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Inhibition of L-type Ca^{2+} channel by mitochondrial Na^{+}–Ca^{2+} exchange inhibitor CGP-37157 in rat atrial myocytes

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Abstract

7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepine-2(*3H*)-one (CGP-37157) inhibits mitochondrial Na^{+}–Ca^{2+} exchange. It is often used as an experimental tool for studying the role of the mitochondrial Na^{+}–Ca^{2+} exchanger in Ca^{2+} signaling. Because the selectivity of CGP-37157 in adult cardiomyocytes has not been confirmed, we tested whether CGP-37157 affects the L-type Ca^{2+} channel using a whole-cell patch-clamp in adult rat atrial myocytes. We found that CGP-37157 suppressed L-type Ca^{2+} current (I_{Ca}) with IC_{50} of ~0.27 μM, without altering the voltage dependence of the current–voltage relationships. CGP-37157 inhibited the Ba^{2+} current (I_{Ba}) through the Ca^{2+} channel with a similar dose–response. The inhibitory effects of CGP-37157 on I_{Ca} or I_{Ba} were resistant to the intracellular Ca^{2+} buffering. Intracellular application of CGP-37157 did not significantly alter I_{Ca}. The combination of CGP-37157 with known Ca^{2+} channel inhibitor diltiazem yielded antagonism consistent with additivity of response. Our data suggest that CGP-37157 directly suppresses the L-type Ca^{2+} channel in intact adult cardiomyocytes.

Keywords: CGP-37157; L-type Ca^{2+} current; Atrial myocyte

1. Introduction

7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepine-2(3H)-one (CGP-37157) is a specific inhibitor of the mitochondrial Na^{+}–Ca^{2+} exchanger, and it is also used as an experimental tool for investigating how the mitochondrial Na^{+}–Ca^{2+} exchanger contributes to Ca^{2+} signaling in many cell types including muscles and neurons (Cox et al., 1993; White and Reynolds, 1996; Baron and Thayer, 1997; Colegrove et al., 2000; Czyz and Kiedrowski, 2003; Cheranov and Jaggar, 2004; Kovacs et al., 2005).

In the heart, CGP-37157 supposedly inhibits the mitochondrial Na^{+}–Ca^{2+} exchanger (IC_{50}=0.36 μM) without affecting either the sarcolemmal Na^{+}–Ca^{2+} exchanger and Na^{+}–K^{+} pump or the Ca^{2+} ATPase in the sarcoplasmic reticulum of the heart (Cox et al., 1993). Nevertheless, controversy surrounds the selectivity of CGP-37157 with respect to the mitochondrial Na^{+}–Ca^{2+} exchanger. For example, CGP-37157 directly inhibits the plasmalemmal Na^{+}–Ca^{2+} exchanger in cerebellar granule cells (Czyz and Kiedrowski, 2003). In addition, the compound inhibits neuronal voltage-gated Ca^{2+} channel (Baron and Thayer, 1997), though it does not change the Ca^{2+} current in isolated neonatal ventricular myocytes (Cox et al., 1993). In different types of neurons, CGP-37157 has shown differential effects on voltage-gated Ca^{2+} channels and the plasmalemmal Na^{+}–Ca^{2+} exchanger (Colegrove et al., 2000; Czyz and Kiedrowski, 2003). Because we need to determine the selectivity of CGP-37157 in adult cardiac myocytes before we can use it to study the role of the mitochondrial Na^{+}–Ca^{2+} exchanger in cardiac Ca^{2+} signaling, we tested whether CGP-37157 alters the function of L-type Ca^{2+} channels in isolated adult atrial myocytes. To this end, we used a whole-cell patch-clamp technique to study how CGP-37157 affects the L-type Ca^{2+} current (I_{Ca}) and the Ba^{2+} current (I_{Ba}) through the L-type Ca^{2+} channel.

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2. Materials and methods

2.1. Single cell isolation

Rat atrial myocytes were enzymatically isolated from male Sprague Dawley rats, (200–300 g) as described previously (Woo et al., 2002). Briefly, rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.), the chest cavity was opened and hearts were excised. The surgical procedures were carried out in accordance with the European Community guidelines for the use of experimental animals and were approved by our University ethics committee. The excised hearts were retrogradely perfused at 7 ml/min through the aorta, first for 5 min with Ca^{2+}-free Tyrode solution composed of (in mM): 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, 10 Glucose, pH 7.3, at 37 °C and then with Ca^{2+}-free Tyrode solution containing collagenase (1.4 mg/ml) and protease (0.16 mg/ml) for 12 min, finally with Tyrode solution containing 0.2 mM $CaCl_2$ for 6 min. The atria of the digested heart were then cut into several sections and subjected to gentle agitation to dissociate the cells.

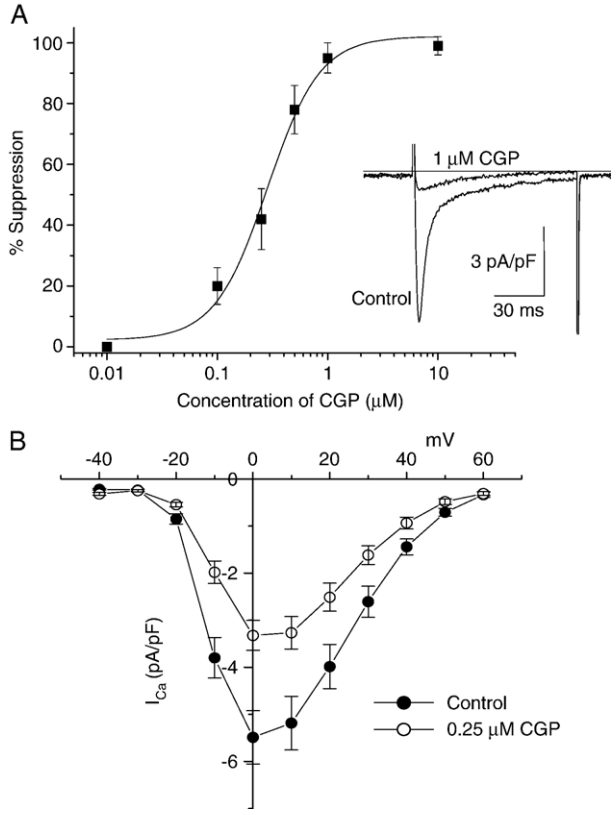

Fig. 1. Inhibition of L-type Ca^{2+} current (I_{Ca}) by CGP-37157 in rat atrial myocytes. (A) Concentration-dependent inhibition of I_{Ca} by the extracellular application of CGP-37157 (CGP) on the amplitude of I_{Ca} ($n=15$). I_{Ca} was activated by depolarizing step pulses from −50 to 0 mV at 0.1 Hz while the other current components were blocked (see Materials and methods). Inset, representative I_{Ca} traces recorded before (Control) and after treatment of 1 μM CGP. (B) Current–voltage relationships of I_{Ca} recorded at different testing potentials ranging from −40 to +60 mV ($V_h=-40$ mV) in the control condition and after exposure to 0.25 μM CGP ($n=7$). Cells were dialyzed with EGTA-free internal solutions.

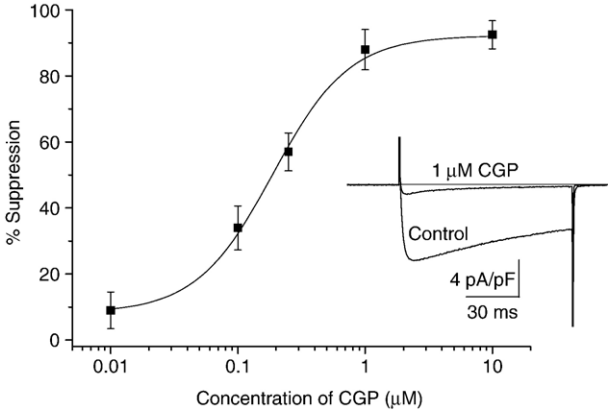

Fig. 2. Suppression of Ba^{2+} current (I_{Ba}) through the L-type Ca^{2+} channel by CGP-37157. The concentration–response curve for CGP-37157 (CGP) obtained after I_{Ba} had reached its steady state level in the presence of given concentrations of CGP ($n=11$). I_{Ca} was activated by depolarizing step pulses from −50 to 0 mV at 0.1 Hz. Inset, representative I_{Ba} traces recorded before (Control) and after treatment of 1 μM CGP.

The freshly dissociated cells were stored at room temperature in Tyrode solution containing 0.2 mM $CaCl_2$.

2.2. Measurement of membrane current

We whole-cell clamped the myocytes (Hamil et al., 1981) with patch pipettes (that had a tip resistance of 2.5 to 3.0 MΩ) and we dialyzed the myocytes with a Cs^+-rich internal solution containing 20 mM tetraethylammonium (TEA) (see below). We also replaced the extracellular K^+ with equimolar Cs^+ to inhibit the remaining K^+ current, and we inhibited Na^+ current by adding tetrodotoxin (20 μM) to the external solution or by holding membrane potential at −40 to −50 mV. Trains of test pulses were to 0 mV for 100 ms with 0.1 Hz. The I_{Ca} was fully sensitive to 20 μM of nifedipine and to 200 μM of Cd^{2+} (data not shown). To measure the Ba^{2+} current, we replaced extracellular Ca^{2+} (2 mM) with equimolar Ba^{2+}. Measurements of I_{Ca} and I_{Ba} were carried out 2–3 min after rupture of the membrane with the patch pipette. After 6–7 min of dialysis, the rundown of Ca^{2+} channels were slowed and stabilized. Membrane currents were measured with a HEKA patch-clamp amplifier (model EPC7, HEKA Elektronik, Germany). Generation of the voltage-clamp protocol and acquisition of the data were carried out using pCLAMP software (version 9.0, Axon Instruments, Foster City, CA) via an A/D converter (model 1322, Axon Instruments). The current signals were filtered at 10 kHz before digitization and storage.

2.3. Solutions

Patch pipettes were filled with a solution containing (in mM) 110 CsCl, 20 TEA-Cl, 20 HEPES, 0.2 cAMP and 5 Mg-ATP, with the pH adjusted to 7.2 with CsOH. In some experiments (see Results) 15 mM EGTA or 5–10 μM CGP-37157 was also included in the pipette solutions. CGP-37157 was purchased from Calbiochem (Darmstadt, Germany). In the experiments to obtain dose–response of the drugs (Figs. 1 and 2) we applied

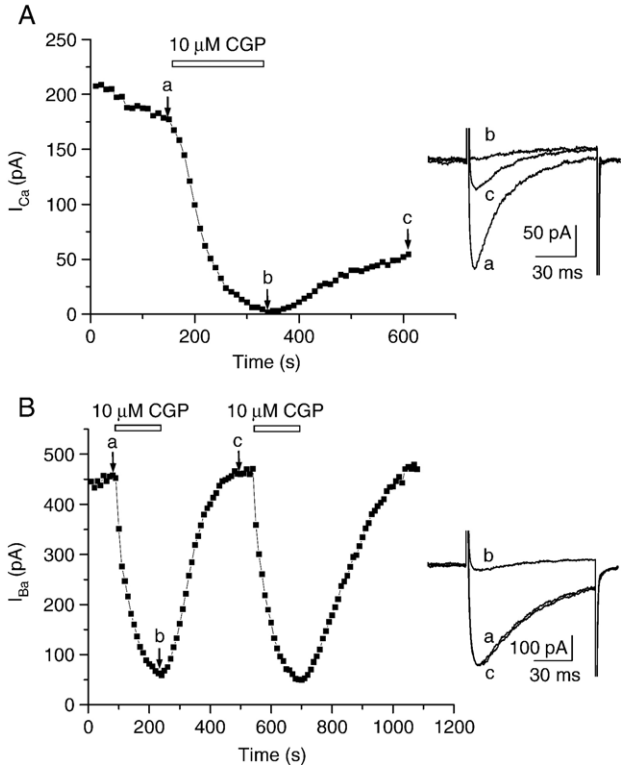

Fig. 3. Effects of CGP-37157 on I_{Ca} and I_{Ba} in highly Ca^{2+}-buffered atrial myocytes. (A) Time course of the effect of CGP-37157 (CGP; 10 μM) on I_{Ca} in a representative cell dialyzed with 15 mM EGTA-containing internal solution. Inset, I_{Ca} traces obtained at the times indicated by the numbers in the time course. (B) Time course of the effect of CGP (10 μM) on I_{Ba} in a representative cell dialyzed with 15 mM EGTA-containing internal solution. Inset, I_{Ba} traces obtained at the times indicated by the numbers in the time course. The *horizontal bars* indicate periods of drug exposures. See text for details.

CGP-37157-containing external solutions rapidly to single cells with the aid of an electronically controlled microbarrelled perfusion system. In other experiments, drug solutions were applied to the cells by exchanging the bathing solution in the chamber (volume, ≈0.65 ml) at the flow rate of ≈1.8 ml/min. All experiments were conducted at room temperature (22−24 °C).

2.4. Data analysis

We used the pCLAMP software to analyze the current data and the Clampfit software to detect the peaks. The magnitude of I_{Ca} was measured as the difference between the peak inward current and the steady-state current at the end of the voltage pulse. The magnitude of I_{Ba} was measured as the peak inward current because the absence of Ca^{2+}-dependent inactivation resulted in no full inactivation of the current. IC_{50} values were calculated by fitting the averaged dose-inhibition curve to the Hill equation. The subsequent analysis and fitting were performed using Origin 6.0 computer program (Microcal™).

The numerical results are given as means±S.E.M. (n =), where S.E.M. is the standard error of the mean and n is the number of cells. For statistical comparisons, we used a Student's t-test; and we considered differences to be statistically significant to a level of $P<0.05$.

3. Results

3.1. Suppression of I_{Ca} and I_{Ba} by CGP-37157 in rat atrial myocytes

Fig. 1A illustrates the CGP-37157 concentration–response curve that was obtained after I_{Ca} had reached its steady-state level in the presence of given concentrations of CGP-37157 in isolated single rat atrial myocytes. The curve shows that CGP-37157 significantly decreases the I_{Ca} at concentrations ≥0.1 μM. The resulting IC_{50} value was 0.27±0.02 μM (n=15). Fig. 1B shows the average I_{Ca}–voltage relationship in the absence and presence of 0.25 μM (that is a concentration close to the IC_{50}) CGP-37157. There was no clear shift in the voltage-dependence of I_{Ca} by CGP-37157 (n=7).

L-type Ca^{2+} current can be decreased by rise in the cytosolic Ca^{2+} concentration (Kass and Sanguinetti, 1984; Lee et al., 1985; Soldatov, 2003). Since CGP-37157 has been shown to elevate basal cytosolic Ca^{2+} concentration in rat atrial myocytes (Woo et al., 2006) we examined, in the next series of experiments, whether CGP-37157 directly inhibits L-type Ca^{2+} channel or indirectly suppresses it by increasing the cytosolic Ca^{2+} concentration using extracellular Ba^{2+} as a charge carrier for the Ca^{2+} channels. The CGP-37157 concentration–response curve on I_{Ba} was not different from that on I_{Ca} at the range of concentrations (Fig. 2; $P>0.05$). CGP-37157 reduced I_{Ba} with IC_{50} value of 0.21±0.01 μM (11 cells). This result suggests that the inhibitory effect of CGP-37157 on I_{Ba} is similar to that on I_{Ca}.

3.2. Effects of CGP-37157 on I_{Ca} and I_{Ba} in myocytes dialyzed with intracellular solutions with EGTA

We further tested the possibility that CGP-37157 indirectly suppresses I_{Ca} through increasing the cytosolic Ca^{2+} level. Fig. 3 shows the effects of 10 μM CGP-37157 on I_{Ca} and I_{Ba} in rat atrial myocytes dialyzed with 15 mM EGTA-containing internal solutions. I_{Ca} measurements were carried out 6–7 min after

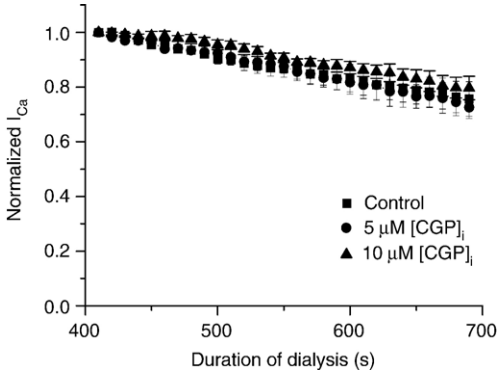

Fig. 4. Effects of the intracellular applications of CGP-37157 (CGP) on I_{Ca}. (A) shows averaged results (±S.E.M.) obtained in 5 control cells (no CGP), in 3 cells dialyzed with 5 μM CGP and in 5 cells dialyzed with 10 μM CGP. I_{Ca} was activated by depolarizing step pulses from −40 to 0 mV at 0.1 Hz, and was normalized by the peak current recorded at 400 s after dialysis. Cells were dialyzed with 15 mM EGTA-containing internal solutions.

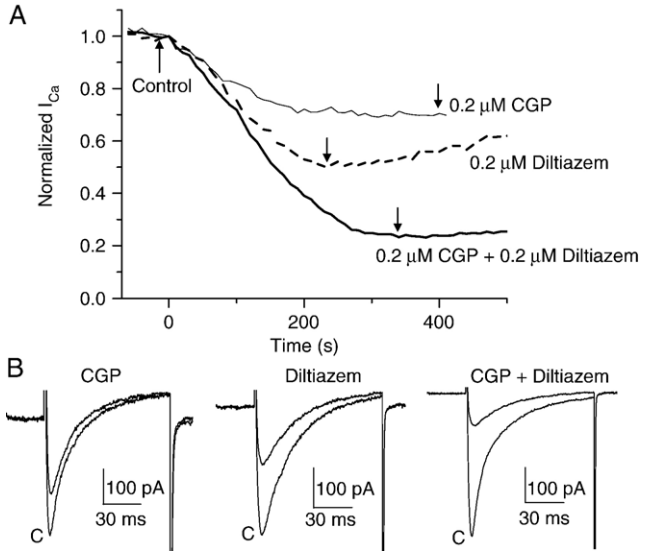

Fig. 5. Effects of the combination of CGP-37157 and diltiazem on I_{Ca}. (A) Time courses of blockade of I_{Ca} by 0.2 μM CGP-37157 (CGP), 0.2 μM diltiazem, or the combination of both drugs. The currents were elicited by applying test pulses to 0 mV for 100 ms from a holding potential of −40 mV at 0.1 Hz. (B) Superimposed current tracings, recorded at the times marked by the arrows in the panel A ("c" indicates control), during the experiments with CGP, diltiazem and the combination of CGP and diltiazem.

rupture of the membrane with the patch pipette. After this period of dialysis, the rate of I_{Ca} inactivation was stable, suggesting that the intracellular EGTA concentration was typically at equilibrium throughout the atrial cells. Superfusion of the cell with CGP-37157 caused almost full loss of I_{Ca}, and the current partially recovered during CGP-37157 washout (Fig. 3A, b–c). Averaged results obtained in cells dialyzed with EGTA using this protocol showed an initial I_{Ca} amplitude of 4.49±1.84 pA/pF (n=4); in the presence of 10 μM CGP-37157, peak I_{Ca} decreased by 97±1.4% (n=4).

Similar experiments were performed using 2 mM Ba^{2+} as charge carrier. In the cell of Fig. 3B, the initial I_{Ba} of 456 pA was reduced in 10 μM CGP-37157 to 57.7 pA. The current quickly recovered during CGP-37157 washout (Fig. 3B, b–c). A second addition of CGP-37157 again caused a reduction of I_{Ba} in a reversible manner. The initial I_{Ba} of another six cells averaged 11.7±1.5 pA/pF, which was reduced by 83.6±2.8% in 10 μM CGP-37157.

3.3. Effect of intracellular applications of CGP-37157 on I_{Ca}

For intracellular drug application, drugs were dissolved in the pipette solution and dialyzed into the myocytes. I_{Ca} was elicited continuously from 2–3 min after the rupture of the membrane at 0.1 Hz. The currents became stable at 6–7 min after the onset of dialysis in the control conditions. Thus I_{Ca}, recorded during 400–700 s following the onset of dialysis with or without CGP-37157, were compared. Fig. 4 shows the effects of intracellular dialyses of 5 and 10 μM CGP-37157 on the normalized I_{Ca}. The time courses of I_{Ca} measured in the myocytes dialyzed with CGP-37157-free (Control, n=5), and CGP-37157-containing (5 μM, n=3; 10 μM, n=5) internal solutions were not significantly different (P>0.05 at all time points).

3.4. Effects of combination of CGP-37157 and diltiazem on I_{Ca}

To further examine whether CGP-37157 inhibits I_{Ca} by directly binding to Ca^{2+} channels we studied the effect of the combination of CGP-37157 with known L-type Ca^{2+} channel inhibitor diltiazem. Fig. 5 shows the effects of 0.2 μM CGP-37157, 0.2 μM diltiazem and the combination of both drugs on I_{Ca}. In order to examine the interaction of the blocking actions of CGP-37157 and diltiazem, we set the concentrations of CGP-37157 and diltiazem at 0.2 μM to elicit moderate block. The application of 0.2 μM CGP or 0.2 μM diltiazem inhibited I_{Ca} by 24±2% (n=6) and 46±4% (n=6), respectively. The combination of CGP-37157 (0.2 μM) and diltiazem (0.2 μM) inhibited I_{Ca} by 73±3% (n=7), which is very close to the sum of % suppression measured in each drug. The additivity of response indicates that CGP-37157 and diltiazem may interact at the same binding sites of L-type Ca^{2+} channel.

4. Discussion

Our results show that benzodiazepine, CGP-37157, directly inhibits the voltage-dependent L-type Ca^{2+} channel in intact adult atrial myocytes. CGP-37157 has been used to selectively inhibit the mitochondrial Na^{+}–Ca^{2+} exchanger in a variety of cells (Cox et al., 1993; White and Reynolds, 1996; Baron and Thayer, 1997; Colegrove et al., 2000; Czyz and Kiedrowski, 2003; Cheranov and Jaggar, 2004; Kovacs et al., 2005). However, our data suggest caution in the use of CGP-37157 as a means of studying the role of mitochondrial Na^{+}–Ca^{2+} exchange in Ca^{2+} signaling in intact adult cardiac myocytes.

We observe that CGP-37157 inhibits I_{Ca} at concentrations similar to those that are normally used to block the mitochondrial Na^{+}–Ca^{2+} exchanger (110 μM: Cox et al., 1993; Cheranov and Jaggar, 2004; Kovacs et al., 2005). Our finding is somewhat consistent with another report that CGP-37157 directly inhibits voltage-gated Ca^{2+} channels in dorsal root ganglion neurons (Baron and Thayer, 1997). However, in different types of neurons (such as sympathetic neurons), the compound does not affect voltage-gated Ca^{2+} channels (Colegrove et al., 2000). Note that, in neonatal ventricular myocytes, CGP-37157 has no apparent effect on I_{Ca} for concentrations ≤10 μM (Cox et al., 1993), and that this result differs from the results we obtained with respect to adult atrial myocytes.

Some other benzothiazepine derivatives, such as 1,5-benzothiazepines (DTZ323 and DTZ417), are known to suppress L-type Ca^{2+} channels in isolated adult cardiac myocytes (Kraus et al., 1996; Kurokawa et al., 1997). The resulting IC_{50} value of CGP-37157 is approximately 0.27 μM (Fig. 1A), which is close to the IC_{50} value of diltiazem (Kurokawa et al., 1997). In addition, at concentrations higher than 1 μM, CGP-37157 almost completely blocks the I_{Ca} (Fig. 1A). CGP-37157 appears to directly inhibit the L-type Ca^{2+} channel by binding competitively to the binding sites of diltiazem (Fig. 5). The present results also suggest that the binding sites of CGP-37157 are located on the extracellular side

of the L-type Ca^{2+} channel (Fig. 4). In this regard, it has been reported that other benzothiazepines including diltiazem affect Ca^{2+} channel much more potently (about 10^3 times) when they were applied from the extracellular side than from the intracellular side (Kurokawa et al., 1997), which is consistent with our results. Although CGP-37157 slightly increases basal Ca^{2+} concentration in rat atrial myocytes (Woo et al., 2006), the change in the intracellular Ca^{2+} concentration does not appear to affect I_{Ca} (or I_{Ba}) since CGP-37157 suppressed I_{Ca} (or I_{Ba}) with a similar potency in the myocytes dialyzed with or without EGTA (Fig. 3).

Acknowledgement

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