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Inhibition of Canine (NCX1.1) and *Drosophila* (CALX1.1) Na^+-Ca^{2+} Exchangers by CGP-37157

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Running Title: Inhibition of Na^{+}-Ca^{2+} exchange by CGP-37157

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ABSTRACT

The electrophysiological effects of the benzothiazepine CGP-37157 (CGP) were investigated on the canine (NCX1.1) and *Drosophila* (CALX1.1) plasmalemmal Na^+-Ca^{2+} exchangers. These exchangers were selected for study as they show opposite responses to cytoplasmic regulatory Ca^{2+}, thereby allowing us to examine the role of this regulatory mechanism in the inhibitory effects of CGP. CGP blocked Na^+-Ca^{2+} exchange current mediated by both transporters with moderate potency (IC_{50}'s= ~3-17 μM) as compared with other recently reported blockers of Na^+-Ca^{2+} exchange (e.g. KB-R7943, SEA0400). Experiments using α-chymotrypsin to remove autoregulation of Na^+-Ca^{2+} exchange showed that block by CGP was reduced, suggesting that part of the effects of this drug may require intact ionic regulatory mechanisms. For NCX1.1, the inhibition produced by CGP was greater for outward Na^+-Ca^{2+} exchange currents as compared with inward currents. When CALX1.1 was examined, the extent of inhibition was similar for both inward and outward exchange currents. While the extent and potency of CGP-mediated inhibition of Na^+-Ca^{2+} exchange are less than those observed with SEA0400 and KB-R7943, our data demonstrate that CGP constitutes a novel class of plasmalemmal Na^+-Ca^{2+} exchange inhibitors. Moreover, the widespread use of CGP as a selective mitochondrial Na^+-Ca^{2+} exchange inhibitor should be reconsidered in light of these additional inhibitory effects.

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Na^+-Ca^{2+} exchangers are ion counter-transporters located in the plasma membrane of most cell types. These electrogenic exchangers are generally believed to transport 3 Na^+ for 1 Ca^{2+}. The role of Na^+-Ca^{2+} exchange is best understood for cardiac, neuronal, and renal tissues as expression is highest in these cell types (Bers, 2001;Blaustein and Lederer, 1999;Philipson and Nicoll, 2000;Hryshko, 2002). In cardiac muscle, for example, Na^+-Ca^{2+} exchange operating in the forward mode (*i.e.*, Ca^{2+} efflux) is the principal mechanism by which Ca^{2+} is extruded from cardiac cells (Bers, 2000). In addition to its role in Ca^{2+} efflux, there are also considerable data indicating that reverse mode (*i.e.*, Ca^{2+} influx) exchange can participate in net Ca^{2+} influx, particularly under pathophysiological conditions (Mochizuki and MacLeod, 1997;Mochizuki and Jiang, 1998). As such, Na^+-Ca^{2+} exchange is integral to Ca^{2+} homeostasis in the heart and any perturbation of inward or outward Na^+-Ca^{2+} exchange currents results in substantial changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cardiac contractility.

To date, there are few pharmacological probes specific to Na^+-Ca^{2+} exchange proteins (Bers, 2001;Shigekawa and Iwamoto, 2001;Hryshko, 2002). Identification of new compounds, particularly those having mode-selectivity (*i.e.,* preferential effects on forward or reverse mode exchange), could have significant clinical utility for a variety of pathophysiological conditions, including ischemia-reperfusion injury, stroke, arrhythmogenesis, and congestive heart failure. Previous work has shown that the benzothiazepine compound, CGP-37157 (CGP), inhibits mitochondrial Na^+-Ca^{2+} exchange with high affinity (IC_{50}= 0.36

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μM) while having no significant effects on sarcolemmal Na^+-Ca^{2+} exchange or Na^+-K^+ ATPase activity (at concentrations up to 10 μM) when measured in isolated sarcolemmal vesicles (Cox et al., 1993;Cox and Matlib, 1993). However, the effects of CGP on Na^+-Ca^{2+} exchange currents were not examined in these experiments. Moreover, other transport systems have been identified where CGP exerts additional inhibitory effects. For example, CGP has been reported to inhibit voltage-gated Ca channels (Baron and Thayer, 1997) in some studies but not others (Lee et al., 2003a).

In the present work, we examined the ability of CGP to inhibit two distinct Na^+-Ca^{2+} exchangers using electrophysiological techniques. The canine cardiac Na^+-Ca^{2+} exchanger (NCX1.1) and a Na^+-Ca^{2+} exchanger from *Drosophila melanogaster* (CALX1.1) were expressed in *Xenopus laevis* oocytes and Na^+-Ca^{2+} exchange activity was measured using the giant excised patch technique. NCX1.1 and CALX1.1 were chosen based upon their distinct responses to regulation by cytoplasmic Ca^{2+} (Hryshko et al., 1996;Omelchenko et al., 1998), allowing us to assess the role of Ca^{2+} regulation on the inhibitory process. We found that CGP inhibits both inward and outward Na^+-Ca^{2+} exchange currents mediated by NCX1.1 and CALX1.1. The extent of current inhibition was reduced upon limited proteolysis of these Na^+-Ca^{2+} exchangers with α-chymotrypsin, a maneuver that eliminates specific ionic regulatory properties (Hilgemann, 1990). Our data indicate that CGP directly inhibits the activity of these plasmalemmal Na^+-Ca^{2+} exchangers. While the potency and efficacy of CGP is lower than that for newer Na^+-Ca^{2+} exchange inhibitors, additional investigation of this class of

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compounds may prove useful towards the development of related inhibitory compounds.

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METHODS

The procedures for oocyte preparation, cRNA synthesis and electrophysiological measurements of Na^+-Ca^{2+} exchange activity have been described in detail previously (Dyck et al., 1998;Dyck et al., 1999). Brief descriptions follow.

Oocyte Preparation and cRNA Synthesis

Xenopus laevis were generally anaesthetized in benzocaine for 30 min. Oocytes were removed, follicles teased apart and the oocytes transferred to buffer containing about 16,000 units of collagenase (Type II; Worthington), followed by incubation for 1.5-2 h at room temperature (RT) with gentle agitation. Oocytes were then de-folliculated in 100 mM K_2HPO_4 (pH 6.5 at RT) for 12-20 min with gentle agitation, after which stage V-VI oocytes were selected and maintained at 18°C until injection the following day. Complementary DNAs encoding NCX1.1 and CALX1.1 were linearized and cRNAs synthesized using mMessage mMachine *in vitro* transcription kits (Ambion). Following injection with ~ 23 ng of cRNA, oocytes were maintained at 18°C for up to 7 days.

Electrophysiological Measurements

Electrophysiological measurements were obtained from days 3-7 post-injection. Unidirectional outward (*i.e.,* reverse) and inward (*i.e.,* forward) Na^+-Ca^{2+} exchange current measurements were obtained using the giant excised patch clamp technique (Hilgemann, 1989). Prior to use in voltage-clamp experiments,

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the vitellin layer of the oocytes was removed by dissection. Oocytes were then placed in a solution containing (in mM): 100 KOH, 100 MES, 20 HEPES, 5 EGTA, 5-10 $MgCl_2$; pH 7.0 at RT (with MES). Gigaohm seals were formed by suction and inside-out membrane patches were excised by gentle movement of the patch pipette.

Rapid solution changes (~200 ms) were accomplished using a computer-controlled, 20-channel solution-switching device. For outward Na^+-Ca^{2+} exchange current measurements, pipette (*i.e.*, extracellular) solutions contained (in mM): 100 NMG-MES, 30 HEPES, 30 TEA-OH, 16 sulfamic acid, 8.0 $CaCO_3$, 6 KOH, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid; pH 7.0 at RT (with MES). Outward currents were elicited by rapidly switching from Li^+- to Na^+-based bath solutions containing (in mM): 100 [Na^+ + Li^+]-aspartate, 20 CsOH, 20 MOPS, 20 TEA-OH, 10 EGTA, 0 - 9.91 $CaCO_3$, 1.0 - 1.5 $Mg(OH)_2$; pH 7.0 at 30°C (with MES or LiOH). For inward Na^+-Ca^{2+} exchange current measurements, the pipette (*i.e.,* extracellular) solution contained (in mM): 100 Na-MES, 20 CsOH, 20 TEA-OH, 10 EGTA, 10 HEPES, 8 sulfamic acid, 4 $Mg(OH)_2$, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid; pH 7.0 at RT (with MES). Inward currents were activated by switching between Ca^{2+}-free and Ca^{2+}-containing, Li^+-based bath solutions, described above. For brevity, only the Na^+ and Ca^{2+} concentrations of experimental solutions are given in the Results section.

Axon Instruments hardware (Axopatch 200a) and software (Axotape) were used for data acquisition and analysis, and Origin software was used for statistical analyses and determination of IC_{50} and I_{MAX} values. Pooled data are

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presented as mean ± SEM. Two-tailed Student's t-tests were used for comparison of unpaired data, and $P < 0.05$ was considered significant. Free Mg^{2+} and Ca^{2+} concentrations were calculated using MAXC software (Bers et al., 1994). All experiments were conducted at 30°C.

CGP-37157 was dissolved in DMSO as 20-40 mM stocks and diluted directly into bath solutions. After each drug concentration change, at least 32 seconds were allowed to lapse prior to re-examining current levels. The concentration of DMSO never exceeded 0.075% and was without effect on inward or outward Na^+-Ca^{2+} exchange current characteristics.

To deregulate Na^+-Ca^{2+} exchange currents, membrane patches were exposed to α-chymotrypsin (Type I-S; Sigma) in some experiments. This procedure eliminates ionic regulation and leaves the Na^+-Ca^{2+} exchanger in a fully activated state (Hilgemann, 1990). α-Chymotrypsin was prepared in bath solution at 1.0 mg/ml and was applied to the cytoplasmic surface of patches. Digestion typically proceeded for 1-2 minutes, after which current amplitudes were stable and maximal.

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RESULTS

We studied the inhibitory action of CGP on Na^+-Ca^{2+} exchange currents generated by the canine (NCX1.1) and *Drosophila* (CALX1.1) Na^+-Ca^{2+} exchangers. Activation of unidirectional outward or inward currents was accomplished under "zero-trans" conditions; that is, keeping the solution on one side of the excised patch free of either Na^+ or Ca^{2+} such that only a single transport mode is possible. For example, to generate unidirectional outward currents, the pipette solution was Na^+-free. Alternatively, unidirectional inward currents were generated by using a Ca^{2+}-free pipette solution.

Block of Outward Na^+-Ca^{2+} Exchange Current

Fig. 1 shows inhibition of NCX1.1-mediated outward Na^+-Ca^{2+} exchange currents by CGP. Outward currents were generated by applying 100 mM Na^+ to the cytoplasmic surface of the patch in exchange for 8 mM pipette Ca^{2+}. In these experiments, 1 μM Ca^{2+} was continuously present in the cytoplasmic solution. For NCX1.1, micromolar levels of Ca^{2+} are required on the cytoplasmic surface of the patch to activate exchange currents (Levitsky et al., 1996;Matsuoka et al., 1995). In response to the application of 100 mM Na^+ to the intracellular surface of the patch, outward Na^+-Ca^{2+} exchange currents peaked rapidly, following by a slow decline towards a steady-state level. This slow decay of outward current reflects the entry of exchanger molecules into an inactive state, a process referred to as Na^+-dependent (or I_1) inactivation (Hilgemann et al., 1992a;Hilgemann et al., 1992b). CGP inhibited both peak and steady-state

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currents in a concentration-dependent manner (Fig. 1B). The IC_{50} values for inhibition of peak and steady-state currents by CGP were 7 ± 3 μM (n = 6) and 5 ± 3 μM (n = 8) for peak and steady-state currents, respectively. Generally, the degree of inhibition of steady-state currents by CGP tended to be greater than for peak currents, particularly at higher drug concentrations. This is reflected in the values calculated for the maximal degree of block (I_{max}), which were I_{max} = 42 ± 6 % (n = 6) and 53 ± 9 % (n = 8) for peak and steady state currents, respectively. Despite this tendency, neither IC_{50} nor I_{MAX} values attained statistically significant differences between peak and steady state values.

Insert Figure 1 here.

Fig. 2A shows the effects of CGP on outward Na^+-Ca^{2+} currents generated by the *Drosophila* exchanger, CALX1.1. As with NCX1.1, outward currents were activated by applying 100 mM Na^+ to the cytoplasmic surface of the patch in exchange for 8 mM pipette Ca^{2+}. However, in this case, there was no Ca^{2+} on the cytoplasmic surface of the patch since, unlike NCX1.1, CALX1.1 is inhibited by cytoplasmic Ca^{2+} (Hryshko et al., 1996). Note that 10 μM CGP decreased both peak and steady state currents. Pooled data in Fig. 2B show that CGP inhibits CALX1.1 in a concentration-dependent manner, with peak and steady-state outward currents blocked to similar degrees (IC_{50} = 17 ± 4 μM (n = 6) and 11 ± 1 μM (n = 7) for peak and steady-state currents, respectively). Comparing the data in Fig. 1B and 2B, the most notable difference is that the extent of block by CGP is substantially greater for CALX1.1 than that observed for NCX1.1.

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Specifically, the fitted I_{max} values for inhibition of CALX1.1 by CGP were 94 ± 4 % (n = 6) and 92 ± 8 % (n = 7) for peak and steady-state currents, respectively.

Insert Figure 2 here.

Block of Inward Na^+-Ca^{2+} Exchange Current

We next tested the effects of CGP on inward Na^+-Ca^{2+} exchange currents mediated by NCX1.1 and CALX1.1. Inward currents were generated by applying 10 µM Ca^{2+} solution to the cytoplasmic surface of the patch in exchange for 100 mM pipette Na^+. As reported previously for NCX1.1 (Elias et al., 2001), there is no decay of inward current in the continued presence of high levels of cytoplasmic Ca^{2+}. Thus, inward current waveforms appear essentially square. Therefore, the effect of CGP was measured only on this steady-state current. The square appearance of inward NCX1.1 currents reflects that fact that Na^+-dependent or I_1 inactivation is absent. Furthermore, the requirement for cytoplasmic regulatory Ca^{2+}, which is necessary to alleviate Ca^{2+} dependent (or I_2) inactivation of NCX1.1, is fulfilled by the high concentration of cytoplasmic Ca^{2+} required to activate transport. Fig. 3 shows that CGP exerts modest inhibitory effects on inward Na^+-Ca^{2+} exchange currents in NCX1.1, with a maximal block of ~12% (n = 5) at 10 µM CGP. Moreover, the pooled data shown in Figure 3B indicate that no obvious concentration dependent effects of CGP are discernible over this limited concentration range. For unknown reasons, we could not obtain reliable data at higher CGP concentrations. In general, patch stability is greatly reduced for inward current measurements as compared to outward.

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Insert Figure 3 here.

The effect of CGP on CALX1.1-mediated Na^{+}-Ca^{2+} exchange inward currents is shown in Fig. 4. Similar to experiments with NCX1.1, inward currents were activated by addition of 10 μM Ca^{2+} solution to the cytoplasmic side of the patch. Unlike NXC1.1 however, CALX1.1-mediated inward Na^{+}-Ca^{2+} exchange currents rapidly peak and then decay to a lower steady-state level, essentially mirroring the behavior of outward currents carried by this exchanger (Fig. 2A). This inactivation of inward Na^{+}-Ca^{2+} exchange currents is believed to reflect the anomalous regulatory response of CALX1.1 (Hryshko et al., 1996) to Ca^{2+}. With the *Drosophila* Na^{+}-Ca^{2+} exchanger, both inward and outward exchange currents are inhibited by cytoplasmic Ca^{2+}, rather than stimulated as occurs for all other exchangers examined to date (Hryshko, 2002;Philipson and Nicoll, 2000). Fig. 4 shows that, notwithstanding this anomalous regulation by Ca^{2+}, exposure of the patch to CGP results in a significant block of both the peak and steady-state components of inward current. As indicated in Fig. 4B, CGP inhibits both peak and steady-state currents in a concentration-dependent manner. Fitted parameters were: IC_{50}= 3 ± 1 (n = 5) and 4 ± 2 μM (n= 6) for peak and steady-state currents, respectively, and I_{max} = 48 ± 4 % and 63 ± 12 % for peak and steady-state currents, respectively.

Insert Figure 4 here.

Figure 5 shows pooled data on the percent inhibition produced by 10 μM CGP for outward and inward Na^{+}-Ca^{2+} exchange currents mediated by NCX1.1 and CALX1.1. Here, a larger database was used as compared with Figures 1-4

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and a single concentration of CGP was utilized. In this case, a small but statistically significant difference was observed when comparing NCX1.1-mediated inward vs. peak outward currents (12 ± 1 % (n = 5) and 23 ± 3 % (n = 14), p = 0.047, for outward peak and steady-state currents, respectively). Also, statistical significance was achieved when comparing peak vs. steady-state NCX1.1-mediated outward currents (23 ± 3 % (n = 14) and 34 ± 3 % (n = 15), p = 0.016, respectively). Several other features are also obvious from analysis of this type. First, the extent of inhibition is typically greater for CALX1.1 compared with NCX1.1 for each type of measurement. Second, when considering CALX1.1, there is relatively little difference in the extent of current inhibition for inward or outward currents. Even though steady-state currents tended to show slightly greater inhibition by CGP (as in Fig. 2), this difference did not achieve statistical significance despite the enlarged database. In contrast, the effects of CGP were clearly greater for outward currents mediated by NCX1.1, with the greatest effects occurring on steady-state currents. Inward currents mediated by NCX1.1 were least sensitive to CGP.

Insert Figure 5 here.

Effects of Deregulation of Na^{+}-Ca^{2+} Exchange

Since the profile of CGP-mediated inhibition was not uniform for these two distinct exchangers, nor was it when examining a single exchanger type undergoing distinct types of transport, we sought to determine whether ionic regulation played a role in the inhibitory process. This was accomplished by re-evaluating the effects of CGP in α-chymotrypsin-deregulated exchangers, where

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ionic regulatory processes (*i.e.,* I_1 and I_2 inactivation) are rendered non-functional for both exchangers (Hilgemann, 1990;Dyck, Maxwell et al., 1998). Figure 6 shows representative outward Na^+-Ca^{2+} exchange current traces for deregulated NCX1.1 (panel A) and CALX1.1 (panel B). Note that in the control tracings, Na^+-dependent or I_1 inactivation is no longer observed and the current waveforms have a square appearance. After proteolytic treatment, outward Na^+-Ca^{2+} exchange currents are also insensitive to regulation by cytoplasmic Ca^{2+}, irrespective of whether regulation was positive (NCX1.1) or negative (CALX1.1). Under these conditions, CGP caused a significantly smaller reduction of outward Na^+-Ca^{2+} exchange currents for both NCX1.1 and CALX1.1 when compared with its effects on intact and fully regulated exchangers. Maximal inhibition of steady-state outward current by 10 μM CGP was 9 ± 1 % (n=4) vs. 20 ± 2 % (n=5), p = 0.003 for NCX1.1 and CALX1.1, respectively. This suggests that CGP may exert at least some of its inhibitory effects through interaction with the intact exchangers' ionic regulatory processes, although other explanations cannot be excluded (see Discussion).

Insert Figure 6 here.

Representative traces showing the effects of CGP on α-chymotrypsin deregulated inward Na^+-Ca^{2+} exchange currents mediated by NCX1.1 and CALX1.1 are shown in Fig. 7A and Fig. 7B, respectively. For control records, α-chymotrypsin produces little or no effect on NCX1.1 exchange currents since Na^+-dependent (I_1) inactivation is absent and Ca^{2+}-dependent (I_2) regulation is already saturated under these recording conditions. Conversely, the

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characteristics of CALX1.1-mediated currents are altered by α-chymotrypsin, as this treatment causes a loss of anomalous or negative Ca^{2+}-regulation. Therefore, CALX1.1-mediated inward currents adopt a square appearance following limited proteolysis. Similar to the results obtained following deregulation of outward currents (Fig. 6), CGP caused a significantly smaller reduction of inward currents for both NCX1.1 and CALX1.1 exchangers. Here, exposure to 10 μM CGP resulted in a small degree of block of inward current generated by NCX1.1 and CALX1.1 (7 ± 2 %, (n = 6) vs. 15 ± 2 %, (n=4), respectively, p = 0.03). Pooled data for block of outward and inward current by CGP in the presence of α-chymotrypsin are shown in Fig. 8.

Insert Figure 7 and 8 here.

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DISCUSSION

The present work was aimed at studying the electrophysiological effects of the benzothiazepine compound CGP-37157 (CGP) on two different plasmalemmal Na^+-Ca^{2+} exchangers with distinct ionic regulatory properties. We found that CGP blocks inward and outward Na^+-Ca^{2+} exchange currents for both canine (NCX1.1) and *Drosophila* (CALX1.1) exchangers. With the mammalian NCX1.1 exchanger, CGP was more effective at blocking outward compared with inward currents. Furthermore, steady-state outward currents were more sensitive to inhibition by CGP than were peak outward currents (Figs. 1, 3 and 5). In contrast, CGP blocked peak and steady-state inward and outward currents with approximately the same efficacy for CALX1.1 (Figs. 2, 4 and 5). For both exchanger types, α-chymotrypsin treatment abolished ionic regulation and led to a reduction in the inhibitory effects of CGP (Figs. 6 and 7). The inhibitory effects of CGP on these plasmalemmal Na^+-Ca^{2+} exchangers are of sufficient magnitude to warrant consideration when CGP is employed as a "selective" blocker of the mitochondrial Na^+-Ca^{2+} exchanger.

Pharmacology of Plasmalemmal Na^+-Ca^{2+} Exchange Proteins

The impetus for our study was to identify novel classes of compounds with inhibitory effects on the cardiac Na^+-Ca^{2+} exchanger. Despite decades of investigation, there are very few pharmacological agents that exhibit any specificity towards the cardiac sarcolemmal Na^+-Ca^{2+} exchanger (Bers, 2001;Hryshko, 2002;Shigekawa and Iwamoto, 2001). This target is of

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considerable therapeutic interest as it has been implicated as a major contributor to ischemia-reperfusion injury in several organs including cardiac, renal and neuronal tissue. Experimental studies provide strong support for the notion that Na^+-Ca^{2+} exchange inhibition will reduce injury in these tissues (Hryshko, 2002). For example, in cardiac muscle, inhibition of Na^+-Ca^{2+} exchange has been shown to offer considerable protection against arrhythmogenesis, contractile dysfunction, and infarct size in response to experimental models of ischemia-reperfusion injury, hypoxia-reoxygenation injury, and digitalis intoxication. Additional examples and possibilities for the spectrum of protective effects achievable with Na^+-Ca^{2+} exchange inhibition have been recently reviewed (Pogwizd, 2003;Hryshko, 2002;Matsumoto et al., 2002).

The most potent Na^+-Ca^{2+} exchange inhibitor described to date is the 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea compound, called SEA0400. This agent, first described in 2001, inhibits the cardiac and neuronal Na^+-Ca^{2+} exchanger at nanomolar concentrations (Matsuda et al., 2001;Tanaka et al., 2002). Moreover, the nature of its inhibitory actions is such that it may exert preferential effects on inhibiting the reverse transport mode of Na^+-Ca^{2+} exchange (Lee et al., 2003b). While experimental studies utilizing this compound are rather limited, it is clear that SEA0400 shows promise in alleviating the degree and extent of ischemia-reperfusion injury in both cardiac and neuronal tissue (Matsuda et al., 2001;Tanaka et al., 2002;Takahashi et al., 2003). Moreover, there is persuasive evidence showing that SEA0400 offers superior protection against this type of injury in cardiac muscle, compared with its

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predecessor, KB-R7943 (Magee et al., 2003).

KB-R7943 (2-[4-[2,5-difluorophenyl)methoxy]phenoxy]phenoxy]-5-ethoxyaniline) was first described in 1996 and was reported to be a selective inhibitor of the reverse mode of Na^{+}-Ca^{2+} exchange (Iwamoto et al., 1996;Watano et al., 1996). While numerous issues remain contentious concerning the details of its inhibitory mechanism, it has been extensively evaluated in tissue injury models (including ischemia-reperfusion, hypoxia-reoxygenation, and digitalis intoxication) and its salutary effects have been consistently demonstrated (Hryshko, 2002). From a mechanistic standpoint, the primary issues of contention concern its site of action, transport mode selectivity, and its specific inhibitory mechanism (i.e., competitive vs. non-competitive, etc.). This topic has also been recently reviewed (Hryshko, 2002;Shigekawa and Iwamoto, 2001).

CGP-37157 is widely utilized as a selective inhibitor of the mitochondrial Na^{+}-Ca^{2+} exchanger and, to our knowledge, has not been described as a plasmalemmal Na^{+}-Ca^{2+} exchange inhibitor. Frequently, the goals of studies of this type have been to ascertain the involvement of this mitochondrial transport system in various aspects of Ca^{2+} homeostasis or Ca^{2+} signaling in a variety of different tissues (Cox and Matlib, 1993). Our data obviously challenge the assertion that CGP functions as a selective mitochondrial Na^{+}-Ca^{2+} exchange inhibitor, particularly at the high concentrations of CGP (≥ 10 μM) that are frequently employed (Haak et al., 2002;Gauchy et al., 2002;Arnaudeau et al., 2001). In fact, it is not uncommon for CGP to be utilized alongside known

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plasmalemmal Na^+-Ca^{2+} exchange inhibitors (e.g., KB-R7943) to dissect the relative contribution of the mitochondrial vs. sarcolemmal Na^+-Ca^{2+} exchange systems (for example, see (Zhong et al., 2001)). The impact of our findings on previous studies utilizing CGP as a selective mitochondrial Na^+-Ca^{2+} exchange inhibitor will require assessment on an individual basis. Fortunately, the lower potency and efficacy of CGP as a sarcolemmal Na^+-Ca^{2+} exchange inhibitor may limit the complications associated with these additional actions.

In many tissues, the plasmalemmal Na^+-Ca^{2+} exchanger may serve a very limited role in Ca^{2+} homeostasis, and therefore the inhibitory effects of CGP on this system would be inconsequential. Alternatively, in systems where the Na^+-Ca^{2+} exchange system is critical (such as in cardiac muscle), it is likely that the Na^+-Ca^{2+} exchanger is present in considerable excess of that required for routine Ca^{2+} homeostasis (Hryshko, 2002). Here, again, the effects of CGP on the mitochondrial Na^+-Ca^{2+} exchanger are likely to represent the dominant functional effect of this agent, as modest inhibition of the cardiac Na^+-Ca^{2+} exchanger is unlikely to have large functional consequences. Nevertheless, our results with CGP on two very distinct plasmalemmal Na^+-Ca^{2+} exchangers highlight the necessity of using this agent cautiously (and at conservative concentrations) as a selective mitochondrial Na^+-Ca^{2+} exchange inhibitor. This is particularly true where high concentrations of CGP are employed (e.g., > 10 μM), which appears to be the case in the majority of studies.

Mechanism of Action of CGP

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CGP is a benzothiazepine derivative that inhibits the electroneutral, mitochondrial Na^+-Ca^{2+} exchanger with sub-micromolar potency. In heart, for example, this transporter is inhibited by CGP with a potency of ~ 400 nM (Cox and Matlib, 1993;Cox, Conforti et al., 1993). Prior to the development of CGP, several related benzodiazepines (e.g., clonazepam and diltiazem) have been utilized as mitochondrial Na^+-Ca^{2+} exchange inhibitors (Cox and Matlib, 1993). In general, there have been very few reports of these compounds inhibiting the cardiac plasmalemmal Na^+-Ca^{2+} exchanger (although see (Hata et al., 1988;Takeo et al., 1985)). While the molecular nature of the mitochondrial Na^+-Ca^{2+} exchanger has not been deduced, the physiology of this transporter is well studied. This protein serves as a Ca^{2+} efflux mechanism operating in opposition to a Ca^{2+} uniporter within the inner mitochondrial membrane. As such, inhibition of the mitochondrial Na^+-Ca^{2+} exchanger leads to an increase in Ca^{2+} levels within this organelle (Cox and Matlib, 1993). Calcium levels within the mitochondria serve as an important regulator of several key enzymes involved in energy metabolism.

Our data demonstrate that CGP can inhibit plasmalemmal Na^+-Ca^{2+} exchangers. From a mechanistic standpoint, this inhibition shares some similarity with the better characterized Na^+-Ca^{2+} exchange inhibitors such as SEA0400 and KB-R7943. For example, both SEA0400 and KB-R7943 exert a preferential inhibition of outward Na^+-Ca^{2+} exchange currents mediated by the cardiac exchanger when investigated using the giant excised patch technique (Elias et al., 2001;Lee et al., 2003b). This was also observed with CGP, although

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this differential effect was far less pronounced than that observed with SEA0400 and KB-R7943. We have also consistently observed a substantial decrease in inhibitory potency for all of these agents when exchangers are de-regulated following limited proteolysis with α-chymotrypsin (Elias et al., 2001;Lee et al., 2003b), a result consistent with a role for ionic regulation in this process. However, we cannot exclude the possibility that proteolysis alters the interaction of CGP and Na^+-Ca^{2+} exchangers by other direct or indirect effects. Finally, the observation that CGP can exert distinct effects on distinct exchangers (in this case NCX1.1 vs. CALX1.1) lends credence to the notion that inhibition by this agent is mediated by direct interactions with Na^+-Ca^{2+} exchanger molecules rather than some non-selective pharmacological effect.

Summary

Our data indicate that CGP inhibits two plasmalemmal Na^+-Ca^{2+} exchangers, namely NCX1.1 and CALX1.1. The primary importance of these results can be summarized as follows:

1) There are no reports demonstrating that CGP-37157 inhibits plasmalemmal Na^+-Ca^{2+} exchange inhibitors. Nevertheless, an extensive number of analogues exist for CGP that could be readily evaluated for their potential as plasmalemmal Na^+-Ca^{2+} exchange inhibitors. An improved pharmacology towards NCX1.1, in particular, is essential towards evaluating this target in cardioprotective strategies.

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2) The utility of CGP as a selective inhibitor of the mitochondrial Na^+-Ca^{2+} exchanger may be influenced by the additional pharmacological actions we have demonstrated in this study. Depending upon the role of plasmalemmal Na^+-Ca^{2+} exchange in the parameter under investigation, it may be prudent (or essential) to consider these effects.

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ACKNOWLEDGEMENTS

none

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Inhibition of NCX1.1-mediated outward Na^+-Ca^{2+} exchange currents by CGP. Panel A shows an example of inhibition exerted by 10 µM CGP. Panel B shows concentration-dependent inhibition by CGP. Data are mean ± SEM % inhibition of outward peak (n = 6) and steady-state (n = 8) currents. Fitted parameters for peak and steady-state currents are, respectively: IC_{50} = 7 ± 3 µM and 5 ± 3 µM; I_{MAX} = 42 ± 6 and 53 ± 9 %.

Figure 2. Inhibition of CALX1.1-mediated outward Na^+-Ca^{2+} exchange currents by CGP. Panel A shows an example of inhibition exerted by 10 µM CGP. Panel B shows concentration-dependent inhibition by CGP-37157. Data are mean ± SEM % inhibition of outward peak (n = 5-6) and steady-state (n = 7) currents, except for 6 µM CGP where only a single measurement was obtained. Fitted parameters for peak and steady-state currents are, respectively: IC_{50}= 17 ± 4 µM and 11 ± 1 µM; I_{MAX} = 94 ± 4 % and 92 ± 8 %.

Figure 3. Inhibition of NCX1.1-mediated inward Na^+-Ca^{2+} exchange currents by CGP. In panel A, representative traces are shown illustrating the effect of 10 µM CGP. Currents were generated by addition of 10 µM Ca^{2+} to the cytoplasmic side of the patch. Pooled data are shown in panel B in mean ± SEM format for % inhibition of inward currents (n = 4-5).

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Figure 4. Inhibition of inward CALX1.1-mediated inward Na^+-Ca^{2+} exchange currents by CGP. Panel A shows an example of inhibition exerted by 10 μM CGP. Panel B shows concentration dependent inhibition by CGP. Data are mean ± SEM % inhibition of inward peak (n = 4-5) and steady-state currents (n = 3-6). Fitted parameters for peak and steady-state currents are, respectively: IC_{50} = 3 ± 1 μM and 4 ± 2 μM; I_{MAX} = 48 ± 4 % and 63 ± 12 %.

Figure 5. Percent inhibition of NCX1.1- and CALX1.1-mediated Na^+-Ca^{2+} exchange currents by 10 μM CGP. Data are mean ± SEM with the numbers of individual patches shown above the corresponding bars.

Figure 6. Inhibition of deregulated outward Na^+-Ca^{2+} exchange currents by CGP. The representative tracings show inhibition of outward Na^+-Ca^{2+} exchange currents by 10 μM CGP for NCX1.1 (Panel A) and CALX1.1 (Panel B). Outward currents were generated by applying 100 mM Na^+ to the cytoplasmic side of α-chymotrypsin treated patches.

Figure 7. Inhibition of deregulated inward Na^+-Ca^{2+} exchange currents by CGP. The representative tracings show inhibition of inward Na^+-Ca^{2+} exchange currents by 10 μM CGP for NCX1.1 (Panel A) and CALX1.1 (Panel B). Inward currents were generated by applying 10 μM Ca^{2+} to the cytoplasmic side of α-chymotrypsin treated patches.

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Figure 8. Percent inhibition of NCX1.1- and CALX1.1-mediated Na^{+}-Ca^{2+} exchange currents by 10 μM CGP in α-chymotrypsin treated patches. Data are mean ± SEM with the numbers of individual patches shown above the corresponding bars.

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FIGURES

[see attached]

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Figure 1.

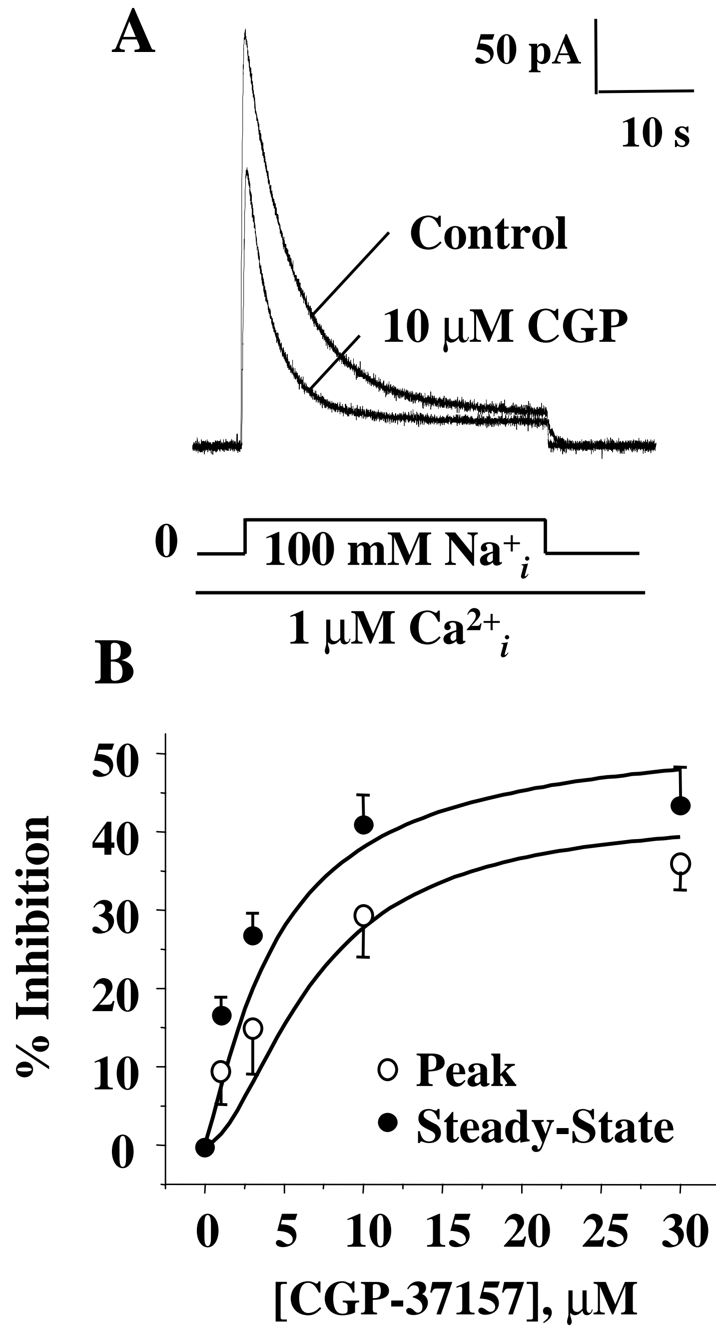

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Figure 2.

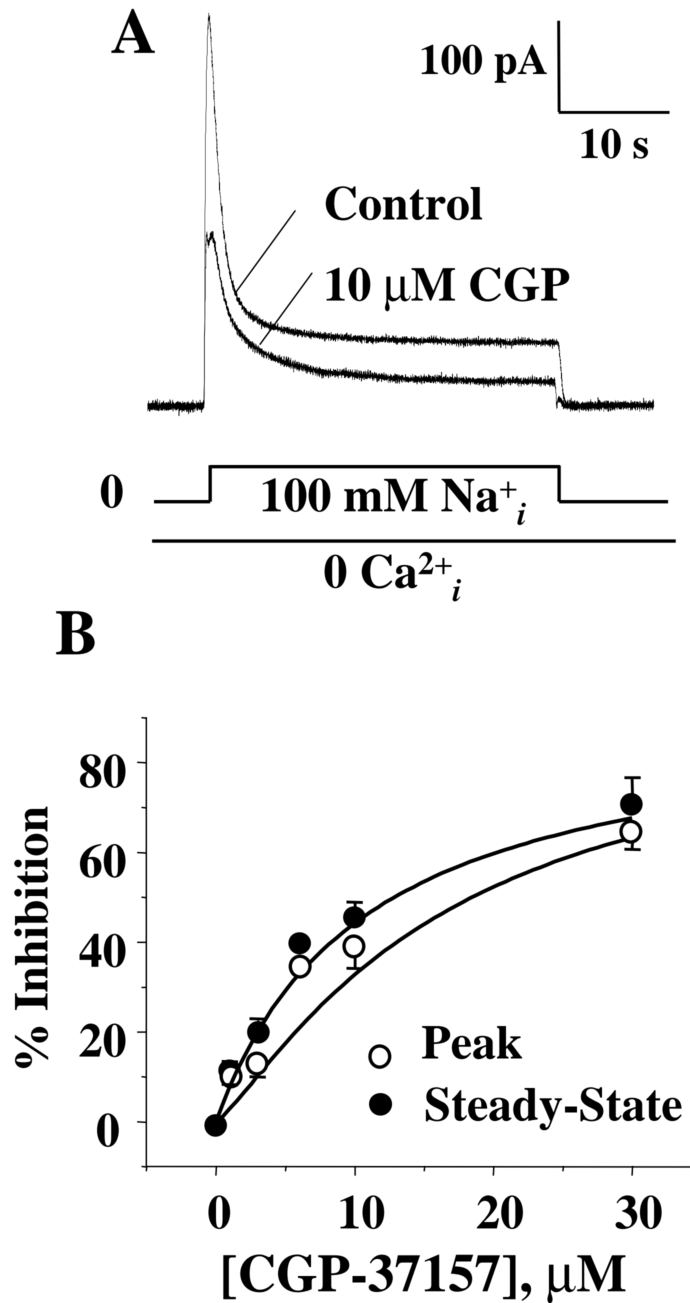

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Figure 3.

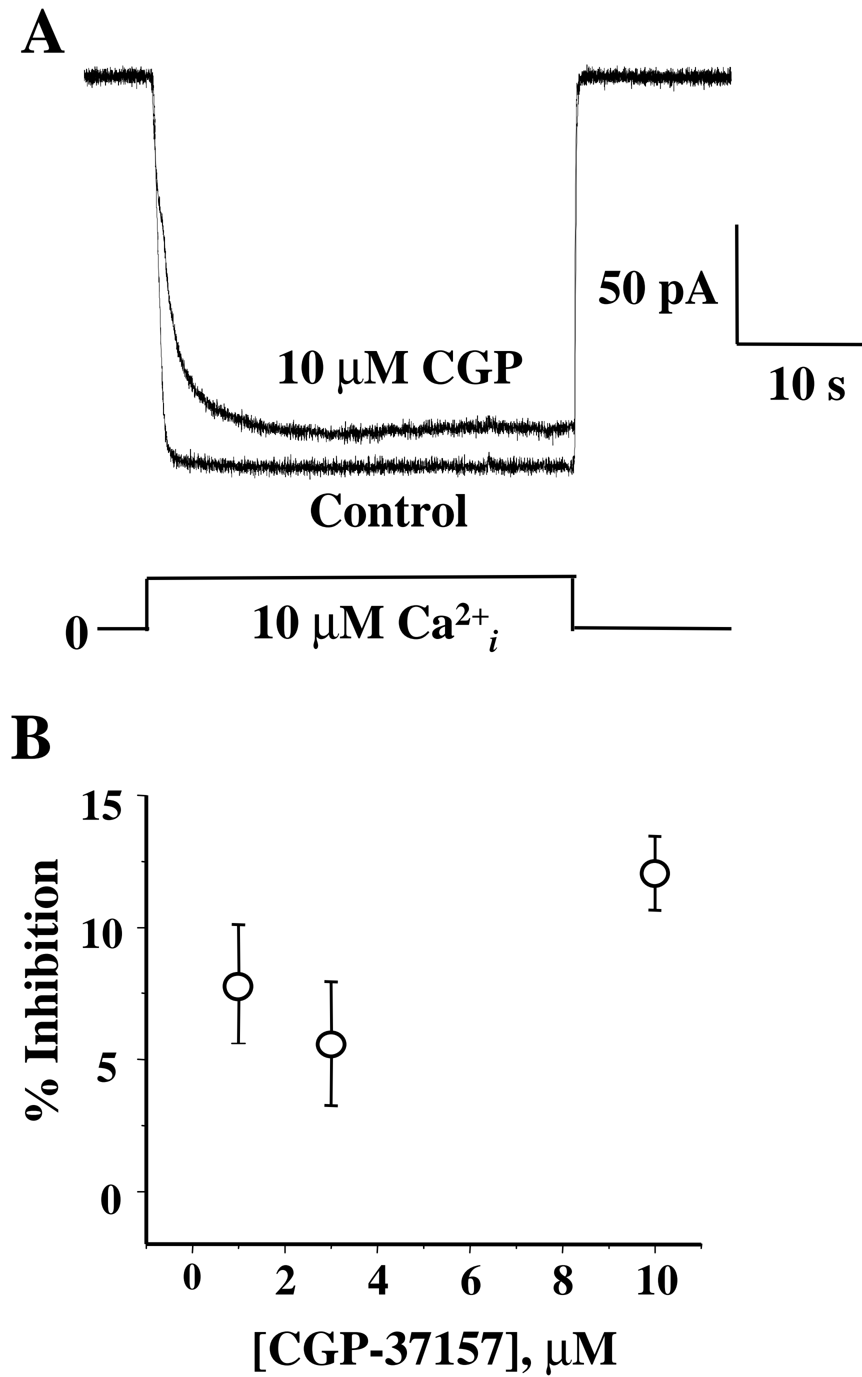

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Figure 4.

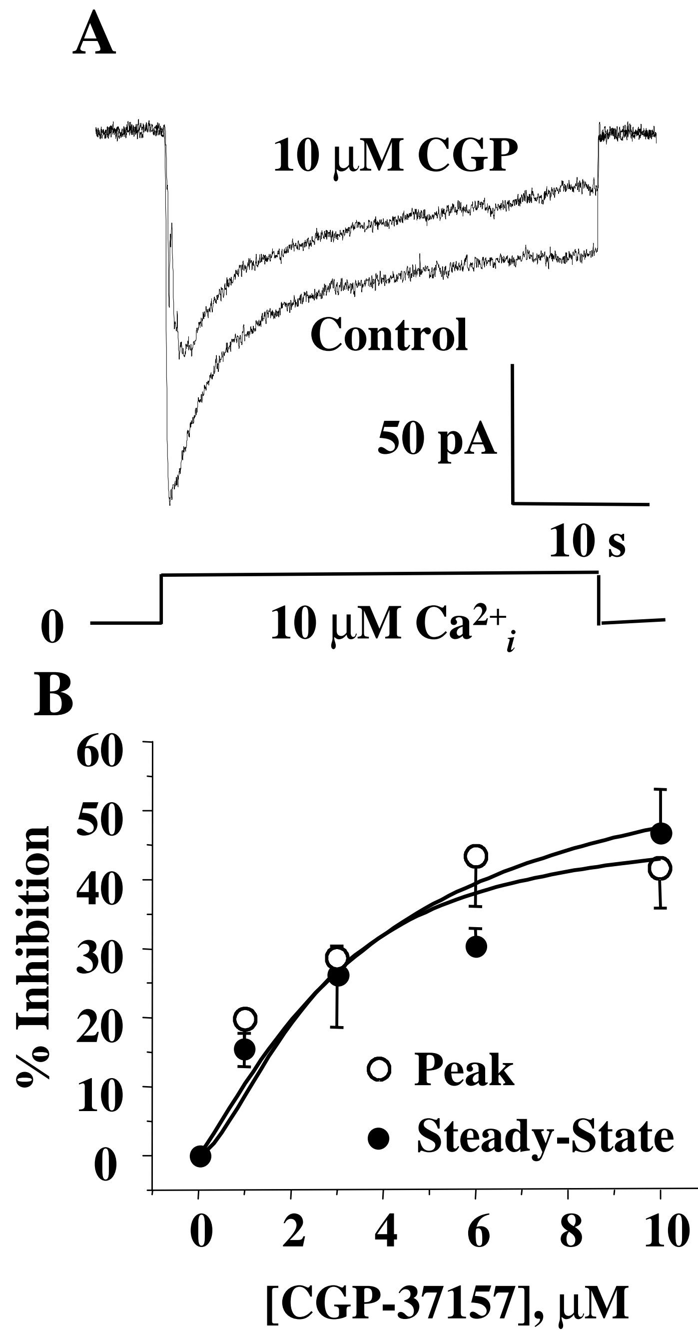

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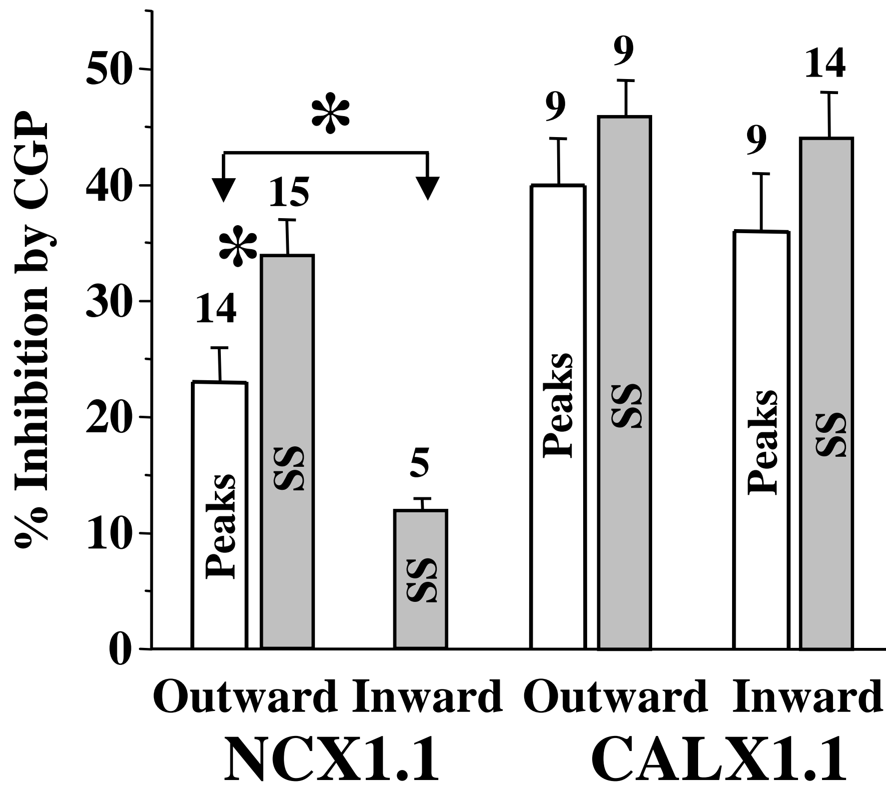
Figure 5.



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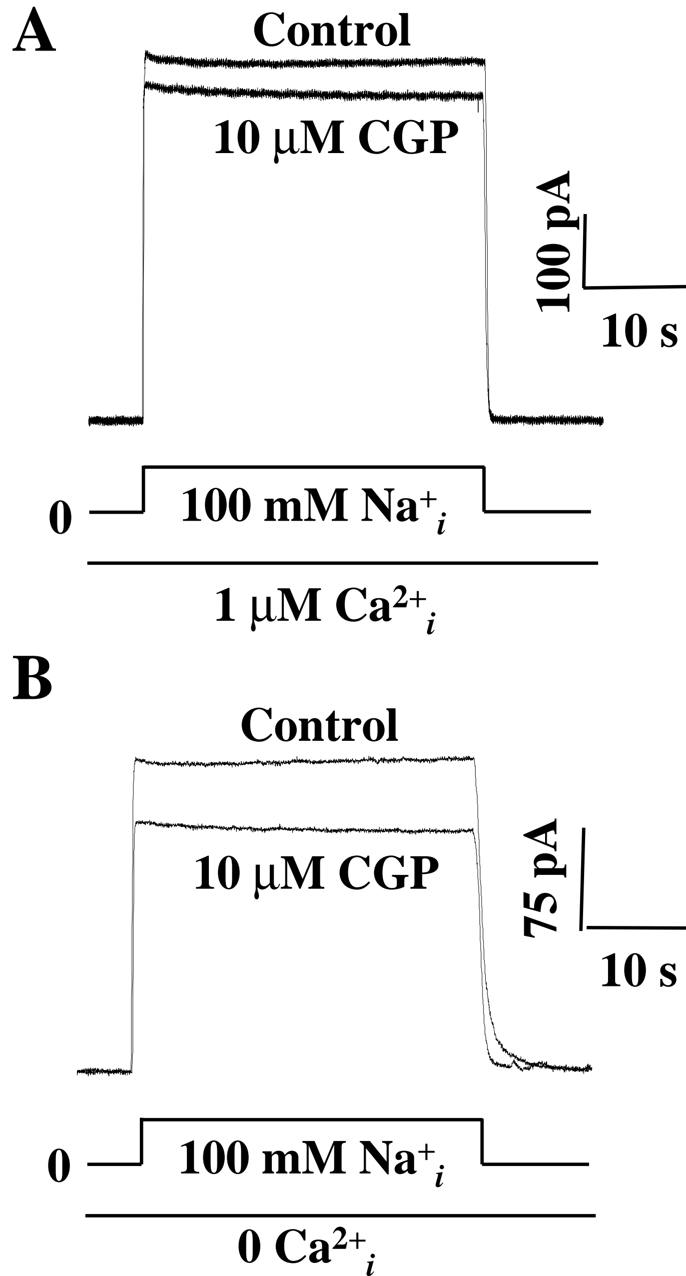

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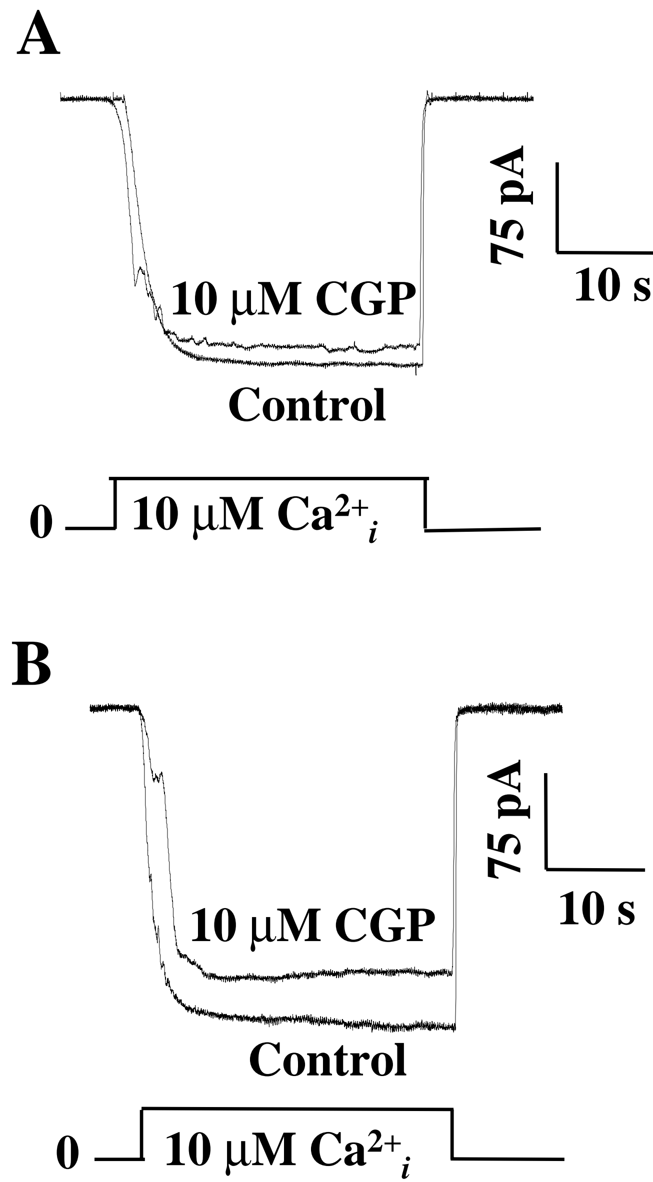

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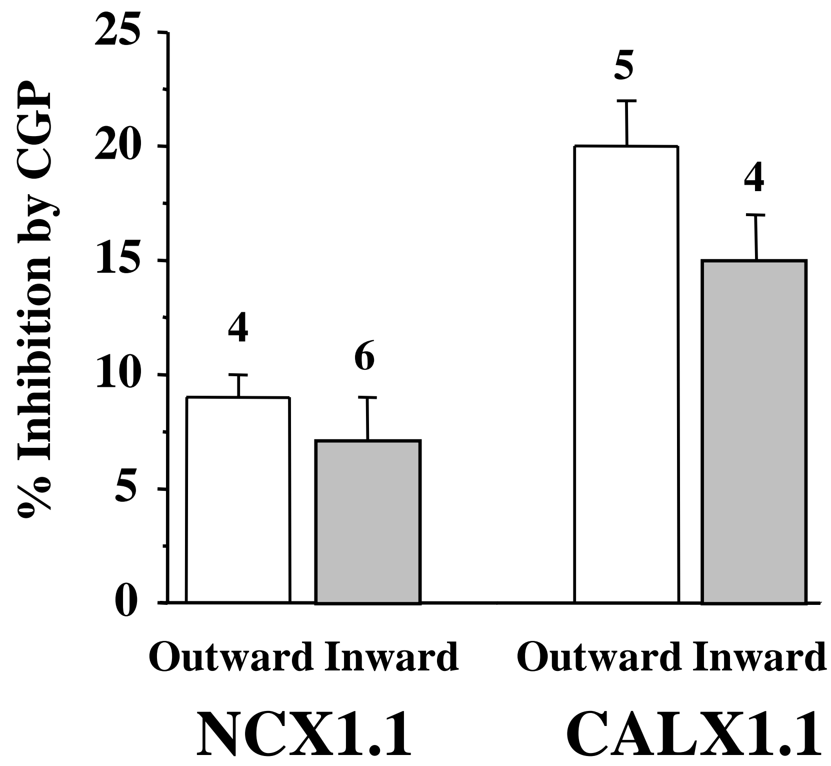

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