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CGP-37157 inhibits the sarcoplasmic reticulum Ca^{2+} ATPase and activates ryanodine receptor channels in striated muscle

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RUNNING TITLE: CGP inhibition of SERCA and activation of RyRs

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Nonstandard Abbreviations:

RyRs – Ryanodine Receptor Channels

SR – Sarcoplasmic Reticulum

SERCA – Sarco/Endoplasmic Reticulum Ca^{2+} ATPase

CGP – CGP-37157

NCX – Na^{+}/Ca^{2+} Exchanger

APIII – Antipyrylazo III

RyR1 – Skeletal RyRs

RyR2 – Cardiac RyRs

CPZ – Cyclopiazonic Acid

EC – Excitation-Contraction

TC – Terminal Cisternae

LT – Longitudinal Tubule

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ABSTRACT

CGP-37157 (CGP), a benzothiazepine derivative of clonazepam, is commonly utilized as a blocker of the mitochondrial Na^+/Ca^{2+} exchanger. Yet, 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}'s = 6.6 and 9.9 μM, respectively). The CGP effects on SERCA activity correlated with a decreased V_{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}'s = 9.4 and 12.0 μM, respectively). In summary, we found that CPG inhibits SERCA and activates RyR channels. Hence, the action of CGP on cellular Ca^{2+} homeostasis reported in the literature of cardiac, skeletal muscle as well as other non muscle systems requires further analysis to take into account the contribution of all CGP-sensitive Ca^{2+} transporters.

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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 (Bers, 2001; Fill and Copello, 2002; Fleischer, 2008; Sitsapesan and Williams, 1998). The Ca^{2+} released into the cytosol is rapidly sequestered back into the SR by the SR Ca^{2+} ATPase (SERCA), 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 (Cheng and Lederer, 2008; Fill and Copello, 2002; Rios et al., 2008; Stern and Cheng, 2004). Mitochondria appear to play an intricate role in the regulation of intracellular Ca^{2+}, which includes structural and functional interactions with the SR and plasmalemma (Csordas and Hajnoczky, 2009; Lukyanenko et al., 2009; Rizzuto et al., 1998). Among mitochondrial transporters, the CGP-37157 (CGP)-sensitive Na^{+}/Ca^{2+} exchanger (NCX) may mediate SR Ca^{2+} load and release (Csordas and Hajnoczky, 2009; Malli et al., 2005; Szalai et al., 2000). Indeed, CGP has been found to significantly affect intracellular Ca^{2+} signaling in smooth, cardiac and skeletal muscle cells as well as in non-muscle systems (Belmonte and Morad, 2008; Chalmers and McCarron, 2009; Csordas and Hajnoczky, 2009; Griffiths et al., 1997; Liu and O'Rourke, 2008; Malli et al., 2005).

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 benzothiazepines with relatively different side chains, like K201 (Hunt et al., 2007;

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Kohno et al., 2003) and KN-326 (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). Consequently, we studied whether the effects of CGP on cellular Ca^{2+} homeostasis could be mediated, at least in part, by CGPs direct interaction with RyRs and SERCA. In order to investigate this possibility, we performed studies on isolated SR microsomes enriched in SERCA and RyRs, as well as on RyRs reconstituted into planar lipid bilayers. Our results demonstrate that CGP can act as both a SERCA antagonist and RyR agonist.

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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 previously described (Chu et al., 1988; Saito et al., 1984). For skeletal muscle, the R4 fraction of SR (TC microsomes) is highly enriched in terminal cisternae of SR, which consists of both the junctional face membrane of SR as well as the calcium pump membrane of SR. The Ryanodine Receptor of SR (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 (Fleischer, 2008; Saito et al., 1984). The R2 fraction of SR (LT microsomes) is referable 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

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SR for use in experiments were separated every month in 15 µl aliquots at a concentration of 5-15 mg protein/ml in 5 mM imidazol-Cl 290 mM sucrose, pH ~ 7), quick frozen and stored at -80°C. For experiments, aliquots were quickly thawed in water, kept on ice and used within 5 hours.

Measurements of Ca^{2+} uptake/leak by SR microsomes

Ca^{2+} uptake by cardiac SR microsomes or R4 fractions of skeletal TC microsomes were measured with a spectrophotometer (Cory 50, Varian, Walnut Creek, CA) using the Ca^{2+}-sensitive dye antipyrylazo III (APIII), as previously described (Chamberlain et al., 1984; Chu et al., 1988; Diaz-Sylvester et al., 2008). Briefly, Ca^{2+} was increased to 40 µM by adding 40 nanomoles $CaCl_2$ to 1 ml of phosphate buffer [in mM: 100 KH_2PO_4, 5 $MgCl_2$ (free Mg^{2+}~0.3 mM), 5 ATP and 0.2 APIII; pH 7.0] containing 40-100 µg SR membranes. The rate of Ca^{2+} uptake by the microsomes was measured in buffer containing ruthenium red (5 µM) and the effect of CGP (0.625-20 µM; preincubated for 5 minutes) was measured as changes in the absorbance (710 - 790 nm) of APIII. Initial rate of uptake (J_{Ca}), in µMole Ca^{2+} · (mg protein)$^{-1}$ · min^{-1} was estimated from fitting

$$J_{Ca^{2+}} = 40\ nMole \cdot \frac{\Delta OD_t}{\Delta OD_0} \cdot \frac{e^{-kt}}{S}$$

to the data. ΔOD_0 is the initial OD change produced by adding 40 µM Ca^{2+} to the cuvette and ΔOD_t is the change in OD as function of time. S is the mass of microsomes added to the cuvette (in mg of protein) and k is the rate of uptake (in min^{-1}) assuming a first order process.

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We also measured the rate of Ca^{2+} leak from R4 fractions of skeletal TC microsomes preloaded with Ca^{2+} (three pulses of 40 μM Ca^{2+}) after addition of cyclopiazonic acid (CPZ) (20 μM), which inhibits SERCA, plus 1 μl DMSO (control) or 5-30 μM CGP in DMSO. In some leak experiments, RyR1-mediated Ca^{2+} release from TC microsomes was blocked with ruthenium red (5 μM).

Measurements of ATPase activity in SR microsomes

The effects of CGP (10 μM) were studied at 50 μM Ca^{2+} which measures the effects of the drug on maximal SERCA ATPase rate (V_{max}). The data allows correlating the effects of CGP on initial rate of Ca^{2+} load with ATPase activity. The effects of the drug were also measured in the presence of 300 nM Ca^{2+}. These levels are close to the half-maximal activating Ca^{2+} levels (K_m) of SERCA pump. We followed a protocol previously used in Dr. S. Fleischer laboratory (Chamberlain et al., 1984; Chu et al., 1988). Briefly, 10-40 μg of R2 fractions enriched in longitudinal tubule were incubated with buffer containing (in mM): 140 KCl, 5 MgCl2, 5 HEPES, 2 phosphoenolpyruvate, 8.4 units/ml of pyruvate kinase and 12 units/ml of 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 hydrolized to ADP by the ATPases. ADP is regenerated to ATP by reactions that induce the oxidation of one molecule of NADH (to NAD^+) per ATP hydrolyzed (Chu et al., 1988). The rate of ATP hydrolysis, in nmole ATP (mg SR protein)$^{-1}$ min^{-1} was estimated from the equation:

$$Rate\ of\ ATP\ hydrolysis\ = \frac{\Delta OD_{340}}{\Delta t \cdot \varepsilon \cdot L \cdot S}$$

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where, ΔOD is the decrease in absorbance at 340 nm (due to NADH consumption) during the interval Δt (in min), ε is the NADH extinction coefficient ($6.22 \cdot 10^6$ ml·Mole^{-1}·cm^{-1}), L the cuvette length (in cm) and S the amount of SR protein added to the cuvette (mg/ml).

RyR channel recordings and data analysis

Cardiac and skeletal RyRs were reconstituted into planar lipid bilayers formed on 80-120 μm diameter circular holes in Teflon septa, separating two 1.2 ml compartments as previously described (Copello et al., 1997). The *trans* bilayer solution contained a HEPES-Ca^{2+} solution [HEPES 250 mM, $Ca(OH)_2$ 50 mM; pH 7.4], and clamped at 0 mV with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). The *cis* compartment (ground) was filled with HEPES-Tris solution (250 mM HEPES, 120 mM Tris, pH 7.4). Fusion of SR vesicles was promoted by subsequently adding, while stirring, 500–1,000 mM CsCl, 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 CsCl and Ca^{2+} was 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 ($[Ca^{2+}]_{cyt}$) (Copello et al., 1997). Free [Mg^{2+}] in mixtures of Mg^{2+} and ATP was estimated using Winmaxc2.5 by Chris Patton, Stanford University (available for free download at http://www.stanford.edu/~cpatton/maxc.html).

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Drug and Chemicals

$CaCl_2$ standard for calibration was from World Precision Instruments Inc. (Sarasota, FL). Phospholipids (phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine) were obtained from Avanti (Alabaster, AL). CGP 37157 was from Tocris Bioscience (Ellisville, MO). All other drugs and chemicals were from Sigma-Aldrich (St. Louis, MO).

Statistical analysis

Data is 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.

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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 Ca^{2+} 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 µM), 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 the dose of CGP that produced a half maximal inhibitory concentration (IC_{50}) of 9.9 ± 2.0 µM (*n* = 4 paired experiments). Likewise, Figs. 1C and 1D suggest that in skeletal muscle TC microsomes, CGP also inhibited the rate of loading with an IC_{50} of 6.6 ± 1.2 µM (*n* = 4 paired experiments).

The effects of CPG (10 µM) 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 µM, where SERCA reaches maximal activity (V_{max}) and the second set was carried out with a Ca^{2+} concentration of 300 nM, which is near the SERCA's half maximal activity (K_m) for Ca^{2+} (Bers, 2001). Figure 1E shows that with 50 µM Ca^{2+}, approximately 95% of the ATPase of LT microsomes was blocked by 20 µM cyclopiazonic acid (CPZ), which inhibits SERCA. We also found that decreasing

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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. 1E and 1F, 10 μM CGP decreased V_{max} (at 50 μM 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. Subsequently, the SERCA pump was inhibited by CPZ and the rate of Ca^{2+} leak was measured in absence or presence of ruthenium red. In absence of ruthenium red, where most of the Ca^{2+} efflux from the vesicles is via RyR1, CGP (5 and 20 μM) significantly increases the rate of Ca^{2+} leak from the TC microsomes. By contrast, the residual leak in the presence of ruthenium red (which inhibit RyR1) was decreased by CGP (20 μM) (Figs. 2A and 2B). 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 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 demonstrates single-channel recordings of a RyR1 under control condition and after the addition of 20 μM CGP to the cytosolic solution. The effect of CGP was observed 5-10 seconds after addition, reached a plateau in ~ 2 min and remained stable during the

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experiments (up to one hour 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 μM. (*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 μM 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 μM CGP significantly activated the channel (P_o = 0.49). With higher doses (20 μM), 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 a EC_{50} of 9.4 ± 2.3 μM (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+}), and as previously reported (Copello et al., 1997). The effect of CGP on the channels was reversed after its removal from the cytosolic solution through superfusion.

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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, CPG 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 (Cox et al., 1993; Gunter and Pfeiffer, 1990). Yet, it was subsequently 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 (Cox et al., 1993; Gershon, 1992; Griffiths et al., 1997). Moreover, clonazepam is a well-

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known agonist of gamma-aminobutyric acid-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, like diltiazem, more specifically targets L-type channels and only affect SERCA and RyRs at much higher doses (Chamberlain et al., 1984). Other compounds of this class, such as KT-362, were termed "intracellular Ca^{2+} channel antagonists" as 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 is still unclear. Two reports found that K201, also known as JTV519, inhibited RyRs, but they differ on the requirement of FK-506 binding protein for inhibition (Hunt et al., 2007; Wehrens et al., 2005). However, an early study suggested that K201 could increase [^{3}H] ryanodine binding (Kohno et al., 2003). In addition, others reported noticeable inhibitory action of K201 on ventricular myocytes whole cell Na^+, Ca^{2+} and K^+ currents, as well as on SERCA (James, 2007; Kimura et al., 1999).

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

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CGP appears to modulate multiple molecular targets in cells

Evidence suggests that mitochondria modulate intracellular Ca^{2+} 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 (Csordas and Hajnoczky, 2009; Malli et al., 2005; Szalai et al., 2000). Reports have suggested that inhibition of the mitochondrial NCX by CGP results in significant depletion of SR Ca^{2+} load (Csordas and Hajnoczky, 2009; Malli et al., 2005; Rizzuto et al., 1998). Yet, in those studies where SR depletion was manifest, and micromolar levels (ranging from 1 to 20 uM) of CGP were utilized.

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 (EC) 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 which has been observed with other L-type Ca^{2+} channel blockers (Hussain and Orchard, 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 sequester into SR by SERCA. However, NCX blockers are known to produce the opposite effect during reperfusion after ischemia, since the NCX works in a reverse mode (Ca^{2+} influx) and inhibition would prevent

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hypercontracture and arrhythmia (reviewed in Bers 2001). Thus, the effects of CGP are complex as 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 (Bonazzola and Takara, 2010; Csordas and Hajnoczky, 2009; Liu and O'Rourke, 2008). A cardioprotective action has been reported for benzothiazepine derivatives (Farber and Gross, 1989; Wehrens et al., 2005), as well as for many other drug classes and processes like 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 utilization of Ca^{2+} sources/sinks (mitochondria, SR stores and/or plasmalemma) during EC coupling. This may lead to a significant decrease in the energy expenditure of cells that may be beneficial under pathological conditions, like ischemia (Bonazzola 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 (Belmonte and Morad, 2008; Griffiths et al., 1997; Malli et al., 2005). 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

Participated in research design: Neumann, Diaz-Sylvester, and Copello.

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, Fleisher, and Copello.

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FIGURE LEGENDS

Figure 1. CGP inhibits SR Ca^{2+} uptake and SERCA-mediated ATPase activity. SR microsomes were incubated in buffer phosphate containing ATP/Mg with 2 µL CGP in DMSO (final CGP levels from 0.625 to 5 and 20 µM) or with 2 µL DMSO (control). SR Ca^{2+} loading was started by increasing Ca^{2+} in the cuvette to 40 micromolar. **(A)** Example of Ca^{2+} uptake by porcine cardiac SR microsomes measured under control conditions and in the presence of various doses of CGP (0.625 µM – 20 µM). **(B)** Percent inhibition of the rate of SR Ca^{2+} loading by porcine cardiac SR microsomes versus CGP concentrations. Experimental data as in (A) were fit by a single-exponential function from which the initial rate of Ca^{2+} uptake was derived (see Methods). The average rate of uptake decreased by 60.2 ± 4.6 % at 20 µM CGP (*n* = 3). From the data in Fig. 1A, a half-maximal inhibitory concentration of 9.9 ± 2.0 µM was estimated. **(C)** Example of CGP-induced inhibition of Ca^{2+} uptake by rabbit skeletal muscle TC microsomes. **(D)** The drug decreased the rate of uptake by skeletal TC microsomes by 62.7 ± 7.0% with 20 µM CGP; IC_{50} = 6.6 ± 1.2 µM (*n* = 4). **(E)** Decrease in NADH absorption versus time (indicative of ATPase activity) by skeletal LT microsomes enriched in SERCA. As shown, CGP (10 µM) partially inhibited the decrease of NADH levels, whereas cyclopiazonic acid (CPZ; 20 µM) completely stopped the reaction. **(F)** CGP-induced inhibition of ATPase activity of skeletal LT microsomes incubated with free [Ca^{2+}] of either 50 µM or 300 nM. The inhibitory action at the two free [Ca^{2+}] was comparable, 31.3 ± 5.3% and 42.7 ± 6.4% respectively (*n* = 4).

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Figure 2. CGP activates skeletal muscle RyR1. (A) Ca^{2+} leak from TC microsomes (R4 fractions) was induced by blocking the SERCA pump with 20 μM CPZ. Measurements were performed under control conditions and upon addition of 5 or 20 μM CGP. Ca^{2+} leak was also measured in presence of 5 μM ruthenium red (which inhibits RyR1). **(B)** Ratio of Ca^{2+} leak rate in presence of CGP versus control. Leak rate significantly increased from control values by 281 ± 20% in presence of CGP. Ruthenium red significantly decreased the rate of leak, where the addition of CGP decreased the leak rate further by 74.4 ± 38.0%. **(C)** CGP (20 μM) activated skeletal RyRs reconstituted into planar lipid bilayers from TC microsomes. Recordings were carried out at 0 mV transmembrane voltage with 100 nM cytosolic free Ca^{2+} concentration (for details see Methods). Openings are shown as discrete upward deflections. **(D)** Dose response of cumulative doses of CGP to RyR1 in planar lipid bilayers with an EC_{50} of 12.0 ± 3.5 μM. Values are means ± S.E.M. *p < 0.05 compared with absence of CGP (n = 4-6 paired experiments).

Figure 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-second recording taken from a representative recording of a channel under control conditions (cytosolic Ca^{2+} = 1μM). The second and third traces are recordings of the same channel after addition of increasing concentrations of CGP to the cytosolic solution (2.5 μM and 20 μM,

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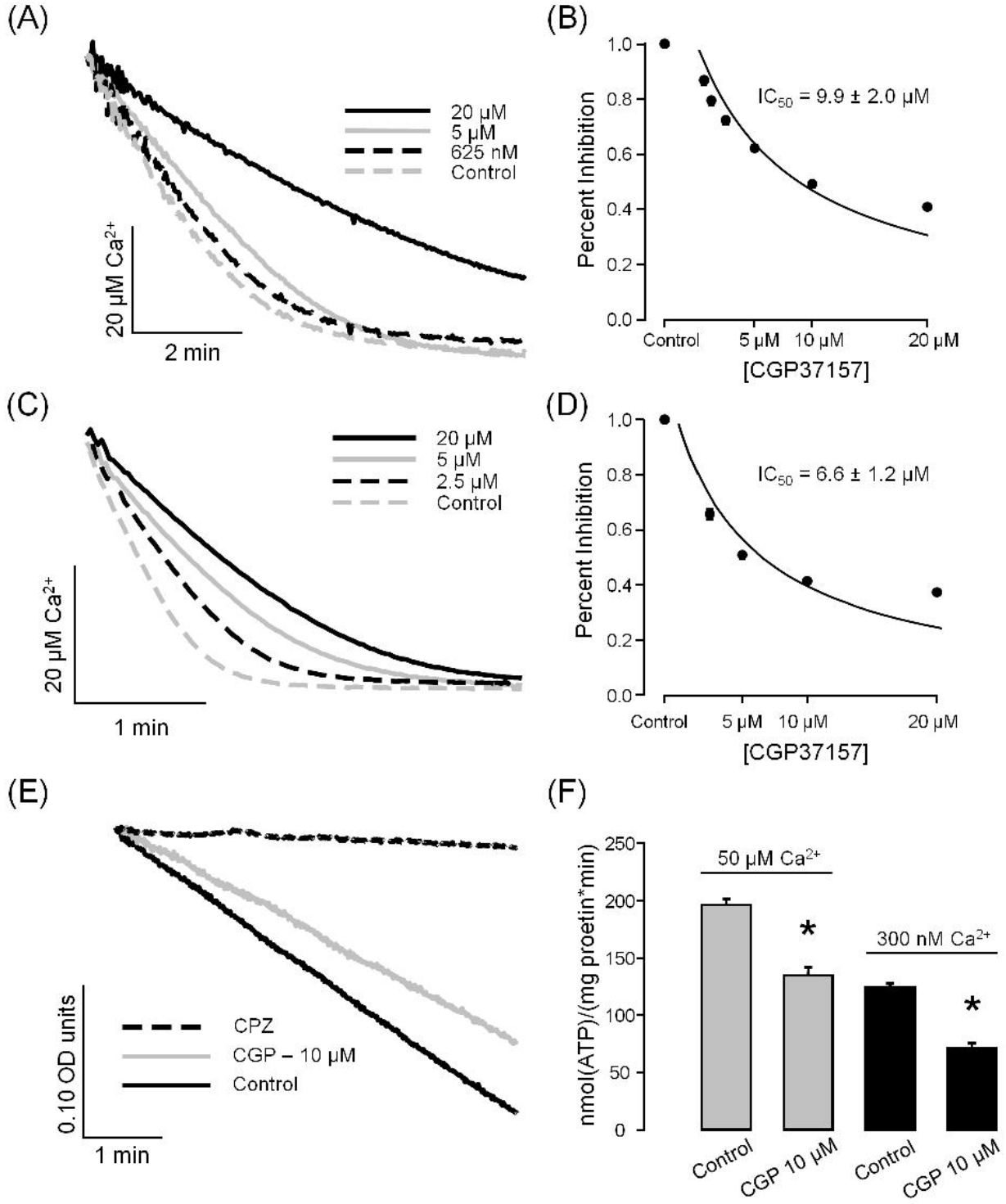
respectively). The fourth trace was recorded after subsequent addition of BAPTA to lower the cytosolic Ca^{2+} concentration down to 100 nM. The bottom trace shows the activity of the same channel after addition of 1 mM $MgCl_2$ to the cytosolic chamber. **(B)** Dose response of cumulative doses of CGP to RyR2 in planar lipid bilayers with an EC_{50} of 9.4 ± 2.3 µM (*n* = 6 experiments). Values are means ± S.E.M. (* $p < 0.05$ vs. control). **(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 µM CGP (grey outlines). Fitting two exponential components to the data produce, under control conditions, dwell open times τ^o_1 = 1.17 ± 0.01 ms (70 ± 0.6%) and τ^o_2 = 3.82 ± 0.07 ms (30 ± 0.7%). Closed times were τ^c_1 = 2.34 ± 0.04 ms (28 ± 0.3%) and τ^c_2 = 19.18 ± 0.25 ms (72 ± 0.4%). For 25 µM CGP dwell open times were much longer: τ^o_1 = 96 ± 5 ms (18 ± 0.5%), τ^o_2 = 633 ± 6 ms (82 ± 0.8%). In contrast, dwell closed times were shorter and distributed with a single τ^c_1 = 1.16 ± 0.12 ms.

FIGURE 1


(A)
20 µM
5 µM
625 nM
Control
20 µM Ca2+
2 min
(B)
IC50 = 9.9 ± 2.0 µM
Percent Inhibition
Control
5 µM
10 µM
20 µM
[CGP37157]
(C)
20 µM
5 µM
2.5 µM
Control
20 µM Ca2+
1 min
(D)
IC50 = 6.6 ± 1.2 µM
Percent Inhibition
Control
5 µM
10 µM
20 µM
[CGP37157]
(E)
CPZ
CGP – 10 µM
Control
0.10 OD units
1 min
(F)
50 µM Ca2+
300 nM Ca2+
nmol(ATP)/(mg proetin*min)
Control
CGP 10 µM
Control
CGP 10 µM

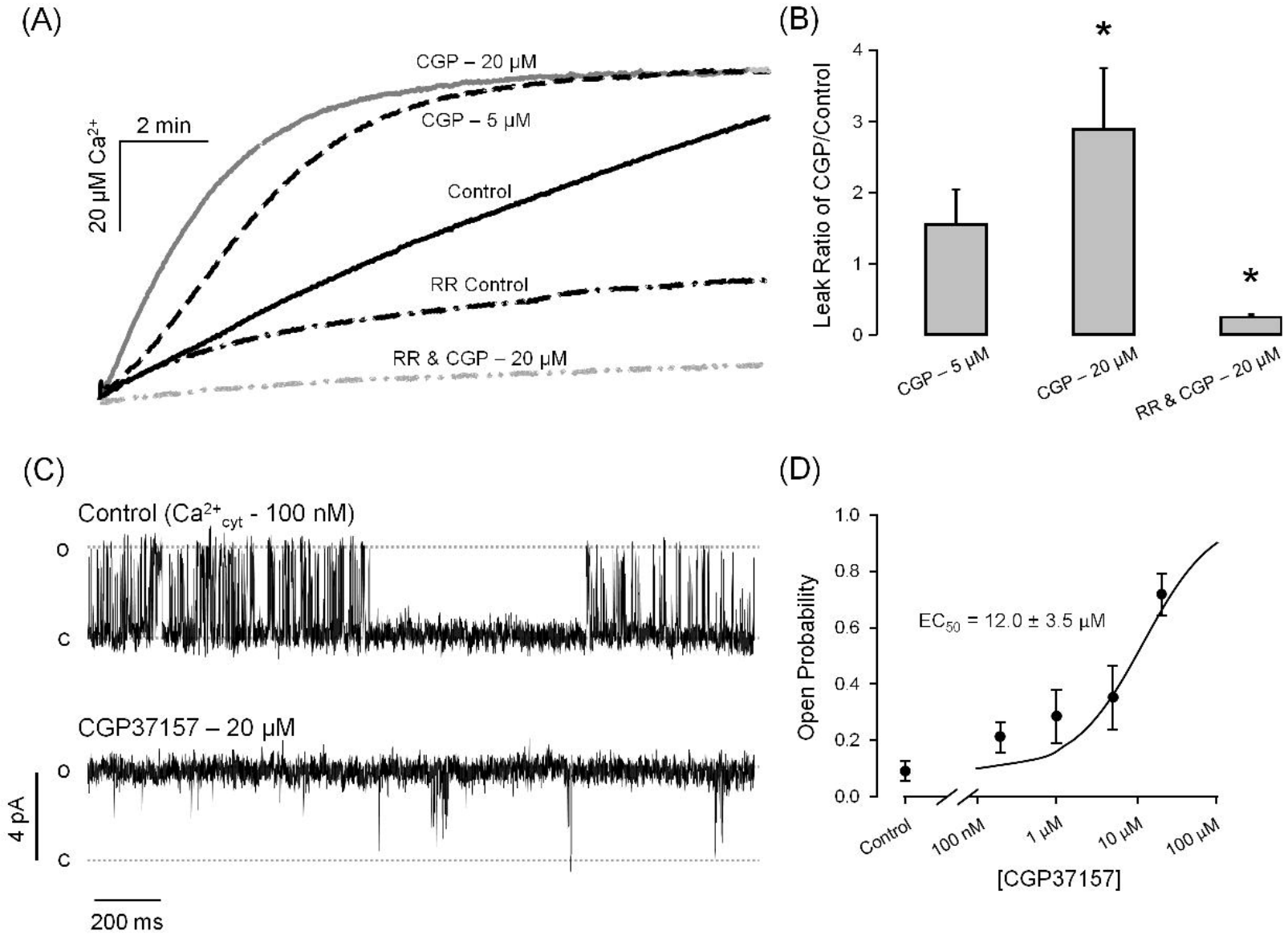

(A)
CGP – 20 µM
CGP – 5 µM
Control
RR Control
RR & CGP – 20 µM
20 µM Ca2+
2 min
(B)
Leak Ratio of CGP/Control
CGP – 5 µM
CGP – 20 µM
RR & CGP – 20 µM
(C)
Control (Ca2+cyt - 100 nM)
o
c
CGP37157 – 20 µM
4 pA
200 ms
(D)
Open Probability
EC50 = 12.0 ± 3.5 µM
Control
100 nM
1 µM
10 µM
100 µM
[CGP37157]

FIGURE 2

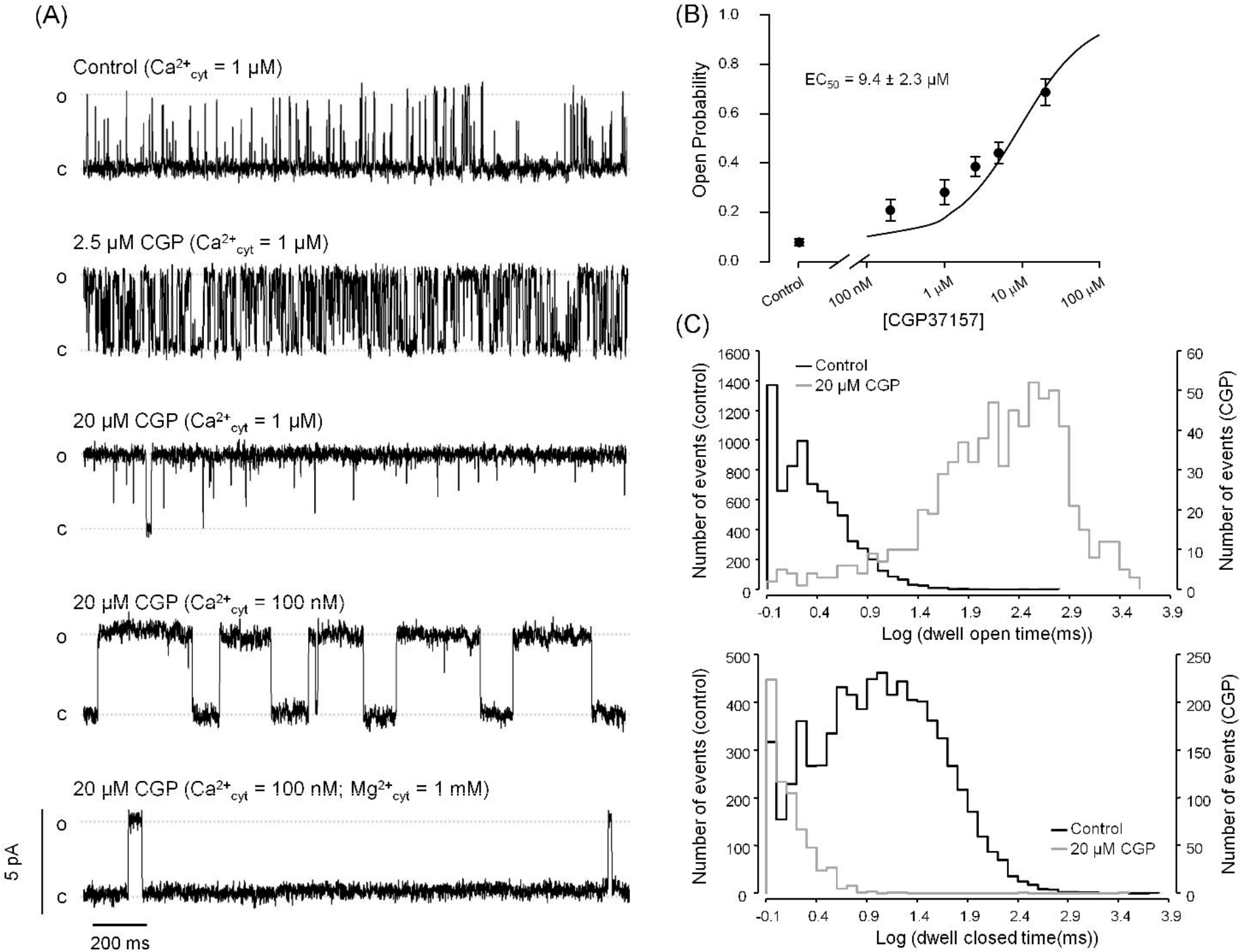

(A)
Control (Ca^{2+}_{cyt} = 1 μM)
2.5 μM CGP (Ca^{2+}_{cyt} = 1 μM)
20 μM CGP (Ca^{2+}_{cyt} = 1 μM)
20 μM CGP (Ca^{2+}_{cyt} = 100 nM)
20 μM CGP (Ca^{2+}_{cyt} = 100 nM; Mg^{2+}_{cyt} = 1 mM)
o
c
5 pA
200 ms
(B)
Open Probability
EC_{50} = 9.4 ± 2.3 μM
Control
100 nM
1 μM
10 μM
100 μM
[CGP37157]
(C)
Control
20 μM CGP
Number of events (control)
Number of events (CGP)
Log (dwell open time(ms))
Log (dwell closed time(ms))

FIGURE 3