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CGP37157 modulates mitochondrial Ca^{2+} homeostasis in cultured rat dorsal root ganglion neurons

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Abstract

The effects of 7-chloro-3,5-dihydro-5-phenyl-1H-4,1-benzothiazepine-2-on (CGP37157), an inhibitor of mitochondrial Na^+/Ca^{2+} exchange, on depolarization-induced intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) transients were studied in cultured rat dorsal root ganglion neurons with indo-1-based microfluorimetry. A characteristic plateau in the recovery phase of the $[Ca^{2+}]_i$ transient resulted from mitochondrion-mediated $[Ca^{2+}]_i$ buffering. It was blocked by metabolic poisons and was not dependent on extracellular Ca^{2+}. CGP37157 produced a concentration-dependent decrease in the amplitude of the mitochondrion-mediated plateau phase ($IC_{50} = 4 \pm 1$ μM). This decrease in $[Ca^{2+}]_i$ was followed by an increase in $[Ca^{2+}]_i$ upon removal of the drug, suggesting that Ca^{2+} trapped in the matrix was released when the CGP37157 was removed from the bath. CGP37157 also inhibited depolarization-induced Ca^{2+} influx at the concentrations required to see effects on $[Ca^{2+}]_i$ buffering. Thus, CGP37157 inhibits mitochondrial Na^+/Ca^{2+} exchange and directly inhibits voltage-gated Ca^{2+} channels, suggesting caution in its use to study $[Ca^{2+}]_i$ regulation in intact cells. © 1997 Elsevier Science B.V.

Keywords: Mitochondrion; CGP37157; Dorsal root ganglion neuron; Ca^{2+}, intracellular

1. Introduction

Mitochondria take up Ca^{2+} in response to an elevation in cytoplasmic Ca^{2+} levels. An increase in intramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mt}$) is thought to stimulate Ca^{2+}-sensitive, rate-limiting dehydrogenases in the matrix, and thus couple the increased energy demand signaled by an elevation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to the aerobic production of ATP (McCormack et al., 1990; McCormack and Denton, 1993). Large, pathologic Ca^{2+} loads result in a significant accumulation of Ca^{2+} within mitochondria and may contribute to the toxicity that results from Ca^{2+} overload (Wang et al., 1994; Reynolds and Hastings, 1995; White and Reynolds, 1995; Wang and Thayer, 1996).

Mitochondrial Ca^{2+} levels are the net result of influx and efflux pathways (Nicholls, 1985). Ca^{2+} enters the matrix via the uniporter which allows Ca^{2+} to run down its electrochemical gradient (Gunter and Pfeiffer, 1990). Thus, agents that affect the mitochondrial membrane potential modulate Ca^{2+} uptake by the mitochondrion (Thayer and Miller, 1990; Friel and Tsien, 1994; Werth and Thayer, 1994; Jouaville et al., 1995). In neuronal tissue an Na^+/Ca^{2+} exchange pathway appears to be the dominant mechanism for removing Ca^{2+} from the matrix. Thus, a reduction in the cytoplasmic Na^+ concentration will slow Ca^{2+} efflux from the mitochondrion (Thayer and Miller, 1990; Kiedrowski and Costa, 1995; Wang and Thayer, 1996). Because Ca^{2+} uptake and release from the mitochondrion are independent processes, the mitochondrion can act as a buffer of variable capacity that modulates the shape of cytoplasmic Ca^{2+} transients (Werth and Thayer, 1994; Herrington et al., 1996). Furthermore, this dynamic process creates the potential for Ca^{2+} to cycle across the inner membrane. Inhibition of Ca^{2+} efflux from the mitochondrion increases $[Ca^{2+}]_{mt}$ and also reduces the potential for cycling of Ca^{2+} across the inner membrane.

Pharmacologic agents that inhibit Ca^{2+} efflux from the mitochondrion might prove useful. Drugs that elevate matrix Ca^{2+} would be anticipated to accelerate aerobic metabolism and be of potential therapeutic benefit in treat-

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ing conditions associated with deficiencies in cellular ATP production. Furthermore, large Ca^{2+} loads may be toxic because of the futile cycling of Ca^{2+} across the inner membrane (Dykens, 1994), slowing Ca^{2+} efflux would reduce the deleterious effects of Ca^{2+} cycling. The Ca^{2+} channel antagonist diltiazem and structurally related compounds inhibit Na^+-induced Ca^{2+} release from isolated heart mitochondria (Vaghy et al., 1982). The most potent inhibitor of mitochondrial Na^+/Ca^{2+} exchange so far reported is 7-chloro-3,5-dihydro-5-phenyl-1H-4,1-benzothiazepine-2-on (CGP37157) (Chiesi et al., 1988; Cox and Matlib, 1993a). In isolated heart mitochondria this drug increased $[Ca^{2+}]_{mt}$, enhanced NADH production and increased the overall rate of oxidative ATP synthesis (Cox and Matlib, 1993b). In cultured cortical neurons White and Reynolds (1995) found that CGP37157 accelerated recovery from glutamate-induced Ca^{2+} loads.

Dorsal root ganglion neurons have a pronounced mitochondrial mediated component to the recovery phase of depolarization-induced $[Ca^{2+}]_i$ transients. Thus, cultured dorsal root ganglion neurons are a useful system to evaluate the effects of drugs on mitochondrion-mediated Ca^{2+} buffering in neuronal cells. In this report we describe the effects of CGP37157 on the $[Ca^{2+}]_i$ during the mitochondrial-mediated plateau phase in dorsal root ganglion neurons. Consistent with its presumed inhibition of mitochondrial Na^+/Ca^{2+} exchange, CGP37157 enhanced the buffering and sequestration of Ca^{2+} by mitochondria.

2. Materials and methods

2.1. Materials

Materials were obtained from the following companies: indo-1 acetoxymethyl ester, Molecular Probes (USA); CGP37157, CIBA-GEIGY (Switzerland); sera, GIBCO (USA); EGTA, Fluka (Germany); and all other reagents, Sigma (USA).

2.2. Experimental procedure

Neurons from the rat dorsal root ganglion were grown on glass coverglasses in primary culture as previously described (Werth and Thayer, 1994). To load cells with indicator, coverglasses were placed in 2 μM indo-1 acetoxymethyl ester in HEPES-buffered Hanks' salt solution containing 0.5% bovine serum albumin for 45–60 min at 37°C. The HEPES–Hanks' solution was composed of the following (in mM): HEPES 20, NaCl 137, $CaCl_2$ 1.3, $MgSO_4$ 0.4, $MgCl_2$ 0.5, KCl 5.4, KH_2PO_4 0.4, Na_2HPO_4 0.3, $NaHCO_3$ 3.0, and glucose 5.6. The coverglass was then mounted in a flow through chamber (Thayer et al., 1988b) and placed on the stage of a dual emission microfluorimeter (Werth and Thayer, 1994). Loading was terminated by washing in HEPES–Hanks' buffer for 15 min prior to starting an experiment. The chamber was superfused at a rate of 3 ml/min and solutions were selected with a multi-port valve coupled to several reservoirs. Depolarization-induced Ca^{2+} influx was produced by changing the superfusing solution from 5 mM K^+ to 50 mM K^+, with K^+ exchanged for Na^+ reciprocally. Ca^{2+}-free experiments were performed in either nominally Ca^{2+}-free buffer (no added Ca^{2+}) or Ca^{2+}-free buffer supplemented with 20 μM EGTA with identical results. CGP37157 was prepared as a 10 mM stock in DMSO and diluted to the concentrations indicated in HEPES–Hanks' solution. The $[Ca^{2+}]_i$ transients studied here were not affected by 1% DMSO. Fluorescence signals were converted to $[Ca^{2+}]_i$ according to previously described calibration procedures (Werth and Thayer, 1994).

3. Results

3.1. Ca^{2+} buffering by mitochondria produces a plateau phase in depolarization-induced $[Ca^{2+}]_i$ transients

Depolarization of cultured rat dorsal root ganglion neurons by superfusion with 50 mM K^+ for 60 s produced large increases in $[Ca^{2+}]_i$ mediated by voltage-gated Ca^{2+} channels (Fig. 1A) (Thayer et al., 1988a). The recovery of these $[Ca^{2+}]_i$ transients was characterized by a prominent plateau phase that we have previously suggested to result from Ca^{2+} uptake into the mitochondrial matrix followed by the gradual release of Ca^{2+} into the cytoplasm (Werth and Thayer, 1994). Except for a modest rundown, the responses were very reproducible from a given cell. The amplitude of the second response was $81 \pm 5\%$ of the first ($N = 14$). The plateau height of the second response, measured 3 min following start of the depolarizing stimulus, was $82 \pm 6\%$ of the first ($N = 14$). Consistent with the idea that mitochondria are responsible for the plateau phase, removal of extracellular Ca^{2+} following the stimulus did not affect the shape of the $[Ca^{2+}]_i$ transient (Fig. 1B). The plateau height (3 min post stimulus) was $81 \pm 6\%$ of the initial control response ($N = 6$). Thus, the plateau elevation in $[Ca^{2+}]_i$ does not result from Ca^{2+} influx. Furthermore, dissipation of the mitochondrial membrane potential with carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP; 1 μM) and inhibition of the ATP synthase with oligomycin (10 μM) completely blocked the plateau phase of the recovery (Fig. 1C). Because a mitochondrial membrane potential is necessary for Ca^{2+} uptake, these data indicate that mitochondrial sequestration of Ca^{2+} is required to produce the plateau

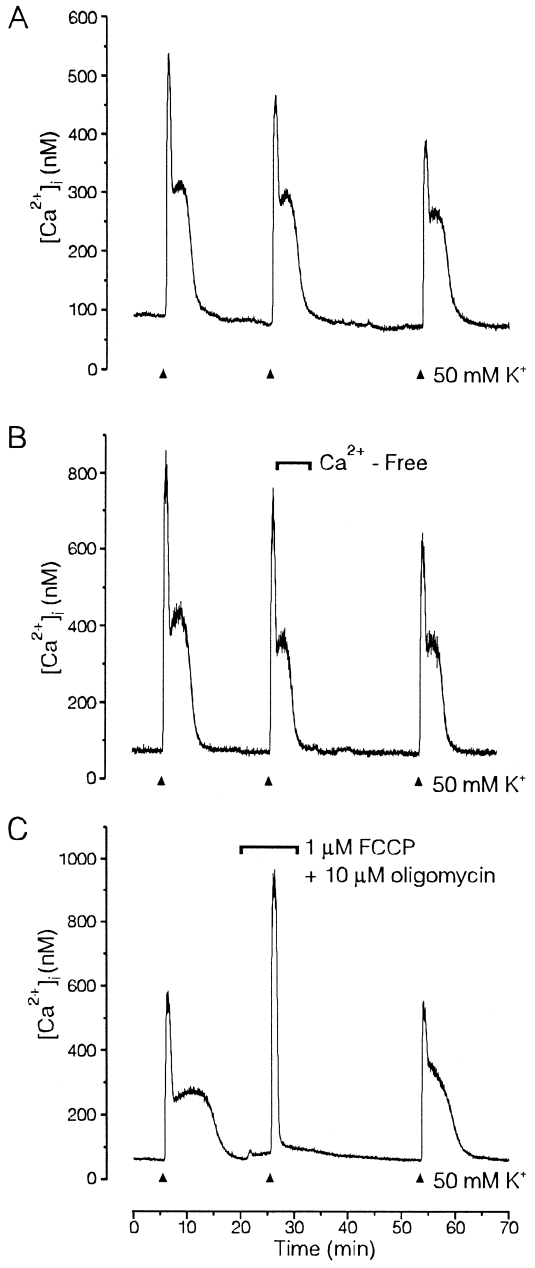

Fig. 1. The plateau in the recovery of 50 mM K^+-induced $[Ca^{2+}]_i$ transients results from Ca^{2+} release from mitochondria. 50 mM K^+ was superfused onto single dorsal root ganglion neurons for 60 s starting at the times indicated by the solid triangles. $[Ca^{2+}]_i$ was monitored with indo-1-based microfluorimetry as described in Section 2. (A) Three reproducible $[Ca^{2+}]_i$ transients could be elicited from a single cell. (B) The plateau phase of the recovery of $[Ca^{2+}]_i$ to basal levels did not require extracellular $[Ca^{2+}]_i$. Nominally Ca^{2+}-free buffer was superfused at the time indicated by the bracket. (C) Superfusion with 1 μM FCCP in combination with 10 μM oligomycin at the time indicated by the bracket completely blocked the mitochondrion-mediated plateau phase.

phase. Furthermore the peak amplitude of the response was increased (peak 2/peak 1 was $81 \pm 5\%$ in control and $145 \pm 12\%$ in the presence of FCCP + oligomycin; $P < 0.001$, Student's t-test) consistent with the loss of a mitochondrial Ca^{2+} buffer.

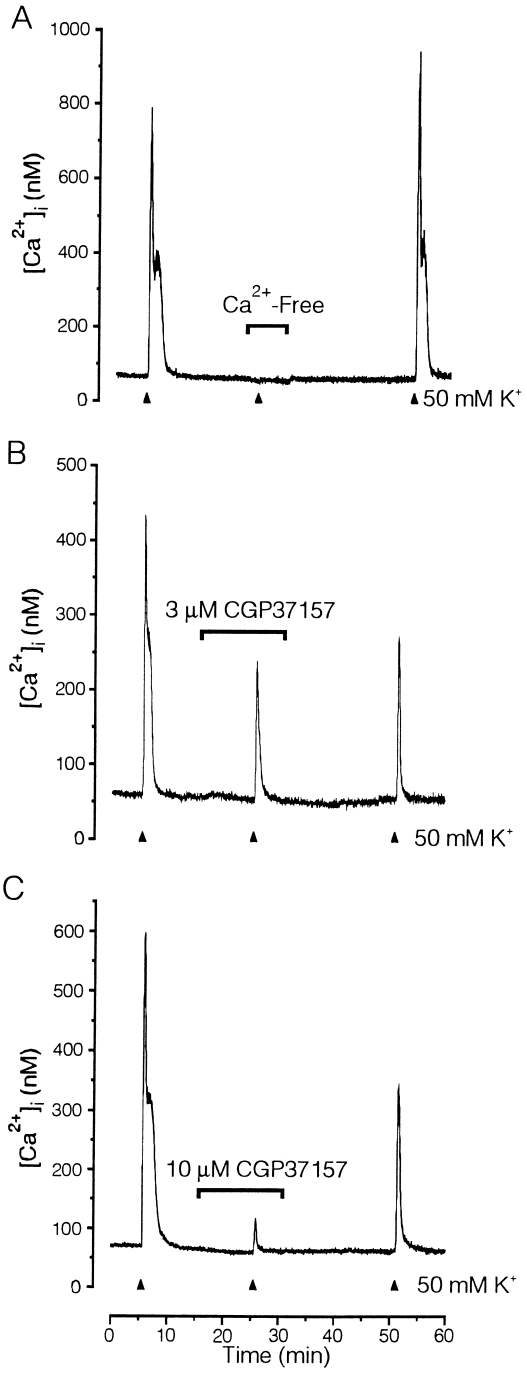

Fig. 2. CGP37157 blocks depolarization-induced Ca^{2+} influx. 50 mM K^+ was superfused onto single dorsal root ganglion neurons for 30 s starting at the times indicated by the solid triangles. $[Ca^{2+}]_i$ was monitored with indo-1-based microfluorimetry as described in Section 2. (A) Superfusion of Ca^{2+}-free buffer 2 min prior to and during the 50 mM K^+ stimulus completely blocked the increase in $[Ca^{2+}]_i$. Ca^{2+}-free (20 μM EGTA) buffer was superfused at the time indicated by the bracket. (B, C) CGP37157 was applied 10 min prior to and during the 50 mM K^+ stimulus as indicated by the bracket. Superfusion of 3 μM (B) and 10 μM (C) CGP37157 reduced the amplitude of the $[Ca^{2+}]_i$ transient in a concentration dependent manner.

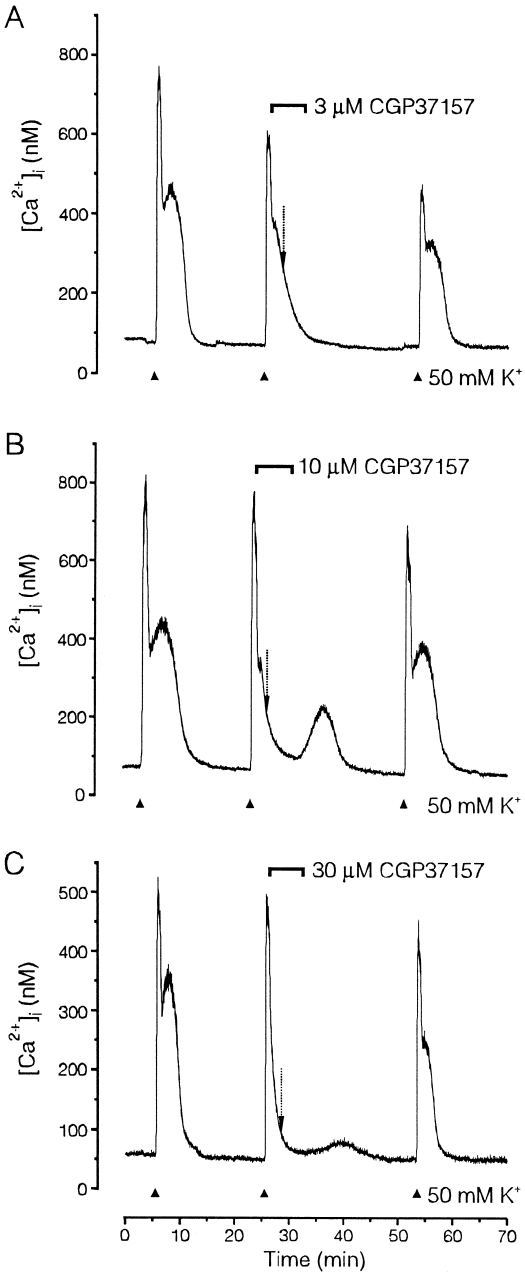

Fig. 3. CGP37157 reduces the amplitude of the mitochondrion-mediated phase of depolarization-induced $[Ca^{2+}]_i$ transients. 50 mM K^+ was superfused onto single dorsal root ganglion neurons for 60 s starting at the times indicated by the solid triangles. $[Ca^{2+}]_i$ was monitored with indo-1-based microfluorimetry as described in Section 2. Buffer containing 3 μM (A), 10 μM (B) or 30 μM (C) CGP37157 was exchanged for the depolarizing buffer at the time indicated by the brackets. Arrows are placed 3 min following start of the stimulus and mark the time at which $[Ca^{2+}]_i$ measurements were taken for determining the concentration–response relationship (Fig. 4).

3.2. CGP37157 inhibits depolarization-induced Ca^{2+} influx

CGP37157 is an analog of the voltage-gated Ca^{2+} channel antagonist, diltiazem. Therefore, prior to characterizing the effects of this drug on the mitochondrial mediated plateau phase we first determined whether it also affected depolarization-induced Ca^{2+} influx. Brief 30 s exposure to 50 mM K^+ elicited small $[Ca^{2+}]_i$ increases that lacked a significant plateau phase. In control recordings, repeated stimulation elicited responses with a modest rundown in amplitude (peak 2/peak 1 = 78 ± 7%, $N = 8$). These $[Ca^{2+}]_i$ transients resulted from Ca^{2+} influx as indicated by their complete block by removal of extracellular Ca^{2+} (Fig. 2A, $N = 5$). CGP37157 produced a concentration-dependent inhibition of the amplitude of the $[Ca^{2+}]_i$ transient (Fig. 2B and C). Treatment with 3 μM and 10 μM CGP37157 ten min prior to and during the second depolarizing stimulus reduced the amplitude of the response to respectively, 44 ± 4% ($N = 3$) and 14 ± 1% ($N = 3$) of the initial depolarization-induced response. 3 and 10 μM CGP37157 significantly inhibited Ca^{2+} influx relative to control (respectively, $P < 0.05$ and $P < 0.001$ by one-way ANOVA, Bonferroni post hoc test). Thus, to study the effects of this drug on the mitochondrial mediated plateau phase we applied the drug immediately following the 50 mM K^+ stimulus and prior to establishment of the plateau.

3.3. CGP37157 produces a concentration-dependent decrease in the amplitude of the mitochondrion-mediated plateau phase of depolarization-induced $[Ca^{2+}]_i$ transients

As shown in Fig. 3, changing directly from the depolarizing 50 mM K^+ buffer to normal 5 mM K^+ buffer

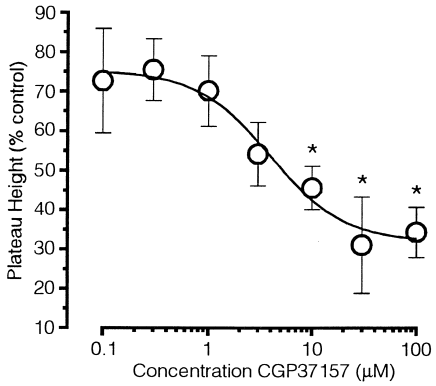

Fig. 4. CGP37157 produces a concentration-dependent inhibition of the height of the mitochondrion-mediated phase of depolarization-induced $[Ca^{2+}]_i$ transients. $[Ca^{2+}]_i$ was recorded 3 min following application of the 50 mM K^+ stimulus as indicated by the arrows in Fig. 3 and basal $[Ca^{2+}]_i$ recorded just prior to the stimulus was subtracted. Net $[Ca^{2+}]_i$ measured for the second response, in the presence of drug, was normalized to the initial control response from that same cell. Data presented are means ± SEM for at least 3 recordings. IC_{50} value (4 ± 1 μM) was calculated by a nonlinear, least-squares curve fitting algorithm (Origin software, Microcal). The curve was fit with a logistic equation of the form % control response $= ((R_{max} - R_{min})/(1 + (X/IC_{50})^b)) + R_{min}$, where X is the drug concentration, R_{max} and R_{min} are the % response calculated for $X = 0$, and for an 'infinite' concentration, respectively, and b is a slope factor that determines the slope of the curve. $^*P < 0.01$, significantly different from untreated control responses by one-way ANOVA, Bonferroni post hoc test.

supplemented with CGP37157 produced marked changes in the shape of the $[Ca^{2+}]_i$ transient. Plateau height was quantified by recording the $[Ca^{2+}]_i$ 3 min following application of the depolarizing stimulus (marked by arrows in Fig. 3). CGP37157 produced a concentration-dependent ($IC_{50} = 4 \pm 1$ μM) decrease in the height of the mitochondrial mediated plateau phase (Fig. 4). This inhibition was significant for CGP37157 concentrations greater than or equal to 10 μM and is consistent with the purported actions of CGP37157 on the mitochondrial Na^+/Ca^{2+} exchanger. Presumably, CGP37157 slowed the release of mitochondrial Ca^{2+} into the cytoplasm, changing the steady state $[Ca^{2+}]_i$ that results from the balance of release and efflux. In support of this interpretation were secondary increases in $[Ca^{2+}]_i$ that occurred following removal of CGP37157 from the bath (Fig. 3B and C). Presumably, the release of Ca^{2+} stored in the mitochondria accelerated upon removal of the inhibitor producing an elevation in $[Ca^{2+}]_i$.

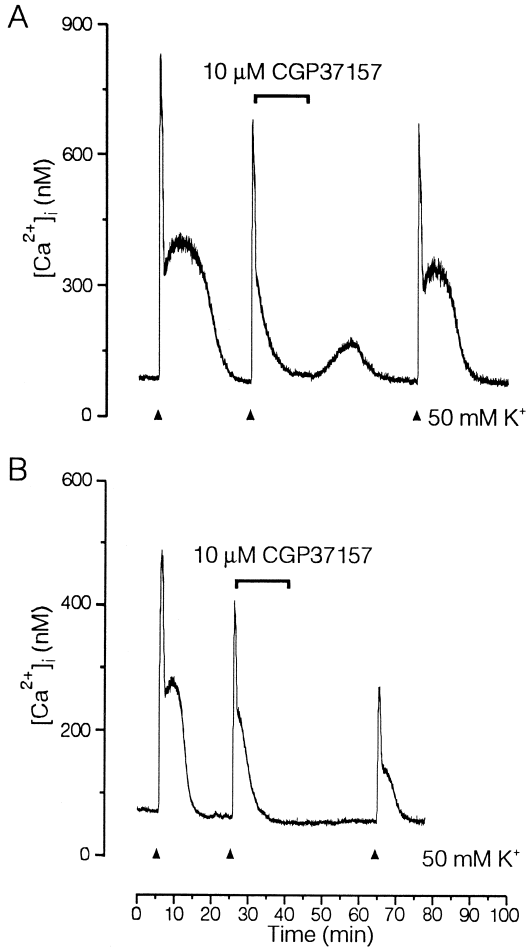

Fig. 5. Prolonged suppression of Ca^{2+} release from mitochondria by CGP37157. 50 mM K^+ was superfused onto single dorsal root ganglion neurons for 60 s starting at the times indicated by the solid triangles. $[Ca^{2+}]_i$ was monitored with indo-1-based microfluorimetry as described in Fig. 2. Buffer containing 10 μM CGP37157 was exchanged for the depolarizing buffer at the time indicated by the brackets (15 min). When drug was washed from the bath some cells (2/4) responded with an increase in basal $[Ca^{2+}]_i$ (A) while in others there was no effect (B).

3.4. CGP37157 can trap Ca^{2+} in the mitochondrial matrix

We attempted to trap Ca^{2+} within the matrix by prolonged exposure to CGP37157. Following depolarization we applied 10 μM CGP37157 for 15 min. The plateau height was greatly reduced in all CGP37157-treated cells although a secondary increase in $[Ca^{2+}]_i$ upon removal of the drug was variable (Fig. 5). Some cells displayed an elevation in $[Ca^{2+}]_i$ (2/4 cells) upon washout of the drug (Fig. 5A) while in other cells there was no elevation in $[Ca^{2+}]_i$ (Fig. 5B). Thus, CGP37157 does not appear to inhibit completely Ca^{2+} efflux from the mitochondrion suggesting that there are alternative routes of Ca^{2+} release from the matrix.

4. Discussion

In this report we have described the use of a well characterized neuronal model of mitochondrion-dependent $[Ca^{2+}]_i$ buffering to evaluate the effects of an inhibitor of mitochondrial Na^+/Ca^{2+} exchange. CGP37157 produced a concentration-dependent inhibition of the height of the plateau phase of depolarization-induced $[Ca^{2+}]_i$ transients which is consistent with the purported site of action of this drug (Cox and Matlib, 1993a). However, CGP37157 also inhibited depolarization-induced Ca^{2+} influx at concentrations required to observe significant effects on $[Ca^{2+}]_i$ buffering. Thus, this drug has two effects on $[Ca^{2+}]_i$ homeostasis in neurons, suggesting caution in its use as a tool to study $[Ca^{2+}]_i$ regulation in intact cells.

In contrast to the results described here, Cox et al. (1993) found CGP37157 to be greater than ten-fold more potent for inhibition of mitochondrial Na^+/Ca^{2+} exchange than for inhibition of L-type Ca^{2+} channels. The difference in selectivity appears to result primarily from the lower potency for modulation of mitochondrion-mediated $[Ca^{2+}]_i$ buffering in dorsal root ganglion neurons described here ($IC_{50} = 4 \pm 1$ μM), relative to the inhibition of Na^+/Ca^{2+} exchange in isolated heart mitochondria ($IC_{50} = 0.36$ μM) (Cox et al., 1993). The discrepancy is likely the result of differences between recording from intact cells versus isolated mitochondria. In intact cells the drug must cross the plasmalemma and may be susceptible to metabolism and excretion. Other studies in which CGP37157 was used to modulate $[Ca^{2+}]_{mt}$ in intact cells employed micromolar concentrations of the drug (White and Reynolds, 1995; Brandenburger et al., 1996).

CGP37157 decreased the plateau $[Ca^{2+}]_i$ following a depolarizing stimulus; this decrease was followed by an abrupt increase in $[Ca^{2+}]_i$ upon removal of the drug. This

observation provides additional support to the concept that the mitochondria act as a Ca^{2+} sink during periods of elevated $[Ca^{2+}]_i$ but do not retain significant levels of free Ca^{2+} in the matrix when cytoplasmic Ca^{2+} levels are at rest. Furthermore, this observation predicts that matrix Ca^{2+} would be elevated during drug exposure, a prediction supported by direct measurement of $[Ca^{2+}]_{mt}$ in adrenal glomerulosa cells during treatment with CGP37157 (Brandenburger et al., 1996).

Because CGP37157 increases $[Ca^{2+}]_{mt}$ while reducing $[Ca^{2+}]_i$ it may be useful in preventing neurotoxicity mediated by elevated $[Ca^{2+}]_i$. CGP37157 would stimulate Ca^{2+}-dependent matrix dehydrogenases to increase ATP production, it would reduce potentially toxic levels of cytoplasmic Ca^{2+} and prevent the deleterious cycling of Ca^{2+} across the inner mitochondrial membrane. Indeed benzothiazepine drugs protect the myocardium from ischemic damage (Nagao et al., 1980), although whether this results from the Ca^{2+} channel blocking activity of these drugs or from direct effects on the mitochondrion is not clear.

The mitochondrion is a potentially useful pharmacologic target that may be of particular relevance to neurodegenerative diseases. CGP37157 is not selective for the mitochondrial Na^+/Ca^{2+} exchanger so its use as a tool to identify the role of mitochondrial Ca^{2+} uptake in intact cells is limited to experimental protocols in which inhibition of voltage-gated Ca^{2+} channels does not confound the interpretation of the results.

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