-110 binds to t-tubules with high affinity to a single class of sites (K_d = 0.6 nM, B_{max} = 16 pmol/mg protein). Ryanodine (60 μM) decreases B_{max} (11 pmol/mg protein) and increases the K_d (1.66 nM) of [^{3}H]PN200-110 binding to the DHP receptor. Binding parameters and IC_{50} values for channels were obtained from least square regression of data from three preparations of t-tubules. The complete dose-response curve for displacement of specifically bound [^{3}H]PN200-110 by ryanodine is shown in fig.3 (triangles) and a Hill plot appears as inset. Ryanodine displaces [^{3}H]-PN200-110 with an IC_{50} = 95 μM and Hill coefficient n_H = 0.75. At the concentration of DHP receptor and [^{3}H]PN200-110 used, receptor occupancy by [^{3}H]PN200-110 was less than 20%. Therefore the IC_{50} for displacement of [^{3}H]-PN200-110 by ryanodine is approximately equal to the apparent affinity of ryanodine for the DHP receptor. The fact that similar concentrations of ryanodine are required to inhibit BAY K 8644-activated Ca channels and to displace specifically bound [^{3}H]PN200-110 (45 μM vs 95 μM, respectively) strongly suggests that the electrophysiological inhibition observed in fig.1 arises from displacement of BAY K 8644 from the DHP receptor. Two blockers of ryanodine receptor channels, ruthenium red [3,23] and a related analog tetramino-palladium [18], also interfere with binding of dihydropyridines to the dihydropyridine receptor. Displacement of [^{3}H]PN200-110 binding by these agents is shown in fig.3, open circles ($Pd(NH_3)_4$) and filled circles (ruthenium red).

The 175 kDa dihydropyridine receptor is a structural component of the dihydropyridine-sensitive Ca channel [14–17]. In skeletal muscle, Ca channels are present exclusively in transverse tubules, the membrane system that originates from foldings of the plasma membrane. High-affinity binding sites for ryanodine in skeletal muscle are present in the junctional SR membrane, the part of the SR that establishes anatomical contacts with the t-

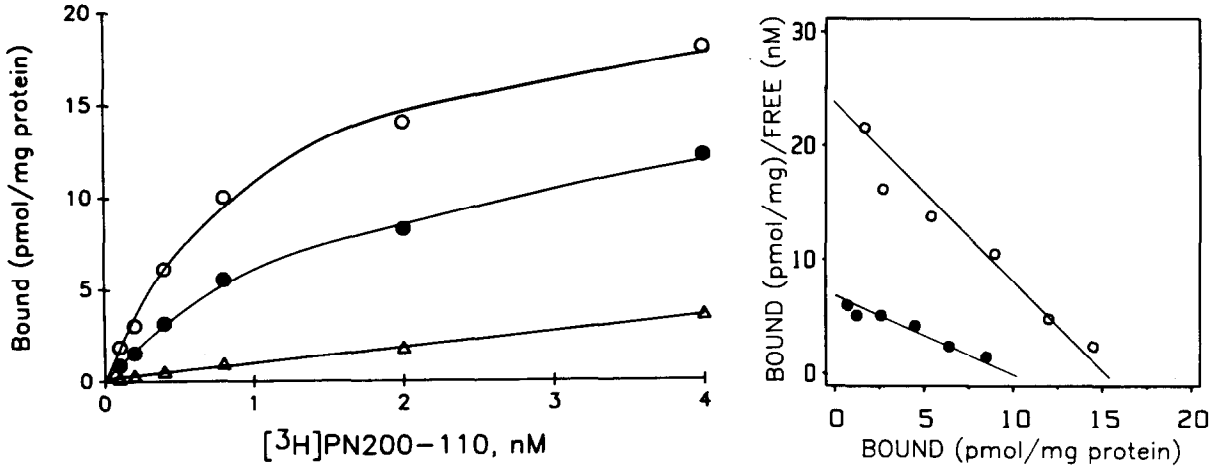

Fig.2. Ryanodine inhibition of [^{3}H]PN200-110 binding to t-tubule vesicles of skeletal muscle. (Left panel) Total binding of [^{3}H]PN200-110 in the absence (open circles) or presence (filled circles) of 60 μM ryanodine in the incubation buffer. T-tubule vesicles were incubated 15 min at 22°C and 60 min at 36°C as described under section 2 with increasing concentrations of [^{3}H]PN200-110. Nonspecific binding (triangles) was defined as the amount of [^{3}H]PN200-110 binding not displaced by 1 μM cold nitrendipine. (Right panel) Scatchard plot of specifically bound [^{3}H]PN200-110 to t-tubule vesicles. K_d and B_{max} for [^{3}H]PN200-110 alone (open circles) were 0.66 nM and 16 pmol/mg protein, respectively. In the presence of 60 μM ryanodine (filled circles), K_d and B_{max} increased to 1.66 nM and 11 pmol/mg protein, respectively.

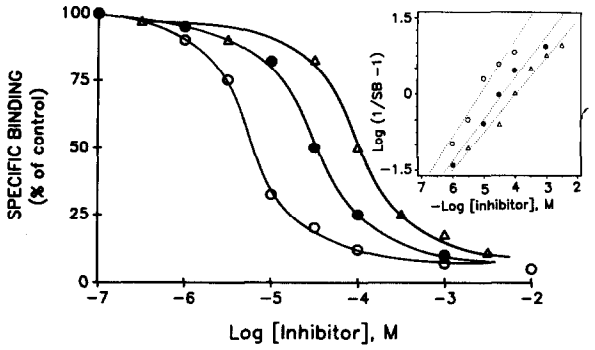

Fig.3. Displacement of [^{3}H]PN200-110 binding to t-tubule vesicles for SR Ca-release channel inhibitors. [^{3}H]PN200-110 (0.2 nM) was incubated 15 min at 22°C and 60 min at 36°C as described in section 2. $Pd(NH_3)_4$ (open circles), ruthenium red (triangles), or ryanodine (filled circles) were present at the indicated concentrations. Nonspecific binding was estimated at each concentration of inhibitor and was defined as the amount of binding in the presence of 1 μM nitrendipine. (Inset) Hill plot of displacement curves. Specific binding (SB) was normalized to 1 and plotted as $\log([1/SB] - 1)$ at the indicated inhibitor concentrations. The slope is n_H, the Hill coefficient, and K_i = antilog − (y-axis intercept/n_H). Values of n_H and K_i for inhibitors are: $Pd(NH_3)_4$, 0.94, 8.6 μM; ruthenium red, 0.80, 41.7 μM; ryanodine, 0.75, 95 μM.

tubules [2,19]. Binding of ryanodine to junctional SR varies widely with Ca [20], adenine nucleotide concentration [20] and ionic strength [9]. In our hands, in 100 μM Ca, 0.15 M NaCl, pH 7.5, binding parameters of ryanodine to its receptor in SR are K_d = 10 nM, B_{max} = 20 pmol/mg protein [21]. Ryanodine specifically binds to a single polypeptide of 350–450 kDa identified as the Ca release channel of sarcoplasmic reticulum [3,20,22,23]. At low concentrations (<1 μM), ryanodine opens the release channel and increases ^{45}Ca efflux from terminal cisternae SR of skeletal and cardiac muscle [4,5]. At higher concentrations (>100 μM), ryanodine inhibits Ca release presumably by closure of Ca release channels [4]. Pessah et al. [9] showed that binding of the alkaloid to its receptor is a slow process. The association rate of ryanodine binding is in the order of 5.5×10^6 $M^{-1} \cdot min^{-1}$ at 30°C which at 5 nM ryanodine translates into a binding reaction with a $t_{1/2}$ of approx. 30 min [9]. Presumably due to this slow onset, micromolar instead of nanomolar concentrations of ryanodine are typically used in electrophysiological experiments in muscle cells [6–8]. Our results suggest that the DHP receptor-Ca channel constitutes a low-affinity binding site for ryanodine as well as for ruthenium red, the classical inhibitor of SR Ca release [4,9,23,24]. We conclude that at high doses, ryanodine may produce in addition to an activation of SR release channels, an inhibition of surface membrane Ca channels. Thus in whole cell experiments that use high concentration of ryanodine, a multiplicity of effects on Ca gradients across the plasma membrane and SR membrane are expected and should be taken into consideration.

Acknowledgements: Supported by NIH grants GM 36852 and HL 37044, Grants In Aid from American Heart Association and Muscular Dystrophy Association, and an Established Investigatorship from American Heart Association to R.C.

REFERENCES

[1] Jenden, D.J. and Fairhurst, A.S. (1969) Pharmacol. Rev. 21, 1–25.
[2] Fill, M. and Coronado, R. (1988) Trends Neurosci. 11, 453–457.
[3] Smith, J.S., Imagawa, T., Ma, J., Fill, M., Campbell, K.P. and Coronado, R. (1988) J. Gen. Physiol. 92, 1–26.
[4] Meissner, G. (1986) J. Biol. Chem. 261, 6300–6306.
[5] Lattanzio, F.A., Schlatteerer, R.G., Nicar, M., Campbell, K.P. and Sutko, J.L. (1987) J. Biol. Chem. 262, 2711–2718.
[6] Ito, K., Kohichi, K. and Sutko, J.L. (1986) Cir. Res. 58, 730–734.
[7] Su, J.-Y. (1988) Pflügers Arch. 411, 371–377.
[8] Sherman, S.J., Chrivia, J. and Catterall, W.A. (1985) J. Neurosci. 5, 1570–1576.
[9] Pessah, I.N., Francini, A.O., Scales, D.J., Waterhouse, A.L. and Casida, J.E. (1986) J. Biol. Chem. 261, 8643–8648.
[10] Ma, J. and Coronado, R. (1988) Biophys. J. 53, 387–395.
[11] Vilven, J. and Coronado, R. (1988) Nature 336, 587–589.
[12] Valdivia, H. and Coronado, R. (1988) J. Gen. Physiol., submitted.
[13] Affolter, H. and Coronado, R. (1985) Biophys. J. 48, 341–347.
[14] Catterall, W.A., Seagar, M.J. and Takahashi, M. (1988) J. Biol. Chem. 263, 3535–3538.
[15] Campbell, K.P., Leung, A.T. and Sharp, A.H. (1988) Trends Neurosci. 11, 425–430.
[16] Ellis, S.B. et al. (1988) Science 241, 1661–1664.
[17] Tanabe, T., Beam, K.G., Powell, J.A. and Numa, S. (1988) Nature 336, 134–139.
[18] Ma, J., Knudson, C.M., Campbell, K.P. and Coronado, R. (1989) Biophys. J., submitted.

[19] Inui, M., Saito, A. and Fleischer, S. (1987) J. Biol. Chem. 262, 1740–1747.
[20] Imagawa, T., Smith, J.S., Coronado, R. and Campbell, K.P. (1987) J. Biol. Chem. 262, 16636–16643.
[21] Valdivia, H.H. and Coronado, R. (1989) Biophys. J., submitted.
[22] Lai, F.A., Erickson, H., Rousseau, E., Liu, Q.Y. and Meissner, G. (1988) Nature 331, 315–319.
[23] Ma, J., Fill, M., Knudson, C.M., Campbell, K.P. and Coronado, R. (1988) Science 242, 99–102.
[24] Volpe, P., Salviati, G. and Chu, A. (1986) J. Gen. Physiol. 87, 289–303.