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2′,3′-Dideoxycytidine Alters Calcium Buffering in Cultured Dorsal Root Ganglion Neurons

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SUMMARY

Mitochondria play a prominent role in shaping intracellular calcium concentration ($[Ca^{2+}]_i$) transients in dorsal root ganglion neurons. Mitochondrial DNA polymerase is inhibited by antiviral compounds such as 2′,3′-dideoxycytidine (ddC). Here, we test the hypothesis that ddC can alter mitochondrially mediated Ca^{2+} buffering in neurons. Chronic treatment of dorsal root ganglion cultures with ddC (1 μM) lowered mitochondrial DNA levels and decreased the mitochondrially mediated component of depolarization-induced $[Ca^{2+}]_i$ transients. The inhibition increased in a time-dependent manner, reaching a maximum at 6 days. ddC did not affect small, action potential-evoked, $[Ca^{2+}]_i$ transients that are predominantly buffered by Ca^{2+}-ATPases, suggesting that ATP levels were not depleted. The drug did not inhibit whole-cell Ca^{2+} currents, indicating that the Ca^{2+} load was not affected. Thus, ddC produces a graded, time-dependent inhibition of mitochondrial function that is reflected, in part, by a decrease in the direct buffering of Ca^{2+} by mitochondria. This effect may contribute to the peripheral neuropathy that results from ddC treatment. Furthermore, ddC promises to be a useful tool to study the role of mitochondria in $[Ca^{2+}]_i$ homeostasis and neurodegenerative processes.

Mitochondria are especially active in excitable cells with high energy demands. In addition to producing ATP, isolated mitochondria are capable of taking up Ca^{2+} (1, 2). Ca^{2+} entry into the mitochondrial matrix and subsequent activation of several key intramitochondrial dehydrogenases may link energy production to energy demands (3, 4). In cultured rat DRG neurons, Ca^{2+} sequestration by mitochondria plays a prominent role in shaping physiological $[Ca^{2+}]_i$ transients (5, 6). Mitochondrial Ca^{2+} uptake may also play a role in neurotoxic phenomena (7–9). Presently it is not clear whether sequestration of pathological Ca^{2+} loads by mitochondria affords protection from Ca^{2+} overload or whether Ca^{2+} accumulation into mitochondria is responsible for Ca^{2+}-dependent toxicity.

Maternally inherited defects in mitochondrial genes lead to various neuropathies and myopathies (10, 11). Such defects have been linked to peripheral neuropathies and neurodegenerative disorders (10–12). For example, mitochondrial abnormalities and decreased cytochrome *c* oxidase activity were reported in patients with clinical signs of axonal degeneration and myopathy (12). A single point mutation in the mitochondrial genome appears to be responsible for 50% or more of cases of Leber's optic neuropathy, a hereditary degeneration of the optic nerve (11, 13). Experimentally, *in vivo* administration of the electron transport inhibitor 3-nitropropionic acid produces selective neurodegeneration of the striatum (10).

Several nucleoside analogs have been shown to inhibit mitochondrial function in cultured cells. These drugs are currently used as anti-HIV treatments (14, 15). Nucleoside analogs inhibit both *in vitro* and *in vivo* replication of HIV by inhibition of reverse transcriptase (16–18). To suppress HIV replication in patients with acquired immunodeficiency syndrome, these drugs must be given continuously, resulting in considerable toxicity. Azidothymidine treatment suppresses bone marrow (19), whereas ddC leads to reversible peripheral neuropathy, a degeneration of sensory and motor nerves that develops after 8–12 weeks of treatment (16, 20).

ddC, dideoxydidehydrothymidine, and dideoxyinosine inhibit mtDNA synthesis in several cell lines, including the neuronal cell line PC-12, at clinically relevant concentrations (14, 15). These compounds preferentially inhibit DNA polymerase γ, which is responsible for replicating the mitochondrial genome, compared with α or β, which are involved in nuclear DNA replication (14, 18). Mitochondrial toxicity is manifested as a decrease in mtDNA levels and an increase in lactate production.

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ABBREVIATIONS: DRG, dorsal root ganglion (or ganglia); $[Ca^{2+}]_i$, intracellular calcium concentration; ddC, 2′,3′-dideoxycytidine; HIV, human immunodeficiency virus; mtDNA, mitochondrial DNA; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HHSS, HEPES-buffered Hanks' balanced salt solution; BAPTA, 1,2-bis-2-aminophenoxyethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Because each mitochondrion contains two to 10 copies of the mitochondrial genome (13), it is not clear whether these drugs decrease mitochondrial number, although the increased lactate production indicates an inhibition of mitochondrial function. The effects of ddC and other nuceloside analogs on mtDNA occur at concentrations lower than those that are cytotoxic. Of the drugs tested, ddC was the most selective for inhibition of mtDNA replication.

Because of the prominent contribution of mitochondria to Ca^{2+} buffering in DRG neurons and the apparently selective effects of ddC on mitochondrial function, we tested the hypothesis that chronic ddC treatment would alter Ca^{2+} buffering kinetics in these cells. Treatment with 1 μM ddC reduced the mitochondrial component of the $[Ca^{2+}]_i$ transient, indicating that drugs that modulate aerobic metabolism can exert direct effects on Ca^{2+} buffering. These effects on $[Ca^{2+}]_i$ regulation may underlie the peripheral neuropathy produced by ddC and suggest that this drug may be useful as a tool to study mitochondrially mediated Ca^{2+} buffering and neurodegeneration.

Some of these results have appeared in abstract form (21).

Materials and Methods

Cell culture. Neurons from the DRG were grown in primary culture as described previously (5). Briefly, the DRG from 1–3-day-old Sprague-Dawley rats were dissected from the thoracic and lumbar regions and incubated at 37° for 20–30 min with collagenase/dispase (0.8 and 6.4 units/ml, respectively). Ganglia were dissociated into single cells by trituration through a flame-constricted pipette. Cells were plated onto laminin-coated (50 μg/ml laminin) glass coverslips (25 mm round). Cells were grown in Ham's F12 medium supplemented with 5% heat-inactivated horse serum, 50 ng/ml nerve growth factor, 44 mM glucose, 2 mM L-glutamine, minimal essential medium vitamins, and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively). Cultures were maintained at 37° in a humidified atmosphere of 5% CO_2. Cells were treated with ddC (1 μM) beginning 1–2 days after plating. Culture medium and ddC were replaced every 2 days.

mtDNA measurement. The mtDNA probe Psp64 was kindly provided by Dr. Susan P. LaDoux (University of South Alabama) and encodes the entire mouse mtDNA sequence. Total cellular DNA was isolated by cell lysis and phenol-chloroform extraction, followed by treatment with ribonuclease A digestion. DNA was digested overnight with *Bam*HI restriction enzyme, which results in two bands, of 10.8 and 4.95 kilobases, on agarose gels. Eleven micrograms of *Bam*HI-digested DNA isolated from control or ddc-treated DRG cells were fractionated on a 0.7% agarose gel and transferred to nitrocellulose. The mtDNA was detected by hybridization of nitrocellulose filters with Psp64 labeled with [^{32}P]dCTP (Amersham Corp.)

$[Ca^{2+}]_i$ measurement. $[Ca^{2+}]_i$ was determined using a microfluorimeter to monitor the Ca^{2+}-sensitive fluorescent chelator indo-1 (22). For excitation of the indo-1, the light from a 75-W xenon arc lamp was passed through a 350 ± 10-nm band pass filter (Omega Optical, Brattleboro, VT). Excitation light was reflected off a dichroic mirror (380 nm) and through a 70× phase-contrast oil immersion objective (numerical aperture, 1.15; Leitz). Emitted light was sequentially reflected off dichroic mirrors (440 and 516 nm) through band pass filters (405 ± 20 and 495 ± 20 nm, respectively) to photomultiplier tubes operating in the photon-counting mode (Thorn EMI, Fairfield, NJ). Cells were illuminated with transmitted light (580-nm long pass) and visualized with a video camera placed after the second emission dichroic mirror. Recordings were defined spatially with a rectangular diaphragm. The 5-V photomultiplier output was integrated by passing the signal through an eight-pole Bessel filter at 2.5 Hz. This signal was then input into two channels of an analog-to-digital converter (Indec Systems, Sunnyvale, CA) sampling at either 1 Hz or 10 Hz.

Cells were loaded with indo-1 by incubation with 2 μM indo-1/acetoxymethyl ester (Molecular Probes Inc., Eugene, OR) for 45 min at 37°, in HHSS, pH 7.45, containing 0.5% bovine serum albumin. HHSS 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; glucose, 5.6. Loaded cells were mounted in a flow-through chamber for viewing (23). The superfusion chamber was mounted on an inverted microscope and cells were superfused with HHSS, at a rate of 1–2 ml/min, for 15 min before the start of an experiment. A suitable cell, defined as a rounded cell body that had extended fine processes and was isolated from other cells, was localized by phase-contrast illumination. $[Ca^{2+}]_i$ transients were elicited by superfusion for 30 sec with 50 mM K^+ (K^+ was exchanged for Na^+ reciprocally) or by evocation of action potentials with field potential stimulation (24). Field potentials were generated by passing current between two platinum electrodes by means of a Grass S44 electrical stimulator and a stimulus isolation unit (Grass, Quincy, MA). Trains of ten 1-msec pulses were delivered at a rate of 10 Hz. The stimulus voltage required to elicit action potentials varied for each individual cell. The threshold voltage for each cell was determined before the beginning of an experiment, and subsequent stimuli were 20 V over this threshold. Cells were stimulated in this manner once every 4 min.

Patch clamp. Whole-cell Ca^{2+} current was measured with a Warner PC501 amplifier. Ca^{2+} currents were isolated from all other currents by ionic substitution. Extracellular recording solution consisted of the following (in mM): tetraethylammonium chloride, 143; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH 7.4. Cells were approached with glass recording electrodes that had been heat polished to a resistance of 1–2 MΩ and filled with the following solution (in mM): CsCl, 137; $MgCl_2$, 1; HEPES, 10; BAPTA, 10; MgATP, 5; Na_2GTP, 1; pH 7.3. After establishment of the whole-cell configuration, capacitance and series resistance were calculated from currents recorded from three 10-mV hyperpolarizing pulses (−80-mV hold). Ca^{2+} currents were elicited by stepping from −80 mV to 0 mV for 80 msec. At the end of each experiment, 0.5–1 mM $CdCl_2$ was applied to the bath to block Ca^{2+} currents; residual current that resulted from depolarization to 0 mV was subtracted from Ca^{2+} current sweeps (off-line).

Data are presented as mean ± standard error. Where appropriate, Student's *t* test was used to determine statistical significance.

Results

ddC significantly inhibited mtDNA synthesis in DRG cultures. As shown in Fig. 1, mtDNA was reduced to undetectable levels in DRG cultures treated with 1 μM ddC for 6 days. These

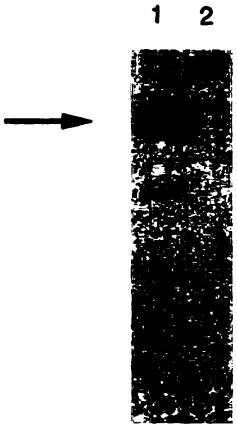

Fig. 1. ddC reduces mtDNA levels in DRG cultures. DRG cultures were treated with 1 μM ddC in culture medium for 6 days (*lane 2*) or were untreated (*lane 1*). Total cellular DNA was isolated and digested with *Bam*HI, and a radiolabeled probe encoding the mitochondrial genome was used in Southern blot analyses. *Arrow*, 10.8-kilobase fragment; the lower band represents the 4.95-kilobase fragment. These cultures contained approximately 10% non-neuronal cells.

results are consistent with the findings of Chen and Cheng (14, 15), showing decreased mtDNA levels in lymphoblastoid cells after treatment with ddC, and extend their findings to primary neuronal tissue.

When depolarized for 30 sec by superfusion with 50 mM K^+, DRG neurons respond with a characteristic $[Ca^{2+}]_i$ transient, recovery from which is composed of three distinct phases (see Fig. 2A), i.e., a rapid decline in $[Ca^{2+}]_i$, a plateau phase, and finally a slow recovery to basal $[Ca^{2+}]_i$. The plateau in the $[Ca^{2+}]_i$ transient is due to cycling of Ca^{2+} ions across the inner mitochondrial membrane (6). $[Ca^{2+}]_i$ transient duration (Fig. 2A, b) was quantified by calculating the time required to recover to one half of the net plateau $[Ca^{2+}]_i$. After depolarization by superfusion for 30 sec with 50 mM K^+, cells treated with 1 μM ddC for 4 or 6 days had a significantly shorter $[Ca^{2+}]_i$ transient duration, compared with cells from untreated sister cultures ($p < 0.05$) (Fig. 2B). This effect was time dependent (Fig. 2C). A significant reduction in $[Ca^{2+}]_i$ transient duration was noted after 4 days in the presence of ddC ($n = 12$), which was more pronounced at 6 days ($n = 10$). However, after 8 days of ddC treatment the trend toward shorter $[Ca^{2+}]_i$ transients appeared to reverse. As shown in Table 1, peak $[Ca^{2+}]_i$ and plateau amplitude did not differ significantly between groups.

To determine whether recovery to basal $[Ca^{2+}]_i$ was altered after more modest Ca^{2+} loads, $[Ca^{2+}]_i$ transients were elicited by field potential stimulation. Field stimulation evokes action potentials in these cells (24). After a train of 10 pulses, $[Ca^{2+}]_i$ increased to 492 ± 42 nM and 463 ± 36 nM in ddC-treated and control cells, respectively. Recovery from this modest Ca^{2+} load does not involve mitochondrial Ca^{2+} buffering and is probably mediated by plasmalemmal Ca^{2+} ATPases (6, 25). The recovery to basal $[Ca^{2+}]_i$ was fit by a single-exponential function, with a time constant of 9.98 ± 3.3 sec for ddC-treated cells, compared with 9.79 ± 1.2 sec for control cells ($p = 0.95$) (Fig. 3), suggesting that nonmitochondrial Ca^{2+} buffering was not altered after ddC treatment.

We next sought to determine whether the difference in $[Ca^{2+}]_i$ transient duration was due to reduced influx of Ca^{2+} in ddC-treated cells. Whole-cell Ca^{2+} current was recorded using the patch-clamp technique. Current was elicited by stepping from −80 mV to 0 mV for 80 msec. Ca^{2+} current amplitude did not differ between the two groups, being 714 ± 153 pA ($n = 15$) for ddC-treated cells and 771 ± 175 pA ($n = 13$) for control cells (Fig. 4A). To normalize for possible variations in cell size, current density was calculated by dividing peak Ca^{2+} current by cell capacitance. Current density for cells treated for 6 days with ddC was 9.61 ± 1.1 pA/pF, which was similar to the value for control cells (10.5 ± 1.9 pA/pF) (Fig. 4B).

Discussion

Treatment of cultured DRG neurons with 1 μM ddC for 6 days decreased mtDNA content, with an apparent impairment of mitochondrial function; the duration of depolarization-induced $[Ca^{2+}]_i$ transients was significantly decreased. This effect appeared to be due to modulation of mitochondrial Ca^{2+} handling, because neither ATP-dependent Ca^{2+} efflux nor Ca^{2+} influx was affected by ddC treatment.

Cheng and co-workers (14, 15) have shown that ddC decreases mtDNA content in differentiated PC-12 cells and decreases mtDNA and increases lactate production in cultured

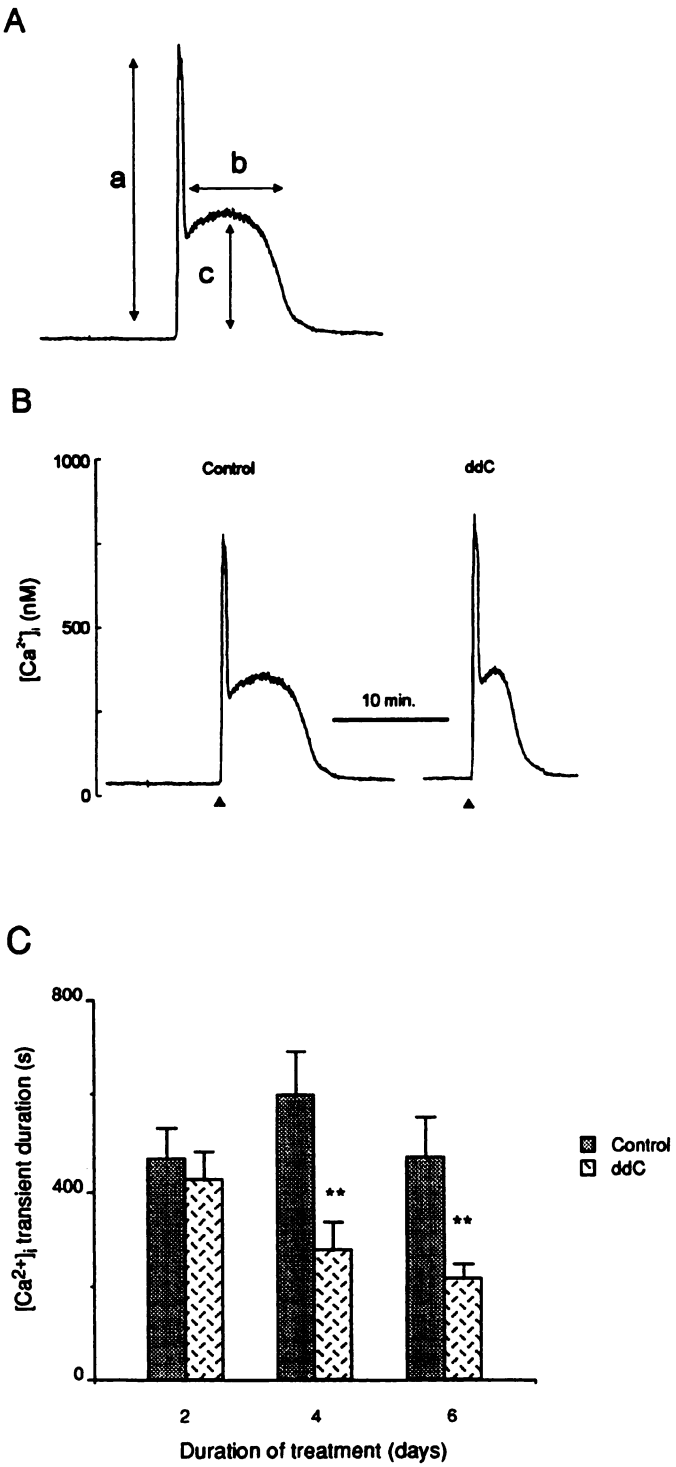

Fig. 2. Treatment with 1 μM ddC shortens the mitochondrially mediated plateau phase of depolarization-induced $[Ca^{2+}]_i$ transients. $[Ca^{2+}]_i$ was measured in single DRG neurons with indo-1-based dual-emission microfluorimetry. A, DRG neurons respond to depolarization, produced by superfusion for 30 sec with 50 mM K^+, with a characteristic $[Ca^{2+}]_i$ transient, recovery from which is composed of three distinct phases, i.e., a rapid decline in $[Ca^{2+}]_i$, a plateau phase, and finally a slow recovery to basal $[Ca^{2+}]_i$. Letters refer to Table 1. B, Representative 50 mM K^+-evoked $[Ca^{2+}]_i$ transients recorded from neurons grown for 6 days in control or ddC-supplemented (1 μM) medium are shown. ▲, Beginning of a 30-sec superfusion with 50 mM K^+. C, The effects of ddC (1 μM) increase with continued exposure. DRG neurons were grown for 1 day before addition of ddC to the medium. **, Statistically significant difference ($p < 0.05$) between ddC-treated and age-matched control cells.

TABLE 1

Comparison of depolarization-induced $[Ca^{2+}]_i$ transients from 6-day ddC-treated and control cells

	Control	ddC
a[a]	2225 ± 289 nM	1829 ± 281 nM
b	455 ± 88 sec	216 ± 31 sec[b]
c	699 ± 97 nM	641 ± 73 nM

[a] Parameters *a*, *b*, and *c* are from Fig. 2A.
[b] $p < 0.05$, control versus ddC-treated.

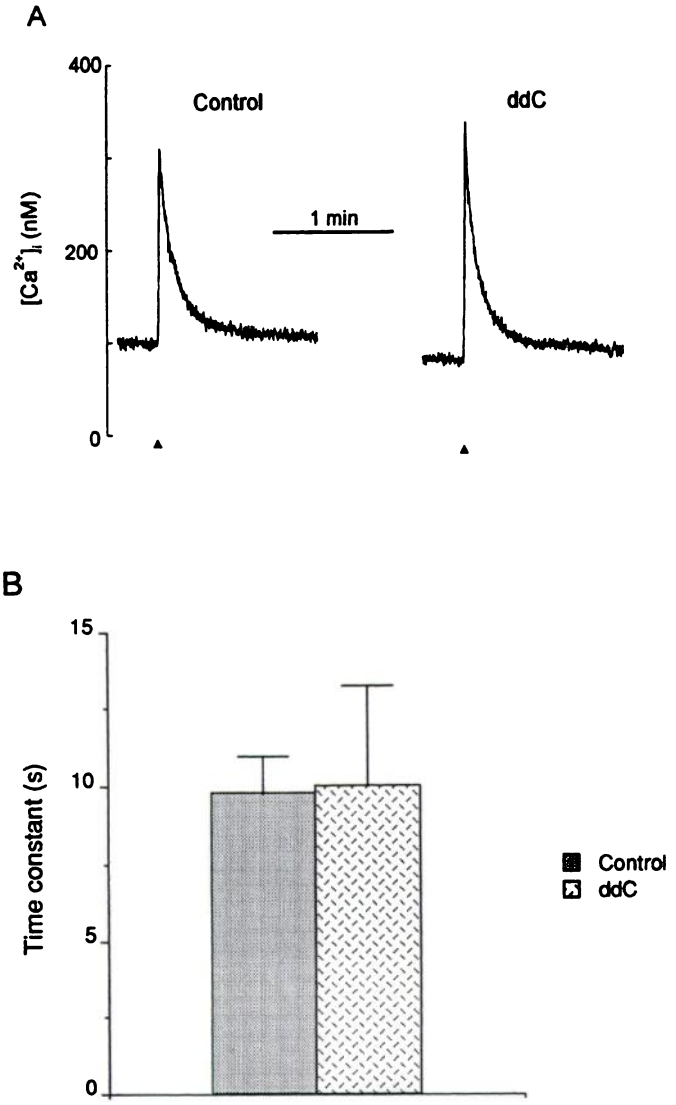

Fig. 3. ddC treatment does not alter recovery from modest Ca^{2+} loads. A, Representative $[Ca^{2+}]_i$ recordings from control and 6-day ddC-treated cells are shown. ▲, Electrical field stimulation with ten 1-msec pulses. B, Time constant of recovery to basal $[Ca^{2+}]_i$ was described by a single-exponential function and did not differ between control and 6-day ddC-treated cells.

lymphoblastoid cells. Here we demonstrate that ddC treatment also decreases mtDNA in primary neuronal tissue. Consistent with the idea that ddC disrupts mitochondrial function was our observation that mitochondrially mediated buffering of large Ca^{2+} loads was decreased after treatment with ddC. This effect on mitochondrial function may underlie the clinically limiting side effects of this drug, which include a sensory and motor neuropathy (16, 20). Interestingly, we have noted that, relative

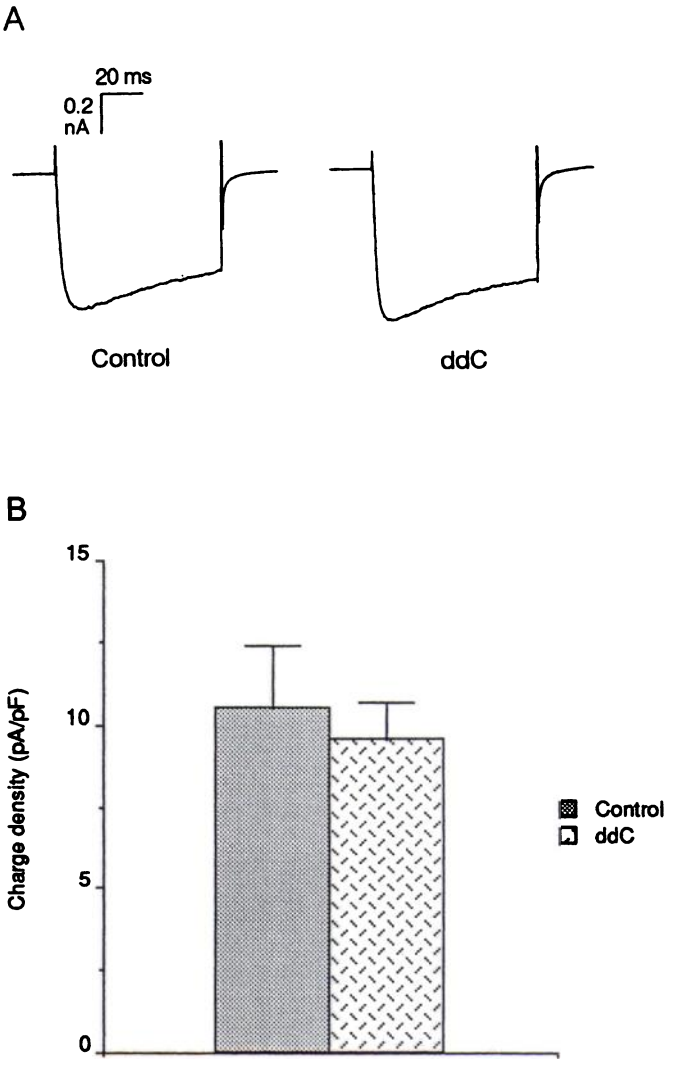

Fig. 4. Whole-cell Ca^{2+} current was not affected by ddC treatment. A, Representative Ca^{2+} currents evoked by stepping to 0 mV from −80 mV in control and 6-day ddC-treated cells are shown. B, Current density (peak Ca^{2+} current/cell capacitance) was not affected by 6 days of ddC treatment.

to sympathetic or central neurons in primary culture (23), sensory neurons have uniquely shaped depolarization-induced $[Ca^{2+}]_i$ transients with a pronounced, mitochondrially mediated, plateau phase (6). Thus, sensory neurons may be uniquely sensitive to ddC because of the significant role mitochondria play in shaping $[Ca^{2+}]_i$ transients in these cells.

Although the link between the changes in mitochondrial Ca^{2+} buffering reported here and the peripheral neuropathy that develops in patients after chronic ddC treatment is speculative, mitochondrial defects have been implicated in peripheral neuropathies (12) and defects in respiratory enzymes have been reported for neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (10, 11). Thus, it is possible that, under conditions in which mitochondrial function is disrupted, Ca^{2+} buffering is compromised, resulting in Ca^{2+} overload or alterations in Ca^{2+} signaling that contribute to toxicity.

The inhibition of mitochondrial function by ddC might be expected to lower cellular ATP levels and thus affect Ca^{2+} buffering. In these studies, however, buffering of small Ca^{2+} loads, which is thought to be mediated by plasmalemmal AT-

Pases, was not affected (Fig. 3). Furthermore, a decrease in the duration of the 50 mM K^+-evoked $[Ca^{2+}]_i$ transient, such as that produced by ddC, is more consistent with increased efflux. Increased Ca^{2+} buffering by mitochondria and Ca^{2+}-binding proteins decreases the amplitude of $[Ca^{2+}]_i$ transients and increases their duration. In contrast, stimulation of efflux pathways decreases the duration of $[Ca^{2+}]_i$ transients. ATP levels may have been maintained in ddC-treated cells by a compensatory increase in glycolysis. ddC treatment increased lactate production in lymphoblastoid cells in culture (14, 15).

Reduced influx of Ca^{2+} could account for the shortened $[Ca^{2+}]_i$ transients in ddC-treated cells. However, peak $[Ca^{2+}]_i$ values were comparable between ddC-treated and control cells after a 30-sec depolarization or a train of 10 action potentials (Fig. 3; Table 1). Additionally, whole-cell Ca^{2+} current and current density did not differ between control and ddC-treated groups (Fig. 4). Thus, the effects of ddC on Ca^{2+} buffering appear to be due to a selective inhibition of mitochondrial function.

We saw a maximum decrease in $[Ca^{2+}]_i$ transient duration after 6 days of ddC treatment and the effect appeared to reverse at 8 days. The reason for the loss of sensitivity is unknown. Chen and Cheng (26) have shown that cytoplasmic deoxycytidine kinase is responsible for converting ddC to its active form, ddC triphosphate. We speculate that these kinases may be down-regulated or that phosphatase acitivity may be increased after ddC treatment. Thymidine kinase-deficient cells have been selected by treatment with other nucleosides (27, 28).

Although we have focused on the $[Ca^{2+}]_i$ response to 50 mM K^+, a nonphysiological stimulus, more physiological stimuli can induce Ca^{2+} loads of sufficient amplitude to involve mitochondrial Ca^{2+} uptake. A train of 30 or more action potentials evokes $[Ca^{2+}]_i$ transients with the characteristic plateau phase indicative of mitochondrial Ca^{2+} buffering (6). Others have shown that mitochondria are capable of taking up Ca^{2+} after increases in $[Ca^{2+}]_i$ evoked by action potentials (5), inositol trisphosphate-coupled receptor stimulation (29, 30), or glucose-induced depolarization (31).

ddC may prove useful as a tool to study mitochondrially mediated neurodegenerative processes and Ca^{2+} buffering. Clearly, any compound that disrupts mitochondrial function may have global effects. However, when administered to rabbits, ddC caused a dose- and time-dependent peripheral neuropathy (32). This animal model of ddC-induced neuropathy may lead to a better understanding of the side effects of ddC. Furtherore, Beal *et al.* (10) have used treatment with the mitochondrial toxin 3-nitropropionic acid to mimic the neuronal degeneration seen in Huntington's disease. ddC is less acutely toxic than are mitochondrial poisons and thus may be useful for the chemical induction of neurodegeneration. Pharmacological agents available for modulating mitochondrial Ca^{2+} buffering are limited to ruthenium red, which is not membrane permeant, and mitochondrial poisons, which are difficult to use because of their acute toxicity. ddC, because it is membrane permeant and inhibits mitochondrial function at concentrations that do not produce cytotoxicity, may be a useful tool for evaluating the role of mitochondria in $[Ca^{2+}]_i$ homeostasis.

In summary, we have demonstrated that chronic treatment of cultured sensory neurons with ddC alters mitochondrial Ca^{2+} buffering. This effect is due to modulation of mitochondrial Ca^{2+} uptake and may contribute to the peripheral neuropathy that is the dose-limiting side effect of ddC. ddC may be a useful tool to study the role of mitochondria in Ca^{2+} buffering and neurodegenerative processes.

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