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The glutathione reductase inhibitor carmustine induces an influx of Ca^{2+} in PC12 cells

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Abstract

We studied the effects of carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea) on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells using fura-2 fluorescence imaging. Carmustine (100 μM) caused a delayed increase in $[Ca^{2+}]_i$ that developed within ~3 h. This effect was enhanced in cells that were pretreated with an inhibitor of glutathione (GSH) synthesis, buthionine sulfoximine (BSO, 200 μM, 24 h), and was suppressed in cells that were treated with an antioxidant deferoxamine (50 μM). The carmustine-induced increase in $[Ca^{2+}]_i$ was absolutely dependent on the presence of extracellular Ca^{2+} and could be inhibited by dihydropyridine blockers of L-type voltage-gated Ca^{2+} channels (nimodipine or nitrendipine, 10 μM). The increase in $[Ca^{2+}]_i$ was also suppressed in Cl^--free solution and in the presence of the Cl^- channel blockers, indanyloxyacetic acid 94 (IAA-94, 100 μM) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 100 μM). The inhibition was complete when the blockers were applied simultaneously with carmustine and was partial when the blockers were applied after the initial increase in $[Ca^{2+}]_i$. We conclude that carmustine induces an influx of extracellular Ca^{2+} through L-type Ca^{2+} channels and that this effect is mediated by oxidative stress that results from the depletion of GSH following the inhibition by carmustine of glutathione reductase.

Keywords: Nitrosourea; Glutathione; Oxidative stress; Ca^{2+} channel; Intracellular Ca^{2+}; Cl^- channel

1. Introduction

Carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea) is an antineoplastic agent that is widely used in the chemotherapy of brain tumors. It is generally accepted that the cytostatic effects of carmustine result primarily from the generation of DNA interstrand cross-links (e.g., Ueda-Kawamitsu et al., 2002). In addition, carmustine has also been shown to inhibit glutathione reductase, the enzyme that catalyzes the recycling of reduced glutathione (GSH) from oxidized glutathione disulfide (GSSG) (Frischer and Ahmad, 1977); this action was proposed to contribute to both the therapeutic and toxic effects of carmustine. The inhibition of glutathione reductase results in the depletion of GSH and the accumulation of GSSG (e.g., Smith and Boyd, 1984; Ma et al., 2003), which is likely to compromise cellular oxidative defenses that rely on antioxidant actions of reduced glutathione (Meister, 1994). Indeed, carmustine-treated cells have been reported to be more vulnerable to oxidative stress (e.g., Ma et al., 2003). In many instances, the link between oxidative stress and cell death is mediated by Ca^{2+} signaling (see reviews by Lounsbury et al., 2000; Ermak and Davies, 2002). In PC12 cells, decreased levels of cellular GSH have been linked to the increased production of reactive oxygen species, calcium disregulation and cell death (Jurma et al., 1997; Pereira and Oliveira, 1997; Froissard et al., 1997).

We recently found that carmustine caused the death of PC12 cells and that this effect depended on the presence of extracellular calcium (Doroshenko and Doroshenko, 2003). This observation suggested that the toxic effect of carmustine may be mediated by an influx of Ca^{2+} ions. The present study was undertaken to directly test this hypothesis and to

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characterize the pathways that are involved in the carmustine-induced influx of Ca^{2+}.

2. Materials and methods

2.1. Cell culture

PC12 cells were grown at 37 °C in 25-cm^2 flasks in the RPMI-1640 cell culture medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum and antibiotics (Sigma, St. Louis, MO) in a humidified 95% air, 5% CO_2 incubator. For the imaging experiments, cells were detached by vigorous shaking, dispersed by 3× passing through a 25G needle, and plated onto poly-L-lysine-coated glass-bottom 35-mm Petri dishes (MatTek, Ashland, MA). Cells were used 24–48 h after plating.

2.2. Fluorescence measurements

For Ca^{2+} imaging, PC12 cells were loaded with the fluorescent Ca^{2+} indicator fura-2 by incubation for 30 min at room temperature in the presence of 2 μM membrane-permeable fura-2/AM. Thereafter, the cells were washed, and the Petri dish with the attached cells was mounted on the stage of a Zeiss Axiovert 100 inverted epifluorescence microscope that was equipped with a Fluar 40×, 1.3 NA oil immersion objective lens and a 75 W xenon light source. Sequential images were acquired at 510 nm after alternative excitation at 340 and 380 nm using an analog integrating CCD camera (Cohu 4915-2000, Cohu, San Diego, CA) connected to a computer via an 8-bit frame grabber (DT3155, Data Translation, Marlboro, MA). Illumination via a Lambda 10-C filter wheel and shutter (Sutter Instruments) was controlled by Axon Imaging Workbench 2.2 software (Axon Instruments). Cells were exposed to excitation light for 200–400 ms per imaging cycle, repeated at 2-min intervals. In each experiment, fura-2 fluorescence was recorded from 10 to 20 cells within the field of view, with each individual cell comprising a separate measuring area (region of interest). Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) are expressed as the ratio between fura-2 fluorescence at 340 and 380 nm (340/380 ratio). All experiments were conducted at room temperature (23–25 °C).

2.3. Solutions

The standard (5-Ca) bath solution contained (in mM): $CaCl_2$ 5, $MgSO_4$ 0.8, KCl 5.36, KH_2PO_4 0.44, $NaHCO_3$ 4.17, NaCl 140, Na_2HPO_4 0.34, glucose 12.5, HEPES 10, at pH 7.4. In the Ca^{2+}-free solution, $CaCl_2$ was replaced with an equimolar concentration of *N*-methyl-D-glucamine (NMDG). In the Cl^--free solution, Cl^- was replaced with an equimolar concentration of membrane-impermeable gluconate. The osmolarity of all solutions was 300–310 mOsm.

2.4. Materials

Carmustine, D,L-buthionine-[S,R]-sulfoximine (BSO), deferoxamine mesylate salt, indanyloxyacetic acid 94 (IAA-94), flufenamic acid (*N*-(3-[trifluoromethyl]phenyl)anthranilic acid), 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), nitrendipine and nimodipine were purchased from Sigma. Fura-2/AM was purchased from Molecular Probes (Eugene, OR). Stock solutions of carmustine (20 mM in H_2O) and the ion channel blockers (in ethanol or dimethyl sulfoxide) were kept as frozen aliquots at −20 °C until use. The final concentrations of ethanol and dimethyl sulfoxide in the test solutions did not exceed 0.1%; in control experiments, neither solvent at this concentration had any effect on fura-2 fluorescence.

2.5. Statistics

Data are presented as the mean±S.E.M. value of 10–20 individual cells in a single experiment. Each experiment was repeated at least three times. The significance of differences between experimental values was determined using Student's *t*-test and one-way analysis of variance (ANOVA), as appropriate (Prism 3.03, GraphPad Software, San Diego, CA).

3. Results

3.1. Carmustine induces an increase in $[Ca^{2+}]_i$ in PC12 cells

Guided by earlier findings on the ion dependence of carmustine toxicity in PC12 cells and the significance of extracellular Ca^{2+} in particular (Doroshenko and Doroshenko, 2003), we investigated how this drug affected the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in PC12 cells. We found that in cells that were treated for several hours with toxic concentrations of carmustine (50–100 μM), $[Ca^{2+}]_i$ was much higher than in control (untreated) cells. Preliminary experiments revealed that this increase in $[Ca^{2+}]_i$ developed faster at higher concentrations of carmustine: 100 μM carmustine caused an elevation of $[Ca^{2+}]_i$ within ~3 h in the majority of exposed cells. The averaged time course of changes in $[Ca^{2+}]_i$ in all cells within the field of view indicated that the carmustine-induced increase in $[Ca^{2+}]_i$ occurred with an ~1 h delay and resulted in a substantial elevation of $[Ca^{2+}]_i$ by the end of the recording period, 3 h (Fig. 1A, ○). In control (untreated) cells, $[Ca^{2+}]_i$ was stable throughout the recording period (Fig. 1A, ●). Reactions to carmustine of individual cells on the same coverslip varied widely, both in the amplitude and in the kinetics of the $[Ca^{2+}]_i$ increase. Some cells had relatively

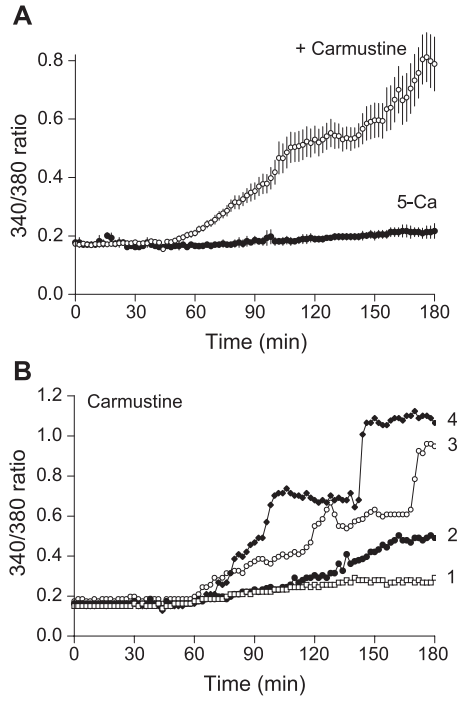

Fig. 1. Carmustine induces an increase in $[Ca^{2+}]_i$ in PC12 cells. (A) Averaged time course of $[Ca^{2+}]_i$ changes in PC12 cells bathed in the 5-Ca solution in the absence (●, n=15) and in the presence of 100 μM carmustine (○, n=12). Changes in $[Ca^{2+}]_i$ here and in all other figures were expressed as the ratio of fura-2 fluorescence intensity at 340 and 380 nm (340/380 ratio); (B) Individual changes in $[Ca^{2+}]_i$ in four (1–4) representative carmustine-treated PC12 cells from the same coverslip as shown in (A).

small increases in $[Ca^{2+}]_i$ (e.g., traces 1 and 2 in Fig. 1B). Others had an increase in $[Ca^{2+}]_i$ that reached an elevated plateau after a slow buildup (e.g., traces 3 and 4 in Fig. 1B). In such cells, a second step-like increase in $[Ca^{2+}]_i$ often occurred after an additional delay. There was no further increase in $[Ca^{2+}]_i$ beyond this second plateau; rather, these cells began to lose fura-2 fluorescence, likely due to a leak. It appears that the variations in the carmustine-induced changes in $[Ca^{2+}]_i$ in individual cells reflect various degrees of cellular impairment, and it is therefore likely that given sufficient time, $[Ca^{2+}]_i$ in all exposed cells would have increased substantially.

3.2. Carmustine-induced increase in $[Ca^{2+}]_i$ is mediated by GSH depletion and is redox-sensitive

Carmustine has been shown to inhibit glutathione reductase, an enzyme that recycles reduced GSH from its oxidized form, GSSG (Frischer and Ahmad, 1977), and therefore is expected to lower the intracellular levels of GSH (e.g., Smith and Boyd, 1984). To test whether the carmustine-induced increase in $[Ca^{2+}]_i$ is indeed mediated by the GSH depletion, we investigated the effects of buthionine sulfoximine (BSO), which also depletes cellular GSH but does so by inhibiting γ-glutamylcysteine synthetase (Griffith, 1982), the key enzyme in the GSH synthesis pathway. BSO has been used frequently to decrease GSH levels both in vitro and in vivo (Mizui et al., 1992; Froissard et al., 1997). In the present study, exposure to carmustine of PC12 cells that were cultured for 24 h in the presence of 200 μM BSO resulted in a much earlier occurrence of the $[Ca^{2+}]_i$ increase than in cells that were not treated with BSO (Fig. 2A and B). To make a quantitative assessment of this effect, we compared the half-rise times of the carmustine-induced $[Ca^{2+}]_i$ increases (as shown in Fig. 2A) in control (no BSO treatment) and BSO-treated cells. In the controls, the mean half-time of the carmustine-induced $[Ca^{2+}]_i$ increase was 97±2 min (n=80 in six experiments, range 57 to 145 min). In the BSO-treated cells, the half-time was significantly smaller (P<0.0001), only 54±6 min (n=30 in three experiments).

Next, we examined the effect of the redox state of the cell on the carmustine-induced $[Ca^{2+}]_i$ increase by applying the antioxidant, deferoxamine. In PC12 cells treated with 50 μM deferoxamine for 30 min before and during exposure to carmustine, the carmustine-induced $[Ca^{2+}]_i$ increases occurred significantly later (Fig. 2C,D), with the mean half-rise time of 137±6 min (P<0.0001, n=31 in three experiments).

3.3. The role of extracellular Ca^{2+} in carmustine-induced $[Ca^{2+}]_i$ increase

An increase in $[Ca^{2+}]_i$ can be brought about by several mechanisms, such as an influx of extracellular Ca^{2+} through Ca^{2+}-permeable ion channels, a decrease in the extrusion of intracellular Ca^{2+} and a release of Ca^{2+} from intracellular stores. In the absence of extracellular Ca^{2+}, both when only Ca^{2+} (Fig. 3A) or all extracellular inorganic cations including Ca^{2+} (Fig. 3B) were replaced by membrane-impermeable *N*-methyl-D-glucamine (NMDG), there was no increase in $[Ca^{2+}]_i$ during exposure of PC12 cells to carmustine. This observation indicates the essential role of Ca^{2+} entering the cells from extracellular solution. Moreover, when extracellular Ca^{2+} was withdrawn during the rising phase of the carmustine-induced $[Ca^{2+}]_i$ increase, in the next few minutes the $[Ca^{2+}]_i$ declined almost to its initial level (Fig. 3C). Reintroduction of extracellular Ca^{2+}, after about 30 min of cell exposure to Ca^{2+}-free solution, resulted in an equally fast recovery of $[Ca^{2+}]_i$ (Fig. 3C).

The presence of extracellular Ca^{2+}, however, does not seem to be required for the carmustine-induced changes in Ca^{2+} permeability of the cell membrane. This is suggested by the observation that the $[Ca^{2+}]_i$ level at the time of reintroduction of extracellular Ca^{2+} was higher than at the time of its removal 30 min earlier (Fig. 3C). Furthermore, when PC12 cells were first exposed to carmustine in the Ca^{2+}-free solution for a prolonged period of time before being transferred into a Ca^{2+}-containing solution (Fig. 3D), $[Ca^{2+}]_i$ began to increase almost immediately. However, without preexposure to carmustine, such a transfer of cells from the Ca^{2+}-free into the Ca^{2+}-containing extracellular

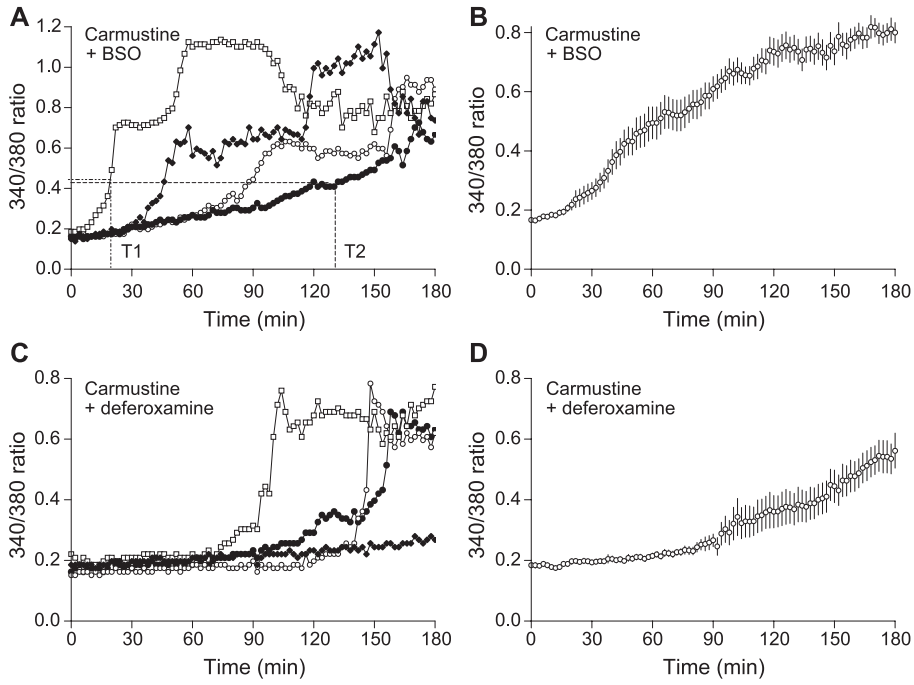

Fig. 2. Redox modulation of the carmustine-induced $[Ca^{2+}]_i$ increase. (A and B) Representative individual (A) and averaged (B, n=18) changes in $[Ca^{2+}]_i$ induced by 100 μM carmustine in PC12 cells cultured overnight in the presence of 200 μM buthionine sulfoximine (BSO). Dotted and dashed lines illustrate the measurements of the half-rise times (T1 and T2) for the $[Ca^{2+}]_i$ signals in individual cells; (C and D) Representative individual (C) and averaged (D, n=14) changes in $[Ca^{2+}]_i$ induced by 100 μM carmustine in PC12 cells treated with 50 μM deferoxamine for 30 min prior and during exposure to carmustine.

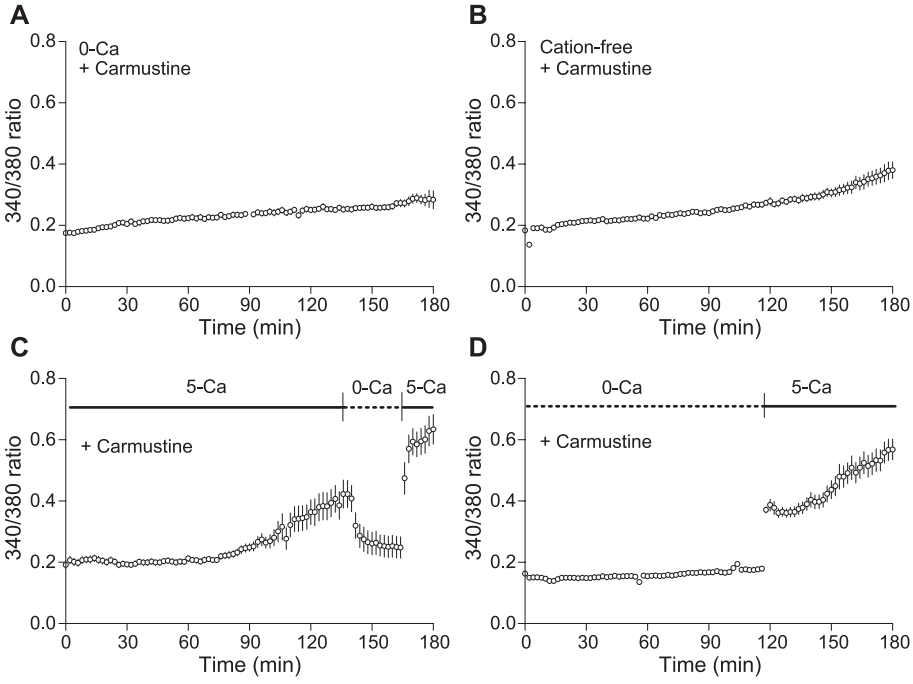

Fig. 3. The role of extracellular Ca^{2+} in the carmustine-induced $[Ca^{2+}]_i$ signals. (A) Averaged changes in $[Ca^{2+}]_i$ in PC12 cells (n=17) bathed in a Ca^{2+}-free solution in the presence of 100 μM carmustine; (B) Averaged changes in $[Ca^{2+}]_i$ in PC12 cells (n=12) bathed in a cation-free solution in the presence of 100 μM carmustine; (C) Reversal of the carmustine (100 μM)-induced increase in $[Ca^{2+}]_i$ in PC12 cells (n=11) by short-term removal of extracellular Ca^{2+}, as indicated by the horizontal bars; (D) Rapid increase in $[Ca^{2+}]_i$ in PC12 cells (n=14) bathed in the Ca^{2+}-free solution in the presence of 100 μM carmustine caused by addition of extracellular Ca^{2+} (5 mM).

solution caused only a small and stable increase in $[Ca^{2+}]_i$ (e.g., see Fig. 4C).

3.4. Elucidation of the carmustine-induced Ca^{2+} influx pathways

Several Ca^{2+}-permeable ion channels have been implicated in oxidative stress-induced Ca^{2+} influx in various types of cells, including PC12 cells. Such channels include: (1) voltage-gated Na^+ channels (Wang and Joseph, 2000); (2) nonselective cation channels (Herson et al., 1999); and (3) voltage-gated Ca^{2+} channels (Jurma et al., 1997; Wang and Joseph, 2000). It is unlikely that the Na^+ channels are involved in the carmustine-induced $[Ca^{2+}]_i$ increase because tetrodotoxin (1 μM) had no effect (not shown). The role of nonselective cation channels is more difficult to address because of the lack of specific blockers for these channels. Flufenamic acid (200 μM), which has been reported to block oxidative stress-induced nonselective cation channels (Meyer et al., 1996; Mukherjee et al., 2002), did not prevent a rise in $[Ca^{2+}]_i$ in the presence of carmustine (not shown). By contrast, dihydropyridine blockers of L-type voltage-activated Ca^{2+} channels effectively suppressed the carmustine-induced $[Ca^{2+}]_i$ increase: both nitrendipine (10 μM, Fig. 4A) and nimodipine (10 μM, not shown), when applied simultaneously with carmustine, completely prevented an increase in $[Ca^{2+}]_i$. When the carmustine-induced $[Ca^{2+}]_i$ increase was already in progress, however, the dihydropyridines prevented only a further increase in $[Ca^{2+}]_i$ (Fig. 4B); in no case did these blockers reverse the $[Ca^{2+}]_i$ rise to the extent that was observed after the removal of extracellular Ca^{2+} (e.g., Fig. 3C). It is likely that this difference between the effects of dihydropyridine blockers and removal of extracellular Ca^{2+} is indicative of the involvement of an additional Ca^{2+} permeability that is insensitive to the dihydropyridines. A possible candidate for such Ca^{2+} permeability is the basal Ca^{2+} permeability described recently in PC12 cells (Bennett et al., 1998). Its presence was revealed by the increase in $[Ca^{2+}]_i$ that occurred when control cells were transferred from the Ca^{2+}-free solution into the solution with 5 mM Ca^{2+} (Fig. 4C). In the presence of 5 mM extracellular Ca^{2+}, $[Ca^{2+}]_i$ was stabilized at an elevated level, which was maintained while the cells were exposed to extracellular Ca^{2+}. The increase in $[Ca^{2+}]_i$ could be reversed by the withdrawal of extracellular Ca^{2+} but not by the addition of Gd^{3+} (10 μM), flufenamic acid (200 μM) or nimodipine (10 μM; Fig. 4D).

3.5. The role of Cl^- in supporting the carmustine-induced Ca^{2+} influx

We showed previously (Doroshenko and Doroshenko, 2003) that the removal of extracellular Cl^- or the addition of Cl^- channel blockers effectively protected PC12 cells from

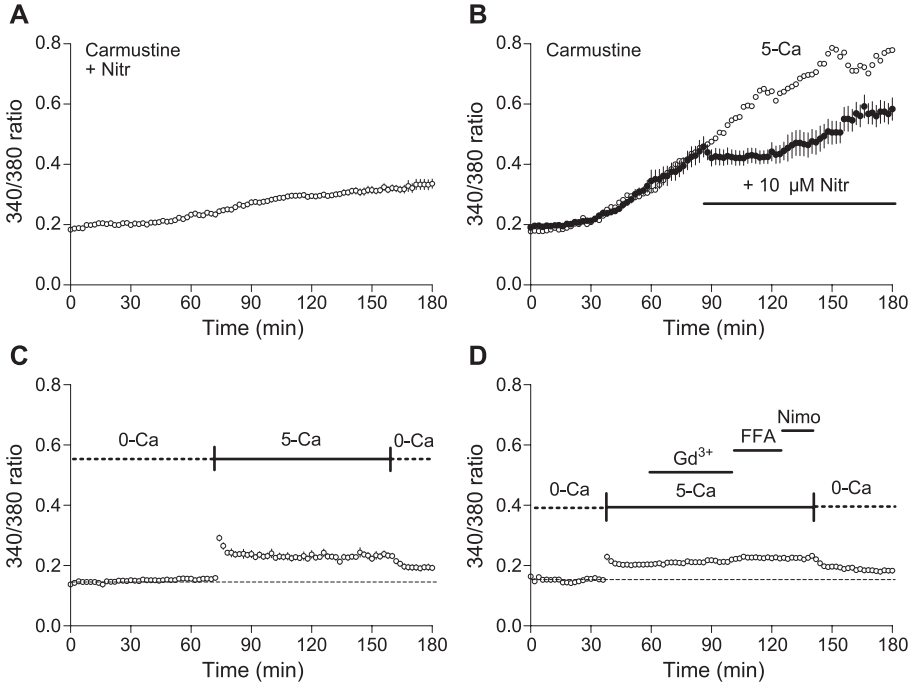

Fig. 4. Pharmacology of the carmustine-induced Ca^{2+} influx pathways. (A) Averaged changes in $[Ca^{2+}]_i$ in PC12 cells (n=14) bathed in the 5-Ca extracellular solution in the presence of 100 μM carmustine and 10 μM nitrendipine (*Nitr*); (B) The effect of a delayed application of 10 μM nitrendipine on the carmustine (100 μM)-induced increase in $[Ca^{2+}]_i$ in PC12 cells (n=12) bathed in the 5-Ca extracellular solution (●). For comparison, the time course in the carmustine (100 μM)-induced increase in $[Ca^{2+}]_i$ in PC12 cells (n=11) from a sister coverslip, without nitrendipine application, is also shown (○); (C) Changes in $[Ca^{2+}]_i$ in PC12 cells (n=11), unexposed to carmustine, following their transfer between the Ca^{2+}-free and the 5-Ca extracellular solutions, as indicated by horizontal bars; (D) The lack of effects of Gd^{3+} (10 μM), flufenamic acid (*FFA*, 200 μM) or nimodipine (*Nimo*, 10 μM) on elevated $[Ca^{2+}]_i$ in PC12 cells (n=15) bathed in the 5-Ca solution. The cells were not exposed to carmustine at any time.

death that normally follows the exposure of these cells to carmustine. In the present study, the replacement of extracellular Cl^- with membrane-impermeable gluconate completely prevented the carmustine-induced increase in $[Ca^{2+}]_i$ (Fig. 5A). To compensate for the effect of chelation of extracellular Ca^{2+} by gluconate, the Ca^{2+} content of the Cl^--free solution in these experiments was increased to 7.5 mM (the calculated free Ca^{2+} concentration was 2.1 mM). Under normal ionic conditions and with 5 mM Ca^{2+} present in the extracellular solution, several Cl^- channel blockers, IAA-94 (100 μM, Fig. 5B) or NPPB (100 μM, not shown), suppressed the carmustine-induced increase in $[Ca^{2+}]_i$ when added simultaneously with or prior to carmustine. When added during the rising phase of the carmustine-induced $[Ca^{2+}]_i$ increase, the aforementioned Cl^- channel blockers clamped $[Ca^{2+}]_i$ at the level that it had reached prior to the application of the blockers (Fig. 5C).

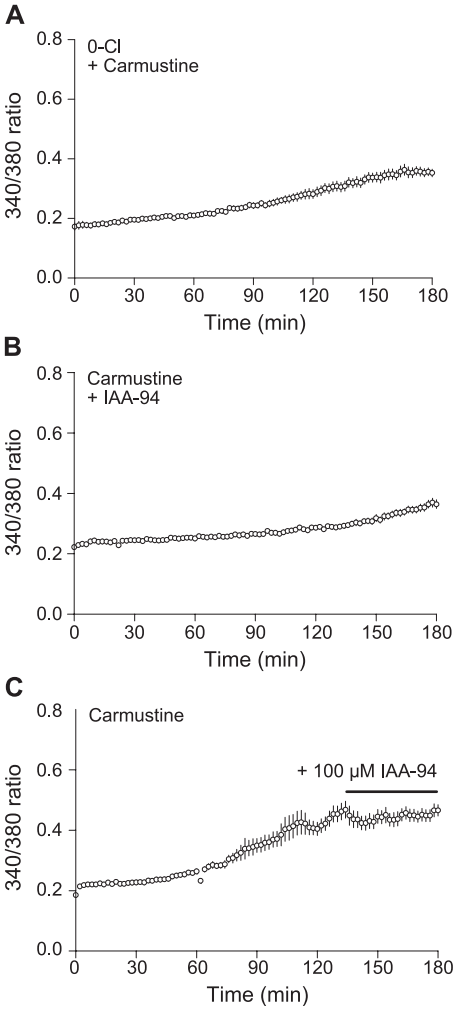

Fig. 5. Carmustine-induced $[Ca^{2+}]_i$ increase depends on Cl^- influx. (A) The lack of the carmustine (100 μM)-induced rise in $[Ca^{2+}]_i$ in PC12 cells (n=20) bathed in the Cl^--free extracellular solution; (B) The lack of the carmustine (100 μM)-induced rise in $[Ca^{2+}]_i$ in PC12 cells bathed in the 5-Ca extracellular solution containing 100 μM indanyloxyacetic acid 94 (IAA-94, n=25); (C) Partial suppression of the carmustine (100 μM)-induced increase in $[Ca^{2+}]_i$ in PC12 cells by a delayed application of 100 μM IAA-94 to the 5-Ca extracellular solution (n=12).

4. Discussion

The results of the present study show that exposure of PC12 cells to carmustine resulted in an increase in the intracellular Ca^{2+} levels. This finding is consistent with the pronounced Ca^{2+} dependence of carmustine toxicity in PC12 cells (Doroshenko and Doroshenko, 2003). We assume that the carmustine-induced $[Ca^{2+}]_i$ increase was caused by depletion of cellular GSH due to the inhibition by carmustine of glutathione reductase (Frischer and Ahmad, 1977; Smith and Boyd, 1984). The recovery of reduced GSH from oxidized GSSG by glutathione reductase and de novo GSH synthesis, whose rate is determined by an enzyme γ-glutamylcysteine synthetase, is the foremost contributor to cellular pool of GSH (Anderson, 1998). Accordingly, when γ-glutamylcysteine synthetase was inhibited by buthionine sulfoximine (BSO), the carmustine-induced $[Ca^{2+}]_i$ increase occurred significantly earlier than in control cells, apparently due to lower GSH levels in BSO-treated cells.

The link between cellular GSH and the $[Ca^{2+}]_i$ remains elusive, although available evidence suggests that it is mediated by changes in cellular redox status. GSH is a potent scavenger of reactive oxygen species and its deficiency is bound to result in the rise in their levels, thereby promoting oxidative stress. An increase in $[Ca^{2+}]_i$ has long been recognized to be a major mediator of oxidative cell death (Beal, 1995; Orrenius et al., 1996; Orrenius and Nicotera, 1994). Indeed, increased levels of reactive oxygen species and intracellular Ca^{2+} have both been found to correlate with decreased GSH levels in PC12 cells with suppressed GSH synthesis (Jurma et al., 1997). Consistent with the hypothesis that oxidative stress is involved in the carmustine-induced $[Ca^{2+}]_i$ increase is the observation in the present study that the carmustine effect was delayed in cells treated with an iron chelator, deferoxamine. Iron is an extremely strong oxidizing agent that can stimulate the production of highly reactive hydroxyl radicals via the iron-catalyzed Fenton reaction and Haber–Weiss cycle (Burkitt and Gilbert, 1990). The effect of deferoxamine may indicate that endogenous iron is required for the toxic effects of carmustine.

The carmustine-induced increase in $[Ca^{2+}]_i$ appears to be mainly due to an influx of extracellular Ca^{2+} ions because it could be completely and reversibly suppressed by the removal of extracellular Ca^{2+}. Also, there was no evidence of Ca^{2+} release from intracellular stores when carmustine was applied to PC12 cells bathed in the Ca^{2+}-free extracellular solution. However, the available data do not exclude its contribution, particularly of the Ca^{2+}-induced Ca^{2+} release, to the subsequent phase(s) of the carmustine-induced increase in $[Ca^{2+}]_i$, e.g., during the secondary increase in $[Ca^{2+}]_i$ observed in some cells.

There are several candidate pathways that can mediate the Ca^{2+} influx. First, the voltage-gated Ca^{2+} channels, whose presence in PC12 cells and contribution to cytotoxic effects of certain agents are well documented (e.g., Kongsamut and Miller, 1986; Janigro et al., 1989; Kim et al., 2000). In addition, PC12 cells possess a measurable basal Ca^{2+} permeability (Bennett et al., 1998), which is responsible for the increase in resting $[Ca^{2+}]_i$ in untreated cells observed in the present study upon the addition of 5 mM Ca^{2+} to the Ca^{2+}-free extracellular solution. Also, distinct redox-sensitive Ca^{2+}-permeable ion channels (e.g., Herson et al., 1999; Mukherjee et al., 2002) induced by GSH depletion/oxidative stress can be involved.

The pharmacological experiments in the present study suggest a role for L-type Ca^{2+} channels in mediating the carmustine-induced increase in $[Ca^{2+}]_i$. Thus, dihydropyridine blockers of L-type Ca^{2+} channels, nimodipine and nitrendipine, prevented the carmustine effects on $[Ca^{2+}]_i$. These channels have earlier been implicated in $[Ca^{2+}]_i$ increase in PC12 cells in which GSH has been depleted by treatment with the γ-glutamylcysteine synthetase antisense oligomers (Jurma et al., 1997). Although the Ca^{2+} channel blockers were able to inhibit the carmustine-induced increase in $[Ca^{2+}]_i$ completely when present during the initial period of exposure to carmustine, their inhibitory effects were only partial when they were applied with a delay, after the initial rise in $[Ca^{2+}]_i$ had occurred. It is likely that this limited ability of the dihydropyridines at saturating concentrations to reverse the carmustine-induced $[Ca^{2+}]_i$ increase reveals the involvement of an additional, pharmacologically distinct Ca^{2+} conductance. It is plausible that the conductance in question is the basal Ca^{2+} conductance, mentioned above. In untreated cells, the influx of Ca^{2+} mediated by this conductance apparently is small, such that the cellular Ca^{2+} regulatory system is able to maintain a stable $[Ca^{2+}]_i$. However, if carmustine suppresses Ca^{2+} regulation or enhances the basal Ca^{2+} permeability, the continuous Ca^{2+} influx associated with the basal Ca^{2+} permeability could result in an increased $[Ca^{2+}]_i$ in carmustine-treated cells. Our experimental observations do not support either of these mechanisms as a principal cause of the carmustine-induced $[Ca^{2+}]_i$ increase. First, the rapid recovery of $[Ca^{2+}]_i$ observed in carmustine-treated cells following withdrawal of extracellular Ca^{2+} indicated that the Ca^{2+} regulatory system remained functional in the presence of carmustine. Second, the basal Ca^{2+} conductance was not affected by any of the ion channel blockers that were tested in the present study, including those (e.g., nimodipine) that inhibited the carmustine-induced increase in $[Ca^{2+}]_i$. However, because it has been reported to be Ca^{2+}-dependent (Bennett et al., 1998), it could be enhanced in carmustine-exposed cells consequent to an early increase in $[Ca^{2+}]_i$ caused by the Ca^{2+} channel-mediated Ca^{2+} influx. The failure of flufenamic acid, a reported blocker of redox-sensitive Ca^{2+}-permeable ion channels (Herson et al., 1999; Mukherjee et al., 2002), to suppress the carmustine effects in PC12 cells argues against a significant role for these channels. As a minimum model, the combined involvement of the L-type Ca^{2+} channels and the basal Ca^{2+} conductance appears to suffice the experimental observations of the present study.

The mechanisms of engagement of Ca^{2+} channels by carmustine are not clear. On the one hand, the anticipated increase in reactive oxygen species in carmustine-treated cells could cause up-regulation of the activity of L-type Ca^{2+} channels, as was described recently in PC12 cells (Green et al., 2002). However, a depolarizing shift of the membrane potential would also be required to open the Ca^{2+} channels. In PC12 cells treated with the γ-glutamylcysteine synthetase antisense oligomers (Jurma et al., 1997), the required membrane depolarization apparently resulted from the inhibition of Na^+/K^+ ATPase activity and subsequent activation of voltage-gated Na^+ channels. In the present study, the carmustine-induced $[Ca^{2+}]_i$ increase was not affected by tetrodotoxin, an inhibitor of the Na^+ channels, but was completely inhibited by several different Cl^- channel blockers or by the removal of extracellular Cl^-. Furthermore, delayed applications of Cl^- channel blockers resulted in stabilizing $[Ca^{2+}]_i$ at the levels attained by the time of applications and preventing its further rise, as did the Ca^{2+} channel blockers. These observations suggest that the required changes in the membrane potential were brought about by transmembrane Cl^- fluxes. The specific mechanisms of Cl^- channel involvement remain unidentified at present.

Taken together, the data obtained in this and an earlier study (Doroshenko and Doroshenko, 2003) strongly support the hypothesis that the carmustine-induced death of PC12 cells is mediated by a Ca^{2+} overload that is caused by a carmustine-induced influx of extracellular Ca^{2+}. The causative link between the carmustine-induced $[Ca^{2+}]_i$ increase and cell death is substantiated by the similarity of the time course of these events and by the finding that each of the experimental treatments that suppressed the carmustine-induced $[Ca^{2+}]_i$ increase also protected PC12 cells against carmustine-induced death. The underlying event supposedly is the inhibition by carmustine of glutathione reductase that results in the depletion of cellular GSH and, consequently, in increased levels of reactive oxygen species.

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