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Inhibition of plasmalemmal Na^+/Ca^{2+} exchange by mitochondrial Na^+/Ca^{2+} exchange inhibitor 7-chloro-5-(2-chlorophenyl)-1, 5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP-37157) in cerebellar granule cells

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Abstract

In the heart, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP-37157) inhibits mitochondrial but not sarcolemmal Na^+/Ca^{2+} exchange. Therefore, CGP-37157 is often used as an experimental tool to study the role of mitochondrial Na^+/Ca^{2+} exchange in Ca^{2+} homeostasis in various cells, including neurons. However, neurons express several K^+-dependent (NCKX) and/or K^+-independent (NCX) isoforms of plasmalemmal Na^+/Ca^{2+} exchange not expressed in the sarcolemma. Because it has never been determined whether CGP-37157 inhibits plasmalemmal NCKX and/or NCX isoforms in neurons, we tested this possibility. As an index of NCKX and/or NCX activity, we studied Na-dependent and gramicidin-induced $^{45}Ca^{2+}$ accumulation in the presence and absence of K^+, respectively. In primary cultures of cerebellar granule cells, CGP-37157 with IC_{50} of 13 μM inhibited over 70% of plasmalemmal NCX activity ($P < 0.01$) but not NCKX activity. Our data suggest that the effects of CGP-37157 on neuronal Ca^{2+} homeostasis include inhibition of certain plasmalemmal NCX isoform(s). Because cerebellar granule cells robustly express NCX3 transcripts, which are not expressed in the heart, it appears that this isoform may be CGP-37157 sensitive.

Keywords: NCX; NCKX; CGP-37157; KB-R7943; Mitochondria; Neurons

1. Introduction

In the heart, benzothiazepine, CGP-37157, with IC_{50} of 0.36 μM inhibits mitochondrial Na^+/Ca^{2+} exchange whereas at concentrations as high as 30 μM it fails to inhibit sarcolemmal Na^+/Ca^{2+} exchange [1]. Therefore, CGP-37157 has been often used as an experimental tool to study the contribution of mitochondrial Na^+/Ca^{2+} exchange to Ca^{2+} homeostasis in a variety of cells, notably cultured neurons [2–4]. However, multiple isoforms of K-independent (NCX) and K-dependent (NCKX) plasmalemmal Na^+/Ca^{2+} exchangers are expressed in neurons [5] whereas in the heart only NCX1 is robustly expressed [6]. Because it has never been determined whether neuronal NCX and NCKX isoforms are sensitive to CGP-37157, in the present report, we tested this possibility. To this end, we studied the effects of CGP-37157 on gramicidin-induced $^{45}Ca^{2+}$ accumulation. Gramicidin is a pore-forming ionophore that permeates alkali cations and protons but not Ca^{2+} [7]. If alternative pathways of Ca^{2+} influx are blocked (see Section 2), the gramicidin-induced and Na-dependent $^{45}Ca^{2+}$ accumulation is an

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Abbreviations: CGP-37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one; KB-R7943, 2-{2-[4-(nitrobenzyloxy)phenyl]ethyl}isothiourea mesylate; NCX, Na^+/Ca^{2+} exchange; NCKX, $Na^+/(Ca^{2+} + K^+)$ exchange; VGCC, voltage-gated Ca^{2+} channels.

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index of Ca^{2+} influx *via* reversed plasmalemmal Na^+/Ca^{2+} exchangers.

2. Materials and methods

2.1. Cell culture

Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague–Dawley rats and cultured in basal Eagle's medium supplemented with 25 mM K^+, 10% fetal bovine serum, 2 mM glutamine, and 50 μg/mL gentamycin. Glial proliferation was curtailed by addition of 10 μM cytosine arabinofuranoside 24 hr after plating. Cultures at 9–11 days *in vitro* were used for the experiments. Other details are given in [8].

2.2. Assay of reverse NCX and NCKX activity

Plasmalemmal Na^+/Ca^{2+} exchange activity was assessed by studying Na-dependent $^{45}Ca^{2+}$ accumulation induced by 5 μM gramicidin in cerebellar granule cells with mitochondria depolarized by 2 μM rotenone and 3 μg/mL oligomycin, and glycolysis blocked by 1 mM iodoacetate [9,10]. To distinguish $^{45}Ca^{2+}$ accumulation mediated by NCKX from that mediated by NCX, we used 30 μM KB-R7943, which inhibits NCX but not NCKX [10,11]. To selectively inhibit NCKX, we removed K^+ from the medium. The K-supplemented medium contained (in mM) 100 NaCl, 2 KCl, 3.6 $KHCO_3$, 57.6 *N*-methyl-D-gluconium-HCl (NMG-Cl), 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4 adjusted with Tris. The K-free medium contained (in mM) 96.4 NaCl, 3.6 $NaHCO_3$, 63.2 NMG-Cl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. Both media also contained Na, K-ATPase inhibitor, 1 mM ouabain, 10 μM nifedipine, 10 μM dizocilpine, and 10 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX), to block L-type VGCC, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors, respectively. Basal $^{45}Ca^{2+}$ accumulation was measured under Na-free conditions (Na^+ replaced by Li^+) and was subtracted from the data. While monitoring reverse NCX and/or NCKX activity we did not attempt to inhibit all VGCC because the gramicidin-induced $^{45}Ca^{2+}$ accumulation is completely inhibited by substitution of Na^+ with Cs^+ [10]. Considering that cytosolic Cs^+, delivered *via* a patch pipette, does not inhibit the VGCC expressed in cerebellar granule cells [12], it is unlikely that these channels would be responsible for the Na-dependent $^{45}Ca^{2+}$ accumulation that we attribute to NCX and/or NCKX reversal.

2.3. Materials

CGP-37157, KB-R7943, dizocilpine and NBQX were obtained from Tocris, $^{45}Ca^{2+}$ from ICN Biomedicals, and other chemicals from Sigma.

3. Results and discussion

CGP-37157 inhibited gramicidin-induced and Na-dependent $^{45}Ca^{2+}$ accumulation in a dose-dependent manner (Fig. 1A), which indicates that the drug inhibits NCX or NCKX or both. To determine whether CGP-37157 inhibits NCX or NCKX, we prevented NCKX reversal by omitting K^+ [13–15] which reduced the Na-dependent $^{45}Ca^{2+}$

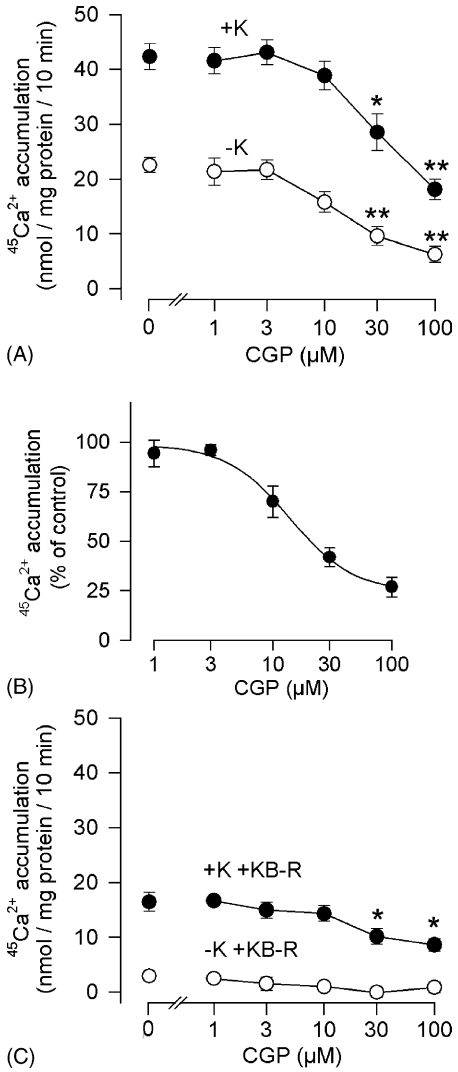

Fig. 1. Inhibition of gramicidin-induced and Na-dependent $^{45}Ca^{2+}$ accumulation in cultured cerebellar granule cells by CGP-37157 (CGP). (A) Effects of CGP on Na-dependent $^{45}Ca^{2+}$ accumulation in the presence of 100 mM Na^+ plus 5.6 mM K^+ (+K) or 100 mM Na^+ without K^+ (−K). (B) The (−K) data from A expressed as percentage of control (without CGP). The curve was fit using a least-square non-linear algorithm (SigmaPlot, SPSS Inc) and a logistic equation: % of control $= ((a-b)/1 + (X/IC_{50})^c) + b$, where X is the CGC concentration, a and b are the percentage of response calculated for $X = 0$ and for an infinite concentration, respectively, and c is the slope of the curve. Based on the data from three independent experiments, the following values were calculated: $a = 98 \pm 3.6$; $b = 28 \pm 7.3$; $IC_{50} = 13 \pm 3.7$; $c = 2 \pm 0.5$. (C) The same as in A but in the presence of 30 μM KB-R7943 (+KB-R). The data are means ± SEM from three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared to respective control (without CGP), one-way ANOVA followed by Student–Newman–Keul's test.

accumulation by 47% (Fig. 1A). This indicates that NCKX mediates about 50% of the Na-dependent $^{45}Ca^{2+}$ accumulation in these cells. When NCKX reversal was prevented, CGP-37157 inhibited 70% of the Na-dependent $^{45}Ca^{2+}$ accumulation with IC_{50} of 13 ± 3.7 μM (Fig. 1A and B). Comparison of the data shown in Fig. 1A vs. C (black circles) shows that 100 μM CGP-37157 inhibited $^{45}Ca^{2+}$ accumulation as effectively as 30 μM KB-R7943, an NCX inhibitor [16,17]. In the presence of 30 μM KB-R7943 and 5 mM K^+, 30 and 100 μM CGP-37157 caused an additional minor inhibition of the Na-dependent $^{45}Ca^{2+}$ accumulation (Fig. 1C).

Cerebellar granule cells cultured at 25 mM K^+, as in this study, robustly express NCX3 transcripts [5,18] that are not expressed in the heart [6]. Therefore, it is likely that the NCX3 isoform might be CGP-37157-sensitive. On the other hand NCKX2, NCKX3 and NCKX4 isoforms expressed in cerebellar granule cells [5] seem to be relatively resistant to CGP-37157.

It is unlikely that the gramicidin-induced $^{45}Ca^{2+}$ accumulation in cerebellar granule cells (Fig. 1A and B) is mediated by a CGP-37157-sensitive mitochondrial Na^+/Ca^{2+} exchange because, when the cells are exposed to 5 μM gramicidin and 100 mM Na^+, this exchanger operates in the forward mode (i.e. it removes Ca^{2+} from the mitochondria to the cytosol) [19]. Inhibition of this process by CGP-37157 should increase the amount of Ca^{2+} stored in the mitochondria and, therefore, enhance the total $^{45}Ca^{2+}$ accumulation in the cells, which is the opposite of what was observed (Fig. 1A and B).

Our data suggest caution in the use of CGP-37157 to study the role of mitochondria in Ca^{2+} homeostasis in intact cells. The drug inhibits not only mitochondrial but also plasmalemmal Na^+/Ca^{2+} exchange in cerebellar granule cells (Fig. 1A and B) and directly inhibits VGCC in dorsal root ganglion neurons [3]. One should note, however, that in frog sympathetic neurons, 2 μM CGP-37157, which does not affect plasmalemmal Na^+/Ca^{2+} exchange (Fig. 1A and B), robustly inhibits mitochondrial Na^+/Ca^{2+} exchange without significantly affecting VGCC [4].

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