CGP-37157 Inhibits the Sarcoplasmic Reticulum Ca\(^{2+}\) ATPase and Activates Ryanodine Receptor Channels in Striated Muscle

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ABSTRACT

7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one [CGP-37157 (CGP)], a benzothiazepine derivative of clonazepam, is commonly used as a blocker of the mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger. However, evidence suggests that CGP could also affect other targets, such as L-type Ca\(^{2+}\) channels and plasmalemma Na\(^+/\)Ca\(^{2+}\) exchanger. Here, we tested the possibility of a direct modulation of ryanodine receptor channels (RyRs) and/or sarco/endoplasmic reticulum Ca\(^{2+}\) -stimulated ATPase (SERCA) by CGP. In the presence of ruthenium red (inhibitor of RyRs), CGP decreased SERCA-mediated Ca\(^{2+}\) uptake of cardiac and skeletal sarcoplasmic reticulum (SR) microsomes (IC\(_{50}\) values of 6.6 and 9.9 μM, respectively). The CGP effects on SERCA activity correlated with a decreased \(V_{\text{max}}\) of ATPase activity of SERCA-enriched skeletal SR fractions. CGP (≥5 μM) also increased RyR-mediated Ca\(^{2+}\) leak from skeletal SR microsomes. Planar bilayer studies confirmed that both cardiac and skeletal RyRs are directly activated by CGP (EC\(_{50}\) values of 9.4 and 12.0 μM, respectively). In summary, we found that CGP inhibits SERCA and activates RyR channels. Hence, the action of CGP on cellular Ca\(^{2+}\) homeostasis reported in the literature of cardiac, skeletal muscle, and other nonmuscle systems requires further analysis to take into account the contribution of all CGP-sensitive Ca\(^{2+}\) transporters.

Introduction

During an action potential in striated muscle, the activation of ryanodine receptor channels (RyRs) induces the global Ca\(^{2+}\) release from sarcoplasmic reticulum (SR) Ca\(^{2+}\) stores triggering contraction (Sitsapesan and Williams, 1998; Bers, 2001; Fill and Copello, 2002; Fleischer, 2008). The SR Ca\(^{2+}\) ATPase (SERCA) rapidly sequesters the Ca\(^{2+}\) released into the cytosol back into the SR, leading to the relaxation of the muscle cells. The dynamics of Ca\(^{2+}\) release events in muscle cells is complex and still not fully understood (Fill and Copello, 2002; Stern and Cheng, 2004; Cheng and Lederer, 2008; Rios et al., 2008). Mitochondria seem to play an intrinsic role in the regulation of intracellular Ca\(^{2+}\), which includes structural and functional interactions with the SR and plasmalemma (Rizzuto et al., 1998; Csordás and Hajnóczky, 2009; Lukyanenko et al., 2009). Among mitochondrial transporters, the 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one [CGP-37157 (CGP)]-sensitive Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) may mediate SR Ca\(^{2+}\) load and release (Szalai et al., 2000; Malli et al., 2005; Csordás and Hajnóczky, 2009). Indeed, CGP has been found to significantly affect intracellular Ca\(^{2+}\) signaling in smooth, cardiac, and skeletal muscle cells and in nonmuscle systems (Griffiths et al., 1997; Malli et al., 2005; Belmonte and Morad, 2008; Liu and O’Rourke, 2008; Chalmers and McCarron, 2009; Csordás and Hajnóczky, 2009).

The ability of the benzothiazepine derivative CGP to react with transporters other than the mitochondrial NCX (i.e., cross-reactivity) has not been fully explored. Reports have suggested that this compound can affect plasmalemma NCX.
(Czyz and Kiedrowski, 2003) and L-type Ca\(^{2+}\) channels (Thu et al., 2006). Various benzoizothiazepines with relatively different side chains, such as K201 (Kohno et al., 2003; Hunt et al., 2007) and KN-362 (Kodama et al., 1988; Tatsukawa and Arita, 1997), are known to modulate RyRs. K201 was also found to block the SERCA (Loughrey et al., 2007). As a consequence, we studied whether the effects of CGP on cellular Ca\(^{2+}\) homeostasis could be mediated, at least in part, by CGP’s direct interaction with RyRs and SERCA. To investigate this possibility, we performed studies on isolated SR CRP’s direct interaction with RyRs and SERCA. To investigate this possibility, we performed studies on isolated SR microsomes enriched in SERCA and RyRs and on RyRs reconstituted into planar lipid bilayers. Our results demonstrate that CGP can act as both a SERCA antagonist and RyR agonist.

Materials and Methods

Cardiac and Skeletal SR Microsomes. All animal procedures were designed to minimize pain and suffering and conformed to the guidelines of the National Institutes of Health. The committee on the Use and Care of Laboratory Animals of Southern Illinois University School of Medicine reviewed and approved the protocols for animal use. R2 and R4 fractions of skeletal muscle SR microsomes (eight different preparations) were isolated from predominantly fast-twitch skeletal muscle (back and leg; adult New Zealand white rabbits), as described previously (Saito et al., 1984; Chu et al., 1988). For skeletal muscle, the R4 fraction of SR (TC microsomes) is highly enriched in terminal cisternae, which consists of both the junctional face membrane as well as the calcium pump membrane of SR. The Ryanodine receptor (RyR1) is localized to the junctional face membrane, whereas the Ca\(^{2+}\) pump protein (SERCA 1a) is localized in the calcium pump membrane of SR (Saito et al., 1984; Fleischer, 2008). The R2 fraction of SR (LT microsomes) is recoverable to the longitudinal tubules of SR, which consists mainly of calcium pump membrane and is practically devoid of Ryanodine receptor (Chu et al., 1988; Fleischer, 2008). The LT microsomes are used to characterize the calcium pump, whereas the TC microsomes contain both RyR1 and SERCA 1a, and therefore specific inhibitors for RyR1 and for the calcium pump must be used to sort out their response (Fleischer, 2008).

Enriched porcine cardiac SR microsomes were prepared following protocols developed for dog heart SR microsomes (Chamberlain et al., 1983). All preparations were stored in liquid nitrogen. Rabbit skeletal TC or LT microsomes or porcine cardiac SR for use in experiments were separated every month in 15-μl aliquots at a concentration of 5 to 15 mg protein/ml in 5 mM imidazole chloride, 290 mM sucrose, pH 7.0, quick-frozen, and stored at −80°C. For experiments, aliquots were quickly thawed in water, kept on ice, and used within 5 h.

Measurements of Ca\(^{2+}\) Uptake/Leak by SR Microsomes. Ca\(^{2+}\) uptake by cardiac SR microsomes or R4 fractions of skeletal TC microsomes was measured with a spectrophotometer (Cory 50; Varian, Walnut Creek, CA) using the Ca\(^{2+}\)-sensitive dye antipyrylazo III (APIII), as described previously (Chamberlain et al., 1984; Chu et al., 1988). In brief, 10 to 40 μg of R2 fractions enriched in longitudinal tubule were incubated with buffer containing 140 mM KCl, 5 mM MgCl\(_2\), 5 mM HEPES, 2 mM phosphoenolpyruvate, 8.4 U/ml pyruvate kinase, and 12 U/ml lactate dehydrogenase. The mixture also contained 150 μM Ca\(^{2+}\) and variable amounts of EGTA (0.1, 0.2, 0.4, and 5 mM) for free Ca\(^{2+}\) levels of approximately 50, 1, 0.25, and 0.01 μM. pH was adjusted to 7.0 by titration with KOH. The reaction starts by adding 1 mM ATP, which is hydrolyzed to ADP by ATPases. ADP is regenerated to ATP by reactions that induce the oxidation of 1 molecule of NADH (to NAD\(^+\)) per ATP hydrolyzed (Chu et al., 1988). The rate of ATP hydrolysis, in nanomoles of ATP per milligram of SR protein per minute was estimated from the equation

\[
\text{OD}_{340}/(e \cdot L \cdot S) = k \times t
\]

where \(e\) is the NADH extinction coefficient (6.22 × 10\(^{-5}\) mol \cdot cm\(^{-1}\)), \(L\) is the cuvette length (in centimeters), and \(S\) the amount of SR protein added to the cuvette (in milligrams per milliliter).

RyR Channel Recordings and Data Analysis. Cardiac and skeletal RyRs were reconstituted into planar lipid bilayers formed on 80- to 120-μm diameter circular holes in Teflon septa, separating two 1.2-ml compartments as described previously (Copello et al., 1997). The trans compartment contained a HEPES-Ca\(^{2+}\) solution [250 mM HEPES and 50 mM Ca(OH)\(_2\), pH 7.4] and clamped at 0 mV with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). The cis compartment (ground) was filled with HEPES-Tris solution (250 mM HEPES and 120 mM Tris, pH 7.4). Fusion of SR vesicles was promoted by subsequently adding, while stirring, 500 to 1000 mM CaCl\(_2\), 1 mM CaCl\(_2\), and SR microsomes (5–15 μg) to the cis solution (Copello et al., 1997). This manipulation allows for reconstitution of RyRs with their cytosolic surface facing the cis chamber. Excess CaCl\(_2\) and Ca\(^{2+}\) were removed by perfusing the cis chamber for 5 min at 4 ml/min with HEPES-Tris solution. A mixture of BAPTA (1 mM) and dibromo-BAPTA (1 mM) was used to buffer free [Ca\(^{2+}\)] on the cytosolic surface of the channel ([Ca\(^{2+}\)_cyt]o) (Copello et al., 1997). Free [Mg\(^{2+}\)] in mixtures of Mg\(^{2+}\) and ATP was estimated using Winmax2.5 by Chris Patton (Stanford University, Palo Alto, CA) (http://www.stanford.edu/~cpatton/max2.html).

Drug and Chemicals. CaCl\(_2\) standard for calibration was from World Precision Instruments Inc. ( Sarasota, FL). Phospholipids (phosphatidylethanolamine, phosphatidylserine, and phosphatidylinol) were obtained from Avanti Polar Lipids (Alabaster, AL). CGP-37157 was from Tocris Bioscience (Ellisville, MO). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. Data are presented as means ± S.E.M. of n measurements. Statistical comparisons between groups were performed with a paired t test. Differences were considered statistically significant at \(p < 0.05\), and figures indicate \(p\) values.
Results

CGP Inhibits SERCA-Mediated Ca\(^{2+}\) Loading and ATPase Activity in Cardiac and Skeletal SR Microsomes. We measured Ca\(^{2+}\) uptake by cardiac SR microsomes and from skeletal TC microsomes. The net Ca\(^{2+}\) uptake is the difference between the active SR Ca\(^{2+}\) influx (which depends on SERCA activity) and the passive efflux of Ca\(^{2+}\) from the SR microsomes (which depends mainly on RyRs activity). The experiments were carried out in the presence of ruthenium red (5 \(\mu\)M), which inhibited the efflux from RyRs. Therefore, under these conditions, changes in the net Ca\(^{2+}\) uptake by the cardiac and skeletal microsomes closely correlate with the SERCA pumping rate.

Figure 1A illustrates an example of how CGP inhibited the process of Ca\(^{2+}\) uptake by cardiac SR microsomes. The dose-response curve (Fig. 1B) suggests that CGP produced a half-maximal inhibition (IC\(_{50}\)) at 9.9 ± 2.0 \(\mu\)M (\(n = 4\) paired experiments). Likewise, Fig. 1C and D, suggests that in skeletal muscle TC microsomes, CGP also inhibited the rate of loading with an IC\(_{50}\) of 6.6 ± 1.2 \(\mu\)M (\(n = 4\) paired experiments).

The effects of CGP (10 \(\mu\)M) on SERCA were also assayed in skeletal muscle LT microsomes with an ATPase assay at two Ca\(^{2+}\) concentrations. The first set was performed at a Ca\(^{2+}\) concentration of 50 \(\mu\)M, where SERCA reaches maximal activity (\(V_{\text{max}}\)) and the second set was carried out with a Ca\(^{2+}\) concentration of 300 mM, which is near the SERCA’s half-maximal activity (\(K_{m}\)) for Ca\(^{2+}\) (Bers, 2001). Figure 1E shows that with 50 \(\mu\)M Ca\(^{2+}\), approximately 95% of the ATPase of LT microsomes was blocked by 20 \(\mu\)M CPZ, which inhibits SERCA. We also found that decreasing Ca\(^{2+}\) to 5 nM fully inhibited the ATPase activity (data not shown). Thus, SERCA seems to mediate most of the ATPase activity in these skeletal LT microsomes, which are highly enriched in longitudinal tubule. As shown in Fig. 1, E and F, 10 \(\mu\)M CGP decreased \(V_{\text{max}}\) (at 50 \(\mu\)M Ca\(^{2+}\)) by 31.3 ± 5.3%. When the ATPase activity was measured at 300 nM Ca\(^{2+}\) (which is near the \(K_{m}\) of SERCA for Ca\(^{2+}\) activation), we also found a comparable inhibition of 42.7 ± 6.4%.

CGP Activates Skeletal and Cardiac RyRs. In another set of experiments, we loaded skeletal muscle TC microsomes with Ca\(^{2+}\) in the absence or presence of ruthenium red. Thereafter, the SERCA pump was inhibited by CPZ, and the rate of Ca\(^{2+}\) leak was measured in the absence or presence of ruthenium red. In the absence of ruthenium red, where most of the Ca\(^{2+}\) efflux from the vesicles is via RyR1, CGP (5 and 20 \(\mu\)M) significantly increases the rate of Ca\(^{2+}\) leak from the TC microsomes. In contrast, the residual leak in the presence of ruthenium red (which inhibit RyR1) was decreased by CGP (20 \(\mu\)M) (Fig. 2, A and B). These results suggest that the increased Ca\(^{2+}\) leak observed in the absence of ruthenium red resulted from the activation of RyR1. The inhibition of the remaining leak in the presence of ruthenium red may represent a secondary Ca\(^{2+}\)-permeable path found in microsomes or inhibition of the reverse mode of SERCA. The activating effect of CGP on RyR1 was confirmed by reconstituting the channels into artificial lipid bilayers. Figure 2C shows single-channel recordings of a RyR1 under control condition and after the addition of 20 \(\mu\)M CGP to the cytosolic solution. The effect of CGP was observed 5 to 10 s after the addition, reached a plateau in ~2 min, and remained stable during the experiments (up to 1 h in duration). CGP was cumulatively added to the cytosolic solution of RyR1 in the planar lipid bilayer to determine a half-maximal effective concentration (EC\(_{50}\)) of 12.0 ± 3.5 \(\mu\)M (\(n = 6\) paired experiments; Fig. 2D). The effects of CGP were also studied on porcine cardiac RyR channels (RyR2). Figure 3A shows one RyR2 incubated with 1 \(\mu\)M cytosolic Ca\(^{2+}\) (control). In this condition, the channel opened with moderate activity (\(P_o = 0.16\), estimated from 8-min recording). As shown in the figure, 2.5 \(\mu\)M CGP significantly activated the channel (\(P_o = 0.49\)). With higher doses (20 \(\mu\)M), the channels were further activated (\(P_o = 0.74\)). Figure 3B shows a dose response to cumulative doses of CGP to the cytosolic solution of RyR2 with an EC\(_{50}\) of 9.4 ± 2.3 \(\mu\)M (\(n = 6\) paired experiments). As
suggested by the recordings, CGP changed the RyR2 kinetics. Dwell-time distributions show that the duration of openings significantly increased, and the duration of closures greatly decreased (Fig. 3C). As shown in the example of Fig. 3A, we found that the RyR2 remained active when [Ca^{2+}]_{cis} was decreased to 100 nM and that this effect was partially counteracted by the addition of 1 mM Mg^{2+}. In absence of CGP, we found that the open probability of RyR2 at 100 nM is less than 5% (absence of Mg^{2+}) or less than 1% (presence of Mg^{2+}), as reported previously (Copello et al., 1997). The effect of CGP on the channels was reversed after its removal from the cytosolic solution through superfusion.

**Discussion**

Our results show that cytosolic CGP has differential direct effects on SERCA pump and RyRs of striated muscle. CGP inhibited both cardiac and skeletal SERCA (putatively SERCA 1a and SERCA 2a), which would decrease SR load. In addition, CGP significantly activated both RyR1 and RyR2, which would increase SR leak. Our results suggest that these direct effects of CGP on SERCA and RyRs may have relevance in explaining the SR depletion observed in myocytes in situ.

**Comparison with Other Benzothiazepines.** This is the first report on CGP direct modulation of RyRs. In this study, we found that CGP increased the activity of RyRs by stabilizing long-lasting opening events (EC_{50} ~ 10–12.0 μM). In addition, CGP was also found to inhibit SERCA V_max both in skeletal and cardiac muscle (IC_{50} ~ 6.6 and 9.9 μM, respectively), without an apparent modification of the K_m for Ca^{2+}. Only one report tested CGP action on SERCA and found the drug at 10 μM was without significant action on ATP-ase activity of SR microsomes (Cox et al., 1993).

Previous studies have shown that CGP, a clonazepam derivative, is an effective (IC_{50} ~ 500 nM) and specific inhibitor of the mitochondrial NCX (Gunter and Pfeiffer, 1990; Cox et al., 1993). Yet, it was reported that CGP inhibits NCX in the plasmalemma (IC_{50} ~ 13 μM) and blocks the L-type Ca^{2+} channels (IC_{50} ~ 0.27 μM) (Czyz and Kiedrowski, 2003; Thu et al., 2006). Inhibition of plasmalemma NCX, L-type channels, and SERCA has also been reported for clonazepam, the parent compound (Gershon, 1992; Cox et al., 1993; Griffiths et al., 1997). Moreover, clonazepam is a well-known agonist of GABA-induced chloride currents (Yakushiji et al., 1989), but effects on RyRs have not been reported.

Various other benzothiazepines have been reported to affect intracellular Ca^{2+} signaling in striated muscle. Some agents, such as diltiazem, more specifically target L-type channels and only affect SERCA and RyRs at much higher doses (Chamberlain et al., 1984). Other compounds of this class, such as 5-3[2-(3,4-dimethoxyphenyl)ethylamino]-1-oxopropyl]-2,3,4,5- tetrahydro-1,5-benzothiazepine fumarate (KT-362), were termed “intracellular Ca^{2+} channel antagonists” because they more specifically impair intracellular Ca^{2+} release in vascular smooth muscle (Shibata et al., 1987; Ueyama et al., 1996). Yet, for many of these compounds, the mechanism of action remains unclear. Two reports found that 4-[-3{1-(4-benzyl)piperidinyl}propionyl]-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine (K201), also known as JTV519, inhibited RyRs, but they differ on the requirement of hexadecahydro-5,19-dihydroxy-3-[-2-(4-hydroxy-3-methoxy)cyclohexyl]-1-methylthienyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclooctin-1,7,20,21(4H,23H)-tetrone (FK-506) binding protein for inhibition (Wehrens et al., 2005; Hunt et al., 2007). However, an early study suggested that K201 could increase ^3H]ryanodine binding (Kohn et al., 2003). In addition, others reported a noticeable inhibitory action of K201 on ventricular myocytes whole-cell Na^+, Ca^{2+}, and K^+ currents and on SERCA (Kikura et al., 1999; James, 2007).

In summary, CGP displays, as do various other benzothiazepines, a complex pattern of interference with transporters.
involved in Ca\(^{2+}\)/H\(^{+}\) signaling (RyRs, SERCA, L-type Ca\(^{2+}\) channels, plasmalemma, and mitochondrial NCXs). These results are quite interesting because CGP acts as a RyR channel agonist and seems to be opposite to K201 or KT-362. This suggests that RyRs may have a domain that binds these compounds and could be targeted for positive/negative modulation of RyR function through drug design.

**CGP Seems to Modulate Multiple Molecular Targets in Cells.** Evidence suggests that mitochondria modulate intracellular Ca\(^{2+}\)/H\(^{+}\)signaling by acting both as a Ca\(^{2+}\) sink when cytosolic levels are high and as a Ca\(^{2+}\) source for repletion of the intracellular Ca\(^{2+}\) stores (Bers, 2001). The process of mitochondrial Ca\(^{2+}\) release that feeds the SR seems to be mediated by various transporters, including the mitochondrial NCX (Szalai et al., 2000; Malli et al., 2005; Csordás and Hajnóczky, 2009). Reports have suggested that inhibition of the mitochondrial NCX by CGP results in significant depletion of SR Ca\(^{2+}\) load (Rizzuto et al., 1998; Malli et al., 2005; Csordás and Hajnóczky, 2009). Yet, in those studies in which SR depletion was manifest, micromolar levels (ranging from 1 to 20 \(\mu M\)) of CGP were used. Overall, this and previous studies demonstrate that micromolar levels of CGP would not only inhibit its most sensitive target (the mitochondrial NCX) but would also affect various other transporters known to significantly modulate intracellular Ca\(^{2+}\) signaling and SR Ca\(^{2+}\) content. From the inhibition of the SERCA pump activity and activation of RyRs, SR depletion and weakened excitation-contraction coupling would be expected (Bers, 2001). Inhibition of L-type Ca\(^{2+}\) currents by CGP (Thu et al., 2006) may also be responsible for the SR Ca\(^{2+}\) depletion, a process that has been observed with other L-type Ca\(^{2+}\) channel blockers (Hussain and Or-

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**Fig. 3.** CGP activates cardiac RyR2. A, single-channel recordings of a RyR2 reconstituted into bilayer from porcine cardiac SR microsomes. All recordings were performed at holding voltage (\(V_m\)) = 0 mV. Lumenal (trans) Ca\(^{2+}\) (50 mM) was the current carrier. Channel openings are observed as positive deflections of the current (\(o\), open state; \(c\), baseline). The top trace shows a 2-s segment representative of a 4-min recording of a channel under control conditions (cytosolic Ca\(^{2+}\) = 1 \(\mu M\)). The second and third traces are recordings of the same channel after the addition of increasing concentrations of CGP to the cytosolic solution (2.5 and 20 \(\mu M\), respectively). The fourth trace was recorded after subsequent addition of BAPTA to lower the cytosolic Ca\(^{2+}\) concentration down to 100 nM. The bottom trace shows the activity of the same channel after the addition of 1 mM MgCl\(_2\) to the cytosolic chamber. B, dose-response of cumulative doses of CGP to RyR2 in planar lipid bilayers with an EC\(_{50}\) of 9.4 ± 2.3 \(\mu M\) (\(n\) = 6 experiments). Values are means ± S.E.M. C, examples of representative dwell-time distributions of channel events. Histograms for open and closed times (top and bottom charts, respectively) were obtained from recordings of channels under control conditions (black outlines) and in the presence of 20 \(\mu M\) CGP (gray outlines). Fitting two exponential components to the data produce, under control conditions, dwell open times \(\tau_1\) = 1.17 ± 0.01 ms (70 ± 0.6%) and \(\tau_2\) = 5.82 ± 0.07 ms (30 ± 0.7%). Closed times were \(\tau'_1\) = 2.34 ± 0.04 ms (28 ± 0.3%) and \(\tau'_2\) = 19.18 ± 0.25 ms (72 ± 0.4%). For 25 \(\mu M\) CGP, dwell open times were much longer: \(\tau'_1\) = 96 ± 5 ms (18 ± 0.5%) and \(\tau'_2\) = 633 ± 6 ms (82 ± 0.8%). In contrast, dwell closed times were shorter and distributed with a single \(\tau'_1\) = 1.18 ± 0.12 ms.
chard, 1997). Inhibition of the plasmalemma NCX by CGP (Czyz and Kiedrowski, 2003) would increase the SR Ca\(^{2+}\) content under normal conditions, because this inhibition would increase the fraction of Ca\(^{2+}\) that is sequestered into SR by SERCA. However, NCX blockers are known to produce the opposite effect during reperfusion after ischemia, because the NCX works in a reverse mode (Ca\(^{2+}\) influx), and inhibition would prevent hypercontracture and arrhythmia (Bers 2001). Thus, the effects of CGP are complex because there are various alternatives to explain its action on SR intracellular Ca\(^{2+}\) levels and potential cardioprotective action.

Reports in the literature suggest that mitochondrial Ca\(^{2+}\) efflux may play a deleterious role during cardiac ischemia, which can be prevented by CGP or clonazepam (Liu and O'Rourke, 2008; Csordás and Hajnóczky, 2009; Bonazzola and Takara, 2010). A cardioprotective action has been reported for benzothiazepine derivatives (Farber and Gross, 1989; Wehrens et al., 2005) and for many other drug classes and processes such as preconditioning (Downey and Cohen, 2009). Although there is large heterogeneity in the mechanisms of action for cardioprotective agents, many of them seem to produce, directly or indirectly, a decrease in the use of Ca\(^{2+}\) sources/sinks (mitochondria, SR stores, and/or plasmalemma) during excitation-contraction coupling. This may lead to a significant decrease in the energy expenditure of cells that may be beneficial under pathological conditions, such as ischemia (Bonazolla and Takara, 2010).

In summary, the literature is replete with studies suggesting that a mitochondrial NCX block by CGP results in intracellular Ca\(^{2+}\) store depletion in various cell systems (Griffiths et al., 2007; Malli et al., 2005; Belmonte and Morad, 2008). In this study, the nature of CGP interactions with SR transporters was explored, and we found that there is direct CGP action on SERCA and RyRs. This report adds to a body of evidence that CGP targets other membrane transporters that play a key role in Ca\(^{2+}\) homeostasis. The significant cross-reactivity of CGP with such a variety of molecular targets confounds the simple interpretation that the action of CGP, in intact cell Ca\(^{2+}\) homeostasis, is due to block of the mitochondrial NCX.

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**Authorship Contributions**

**Conducted experiments:** Neumann, Diaz-Sylvester, and Copello.

**Contributed new reagents or analytic tools:** Fleischer and Copello.

**Performed data analysis:** Neumann, Diaz-Sylvester, and Copello.

**Wrote or contributed to the writing of the manuscript:** Neumann, Diaz-Sylvester, Fleischer, and Copello.

**References**


Csordás G and Hajnóczky G (2009) SR/ER-mitochondrial local communication: calcium release and 


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