K201 (JTV519) is a Ca\(^{2+}\)-Dependent Blocker of SERCA and a Partial Agonist of Ryanodine Receptors in Striated Muscle

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ABSTRACT

K201 (JTV519) may prevent abnormal Ca\(^{2+}\) leak from the sarcoplasmic reticulum (SR) in the ischemic heart and skeletal muscle (SkM) by stabilizing the ryanodine receptors (RyRs; RyR1 and RyR2, respectively). We tested direct modulation of the SR Ca\(^{2+}\)-stimulated ATPase (SERCA) and RyRs by K201. In isolated cardiac and SkM SR microsomes, K201 slowed the rate of SR Ca\(^{2+}\) loading, suggesting potential SERCA block and/or RyR agonism. K201 displayed Ca\(^{2+}\)-dependent inhibition of SERCA-dependent ATPase activity, which was measured in microsomes incubated with 200, 2, and 0.25 μM Ca\(^{2+}\) and with the half-maximal K201 inhibitory doses (IC\(_{50}\)) estimated at 130, 19, and 9 μM (cardiac muscle) and 104, 13, and 5 μM (SkM SR). K201 (≥5 μM) increased RyR1-mediated Ca\(^{2+}\) release from SkM microsomes. Maximal K201 doses at 80 μM produced ~37% of the increase in SkM SR Ca\(^{2+}\) release observed with the RyR agonist caffeine. K201 (≥5 μM) increased the open probability (P\(_o\)) of very active (“high-activity”) RyR1 of SkM reconstituted into blayers, but it had no effect on “low-activity” channels. Likewise, K201 activated cardiac RyR2 under systolic Ca\(^{2+}\) conditions (~5 μM; channels at P\(_o\) ∼0.3) but not under diastolic Ca\(^{2+}\) conditions (~100 nM; P\(_o\) < 0.01). Thus, K201-induced the inhibition of SR Ca\(^{2+}\) leak found in cell-system studies may relate to potentially potent SERCA block under resting Ca\(^{2+}\) conditions. SERCA block likely produces mild SR depletion in normal conditions but could prevent SR Ca\(^{2+}\) overload under pathologic conditions, thus precluding abnormal RyR1-mediated Ca\(^{2+}\) release.

Introduction

K201 (JTV519, a 1-4 benzothiazepine) cardioprotective properties were first reported by Kaneko and coworkers (Kaneko, 1994; Hachida et al., 1997; Kaneko et al., 2009). Other groups have further confirmed K201’s cardioprotective properties in various heart pathologies, including ischemia and arrhythmogenesis in various animal models (Kaneko, 1994; Ito et al., 2000; Kawabata et al., 2002; Wehrens et al., 2004; Lisy and Burnett, 2006; Loughrey et al., 2007; Otani et al., 2013), in addition to human cardiac cells (Toischer et al., 2013), in smooth muscle cells (Inagaki et al., 2000; Chen et al., 2008), an unexpected property for a drug that prevents RyR2-mediated leak. There are no reports on the direct effect of K201 at the single RyR2 channel level, but [\(^{3}H\)] ryanodine binding studies suggest that K201 may have a mild effect on RyR2 (Hunt et al., 2007). Furthermore, other targets have been proposed to explain SR depletion or the lack of SR overload observed with K201, including SERCA, the Ca\(^{2+}\) ATPase of SR (Loughrey et al., 2007).

K201 is known to improve the function of the ischemic skeletal muscle (SkM) (Wehrens et al., 2005). Two studies assessed the effects of K201 on the RyR isoform 1 (RyR1). In one study, K201 was found to slightly decrease or increase [\(^{3}H\)] ryanodine binding to purified and solubilized RyR1 in the absence or presence of physiologic levels of Mg\(^{2+}\), respectively (Blayney et al., 2010). The other study described a K201-induced increase in SR Ca\(^{2+}\) release from microsomes (Almassy et al., 2008). Additionally, some studies have a mild effect on RyR2 (Hunt et al., 2007). Furthermore, other targets have been proposed to explain SR depletion or the lack of SR overload observed with K201, including SERCA, the Ca\(^{2+}\) ATPase of SR (Loughrey et al., 2007).

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ABBREVIATIONS: [Ca\(^{2+}\)]\text{cyt}, cytosolic Ca\(^{2+}\) concentration; CGP, CGP-37157; CPZ, cyclopiazonic acid; DMSO, dimethyl sulfoxide; EGCG, epigallocatechin-3-gallate; FKBP, FK506 binding protein; HA, high activity; K\(_m\), dose that includes half-maximal rate; K201, JTV 519, 4-[3-(4-benzylpiperidin-1-yl)propionyl]-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine; LA, low activity; RR, ruthenium red; ryanodine, 1H-pyrimido[2,3-b]carbazole acid [3S,4R,4aR,6S,6aS,6aR]-dodecahydro-4,6,7,8a,9-trimethyl-7-(1-methylethyl)-6,9-methanobenz[1,2]pentaleno [1,6-bc]uran-8-yl ester; RyR, ryanodine receptor; RyR1, skeletal muscle RyR isoform 1; RyR2, cardiac muscle RyR isoform 2; SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase; SkM, skeletal muscle; SR, sarcoplasmic reticulum; V\(_{\text{mem}}\), membrane voltage; V\(_{\text{max}}\), maximal activity.
Materials and Methods

Isolation of Cardiac and SkM SR Microsomes. All procedures involving animals were designed conformed to the guidelines of the National Institutes of Health to minimize pain and suffering and using protocols (196-05-021 and 196-11-010) reviewed and approved by the Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine, whose animal research procedures are certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC 000551) and by the Public Health Service (PHS A3209-1).

SkM SR microsomal fraction R2, enriched in longitudinal tubule containing high density of SERCA1a (LT fractions) and R4 enriched in terminal cisternae, where the RyR1 localizes (TC fractions), were isolated from adult New Zealand male white rabbits, as previously described by others (Saito et al., 1984; Chu et al., 1988). Cardiac SR microsomes containing RyR2 and SERCA2a were prepared from ventricular tissue from male Yorkshire-Landrace crossed breed pigs (age 3 months; weight from 30 to 40 kg) following standard protocols (196-05-021 and 196-11-010) reviewed and approved by the Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine, whose animal research procedures are certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC 000551) and by the Public Health Service (PHS A3209-1).

Measurements of Ca2+ Loading/Leak by SR Microsomes. Ca2+ uptake by cardiac or SkM SR microsomes was measured following published protocols (Chamberlain et al., 1984; Chu et al., 1988; Neumann and Copello, 2011; Neumann et al., 2011). Ca2+ uptake was initiated by adding 40 nm CaCl2 to a cuvette containing 40–100 μg SR membranes resuspended in 1 ml of buffer in mM: 100 KHP04, 5 MgCl2 (free Mg2+ ~0.3 mM), 5 ATP and 0.2 antipyrilazolo III; pH 7.0. Ca2+ uptake was measured in SkM (R4 TC fractions) and cardiac SR microsomes with a spectrophotometer (Cory 50, Varian, Walnut Creek, CA) following changes in antipyrilazolo III absorbance (710–790 nm). Uptake rates (in nMole Ca2+ (mg protein)−1 min−1) were measured in the absence (control) or presence of K201 and/or 5 mM ruthenium red (RR), as previously described (Neumann et al., 2011). Ca2+ leak from SkM R4 fractions preloaded with Ca2+ (three pulses of 50 μM Ca2+) was measured after stopping SERCA activity with 20 μM cyclopiazonic acid (CPZ) and observing the effects of 1 μM dimethyl sulfoxide (DMSO; control) versus K201, both in the absence or presence of 5 μM RR.

Measurements of ATPase Activity in SR Microsomes. Studies of ATPase activity in SR microsomes used a previously described assay (Chu et al., 1988; Neumann and Copello, 2011; Neumann et al., 2011). SkM SR fractions (10–40 μg) enriched in longitudinal tubule (R2 fractions) or cardiac SR microsomes (300 μg) were incubated with buffer containing (in mM): 140 KCl, 5 MgCl2, 5 HEPES, 2 phosphoenolpyruvate, 0.3 CaCl2, and BAPTA/BromoBAPTA to obtain free Ca2+ levels of 250, 3, and 0.2 μM, for representation of complete saturation for SERCA binding sites, ~95% maximal activity (Vmax) and near half-maximal rate (Km), respectively. The buffer also contained 3.5 μM A23187, a Ca2+ ionophore, to prevent increase in SR lumen Ca2+ levels with time owing to SERCA activity. The pH was adjusted to 7.0 with KOH. The assay also used pyruvate kinase (8.4 U/ml) and lactic dehydrogenase (12 U/ml). The addition of 1 mM ATP activates ATPase hydrolysis to ADP. This process is coupled to reactions that regenerate ATP from ADP, consuming one equivalent of NADH (oxidation to NAD+) for every ATP hydrolyzed. Thus, monitoring NADH depletion at 340 nm allowed determination of the rate of ATP hydrolysis (Chu et al., 1988; Neumann and Copello, 2011; Neumann et al., 2011). In SkM R2 fractions, ~99% of the ATPase activity is inhibited by addition of the SERCA blocker CPZ (20 μM). In cardiac microsomes, there is a significant CPZ-independent (non-SERCA mediated) component of ATPase activity, which was inhibited by addition of sodium azide (1 mM) and ouabain (100 μM).

In the presence of 250 μM Ca2+, 75%–85% of the total ATPase activity was CPZ sensitive; at 0.25 μM (approximately Km for SERCA) Ca2+, 25%–50% of the ATPase activity is independent of CPZ. At cell resting levels, SERCA activity is very small (3%–7% of Vmax). Consequently, the CPZ-independent component dominates and prevented quantitative analysis of K201 effects on SERCA.

RyR Channel Measurements in Planar Lipid Bilayers. Planar lipid bilayers made of 50% phosphatidylethanolamine, 40% phosphatidylinerine, and 10% phosphatidylcholine (Avanti Polar Lipids; total 50 mg/ml), were formed on teflon septa, separating two 1.2-ml hemichambers (Copello et al., 1997). The trans hemichamber contained 250 mM HEPES and 50 mM Ca(OH)2 (pH 7.4) and was clamped at 0 mV (Axopatch 200B; Molecular Devices, Sunnyvale, CA). The cis compartment (ground) is initially filled with 250 mM HEPES and 120 mM Tris (pH 7.4). While stirring, 500–1000 mM CsCl, 1 mM CaCl2, and SR microsomes (5–15 μg) are added to the cis solution. After a few minutes, RyRs from SR are reconstituted into bilayers with their cytosolic surface facing the cis chamber (Copello et al., 1997). Subsequently, the cis chamber was superfused (5 minutes at 4 ml/min with HEPES-Tris solution, and studies of RyR function were carried out in the presence of 1 mM BAPTA and dibromo-BAHP to buffer the free [Ca2+]cyt on the cytosolic surface of the channel ([Ca2+]cyt). Channels used in our experiments displayed conductance (40–110 pS), gating (channels are activated by Ca2+, ATP, and caffeine, and/or inhibited by Mg2+ or tetracaine), and sensitivity to conductance modifiers (peptide blockers, ryanoïd, or imiperon[zj] that are characteristics of RyRs (Copello et al., 1997; Sitsapesan and Williams, 1998; Fill and Copello, 2002).

Channel recordings (4–8 minutes in duration) were filtered at 1 kHz, digitized at 20 kHz with a Digidata 1360 (Molecular Devices), stored, and analyzed using cplan10 software (Axon Instruments) as described before (Copello et al., 1997; Diaz-Sylvester et al., 2011). The presence and abundance of substates and/or flicker blocker were estimated as previously described (Porta et al., 2008). Briefly, all points current-amplitude histograms (bandwidth = 0.01 pA) were obtained from digitally filtered (500 Hz) single-channel recordings (2 minutes each, obtained at voltage ranging from +40 to −60 mV). In all control conditions, we detected only peaks corresponding to the baseline and full openings. Each component was fitted with a Gaussian function using the Levenberg-Marquardt method, and an estimation of probability was obtained. Only with K201 and −60 mV, intermediate states were observed and their probability was estimated by subtracting from the area of the curve the fits to the baseline and full openings from control experiments.

Drugs and Chemicals. CaCl2 standard for calibration was from World Precision Instruments Inc. (Sarasota, FL). Phospholipids were obtained from Avanti (Alabaster, AL). Cyclopiazonic acid was from Tocris Bioscience (Avonmouth, Bristol, UK). One set of experiments was carried out with K201 (Aetas Pharma Co. Ltd., Tokyo, Japan), but most of the studies were carried out with K201 from Torcis Biosience (Bristol, UK) or Sigma-Aldrich (St. Louis, MO). No significant differences...
were observed in data collection from the K201 drug batches. All other agents were obtained from Sigma-Aldrich-Fluka.

**Statistical Analysis.** Data are presented as means ± S.E.M. of n measurements. Statistical comparisons between groups were performed with a paired t test or with analysis of variance test for comparison of multiple groups versus a control. Differences were considered statistically significant at P < 0.05. The theoretical curves of Ca2+ loading and ATPase activity as a function of K201 dose were obtained from the fit, using Sigmaplot10 (Systat Software Inc., San Jose, CA), of eq.1:

\[ V_{\text{ATPase or loading rate}} = \frac{V_{\text{max}} [\text{K201}]}{[\text{IC}_{50} + [\text{K201}]]} \]  

Previously, the Hill equation was used to fit the data, but the estimates of Hill coefficient (n) for K201 inhibition of SR Ca2+ loading or ATPase activity only varied from 0.76 to 1.2. The n ~ 1 coefficient value in all curve fittings suggests minor, if any, cooperativity in K201 action. Consequently, a Hill coefficient of n = 1 was assumed for all curves.

**Results**

**K201 Inhibits Ca2+ Loading and SERCA-Mediated ATPase Activity in Cardiac and SkM SR Microsomes.** Net SR Ca2+ uptake by SR microsomes represents the numerical difference between the active, SERCA-mediated, Ca2+ influx and the passive Ca2+ efflux, dependent mainly on RyRs activity (Burg et al., 1997; Chamberlain et al., 1984). Figure 1A represents a sample UV recording with SkM SR microsomes. Under control conditions (DMSO), a Ca2+ pulse produced a rapid spike in the absorbance value, followed by a slow, gradual decrease in Ca2+ as it is uptaken by the SR microsomes. Figure 1, A (top panel) and B, shows that K201 (80 μM) decreased SR Ca2+ loading rates similarly to the RyR agonist caffeine (10 mM). Figure 1D shows changes in the rate of SR Ca2+ loading into Skm microsomes in response to increasing doses of K201, from where the IC50 was estimated at 27.5 μM. Figure 1, C and E, shows similar findings in cardiac SR microsomes; K201 inhibited SR Ca2+ loading with an IC50 of 40.5 μM. To discern whether K201 decreases SR Ca2+ loading rates by stimulating Ca2+ efflux through RyR1, inhibiting Ca2+ influx through SERCA or a combination of both mechanisms, we conducted our experiments in the absence and presence of the well known RyR blocker RR (5 μM). Figure 1A (lower panel, RR) as well as Fig. 1, B and C (RR), show that RR did not prevent the inhibitory action of 80 μM K201 on the rate of loading while, as expected, it abolished the effect of 10 mM caffeine. These results may indicate that K201 either renders the RyRs insensitive to block by RR or that K201 might directly inhibit SERCA.

**Effects of K201 on SERCA-Mediated ATPase Activity of SkM and Cardiac SR Microsomes.** The effects of K201 on SERCA were studied by measuring ATPase activity using an assay that follows the decrease of NADH absorbance, which is coupled mole to mole to the consumption of ATP by ATPases (see Materials and Methods). Figure 2 shows examples of recordings of [NADH] versus time (A) and summary data (B) of the rate of ATPase activity. These assays were performed by incubating cardiac SR microsomes with bathing solutions containing either 200 μM, 2 μM, or 0.25 μM Ca2+. Figure 2a shows recordings with 200 μM Ca2+ under control conditions, in the presence of K201 (at 4, 16, or 80 μM), as well as in the presence of cyclopiazonic acid (CPZ), a SERCA inhibitor. As shown, the decreases in NADH absorbance with K201 are more similar to those under control conditions than to CPZ. Figure 2b shows that at 200 μM Ca2+, where SERCA Ca2+ binding sites are saturated, K201 (at doses up to 8 μM) had minor effects on ATPase activity of cardiac SR. Figure 2c shows analogous effects of K201 on ATPase activity of Skm SR microsomes in the presence of 200 μM Ca2+. We estimated similar IC50 values for cardiac and SkM SERCA-mediated ATPase activities (130 and 104 μM, respectively; Fig. 2, B and C). At 2 μM Ca2+, where SERCA still retains ~90% of maximal activity (Vmax), K201 was much more efficacious, as suggested in the recordings (Fig. 2a2) and summary data (Fig. 2, B and C, triangles). The IC50 values estimated from these data were 17.8 μM and 13.3 μM for cardiac and SkM microsomes, respectively. At 0.25 μM Ca2+, slightly above cell resting levels, K201 efficacy to inhibit SERCA increased further, and the ATPase activity was significantly reduced at all K201 concentrations tested (Fig. 2, Aa3, B, and C, circles). IC50 values were 8.8 and 4.9 μM for cardiac and SkM microsomes respectively. Overall, these results indicated that K201 is a Ca2+-dependent blocker of SERCA, having a similar potency in both SkM and cardiac SR microsomes. Inferring from the data, K201 inhibition of SERCA could be further magnified under low, resting cell, Ca2+ conditions (40–80 nM).

**K201 is a Partial Agonist of SkM RyR1 and Cardiac RyR2.** We tested the effects of K201 on RyR1-mediated SR leak from SkM TC microsomes (R4 fractions). SR microsomes were loaded with Ca2+ (~1.5 μM Ca2+/mg SR protein). We measured the effects of K201 on the release (or leak) of Ca2+ after the addition of 20 μM of the SERCA inhibitor CPZ. Figure 3A showed a sample assay of K201 effects on Ca2+ leak at various conditions (summarized in Fig. 3B). K201 was a partially effective RyR agonist since the maximal concentrations of K201 tested (80 μM) did not reach the Ca2+ release values observed with 10 mM caffeine (Fig. 3B). K201 partial agonism on RyR1-mediated Ca2+ leak was not significantly affected by the addition of calmodulin (1 μM; n = 4; results not shown). K201 did not affect the action of caffeine (Fig. 3B), whereas agents that induce subconductance states (“substates”), such as ryanodine or imperatorin-A, decreased the rate of Ca2+ release since these substates decrease the current amplitude of caffeine-activated RyR1. Our results also demonstrated that K201 is unable to counteract RyR1 block by RR. Moreover, K201 was also unable to counteract RyR1 block by 500 μM Tetracaine (n = 3; results not shown).

The effects of K201 on channel activity of RyR1 from rabbit SkM and RyR2 from pig heart were studied after reconstitution of the channels from SR membranes into planar lipid bilayers. In all experiments, we used 50 mM Ca2+ (in the trans chamber) at a pH of 7.4. In most cases, the bilayer membrane was clamped at 0 mV. For SkM RyR1, we carried out experiments in the presence of Mg2+/ATP and ~2 μM cytosolic [Ca2+]. Under these conditions, RyR1 had been classified into two groups: high affinity (HA) channels, with open probability, Popen, ranging from 0.1 to 0.6, and low activity (LA) channels with Popen < 0.01 (Copello et al., 1997, 2002). Fig. 3C presented an example of HA RyR1 recordings in the absence or presence of K201. Figure 3D shows summary data indicating that with the addition of K201, the Popen of HA-RyR1 channels increased. In contrast, LA-RyR1 remained inactive. Figure 3, E and F, shows that the increase in Popen induced by K201...
in HA-RyR1 is associated with subtle changes in channel kinetics. As previously done, we fit the distribution of RyR2 openings with two open and two closed dwell time components, which is an approximation (Diaz-Sylvester et al., 2011). In the presence of K201, the two dwell open times ($t_{o1}$ and $t_{o2}$) are similar in value to those under control conditions. Still, the probability of the longer openings increases (Fig. 3). K201 also induces a significant decrease in the closed dwell times, $t_{c1}$ (from 13.9 to 3.11 milliseconds) and $t_{c2}$ (from 71.6 to 22.8 milliseconds) (Fig. 3).

Figure 4 summarizes our results with RyR2 channels. A set of experiments was carried out in the presence of cytosolic $\sim 5$ mM $\text{Ca}^{2+}$, where channels displayed moderate $P_o$. Here, we were able to determine that K201 at doses $\geq 5$ $\mu$M increases the activity of RyR2 (Fig. 4, A and C, triangles). At holding voltages of 0 mV, K201 did not affect channel current amplitudes and did not induce any type of channel openings to subconductance states (Fig. 4, A, D, and E). Therefore, we conclude that, under these conditions, K201 only affected RyR2 channel gating characteristics. Still, the highest dose of K201 we tested (80 $\mu$M; close to the maximal solubility of K201 in aqueous solutions) resulted in a $P_o$ $\sim 0.8$, a value significantly lower than the $P_o$ $\sim 0.99$ obtained with caffeine under the same conditions (Fig. 4, A and C). We also tested the effect of K201 on channels incubated with cytosolic $\sim 100$ nM in the presence of $\text{Mg}^{2+}/\text{ATP}$ 5 mM. As previously reported (Copello et al., 1997, 2002; Porta et al., 2011), RyR2 display sporadic openings and are quiescent most of the time under these conditions ($P_o$, 0.01). Aliquot additions of K201 (from 1.5 to 80 $\mu$M) did not alter RyR2 gating kinetics or channel activity.

**Fig. 1.** K201 inhibits SR $\text{Ca}^{2+}$ uptake. SR microsomes (from rabbit SkM and pig heart) were incubated in phosphate buffer containing ATP/Mg with 2 $\mu$l of K201 in DMSO (final K201 levels range from 1.5 to 80 $\mu$M) or with 2 $\mu$l of DMSO (control) and in the absence or presence of 5 $\mu$M RR or 10 mM caffeine. SR $\text{Ca}^{2+}$ loading was started by increasing $\text{Ca}^{2+}$ in the cuvette to 30 $\mu$M. (A) An example of $\text{Ca}^{2+}$ uptake by rabbit SkM microsomes measured in the absence (top panel) or presence (lower panel) of 5 $\mu$M RR under control conditions, 80 $\mu$M K201, or 10 mM caffeine. (B and C) Effect of 80 $\mu$M K201 and of 10 mM caffeine on the rate of SR $\text{Ca}^{2+}$ loading by rabbit SkM (B) and cardiac (C) SR microsomes in the absence or presence of RR; data are mean $\pm$ S.E.M. of $n$ = 4 experiments. (D and E) Loading rates of SkM (D) and cardiac (E) SR microsomes as a function of K201 concentration. Experimental data ($n$ = 4 experiments in each condition) as in (A) were fitted by a single exponential function from which the initial rate of $\text{Ca}^{2+}$ uptake was derived (see Materials and Methods). From the data in (D), an $IC_{50}$ of 27.5 $\pm$ 2.4 $\mu$M was estimated for K201 in SkM microsomes. From the data in (E), we estimated an $IC_{50}$ of 40.5 $\pm$ 4.3 $\mu$M for K201 in cardiac microsomes.
(values of \(P_0 < 0.01\) were recorded even in the presence of 80 \(\mu M\) K201; Fig. 4, B and C, filled circles). Caffeine (10 mM) was still capable of activating the channels to \(P_0 \sim 0.9\) (Fig. 4, B and C). These results indicate that K201 has no effect on RyR2-dependent \(Ca^{2+}\) release under resting cell conditions.

Previous reports indicated that K201 strengthens FKBP12.6 binding to RyR2 and FKBP12.6 is a requirement for K201-induced channel gating stabilization of RyR2 in myocytes (Yano et al., 2003; Wehrens et al., 2005). In vitro studies, however, suggest that K201 increasing FKBP12.6 rate of dissociation from RyRs (Blayney et al., 2010). Here, we tested if the effect of K201 on RyRs could be related to FKBP12.6 dissociation. As seen in Fig. 4D, with the addition of 20 \(\mu M\) K201, RyR2 \(P_0\) increased; however, after the addition of FKBP12.6 (20 \(\mu M\)) channel activity did not change, suggesting that the action of K201 is independent of FKBP12.6.

K201 induced subconductance states (substates) during openings of purified RyR1, but only when channels are held at negative SR voltages and not with positive voltages; \(V_m \sim 0\) mV (the putative SR voltage in cells) could not be tested due to the symmetrical ionic conditions (Almassy et al., 2008). Most of our experiments were conducted at 0 mV, but we occasionally tested voltages ranging from \(-40\) to \(+15\) using our usual cations (Tris \(1\) in \(cis\) and \(Ca^{2+}\) \(2\) in \(trans\) solution). We have previously described a subpopulation of RyR2 that gates mostly to long openings (i.e., they do not display short flickering events) (Diaz-Sylvester et al., 2011). We performed a set of experiments using this population to increase the

![Fig. 2. K201 inhibits SERCA in a Ca\(^{2+}\)-dependent manner. (A) Decrease in NADH absorption at 340 nm as a function of time (indicative of equimolar ATP consumption by ATPase activity) by cardiac SR microsomes and at three \(Ca^{2+}\) concentrations: 200 \(\mu M\) (a1), 2 \(\mu M\) (a2), and 0.25 \(\mu M\) (a3). As shown, K201 inhibited the decrease in NADH levels (\(\Delta\)NADH) more strongly at low \(Ca^{2+}\). On the contrary, 20 \(\mu M\) CPZ is a strong SERCA inhibitor at all \(Ca^{2+}\) concentrations, which leaves only a small residual fraction of SERCA-independent ATPase activity (similar in magnitude at the three [\(Ca^{2+}\)] concentrations tested) (B and C) ATPase activity of cardiac SR microsomes (B) and SkM R2 microsomal fraction enriched in longitudinal tubule (C) as a function of K201 concentrations (ranging from 1.5 to 80 \(\mu M\). Dose-response curves to K201 were built at 200 \(\mu M\) (squares) 2 \(\mu M\) (triangles), and 0.25 \(\mu M\) \(Ca^{2+}\) (circles). Data are shown as means \(\pm\) S.E.M of \(n = 4\) experiments. From the data, we estimated K201 IC\(_{50}\) for SERCA at high \(Ca^{2+}\) concentrations (130 \(\pm\) 11.8 \(\mu M\) and 104 \(\pm\) 10 \(\mu M\), respectively, for cardiac and SkM), mid- \(Ca^{2+}\) levels (17.8 \(\pm\) 1.5 \(\mu M\) and 13.3 \(\pm\) 2.5 \(\mu M\)), and low \(Ca^{2+}\) (8.8 \(\pm\) 1.0 \(\mu M\) and 4.9 \(\pm\) 0.7 \(\mu M\)).]
Fig. 3. K201 activates skeletal muscle RyR1. (A) Ca\(^{2+}\) leak from SkM microsomes (R4 fractions enriched in terminal cisternae) was induced by blocking the SERCA pump with cyclopiazonic acid (CPZ, 20 μM). Measurements were performed under control conditions and upon the addition of 0.4, 2, 4, 16, and 80 μM K201. SR Ca\(^{2+}\) leak was also measured in the presence of the RyR1 agonist caffeine (10 mM) or with 10 μM RR (which inhibits RyR1). (B) SR Ca\(^{2+}\) leak rate in the presence of K201 versus control (n = 4 experiments in each condition). Leak rate significantly increased from control values by 107% ± 8% in the presence of 80 μM K201. Still, the leak increased by 292% ± 25% with caffeine, a significantly stronger agonist than K201. Note also that K201 does not interfere with the effects of caffeine. RR significantly decreased the rate of SR Ca\(^{2+}\) leak, in which the addition of K201 has no effects. (C) K201 (40 μM but not 2 μM) activated a skeletal HA RyR1 reconstituted into planar lipid bilayers from TC microsomes. Recordings were carried out at 0-mV transmembrane voltage with 2 μM cytosolic free Ca\(^{2+}\) concentration and 5 mM of Mg\(^{2+}\)/ATP (for details, see Materials and Methods). Openings are shown as discrete upward deflections of the current. (D) Dose-response of K201 action on LA (n = 4) and HA RyR1 (n = 5) in planar lipid bilayers. Values are means ± S.E.M. *P < 0.05 compared with absence of K201 (n = 4–6 paired experiments). For HA, we estimated an EC\(_{50}\) of 29.0 ± 3.5 μM. (E and F) Histograms for open and closed dwell-time distribution (left and right charts, respectively) were obtained from 4-minute recordings of channels under control conditions (black outlines) and in the presence of 40 μM K201 (gray outlines). Two exponential components were used to fit openings and closures. Under control conditions, dwell open times were t\(_{1}\) = 3.52 ± 0.08 millisecond (71% ± 4%) and t\(_{2}\) = 26.8 ± 0.2 millisecond (29% ± 4%).
visibility of any K201-induced changes in conductance. Even minor K201 effects would be visible when minimizing noise from flicker short openings/closures, which is found under control conditions in most other RyRs (Copello et al., 1997; Diaz-Sylvester et al., 2011). We used Ca\(^{2+}\) (instead of Tris\(^{+}\)) in our cytosolic (cis) solution to increase RyR2 channel amplitude at 0 and −15 mV, as well as to generate measurable cis-to-trans Ca\(^{2+}\) currents (i.e., 2.3 pA cytosol to lumen) at the SR voltage (V\(_{m}\)) of −60 mV. Figure 4E shows current-amplitude plots that do not evidence any K201 induced change in conductance, substrates, or increase in flickering in the range of +0 mV to −15 mV. No substrates were observed with K201 at +20 and +40 mV (results not shown). In the amplitude distribution of channel openings (filtered at 500 Hz), well defined open and closed conductance states generate two Gaussian distributions. The use of different scales at different voltages (Fig. 4E) makes the standard deviation of the Gaussian distributions. The use of different scales at different

Discussion

We found that K201 blocks SERCA ATPase activity with similar characteristics and affinity in cardiac and SkM SR microsomes. K201 acts as a Ca\(^{2+}\)-dependent SERCA blocker, which has increased potency when Ca\(^{2+}\) levels decrease to those observed in resting cells. K201 also acts as a partial RyR1 and RyR2 agonist, which would increase the P\(_o\) of partially active channels but would not activate channels that are in the closed state (such as LA RyR1 or RyR2 under Ca\(^{2+}\) resting conditions). K201 also induces RyR2 flicker block at negative SR voltages, which has unclear significance for the drug action in the cellular environment.

Ca\(^{2+}\)-Dependent Blocker of SERCA is a Novel Characteristic of K201. With incubating Ca\(^{2+}\) levels of 100, 2, and 0.25 μM, we estimated that the corresponding IC\(_{50}\) values of K201 ATPase inhibition were within ~130–104, 18–13, and 9–5 μM (cardiac vs. SkM respectively). Previous studies with cardiomyocytes have shown that K201 induces SR Ca\(^{2+}\) depletion (Inagaki et al., 2000; Loughrey et al., 2007; Chen et al., 2008). The decrease in SR content could be attributed to other K201 targets in plasmalemma, including block of L-type Ca\(^{2+}\) channels (Kimura et al., 1999; Inagaki et al., 2000) and/or inhibition of SERCA-mediated Ca\(^{2+}\) uptake (Inagaki et al., 2000; Loughrey et al., 2007). Indeed, K201 (3 μM) inhibited by ~30% the V\(_{max}\) of Ca\(^{2+}\) uptake in aggregates of permeabilized cardiomyocytes, which was measured in the range of 0.2 and 2.5 μM Ca\(^{2+}\) (Loughrey et al., 2007). These data correlate relatively well with the results we obtained when measuring ATPase activity carried out in the presence of 2 μM Ca\(^{2+}\), indicating that SERCA is also an important cellular target for K201. Our data suggest that K201 potency would be even higher to inhibit SERCA ATPase activity in the presence of 0.05 μM Ca\(^{2+}\) (resting cell levels).

Many SERCA blockers have been previously identified, including the widely used thapsigargin and cyclopiazonic acid; however, most display much milder Ca\(^{2+}\) dependency in their effects on ATPase activity, despite the marked Ca\(^{2+}\) dependence of normal SERCA function (Seidler et al., 1989; Wuytack et al., 2002; Zafar et al., 2008; Michelangeli and East, 2011; Soler et al., 2012). It is possible that the mechanism of action of K201’s is similar to that of epigallocatechin-3-gallate (EGCG), a drug that also shows marked Ca\(^{2+}\) dependence in its SERCA block, attributed to EGCG interfering with Ca\(^{2+}\) binding being counteracted by accumulation of Ca\(^{2+}\)-bound SERCA conformations with increasing Ca\(^{2+}\) levels (Soler et al., 2012). Various benzothiazepines, including K201, are known to target cellular paths that modulate intracellular Ca\(^{2+}\) homeostasis, as previously discussed (Neumann et al., 2011). Still, there is only one report from our group where CGP37157 (which is a 4,1 benzothiazepine) was found to be a SERCA blocker (Neumann et al., 2011). More recent ATPase testing has revealed that CGP and various CGP analogs are also Ca\(^{2+}\) dependent SERCA blockers (Darcy YL and Copello JA, unpublished results).

K201 inhibits SERCA1a (main isoform in fast twitch SkM) and SERCA2a (main isoform in heart SR microsomes) with similar potency, suggesting that the action of K201 is not mediated by phospholamban, which is not expressed in the fast-twitch SkM SR used here (Jorgensen and Jones, 1986); however, a previous study conducted with SkM SR microsomes found an IC\(_{50}\) ~110 μM for K201 inhibition of SERCA1a ATPase activity in 2 μM Ca\(^{2+}\) (Almassy et al., 2008). These values are almost 10 times higher compared with the results reported here and the estimates of IC\(_{50}\) based on extrapolation from the effect of K201 on SERCA mediated Ca\(^{2+}\) uptake in cardiomyocytes (Inagaki et al., 2000; Loughrey et al., 2007). The cause of this discrepancy is unknown, but the pH = 7.5 in Almassy et al. (2008) was much higher than in our experiments and in the cytosol of cells (pH ~7.0), which may affect the action of K201.

K201 Modulation of RyR1 and RyR2: A Review of the Literature. Studies with SkM SR microsomes suggest that K201 increases RyR1-mediated SR leak, which was prevented

r1 = 13.9 ± 0.1 millisecond (63% ± 4%) and r2 = 71.6 ± 0.2 millisecond (37% ± 4%). For 40 μM K201, dwell open times were similar but had different probability: r1 = 4.15 ± 0.11 millisecond (38% ± 3%) and r2 = 27.6 ± 0.2 millisecond (62% ± 3%). In contrast, dwell closed times were significantly shorter and distributed with r1 = 3.11 ± 0.13 millisecond (51% ± 3%) and r2 = 22.8 ± 1.2 milliseconds (49% ± 4%).
by RyR blockers (RR and tetracaine). K201 is clearly a less potent agonist of RyRs than caffeine, and its action was not significantly affected by the addition of calmodulin. Our bilayer data indicate that K201 preferentially acts on channels that are moderately active (RyR2 channels incubated with diastolic Ca\textsuperscript{2+} levels or HA RyR1 channels). In contrast, K201 does not affect channels that are in the closed state (RyR2 under resting Ca\textsuperscript{2+} conditions or LA RyR1). Our data also indicate that K201 would have no direct effects on RyR2 at doses that have been commonly used in cellular studies (0.3–2 μM). Nonetheless, K201 is a positively charged and cell permeable molecule. Because of more negative potential of the cytosol (−80 to −90 mV), K201 could accumulate there at 10–20 times higher concentrations than those added to the bathing solution.

Some aspects of K201-mediated RyR2 stabilization and prevention of SR leak are controversial, including the ideas that K201 promotes FKBP12.6 binding to the channels and/or modulates phosphorylation status (Yano et al., 2003; Wehrens et al., 2005; Xiao et al., 2007; Blayney et al., 2010; Sacherer et al., 2012). In our hands, the effects of K201 on RyR2 open probability were not directly affected by adding FKBP12.6, which is in line with our previous reports of FKBP12.6 not being a direct modulator of RyR2 (Barg et al., 1997; Xiao et al., 2007). Still, there are various conflicting opinions on the direct modulation of RyRs by FKBP1s (Fill and Copello, 2002). Furthermore, our results do not rule out the possibility of an indirect modulation of RyR-FKBP interactions by K201.

A previous study conducted on reconstituted purified RyR1 under full activation conditions (which allows only
measurement of the effect of antagonist blockers) reported that K-201 induced conductance substates in a voltage-dependent manner and at negative but not at positive SR voltages (Almassy et al., 2008). Here, we used a different model (RyRs reconstituted from native SR microsomes) and experimental conditions where RyR1 and RyR2 had low or moderate activity (which allowed detecting agonistic effects). Under these conditions, K201 acted as a mild agonist, and we did not distinguish any blocking events or substates within the range of +40 to −15 mV. However, at a SR voltage of −60 mV (similar as in Almassy et al., 2008), we observed flicker block and intermediate states of conductance (which could be attributed to short-lived substates) in RyR2 induced by K201. The blocking effect of K201 is intriguing, but the role of voltage dependence in RyRs has unknown physiologic significance as channels are thought to operate at a nearly constant SR voltage of −60 mV (Fill and Copello, 2002; Diaz-Sylvester et al., 2011).

Only a few studies tested the direct effects of K201 on RyR2. Two articles reported a minor decrease in [3H] ryanodine binding by SR microsomes (which is used as an index of RyRs activity) induced by 1 or 50 μM K201 (Hunt et al., 2007; Blayney et al., 2010). In principle, these differences with our results may relate to the lack of ATP/Mg2+ and the very low luminal Ca2+ in the SR in [3H] ryanodine binding studies, which made RyRs less sensitive to agonists (Sitsapesan and Williams, 1990; Ogawa, 1994; Fill and Copello, 2002). It is possible that the experimental conditions used in the [3H] ryanodine binding studies are similar to those in our low cytosolic Ca2+ conditions, where K201 is not effective as an agonist.

A SERCA Role for the Cell-Protective Action of K201? There is consensus that the role of voltage dependence in RyRs has unknown physiologic significance, but in hypothermia and heart failure (Yano et al., 2003; Wehrens et al., 2005; Cobbold and Sigworth, 1995) Fitting and statistical analysis of Single-Channel Records, in Single-Channel Recording (Sakmann B and Neher E, eds) pp 483–587, Springer US, Boston, MA. Darcy, Diaz-Sylvester, Copello. Contributed new reagents or analytic tools: Copello. Conducted experiments: Darcy, Diaz-Sylvester, Copello. Contributed new reagents or analytic tools: Copello. Performed data analysis: Darcy, Diaz-Sylvester, Copello. Wrote or contributed to the writing of the manuscript: Darcy, Diaz-Sylvester, Copello.

In conclusion, K201 is a novel Ca2+ dependent inhibitor of SERCA that may be highly potent under cell resting conditions and may play an important role in preventing resting RyR-mediated SR Ca2+ leak in muscle and heart pathologies. K201 also modulates RyRs, acting as an agonist at the putative SR voltage, but with much lower potency under cell resting conditions.

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Authorship Contributions

Participated in research design: Darcy, Diaz-Sylvester, Copello.

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Contributed new reagents or analytic tools: Copello.

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References


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