A new cardioprotective agent, JTV519, improves defective channel gating of ryanodine receptor in heart failure

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Kohno, Masateru, Masafumi Yano, Shigeki Kobayashi, Masahiro Doi, Tetsuro Oda, Takahiro Tokuhisa, Shinichi Okuda, Tomoko Ohkusa, Michihiro Kohno, and Masunori Matsu. A new cardioprotective agent, JTV519, improves defective channel gating of ryanodine receptor in heart failure. Am J Physiol Heart Circ Physiol 284: H1035–H1042, 2003. First published November 14, 2002; 10.1152/ajpheart.00722.2002.—Defective interaction between FKBP12.6 and ryanodine receptors (RyR) is possible cause of cardia dysfunction in heart failure (HF). Here, we assess whether the new cardioprotective agent JTV519 can correct it in tachycardia-induced HF. HF was demonstrated that in a canine model of pacing-induced heart failure, a decrease in the activity of sarcoplasmic reticulum (SR) Ca2+ was significantly larger as a fraction of the total Ca2+ uptake in failing SR vesicles than in normal SR vesicles (17). These findings suggest that the gating function of the SR Ca2+ release channel is altered in heart failure. Recently, we (21) reported that in a canine model of heart failure, a prominent abnormal Ca2+ leak occurs through RyR, presumably after a partial loss of RyR-bound FKBP12.6 and the resultant conformational change in RyR. This abnormal Ca2+ leak might cause Ca2+ overload and consequent diastolic dysfunction as well as systolic dysfunction. Moreover, we showed that the conformational change in RyR due to a partial loss of RyR-bound FKBP12.6 might also be a cause of the abnormal Ca2+ release function of RyR seen in the failing heart (14). This was based on two findings: 1) in normal SR vesicles, the polylsine-induced enhancement of [3H]ryanodine binding was reduced after the addition of FK506 (which dissociates FKBP12.6 from RyR), but no significant change was seen in failing SR vesicles; and 2) the rate of polylsine-induced Ca2+ release was decreased after the addition of FK506 to normal SR vesicles, but unchanged in failing SR vesicles.

A new 1,4-benzothiazepine derivative, JTV519, developed a few years ago by Kaneko (8), has a protective effect against Ca2+ overload-induced myocardial injury. This cardioprotective effect may be attributable to an inhibition of the Ca2+ overload that results from an enhanced supply by the intracellular Ca2+ store (8), although this is still unclear. In this regard, we (20) recently observed that JTV519 completely inhibited the FK506-induced Ca2+ leak in normal SR and also inhibited the spontaneous Ca2+ leak in failing SR by restoring the conformational state of RyR2. In the present study, we investigated whether JTV519 improves the RyR Ca2+ release function by restoring the interaction of FKBP12.6 with RyR2 in a canine model of heart failure.

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MATERIALS AND METHODS

**Chemicals.** Fluo 3 was obtained from Molecular Probes, and sulfosuccinimidyl 3-[N-(7-azido-4-methylcoumarin-3-acetamido)ethyl]dithio]propionate was purchased from Pierce. [3H]Rykyanodine was purchased from DuPont-New England Nuclear. Anti-FKBP12 (C-19), an antibody that cross-reacts with FKBP12.6 (15), was purchased from Santa Cruz Biotechnology. Anti-RyR antibody was from Oncogene Research Products. Human recombinant FKBP12.6 was produced in our own laboratory. FK506 was provided by Fujisawa Pharmaceutical (Osaka, Japan), and JTV519 was provided by Japan Tobacco (Tokyo, Japan).

**Production of pacing-induced heart failure.** In beagle dogs weighing 10–14 kg, we induced heart failure by 28 days of rapid ventricular (RV) pacing at 250 beats/min with an externally programmable miniature pacemaker (Medtronic; Minneapolis, MN), as described previously (14, 17). While the dogs were under anesthesia, we chronically implanted a 5-Fr micromanometer (Millar) in the left ventricle (LV) via the apex for the measurement of LV pressure. After a recovery period of 1 wk was allowed, we measured LV pressure and recorded two-dimensional short-axis echocardiograms at the level of the head of the papillary muscle in the conscious state, ~1 h after the termination of rapid RV pacing. These hemodynamic parameters were also measured at 1 and 4 wk in the period of chronic RV pacing.

The care of the animals and the protocols used were in accord with guidelines laid down by the Animal Ethics Committee of Yamaguchi University School of Medicine.

**Preparation of SR vesicles.** We prepared SR vesicles essentially by the method of Kranias et al. (9), with the modifications described elsewhere (14, 17, 22).

**Ca2+ release assay.** We performed the Ca2+ release assay as described previously (14, 17, 22). The SR vesicles (0.5 mg/ml) were incubated for 5 min in a solution containing 0.15 M KCl, 10 mM NaN3, 2.5 mM MgATP, and 20 mM MES, pH 6.8 (adjusted with KOH) (solution A), to load the SR with Ca2+. Solution A (1 vol) was then mixed with 1 volume of solution B (containing 0.15 M KCl and 20 mM MES; adjusted to pH 6.8 using KOH). Polylysine (a RyR-specific Ca2+ release trigger: 0.74 μM, MW 27,000) was added to solution B, with a final concentration of polylysine after being mixed of 0.37 μM, a concentration at which a half-maximal stimulation of Ca2+ release was obtained (17). The Ca2+ concentration ([Ca2+]i) in each of the two solutions was buffered at 3 μM with an EGTA-Ca2+ buffer (0.212 mM CaCl2 and 0.25 mM EGTA, pH 6.8). In the presence or absence of 30 μM FK506, 0.3 μM JTV519, or 10 μM of Ca2+ antagonist (verapamil, nifedipine, or diltiazem), the time course of the polylysine-induced Ca2+ release was monitored with the use of a stopped-flow apparatus (model RSP-601S, Unisoku; Osaka; Japan), with 5 μM arsenazo III as a Ca2+ indicator (5, 14, 17, 22). All of the reactions mentioned above were carried out at 22°C. Twenty to twenty-five traces (each representing 1,000 data points) of the arsenazo III signal were averaged for each experiment. The arsenazo III signal was converted to nanomoles of Ca2+ released per milligram of protein by determining the change in (Δ) arsenazo III signal/Δ[Ca2+]i coefficient from a Ca2+ calibration curve (5, 6, 22).

**Site-directed labeling of RyR with fluorescent conformational probe.** We performed specific fluorescent labeling of RyR in SR vesicles with the cleavable heterobifunctional cross-linking reagent sulfosuccinimidyl 3-[N-(7-azido-4-methylcoumarin-3-acetamido)ethyl]dithio]propionate with polylysine as a site-specific carrier, as described elsewhere (5, 18, 19). Fluorometric scanning revealed that methylcoumarin acetamide (MCA) fluorescence was localized specifically to the RyR in both normal and failing SR vesicles because no other bands were fluorescently labeled (see Fig. 3 in Ref. 21).

**Fluorescence assays of protein conformational change.** The time course of the rapid changes in the fluorescence intensity of the RyR-bound MCA probe (excitation at 360 nm, emission at 440 nm) was monitored under the same conditions as those used for the Ca2+ release assay (except that there was no arsenazo III in the reaction solution) and with the use of the same stopped flow apparatus (model RSP-601S, Unisoku) described previously (5, 18, 19). About 15 traces (each representing 1,000 data points) of the MCA fluorescence signal were averaged for each experiment and noted after subtracting the background signal present in unlabeled samples. The magnitude of the MCA fluorescence change was expressed in percentage terms [(F – F0)/F0] 100%, where F0 is the fluorescence value just before the reaction (baseline) and F is the fluorescence value at 200 ms after the reaction.

**[3H]Rykyanodine-binding assay.** To assess the effect of JTV519 on Ca2+ dependence of [3H]rykyanodine binding (4), cardiac SR vesicles (0.1 mg/ml) were incubated in 1 ml of a reaction solution containing 10 nM [3H]rykyanodine (68.3 Ci/ml, DuPont-New England Nuclear), 0.3 M KCl, 1 mM EGTA, various amounts of CaCl2 to create various levels of [Ca2+], and 20 mM MOPS, pH 7.2, for 120 min at 36°C in the presence or absence of 1 μM JTV519. The incubated reaction mixture was filtered through Millipore filters (type HA, pore size 0.45 μm) and washed twice with 5 ml of the same reaction solution devoid of both microsomes and [3H]rykyanodine. The specific binding was calculated as the difference between the levels of binding observed in the absence (total binding) and presence (non-specific binding) of 10 μM unlabeled ryanodine. Each datum point was obtained by averaging the values from duplicate experiments.

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Values are means ± SD, n, no. of dogs. HR, heart rate; LVESP, left ventricular (LV) end-systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt, peak positive LV pressure over time; v, time constant of LV pressure decay during isovolumic relaxation period; LVESD, LV end-diastolic diameter; FS, fractional shortening ([LVESD – LVESD]/LVESD × 100). *P < 0.01 vs. control; †P < 0.05 vs. 1-wk pacing; ‡P < 0.01 vs. 1-wk pacing.
**Dissociation and reconstitution of FKBP12.6.** We achieved dissociation of FKBP12.6 from, and its reconstitution into, SR vesicles by the method of Timerman et al. (16), with slight modifications. Briefly, SR vesicles (2 mg/ml) were preincubated for 1 h at room temperature in imidazole homogenization medium (IHM) (5 mM imidazole-Cl, pH 7.4, and 0.3 M sucrose) containing 5 μM FK506. The samples, each 50 μl, were then centrifuged in a rotor (model TL-100.1, Beckman) at 95,000 g for 10 min at 2°C to yield sedimentable and supernatant fractions. The supernatant contains the soluble FKBP-FK506 complex. The pellet was washed by resuspension in 200 μl of IHM, resedimented, and resuspended in 50 μl of IHM buffer, and is referred to as FKBP12.6-deficient SR. FKBP12.6 was coimmunoprecipitated from SR using anti-RyR antibody, followed by immunoblotting with anti-FKBP12 antibody. Reconstitution of recombinant FKBP12.6 was performed by adding FK506 and JTV519 (30 μM and 0.3 μM) to the SR vesicles, followed by incubation at room temperature for 1 h. The reconstituted SR was then compared to the original SR for Ca²⁺ release function.

**Fig. 1.** Effects of FK506 (30 μM) and JTV519 (0.3 μM) on polylysine (PL; 0.37 μM)-induced Ca²⁺ release in normal and failing sarcoplasmic reticulum (SR) vesicles. A: representative time courses of Ca²⁺ release. B: comparisons of rate constants and initial rates of Ca²⁺ release between the presence and absence of the above reagents. Kinetic parameters shown were calculated by fitting a single exponential model, \( y = A(1 - e^{-kt}) \), to the Ca²⁺ release time courses shown in A (where \( k \) is the rate constant and \( A \cdot k \) is the initial rate of Ca²⁺ release). Data represent means ± SD of 5 experiments, each performed on a different pair of normal and failing SR vesicle preparations. **ns, Not significant.**
into failing SR vesicles was achieved by mixing FKBP12.6 (1 μg/ml) with failing SR vesicles (2 mg/ml) at room temperature for 12 h. We then performed the same centrifugation procedures as in the dissociation experiments to separate the free FKBP12.6 from the FKBP12.6 reconstituted into RyR. Finally, FKBP12.6 was again coimmunoprecipitated from SR using anti-RyR antibody, followed by immunoblotting with anti-FKBP12 antibody.

**Ca^{2+}** uptake assay. SR vesicles (0.2 mg/ml) were first incubated in 0.5 ml of solution containing 0.15 M potassium gluconate, 1 mM MgCl₂, 0.2 mM EGTA-calcium buffer (free [Ca^{2+}] = 0.3 μM), 10 mM Na₃HPO₄, 10 μM ruthenium red, and 20 mM MOPS, pH 6.8. Ca^{2+} uptake was initiated by the addition of 0.5 mM ATP into the cuvette. JTV519 (1 μM) was added before the initiation of the Ca^{2+} uptake. The time course of the Ca^{2+} uptake was monitored spectrophotometrically with the use of fluo 3 (Molecular Probes) as a Ca^{2+} indicator (excitation 480 nm, emission 530 nm), as described previously (21).

**Statistics.** Intragroup comparisons were carried out with the use of a paired t-test. Intergroup analysis was performed by ANOVA with a post hoc Scheffe test. Data represent means ± SD. A P value <0.05 was accepted as statistically significant.

**RESULTS**

**Hemodynamic data.** In 1- and 4-wk paced dogs, LV end-diastolic pressure was significantly elevated and the rate of peak LV pressure over time (+dP/dt) was decreased, whereas the time constant of the LV pressure decay during the isovolumic relaxation period was increased (Table 1). These data indicate that both systolic and diastolic functions were impaired in the heart failure group, in line with our previous findings (14, 17, 21).

**Effect of JTV519 on polylysine-induced Ca^{2+} release.** In confirmation of our previous finding (14, 17), the rate of polylysine (0.37 μM)-induced Ca^{2+} release was decreased after the addition of 30 μM FK506 to normal SR vesicles, but it was unchanged in the failing SR vesicles prepared from 4-wk paced dog hearts (Fig. 1A). In normal SR, JTV519 (0.3 μM) prevented the decrease in the rate of Ca^{2+} release due to FK506. The various parameters characterizing the kinetics of Ca^{2+} release in both normal and failing SR vesicles are shown in Fig. 1B. In normal SR, FK506 decreased both the rate constant (k) and the initial rate (A·k) of Ca^{2+} release, both of which were restored by JTV519. In failing SR, in which these kinetic parameters were smaller in magnitude than in normal SR, they were restored by JTV519 both in the absence and in the presence of FK506.

**Effects of JTV519 on Ca^{2+} dependence of [3H]ryanodine binding.** Figure 2 shows the Ca^{2+} dependence of ryanodine binding to RyR2 in the absence or presence of 1 μM JTV519. In failing SR, Ca^{2+} dependence was shifted downward, indicating the reduced Ca^{2+}-induced activation of RyR compared with normal SR. In the presence of JTV519, the Ca^{2+} dependence was partially restored back to normal in failing SR. In contrast, it was unchanged by JTV519 in normal SR.

**Effect of JTV519 on protein conformational change in RyR during Ca^{2+} release.** We then examined the polylysine (0.37 μM)-induced rapid change in MCA fluorescence preceding the Ca^{2+} release (Fig. 3A). The MCA fluorescence change occurred much faster than SR Ca^{2+} release, suggesting that the conformational change is a causative mechanism for channel opening and Ca^{2+} release. Thus MCA fluorescence change indicates a rapid conformational change in RyR presumably due to channel opening, as described previously (5, 18, 19). In normal SR, FK506 decreased the polylysine-induced MCA fluorescence change, and this effect of FK506 was inhibited by 0.3 μM JTV519. In failing SR, the MCA fluorescence change induced by polylysine was smaller than in normal SR, and it was increased by JTV519 both in the presence and in the absence of FK506.

There was no MCA fluorescence change during ionomycin-induced Ca^{2+} leak (Fig. 3B), suggesting that MCA fluorescence change triggered by polylysine was indeed mediated through RyR, and it was not under the control of intraluminal or extravesicular [Ca^{2+}].

**Effect of JTV519 on SR Ca^{2+} uptake.** In both groups (normal and heart failure), JTV519 (1 μM) had no effect on the magnitude of the Ca^{2+} uptake seen in the presence of 10 μM ruthenium red (normal (in nmol·min⁻¹·mg⁻¹): JTV519 (−) 16.0 ± 1.7, JTV519 (+) 16.1 ± 1.6, P = not significant (NS); heart failure (in nmol·min⁻¹·mg⁻¹): JTV519 (−) 8.7 ± 1.2, JTV519 (+) 8.8 ± 1.3, P = NS). These data indicate that JTV519 does not have a direct effect on the SR Ca^{2+} uptake function.

**Effects of various Ca^{2+} antagonists on the rapid Ca^{2+} release.** The effects of verapamil, nifedipine, and diltiazem on the initial rate of polylysine-induced Ca^{2+} release were assessed in normal and failing SR vesicles. In normal SR vesicles, diltiazem partially restored the rate of Ca^{2+} release, which was decreased by
FK506 [diltiazem(-)] 9.4 ± 1.8 nmol·s⁻¹·mg⁻¹, diltiazem(+) 12.2 ± 1.9 nmol·s⁻¹·mg⁻¹, P < 0.05]. However, neither verapamil nor nifedipine had any such effect. In failing SR, in which the rate of Ca²⁺ release was slower than the rate seen in normal SR vesicles, diltiazem also tended to restore the rate of Ca²⁺ release [diltiazem(-)] 11.0 ± 1.9 nmol·s⁻¹·mg⁻¹, diltiazem(+) 12.9 ± 1.5 nmol·s⁻¹·mg⁻¹, P = 0.16]. Again, neither verapamil nor nifedipine had any such effect. Effects of dissociation of FKBP12.6 from, and its reconstitution into, SR vesicles on Ca²⁺ release and MCA fluorescence change. In normal SR, dissociation of FKBP12.6 from RyR was induced by FK506 (FKBP12.6-deficient normal SR), and reconstitution of FKBP12.6 into RyR was induced by the addition of rFKBP12.6 (FKBP12.6-reconstituted SR) (Fig. 4, insets). In FKBP12.6-deficient normal SR, both the Ca²⁺ release rate and the MCA fluorescence change induced by polylysine were
smaller than in normal SR, whereas in FKBP12.6-reconstituted failing SR both the Ca\(^{2+}\) release rate and the MCA fluorescence change were increased (Fig. 4). These findings support the view that JTV519 exerts an FKBP12.6-like channel-stabilizing effect mediated through restoration of the RyR conformational state.

**DISCUSSION**

An abnormal regulation of intracellular Ca\(^{2+}\) by the SR is the chief pathogenic mechanism responsible for the various types of dysfunctions seen in heart failure. It is therefore important to clarify the molecular mechanisms governing the highly regulated excitation-contraction coupling process and their alterations in heart failure if we are to develop new therapeutic strategies against this disease. Recently, we demonstrated the following in a series of experimental studies using a canine model of pacing-induced heart failure: 1) that the Ca\(^{2+}\) release function of the RyR is defective in heart failure (17), presumably due to a partial loss of FKBP12.6 from RyR (14), and 2) that this defective regulation of RyR causes an abnormal Ca\(^{2+}\) leak mediated through a conformational change in the RyR itself (21). In an investigation of the partial loss of FKBP12.6 in heart failure, Marx et al. (12) demonstrated that protein kinase A-mediated hyperphosphorylation of RyR causes dissociation of FKBP12.6 from RyR, resulting in a defective channel function due to an increased sensitivity to Ca\(^{2+}\)-induced activation. Conceivably, this mechanism might be involved in the partial loss of FKBP12.6 seen in heart failure.

The present study permits insight into the mechanism underlying the altered RyR function in heart failure. This greater insight relies on the following findings: 1) in normal SR vesicles, JTV519 attenuated the decrease induced by FK506 in the rate of polylysine-induced Ca\(^{2+}\) release as well as the FK506-induced rapid conformational change in RyR; and 2) in failing SR vesicles, JTV519 restored toward normal all the above parameters (which showed deterioration in failing vesicles).

The decreased rate of Ca\(^{2+}\) release in failing SR may be explained by the reduced SR Ca\(^{2+}\) loading. In this respect, the MCA fluorescence change was also smaller in failing SR than in normal SR, and moreover its decrease was reversed by JTV519, in conjunction with the increase in the rate of Ca\(^{2+}\) release. Also, the Ca\(^{2+}\)-induced increase in RyR binding, which was decreased in failing SR, was restored back to normal SR (Fig. 2). These findings strongly suggest that the decreased rate of Ca\(^{2+}\) release seen in failing SR was indeed partly mediated through the conformational change of RyR, which precedes Ca\(^{2+}\) release. Although JTV519 reversed the detrimental effects of FK506 on Ca\(^{2+}\) release in normal SR vesicles and improved Ca\(^{2+}\) release function in failing SR vesicles, we cannot say that the mechanism of action of the drug is the same as FKBP12.6 in terms of the channel stabilization of RyR2.

Major alterations in excitation-contraction coupling of heart failure have been characterized and related to contractile and relaxation functions. These dysfunctions are described by reduced twitch amplitude, delayed relaxation, and disturbed relaxation function. Also, these alterations are associated with parallel changes in the intracellular whole Ca\(^{2+}\) transient. The delayed fall of the descending portion of the intracellular Ca\(^{2+}\) transient might be caused by the decrease in the expression and/or activity of the SR Ca\(^{2+}\)-ATPase (1). The decreased acceleration of the Ca\(^{2+}\) transient (prolongation of time to peak Ca\(^{2+}\) transient) may be due mainly to the altered Ca\(^{2+}\) release function of...
the RyR because no other protein or receptor can induce faster Ca\(^{2+}\) release than the RyR. Because cross-bridge cycling in myofilaments occur very rapidly from the beginning of the rise of the Ca\(^{2+}\) transient (2), it is likely that as cytosolic [Ca\(^{2+}\)] elevates faster, the cross-bridge attachment occurs faster, resulting in faster and/or higher tension development. In heart failure, the restored gating function of RyR through amelioration of FKBP12.6-RyR interaction by JTV519 may then lead to an improvement of cardiac contractile and relaxation function. When JTV519 (1 mg/kg iv) was acutely administered to 4-wk paced conscious dogs, peak +dP/dt of LV pressure was increased by 19%, LV end-diastolic pressure was decreased by 11%, and time constant of LV pressure decay was shortened by 12%, whereas there was no significant change in any of the hemodynamic parameters in the normal conscious dog (M. Kohno and M. Yano, unpublished data).

However, before we can be sure about the above point, several questions remain to be answered. First, we should clarify the specificity of polylysine on Ca\(^{2+}\) release function through RyR in cardiac SR. With regard to this, we have demonstrated that, like skeletal SR, polylysine (submicromolar)-induced Ca\(^{2+}\) release was completely inhibited by 10 \(\mu\)M ruthenium red in cardiac SR (22). Also, in cardiac SR, polylysine (submicromolar) increased the [\(\mathrm{H}\)]RyR binding like skeletal SR, and this increase in the [\(\mathrm{H}\)]RyR binding was again completely inhibited by 10 \(\mu\)M ruthenium red (22). Moreover, as shown in our previous study (see Fig. 3 in Ref. 21), site-specific fluorescent labeling for RyR2 was successfully done with the use of 0.5 \(\mu\)M polylysine as a carrier. Our previous findings suggest that polylysine-induced Ca\(^{2+}\) release is indeed a simplified model suitable for studying the fundamental channel gating mechanism underlying various types of Ca\(^{2+}\) release through cardiac and skeletal RyR. Second, although the present model of tachycardia-induced heart failure involves a well-defined, predictable, and progressive LV dilatation as well as contractile dysfunction and neurohormoral activation, it is not identical to human chronic heart failure. Finally, because the present findings only show the pharmacological action of JTV519 on channel gating of RyR, it remained to be elucidated whether the beneficial effect of JTV519 on SR Ca\(^{2+}\) release makes it a useful drug for the treatment of heart failure in humans.

In summary, the defective channel gating of RyR was restored (increase in Ca\(^{2+}\) release rate and ryanodine binding activity) by the new 1,4-benzothiazepine derivative JTV519. This finding may advance the understanding of the mechanism for contractile and relaxation dysfunction in heart failure and hence provide a clue for the development of new methods of treatment to prevent and cure heart failure.

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