Calcium Entry through L-type Calcium Channels Causes Mitochondrial Disruption and Chromaffin Cell Death*

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Maria F. Cano-Abad†§, Mercedes Villarroya‡¶, Antonio G. García†§**, Nelson H. Gabilan†‡, and Manuela G. López†§§

From the †Instituto de Farmacología Teófilo Hernando, Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, C/Arzobispo Morcillo 4, Madrid 28029, Spain, ‡Servicio de Farmacología Clínica, Hospital de la Princesa, C/Diego de León 62, Madrid 28006, Spain, §§Instituto Universitario de Investigaciones Gerontológicas y Metabólicas, Hospital de la Princesa, C/Diego de León 62, Madrid 28006, Spain, and ¶¶Departamento de Bioquímica, Universidad Federal de Santa Catarina, Florianópolis 88049SC, Brasil

Sustained, mild K+ depolarization caused bovine chromaffin cell death through a Ca2+-dependent mechanism. During depolarization, Ca2+ entered preferentially through L-channels to induce necrotic or apoptotic cell death, depending on the duration of the cytosolic Ca2+ concentration ([Ca2+]i) signal, as proven by the following. (i) The L-type Ca2+ channel activators Bay K 8644 and FPL64176, more than doubled the cytotoxic effects of 30 mM K+; (ii) the L-type Ca2+ channel blocker nimodipine suppressed the cytotoxic effects of K+ alone or K+ plus FPL64176; (iii) the potentiation by FPL64176 of the K+-evoked [Ca2+]i elevation was totally suppressed by nimodipine. Cell exposure to K+ plus the L-type calcium channel agonist FPL64176 caused an initial peak rise followed by a sustained elevation of the [Ca2+]i, that, in turn, increased [Ca2+]m and caused mitochondrial membrane depolarization. Cyclosporin A, a blocker of the mitochondrial transition pore, and superoxide dismutase prevented the apoptotic cell death induced by Ca2+ overload through L-channels. These results suggest that Ca2+ entry through L-channels causes both calcium overload and mitochondrial disruption that will lead to the release of mediators responsible for the activation of the apoptotic cascade and cell death. This predominant role of L-type Ca2+ channels is not shared by other subtypes of high threshold voltage-dependent neuronal Ca2+ channels (i.e. N, P/Q) expressed by bovine chromaffin cells.

Alteration of calcium homeostasis is hypothesized to contribute to neuronal death following ischemia-reperfusion (1–5) or neurodegenerative diseases like Alzheimer’s disease (6–11). An overload of neuronal Ca2+ may activate a number of pathological processes like disruption of the mitochondrial membrane potential, free radical production, stimulation of catabolic enzymes, and enhancement of excitatory amino acid release that will lead to cell death (12). Considerable attention has been focused on the possibility of Ca2+ entry through the N-methyl-D-aspartate-glutamate receptor gated channel as responsible for Ca2+ overload, but other sources are also being considered, such as release from intracellular stores or Ca2+ influx through high threshold voltage-dependent calcium channels (VDCC).1

In neurons, several subtypes of high threshold and low threshold VDCC (L-, N-, P/Q-, R- and T-type) have been described (16–19). L-type VDCC have been localized predominantly in the soma and proximal dendrites of neurons throughout the brain (19–21) and are sensitive to dihydropyridine activators (e.g. Bay K 8644) and blockers (e.g. nimodipine).

At the moment, there are enough pieces of evidence that support the participation of Ca2+ entry through L-channels during ischemia, to cause cell death (13, 14, 21–24). However, the mechanism by which cell death is orchestrated by such Ca2+ entry has not been defined precisely. In this work, we attempt to clarify such a mechanism using the bovine adrenal medullary chromaffin cell as a model. This cell expresses the same set of Ca2+ channel subtypes described in neurons (25). Bovine chromaffin cells in primary cultures constitute a homogeneous cell population that expresses various subtypes of Ca2+ channels at relative densities similar to certain types of neurons (i.e. granular cerebellar neurons (about 20% L-type, 30% N-type, and 50% P/Q-type) (26)). In addition, R-type channels have been recently discovered in chromaffin cells using the perforated configuration of the patch-clamp technique (27). Hence, bovine chromaffin cells constitute an adequate model to investigate the problem posed here (i.e. (i) to know whether Ca2+ entry through these channels during sustained depolarization produced apoptotic cell death; (ii) to define why the L-channel pathway is more efficacious to activate this lethal signal; and (iii) to determine which is the role of mitochondria in the activation of cell death.

EXPERIMENTAL PROCEDURES

Materials and Solutions—Dulbeco’s modified Eagle’s medium and fetal calf serum were obtained from Life Technologies, Inc.; nimodipine, FPL64176, Bay K 8644, and superoxide dismutase (SOD) were from Sigma; ω-conotoxin GVIA was from Bachem Feinchemikalien (Switzer-

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1 The abbreviations used are: VDCC, voltage-dependent calcium channels; SOD, superoxide dismutase; LDH, lactate dehydrogenase; [Ca2+]o, cytosolic Ca2+ concentration; [Ca2+]i, mitochondrial Ca2+ concentration; Δψm, mitochondrial transmembrane potential; LDHo, extracellular LDH; [K+]o, and [Ca2+]o, external K+ and Ca2+ concentration, respectively.
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land; and \(\omega\)-conotoxin MVIIIC and \(\omega\)-agatoxin IVA were from the Peptide Institute (Osaka). The assay kit for measuring the activity of lactate dehydrogenase (LDH) and Cell Death Detection ElisaPlus Kit were purchased from Roche Molecular Biochemicals. Fura-2/AM, rhodamine 123, Vybrant Apoptosis Kit, and Mitotracker Red were purchased from Invitrogen (Carlsbad, CA) or Probes, Inc.

Concentrated solutions of drugs were prepared in water (\(\omega\)-conotoxin GVIA, \(\omega\)-conotoxin MVIIIC, \(\omega\)-agatoxin IVA, superoxide dismutase), dimethyl sulfoxide (nimodipine, FPL64176), or ethanol (Bay K 8644). Appropriate dilutions were then made in Krebs-Hepes solution containing 144 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose, pH 7.5, titrated with NaOH. Measuring at the final concentration used (less than 0.1%), had no effect on any of the parameters tested.

Preparation and Culture of Bovine Chromaffin Cells—Bovine adrenal medulary chromaffin cells were isolated as previously described (28) with some modifications (29). To reduce the number of endothelial cells in the culture that could alter LDH measurements, cells were preplated for 30 min, and proliferation inhibitors (cytisine arabinoside, l-arginine, l-leucine methyl ester, and fluoro-deoxy-xouridine) were used during the maintenance of the culture in the Dulbecco’s modified Eagle’s medium. For cell death studies, cells were plated at a density of 5 \(\times\) 10\(^5\)/well on 24-well Orange plates coated with 0.01 mg/ml poly-L-lysine, 10 \(\mu\)g/ml l-arginine, 10 \(\mu\)l/ml l-leucine methyl ester, and 10 \(\mu\)l/ml fluoro-deoxy-xouridine. After 2–3 days, the medium was replaced by 1 ml of serum-free fresh medium and subsequently changed every 2–3 days. Cells were normally used during days 2–3, to avoid excessive growth of endothelial cells that could interfere with LDH measurements.

LDH Assay—Extracellular and intracellular LDH activities were spectrophotometrically measured by following tetrazolium reduction at 600 nm. Results were expressed as the absorbance at 600 nm.

Statistical Analysis—Data were expressed as means \(\pm\) S.E. Statistical significance of differences between means was determined by an analysis of variance test. If significant differences were found, an appropriate multiple comparison test (Fisher PLSD) was done. In some cases, Student’s t test was used (see Figs. 1 and 3–6 legends). Differences were considered significant at the level of p < 0.05.

RESULTS

Cell Death as a Function of Depolarization and Ca\(^{2+}\) Entry through L-type Calcium Channels: Protection by Nimodipine—To facilitate ionic manipulations, all experiments were performed in Krebs-Hepes solutions. For instance, in the experiment shown in Fig. 1a, cells were incubated in Krebs-Hepes solution containing increasing concentrations of KCl (5.9–50 mM), with isosmotic reduction of NaCl, for 24 h at 37 °C. At the end of this incubation period, the medium was collected to measure LDH released from dead cells (extracellular LDH; LDH\(_e\)), the cells that remained attached to the well were then lysed, and their LDH content was measured. The expression of LDH\(_e\) as a percentage of total LDH provides an indication of the fraction of cells dying as a consequence of a given treatment, in this case depolarization of increasing strengths. Incubation of the cells for 24 h with increasing [K\(^+\)]\(_o\) gave a gradual release of LDH\(_e\); maximum cell damage was observed at 30 mM K\(^+\), which caused 25% cell loss; a further increase to 50 mM K\(^+\) did not enhance cell death. Hence, 30 mM K\(^+\) was chosen to perform subsequent experiments.

Cell damage was not only a function of the degree of VDCC opened upon depolarization with high [K\(^+\)], but also of the quantity of external Ca\(^{2+}\) offered to the cells ([Ca\(^{2+}\)]\(_{o}\)), in a second experiment (Fig. 1b), the effects of increasing [Ca\(^{2+}\)]\(_{o}\), on cell death, at a fixed level of depolarization (30 mM K\(^+\) were assessed. Cell death after a 24-h incubation was close to basal at 0.2–0.5 mM [Ca\(^{2+}\)]\(_{o}\), (13% LDH\(_e\) release); at 2–10 mM [Ca\(^{2+}\)]\(_{o}\), cell death increased to around 25%, and it declined to 20% at 20 mM [Ca\(^{2+}\)]\(_{o}\); this effect could be due to inactivation by excess Ca\(^{2+}\) of VDCC (38–40).

To increase the cell damage caused by Ca\(^{2+}\) entry through L-type VDCC, we used different L-type agonists like FPL64176, (+)Bay K 8644, and (-)Bay K 8644 (41) under mild depolarizing conditions (30 mM K\(^+\)) for 24 h. Fig. 1c shows the cytotoxic consequence of the incubation with these agonists at concentrations that ranged from 0.3 to 10 \(\mu\)M. Maximum...
cytotoxic effects, for all drugs tested, were observed at 0.3–1 μM; higher concentrations of the agonists (3–10 μM) presented a decrease in LDH; this effect can be explained by their Ca\(^{2+}\) antagonist action at high concentrations (41, 42). Out of the three L-type agonists used, FPL64176 presented the highest cytotoxic effects. Therefore, the combination of 0.3 μM FPL64176 in 30 mM K\(^+\) was used to induce cell death via Ca\(^{2+}\) entry through L-type VDCC.

The cytotoxic effect of Ca\(^{2+}\) entry through L-channels induced by FPL64176 was dependent on [Ca\(^{2+}\)]\(_e\). In Fig. 1d, basal LDH\(_e\) (24-h incubation in 5.9 mM K\(^+\)) amounted to 8.4 ± 0.9%; 0.3 μM FPL64176 in the presence of 30 mM K\(^+\) increased the basal LDH\(_e\) at all [Ca\(^{2+}\)]\(_e\), studied. For instance, at 0.2 mM [Ca\(^{2+}\)]\(_e\), LDH\(_e\) increased from 12.3 ± 2.6% (Fig. 1b) to 25.9 ± 2.6% in the presence of FPL. Maximum cell death was achieved in the presence of 5–10 mM [Ca\(^{2+}\)]\(_e\) (41.5 ± 2.0 and 43.5 ± 1.8%, respectively). It is interesting that this sharp increase in cell death could be completely reversed by a 1 μM concentration of the L-type Ca\(^{2+}\) channel blocker nimodipine (Fig. 1d); LDH\(_e\) after 24 h was in the range of 10–15% (i.e. similar to that found in basal conditions (5.9 mM K\(^+\))). It is worth noting that nimodipine counteracted both the LDH\(_e\) increase evoked by 30 mM K\(^+\) in the absence (about 25%) and in the presence of FPL64176 (about 50%), suggesting that the increase in cell death was associated to increased Ca\(^{2+}\) entry mostly through L-type Ca\(^{2+}\) channels.

Ca\(^{2+}\), Signals and Cell Damage Generated by K\(^+\) at Low and High [Ca\(^{2+}\)]; Effects of FPL64176—Since cell damage caused by depolarization was minimum at 0.2 mM [Ca\(^{2+}\)]\(_e\), and maximum at 5 mM [Ca\(^{2+}\)]\(_e\), we selected these extreme [Ca\(^{2+}\)]\(_e\) values to analyze the time course of the [Ca\(^{2+}\)]\(_e\) signals in Fura-2-loaded single cells exposed to 30 mM K\(^+\). Cells superfused with 0.2 mM [Ca\(^{2+}\)]\(_e\) gave an average initial basal [Ca\(^{2+}\)]\(_e\) of 55 ± 4.8 nM. Exposure to 30 mM K\(^+\), 0.2 mM Ca\(^{2+}\) caused an initial mean peak of 198 ± 39 nM (n = 8) (Fig. 3a). Despite the sustained depolarization with K\(^+\), the [Ca\(^{2+}\)]\(_e\) peak declined quickly to basal levels and remained stable along the rest of the 30-min period of recording. In 5 mM [Ca\(^{2+}\)]\(_e\), the resting [Ca\(^{2+}\)]\(_e\) was 61 ± 5.9 nM (n = 7); upon superfusion with 30 mM K\(^+\), 0.2 mM Ca\(^{2+}\), an initial [Ca\(^{2+}\)]\(_e\) peak of 560 ± 45 nM was reached (n = 7); then the peak [Ca\(^{2+}\)]\(_e\), declined gradually and reached a stable plateau of 242 ± 79 nM.

The transient nature of the [Ca\(^{2+}\)]\(_e\) signal was probably due to voltage- and [Ca\(^{2+}\)]\(_e\)-dependent inactivation of Ca\(^{2+}\) channels upon sustained cell depolarization (38–40). This transient response of [Ca\(^{2+}\)]\(_e\), might explain the scarce cell damage observed in the experiments of Fig. 2b. Hence, we tried to delay the inactivation of Ca\(^{2+}\) channels by using the L-type Ca\(^{2+}\) channel activator FPL64176. In the presence of 0.3 μM FPL64176, 30 mM K\(^+\), 0.2 mM Ca\(^{2+}\), the initial peak of [Ca\(^{2+}\)]\(_e\) reached 432 ± 130 nM (n = 4) and then declined to reach a stable Ca\(^{2+}\) entry of 298 ± 8 nM. Thus, FPL64176 converted the [Ca\(^{2+}\)]\(_e\) signal obtained in 0.2 mM [Ca\(^{2+}\)]\(_e\) into a response similar to that produced by 5 mM [Ca\(^{2+}\)]\(_e\). When FPL64176 was applied in the presence of high [Ca\(^{2+}\)]\(_e\) (5 mM), the [Ca\(^{2+}\)]\(_e\) rose initially to a peak of 905 ± 120 nM (n = 9) and then declined to a sustained plateau that was 1.4-fold higher (409 ± 91 nM) than in low [Ca\(^{2+}\)]\(_e\) (0.2 mM), along the rest of the 30-min depolarization period (Fig. 2d).

When measuring the total quantity of Ca\(^{2+}\) entering the cells...
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During the 30-min exposure period in low (0.2 mM) or high (5 mM) [Ca\(^{2+}\)]\(_{e}\), in the presence or absence of FPL64176, we observed that the maximum levels of [Ca\(^{2+}\)]\(_{e}\), were achieved when using 5 mM [Ca\(^{2+}\)]\(_{e}\), plus FPL64176 (496 ± 77 nM/s) (n = 8). Ca\(^{2+}\) entry at 0.2 mM [Ca\(^{2+}\)]\(_{e}\), plus FPL64176 and 5 mM [Ca\(^{2+}\)]\(_{e}\), alone were rather similar, 317 ± 8 nM/s (n = 4) and 302 ± 62 nM/s (n = 8), respectively. The same pattern was also observed when measuring the initial peak of [Ca\(^{2+}\)]\(_{e}\), (see Fig. 3a).

When analyzing cell lesions as LDH\(_{e}\), released, we observed that the amount of Ca\(^{2+}\) entering the cell during 30 min, measured as the integral of the curve of [Ca\(^{2+}\)]\(_{e}\), correlated well with the extent of cell death observed 24 h later (see Fig. 3b). These results prove that cell lesion is a function of the quantity of Ca\(^{2+}\) entering through VDCC and that it depends not only on the [Ca\(^{2+}\)]\(_{e}\), but also on the inactivation state of those channels.

**Cytoprotection Afforded by Nimodipine Is Related to the Reduction of [Ca\(^{2+}\)]\(_{e}\).**—We had previously observed (Fig. 1d) that 1 \(\mu\)M nimodipine completely counteracted LDH release induced by Ca\(^{2+}\) overload through L-type Ca\(^{2+}\) channels. In order to determine if the protection afforded by nimodipine was related to a reduction in the levels of [Ca\(^{2+}\)]\(_{i}\), we performed experiments in single Fura-2/AM-loaded cells. Fig. 4 shows how nimodipine restored the [Ca\(^{2+}\)]\(_{i}\), to basal levels after the [Ca\(^{2+}\)]\(_{i}\), was raised by FPL64176, 30 mM K\(^{+}\), both at low (0.2 mM), (Fig. 4a) and high (5 mM) (Fig. 4b) [Ca\(^{2+}\)]\(_{e}\). Total Ca\(^{2+}\) entry, analyzed as the area under the curve, in the presence and absence of nimodipine was significantly reduced from 80 ± 8 \(\mu\)M/s to 10 ± 1 \(\mu\)M/s (n = 5) at 0.2 mM [Ca\(^{2+}\)]\(_{e}\), and from 120 ± 12 \(\mu\)M/s to 15 ± 1 \(\mu\)M/s (n = 5) at 5 mM [Ca\(^{2+}\)]\(_{e}\), (Fig. 4c). This drastic reduction in the [Ca\(^{2+}\)]\(_{i}\), could explain the total cytoprotection observed previously with nimodipine (see Fig. 1d).

Since nimodipine is selectively blocking L-type Ca\(^{2+}\) channels, it seemed obvious that N- and P/Q-type Ca\(^{2+}\) channels would not be contributing to the Ca\(^{2+}\) signals or to the LDH\(_{e}\) release in cells exposed to Ca\(^{2+}\) overload through L-channels. This possibility was verified by measuring both [Ca\(^{2+}\)]\(_{e}\), changes and cell death in the presence of non-L-type Ca\(^{2+}\) channel blockers (\(\omega\)-conotoxin GVIA for N-channels and \(\omega\)-conotoxin MVIIC for N/P/Q-channels). When measuring cell damage as a function of LDH release in depolarized cells treated with FPL64176 at different [Ca\(^{2+}\)]\(_{e}\), (0.2–20 mM), we curiously observed significant cytoprotection by N/P/Q-type blockers at low [Ca\(^{2+}\)]\(_{e}\), (0.2–2 mM) but not at higher concentrations (i.e. above 5 mM; see Fig. 1d). However, the increase of [Ca\(^{2+}\)]\(_{e}\), induced by FPL64176, 30 mM K\(^{+}\) in low and high [Ca\(^{2+}\)]\(_{e}\), was not significantly modified in the presence of the non-L-type channel blockers (data not shown).

**Prolonged Ca\(^{2+}\) Entry through L-type Channels Leads Primarily to Necrotic Cell Death.—**To further understand the basic mechanism by which chromaffin cells were dying upon their sustained depolarization, we compared the amount of necrosis measured as LDH release and apoptosis measured as histone-associated DNA fragments in cells exposed for 24 h to FPL64176, 30 mM K\(^{+}\), 5 mM Ca\(^{2+}\). Fig. 5a shows that the cytotoxic effect of Ca\(^{2+}\) entry through L-channels induced significant LDH\(_{e}\) release but no significant increase in the number of apoptotic cells. These results suggest that the elevation of [Ca\(^{2+}\)]\(_{e}\), during prolonged depolarizations, in the presence of an L-type channel activator, constitutes predominantly a necrotic signal.

During cell necrosis, dramatic changes can be observed in the mitochondrial structure (9). The morphological changes in the mitochondria network induced by prolonged (24-h) Ca\(^{2+}\) entry through L-channels could be observed in cells stained with the mitochondrial dye Mitotracker red. Chronic treatment with FPL64176 induced total disruption of the mitochondrial mesh (Fig. 5c), indicating cell necrosis. In contrast, the control...
Cell in Fig. 5b presents the mitochondrial network completely preserved.

Transient \(\text{Ca}^{2+}\) Overload through L-channels Mediates Apoptotic Cell Death Secondary to the Release of Cytochrome c and Free Radicals from the Mitochondria—During an ischemic episode, a transient lack of oxygen normally occurs. Therefore, we tried experimental conditions where \(\text{Ca}^{2+}\) overload through L-channels (0.3 \(\mu\)M FPL64176, 30 mM \(K^+\), 5 mM \(\text{Ca}^{2+}\)) had a duration of 30 min; we evaluated the cytotoxic consequence of such stimulus, measuring cell death as release of LDH (immediately after the stimulus) and the number of apoptotic nuclei (48 h after the stimulus) with the fluorescent dye Hoechst. We found that \(\text{Ca}^{2+}\) entering through L-channels for 30 min almost doubled the basal release of LDH and increased the number of apoptotic nuclei from 11 \(\pm\) 0.7% (basal) to 26.8 \(\pm\) 3.3% (depolarizing pulse) after 48 h (Fig. 6a). These results contrast with those obtained when the toxic stimulus (0.3 \(\mu\)M FPL64176, 30 mM \(K^+\), 5 mM \(\text{Ca}^{2+}\)) was kept for 24 h (Fig. 5). In the latter case, no significant increase in the number of apoptotic nuclei was observed; the main lesion found was necrotic. These results indicated that shorter increases in the \([\text{Ca}^{2+}]_c\) were capable of activating the apoptotic cascade; the question now was to verify how this mechanism was taking place.

Transient peaks in \([\text{Ca}^{2+}]_c\) can secondarily increase mitochondrial \(\text{Ca}^{2+}\) \([\text{Ca}^{2+}]_m\), lead to the opening of the mitochondrial transition pore (43), release of cytochrome c (36) with the resultant release of oxygen free radicals (like superoxide) from the electron transport chain (44, 45), and initiate the apoptotic cascade (46). To determine if the cells were dying through this mechanism, we performed two types of experiments: (i) block-
Nimodipine returned to basal levels the \([Ca^{2+}]_c\) rise induced by FPL64176. In single Fura-2-loaded cells stimulated with 0.3 \(\mu\)M FPL64176, 30 mM K\(^+\), nimodipine (1 \(\mu\)M) reduced the \([Ca^{2+}]_c\), to basal levels (a); this effect was reversible (b). \(\Delta\) represents total mean \(Ca^{2+}\) entry obtained during 5 min with 0.3 \(\mu\)M FPL64176/30 mM K\(^+\) at low (0.2 mM) or high (5 mM) \([Ca^{2+}]_c\), alone or in the presence of nimodipine. ***, \(p < 0.001\) with respect to the depolarizing pulse in the absence of nimodipine (Student's \(t\) test).
ade of the opening of the transition pore by CsA (3 μM) and the subsequent release of cytochrome c and (ii) use of the antioxidant enzyme SOD to prevent the action of superoxide free radicals that could be released from mitochondria. When CsA and SOD were present during the 30-min depolarizing pulse (0.3 μM FPL64176, 30 mM K⁺, 5 mM Ca²⁺), the number of apoptotic nuclei returned to basal levels (10.8 ± 2.3 and 7.5 ± 2.4%, respectively), indicating that release of cytochrome c and free radicals was involved in inducing apoptosis via Ca²⁺ entry through L-channels. When Ca²⁺ entry was prevented with nimodipine (1 μM), apoptotic cell death was also prevented (Fig. 5a).

Cell death measured as LDH released in the first 30 min was completely counteracted when SOD and nimodipine were present during the 30-min depolarizing pulse, but not by CsA (Fig. 5b).

These results suggest that the amount of Ca²⁺ entering the cell with FPL64176, 30 mM K⁺, 5 mM Ca²⁺ during 30 min, that corresponds to 496 ± 77 nM·s of [Ca²⁺], mediates early necrotic cell death (LDHe) and late apoptosis. Early necrotic cell death and late apoptosis could be prevented by nimodipine and SOD. Therefore, blockade of L-channels and superoxide radicals are involved both in early necrotic and late apoptotic cell death. The fact that CsA did not protect against early necrosis suggests that release of cytochrome c, under these experimental conditions, is primarily activating the apoptotic cascade and cell death at later stages.

Calcium Overload through L-type Ca²⁺ Channels Increases Mitochondrial [Ca²⁺], Mitochondrial Membrane Depolarization, and Release of Free Radicals—Mitochondria are involved in Ca²⁺ sequestration during an excytotoxic insult (47). Recent

Fig. 5. Sustained Ca²⁺ overload through L-channels mediates necrotic cell death. a, cell death measured as apoptosis (black columns) or necrosis (white columns), at low (0.2 mM) or high (5 mM) [Ca²⁺], in the presence of the depolarizing mixture (0.3 μM FPL64176/30 mM K⁺) for 24 h. Data correspond to the mean ± S.E. of four experiments from three different batches of cells. Statistical differences were observed among the LDHe measurements but not for the apoptotic measurements. **, p < 0.01 with respect to basal level (Student’s t test). Shown are mitochondria stained with the fluorescent dye Mitotracker red, in a resting cell (b) and in a cell exposed to calcium overload through L-channels (0.3 μM FPL64176, 30 mM K⁺, 5 mM Ca²⁺) (c).

Fig. 6. Transient Ca²⁺ entry through L-channels mediates necrotic and apoptotic cell death. A depolarizing period of 30 min (0.3 μM FPL64176, 30 mM K⁺, 5 mM Ca²⁺) induced significant increase in the percentage of apoptotic nuclei measured with the fluorescent dye Hoechst 48 h later (a) and necrotic cell death measured as percentage of LDHe immediately after the cytotoxic pulse (b). SOD (1500 units), nimodipine (1 μM), and CsA (3 μM), during the 30-min depolarization period, prevented the delayed apoptotic cell death (a). Early necrotic cell death, measured as LDHe, was prevented by nimodipine and SOD but not by CsA (b). Data correspond to the mean ± S.E. of three experiments from three different batches of cells. *, p < 0.05; **, p < 0.01 with respect to 0.3 μM FPL64176, 30 mM K⁺ (Student’s t test).
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Studies have begun to clarify the deleterious effects that may result from mitochondrial Ca\(^{2+}\) overload. Rapid Ca\(^{2+}\) uptake causes mitochondrial depolarization (48), impairment of energy metabolism (49), and uncoupling of electron transport from ATP production (50, 51) and cell death.

With this background in mind, we carried out experiments to determine whether Ca\(^{2+}\) driven through L-type Ca\(^{2+}\) channels was being seen primarily by the mitochondria and if this Ca\(^{2+}\) was able to depolarize the mitochondrial membrane that, in turn, would lead to cell death. Fluorescence measurements of [Ca\(^{2+}\)]\(_i\) were performed in single loaded cells with Fura-2/AM. Fig. 7a shows an original trace of an experiment where two initial control (0.3 \(\mu\)M FPL64176, 30 mM K\(^+\), 5 mM Ca\(^{2+}\)) depolarizing pulses (10 s) were applied, followed by a pulse in the presence of the protonophore CCCP (2 \(\mu\)M) or after pretreatment with CCCP for 20 s. In both cases, when Ca\(^{2+}\) uptake by mitochondria was prevented with CCCP, the [Ca\(^{2+}\)]\(_i\) signal almost doubled that of control pulses.

A similar experimental procedure was carried out to test the contribution of the endoplasmic reticulum to Ca\(^{2+}\) buffering. The same protocol was performed in single loaded cells with Fura-2/AM. After the application of two control pulses (0.3 \(\mu\)M FPL64176, 30 mM K\(^+\), 5 mM Ca\(^{2+}\)), the endoplasmic reticulum was depleted using the combination of 10 mM caffeine, 10 mM ryanodine, and 1 \(\mu\)M thapsigargin. The depolarizing pulse given in the presence of the endoplasmic reticulum-depleting solution did not increase the intracellular Ca\(^{2+}\) signal, indicating little or no contribution of the endoplasmic reticulum to the buffering of cytosolic Ca\(^{2+}\) in these experimental conditions. Therefore, these results suggest that mitochondria are the main buffering system for Ca\(^{2+}\) overload through L-channels.

In order to determine whether [Ca\(^{2+}\)]\(_i\) elevations mediated by activation of L-channels (FPL64176, 30 mM K\(^+\), 5 mM Ca\(^{2+}\)) were able to depolarize the mitochondria, we measured the \(\Delta\psi\)m with the fluorescent dye rhodamine 123. Confocal images were taken every 0.372 s. Fig. 8 shows experiments that illustrate how FPL64176, 30 mM K\(^+\), 5 mM Ca\(^{2+}\) induced increases in the fluorescence of rhodamine 123, indicating mitochondrial membrane depolarization; when the same cell was perfused with FPL, 30 mM K\(^+\), 5 mM Ca\(^{2+}\) in the presence of nimodipine, mitochondrial depolarization was prevented. These results...
demonstrate that depolarization of the mitochondrial membrane occurs when an elevation of \([\text{Ca}^{2+}]_m\) occurs, in this case after the opening of L-type \(\text{Ca}^{2+}\) channels.

**DISCUSSION**

We demonstrate in this study that sustained, mild \(\text{K}^+\) depolarization causes chromaffin cell death (Fig. 1b) through a \(\text{Ca}^{2+}\)-dependent mechanism. Under these conditions, \(\text{Ca}^{2+}\) can gain the cell cytosol through several of the voltage-dependent \(\text{Ca}^{2+}\) channels described in bovine chromaffin cells. As indicated by numerous patch-clamp studies, external \(\text{Ca}^{2+}\) enters the voltage-clamped bovine chromaffin cell during depolarization through L-type (20%), N-type (30%), and P/Q-type (50%) \(\text{Ca}^{2+}\) channels (see Ref. 18). Despite this, it was curious that most of the \(\text{Ca}^{2+}\) entering through L-channels, but not through N- or P/Q-channels, was responsible for the activation of the death signal, as proven by the following observations. (i) The L-type \(\text{Ca}^{2+}\) channel activators Bay K 8644 and FFPL64176 more than doubled the cytotoxic effects of 30 mM \(\text{K}^+\); (ii) the L-type \(\text{Ca}^{2+}\) channel blocker nimodipine suppressed the cytotoxic effects of 30 mM \(\text{K}^+\) alone or 30 mM \(\text{K}^+\) plus FFPL64176; (iii) conversely, toxin blockade of N- and P/Q-channels caused partial cytoprotection at 0.2–2 mM \([\text{Ca}^{2+}]_c\), and no protection at 5–20 mM \([\text{Ca}^{2+}]_c\); and (iv) the potentiation by FFPL64176 of the \(\text{K}^+\)-evoked \([\text{Ca}^{2+}]_c\) elevation was suppressed by nimodipine.

The partial cytoprotection afforded by combined toxins at the lower \([\text{Ca}^{2+}]_c\) is puzzling but can surely be interpreted in the context of the recent finding of our laboratory showing the \(\text{Ca}^{2+}\)-dependent inactivation of L-, N-, and P/Q-channels in voltage-clamped bovine chromaffin cells (39). The elevation of \([\text{Ca}^{2+}]_c\) elicited by the mitochondrial uncoupler CCCP during cell depolarization causes a faster inhibition of N- and P/Q-channels, as compared with L-channels. This might explain why, in the present study, \(\text{Ca}^{2+}\) entry through L-type \(\text{Ca}^{2+}\) channels caused greater cell lesion than that gained through N- and P/Q-channels. Despite the fact that L-channels account for only one-fifth of the total \(\text{Ca}^{2+}\) entering the cell during depolarization, they are capable of triggering a cell death signal with more efficacy than N- or P/Q-channels. Now the question arises as to how such \(\text{Ca}^{2+}\) entry through L-channels causes cell death.

Prolonged depolarization induced by \(\text{K}^+\) caused a transient sharp rise of \([\text{Ca}^{2+}]_m\), followed by a sustained plateau. Although this bulk \([\text{Ca}^{2+}]_m\), elevation reached only around 1 \(\mu\text{M}\), it is certain that mitochondria see greater \(\text{Ca}^{2+}\) transients at sub-plasmalemmal sites near the \(\text{Ca}^{2+}\) channels; thus, using mitochondrially targeted aequorin, we have recently shown that \(\text{Ca}^{2+}\) inside the mitochondria can reach near millimolar concentrations during cell depolarization (52). If these elevations of \([\text{Ca}^{2+}]_m\) are sustained, then the mitochondrial transition pore will open (49, 53, 54), and the apoptotic cascade will be activated. These findings suggest the following. (i) Cell exposure to \(\text{K}^+\) plus FFPL64176 caused depolarization of mitochondria, surely due to mitochondrial \(\text{Ca}^{2+}\) accumulation; (ii) CaA, a blocker of the mitochondrial transition pore, prevented the apoptotic cell death induced by \(\text{K}^+\) plus FFPL64176; (iii) and SOD also suppressed this apoptotic signal, suggesting that mitochondrial \(\text{Ca}^{2+}\) overload was generating free radicals to cause cell death (13, 55, 56).

It is interesting that nimodipine provided full protection against both necrotic and apoptotic cell death. This finding reinforces the view that nimodipine might have direct neuroprotectant effects on neurons subjected to an ischemic insult (14, 57) in addition to its well known cerebrovascular vasodilatory effects (58). The neuroprotectant actions of nimodipine in clinical trials performed in patients suffering a thrombotic stroke have proven difficult to demonstrate (59–61). However, in experimental animal models of cerebral ischemia, a clear nimodipine-induced neuroprotection has been shown (22, 62), which is in line with the results of the experiments shown here.

In conclusion, our data suggest that N- and P/Q-type \(\text{Ca}^{2+}\) channels, which suffer rapid \(\text{Ca}^{2+}\)-dependent inhibition after cell depolarization, are unlikely to contribute to cell death upon a depolarizing stimulus. This observation is in line with previous data from our laboratory indicating that blockers of N- and P/Q-type \(\text{Ca}^{2+}\) channels did not protect against veratridine-induced cell death (64, 65). However, they disagree with the observation that N-type \(\text{Ca}^{2+}\) channel blockers afforded protection in a rat model of cerebral ischemia (15). In contrast, L-type channels that are localized preferentially at the neuronal soma (66) inactive more slowly and are clearly associated with cell \(\text{Ca}^{2+}\) overload, mitochondria depolarization, generation of free radicals, and cell death. These data strengthen the view that \(\text{Ca}^{2+}\) entry through L-channels during a cerebral ischemic condition causing neuronal depolarization (67) might be a critical determinant of delayed death of neurons located in the penumbra area. Hence, dihydropropyridine blockers of L-channels should have pronounced neuroprotectant actions if given with an adequate therapeutic window to stroke patients.

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33. Neher, E. (1989) in Neuromuscular Junction (Sellin, L. P., Libelius, R., and Theileff, S., eds) pp. 65–76, Elsevier, Amsterdam

34. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450

35. Mattson, M. P., Zhang, Y., and Buse, S. (1993c) Exp. Neurol. 121, 1–13

36. Krajeweski, S., Krajeweski, M., Ellerby, L. M., Welsh, K., Xie, Z., Deveraux, Q. L., Salvesen, G. S., Breeden, D. E., Rosenhal, R. E., Fiskum, G., and Reed J. C. (1999) P. Natl. Acad. Sci. U. S. A. 96, 5752–5757

37. Gruisse, M., Tieuex, O., Dabadjie, P., Georgescand, D., and Mazat, J. P. (1990) Biochem. J. 271, 269–272

38. Villarroya, M., Olivares, R., Ruiz, A., Cano-Abad, M., De Pascual, R., Lomax, R. B., Lopez, M. G., and Garcia, A. G. (1996) J. Physiol. 516, 421–432

39. Henandez-Guijio, J. M., Manueu-Flores, V. E., Ruiz-Nuio, A., Villarroya, M., Garcia, A. G., and Gandia, L. (2001) J. Neurosci. 21, 2553–2560

40. Michealena, P., Garcia-Perez, L. E., Artalejo, A. R., and Garcia, A. G. (1993) P. Natl. Acad. Sci. U. S. A. 90, 3284–3288

41. Garcia, A. G., Sala, F., Reig, J. A., Vinuegra, S., Frias, J., Fontiz, R., and Gandia, L. (1984) Nature 309, 69–71

42. Fontiz, R., Gandia, L., Lopez, M. G., Artalejo, C. R., and Garcia, A. G. (1987) Brain Res. 408, 359–363

43. Krumn, I. I., and Mattson, M. P. (1999) J. Neurochem. 72, 529–540

44. Turrens, J. F., Alexander, A., and Lehninger, A. L. (1985) Arch. Biochem. Biophys. 237, 408–414

45. Coyle, J. P., and Puttfarden, P. (1993) Science 262, 689–696

46. Szalai, G., Krishnamurthy, R., and Hajneczky, G. (1999) EMBO J. 18, 6349–6361

47. Griffiths, E. J., and Halestrap, A. P. (1995) Biochem. J. 307, 93–98

48. Schinder, A. F., Olsen, E. C., Spitzer, N. C., and Montal, M. (1996) J. Neurosci. 16, 6125–6133

49. Duchen, M. R. (1999) J. Physiol. 516, 1–17

50. Beatrice, M. C., Palmer, J. N., and Pfeiffer, D. R. (1980) J. Biol. Chem. 255, 8663–8671

51. Gunter, T. E., and Pfeiffer, D. R. (1990) Am. J. Physiol. 258, C755–C786

52. Montero, M., Alonso, M. T., Carnicerio, E., Cuchillo, I., Alhilios, A., Garcia, A. G., Garcia-Sanchez, J., and Alvarez, J. (2000) Nat. Cell Biol. 2, 57–61

53. Green, D. R., and Reed, J. C. (1998) Nature 281, 1309–1312

54. Hengartner, M. O. (2000) Nature 407, 770–776

55. Carriedo, S. G., Yin, H. Z., Senis, S. L., and Weiss, J. H. (1998) J. Neurosci. 18, 7727–7738

56. Yuan, J., and Yankner, B. A. (2000) Nature 407, 802–808

57. Ahmed, N., Nasman, P., and Wahlgren, N. G. (2000) Stroke 31, 1250–1255

58. Allen, G. S., and Banghart, S. B. (1979) Neurosurgery 4, 37–42

59. Mehr, J. P., Orgogozo, J. M., Harrison, M. J. G., Hennerici, M., Wahlgren, N. G., Gelmers, J. H., Matinez-Vila, E., Dycka, J., Tetternborn, D. (1994) Cerebrovasc. Dis. 4, 197–203

60. Fugelholm, R., Erila, T., Palomaki, H., Murros, K., and Kaste, M. (2001) Cerebrovasc. Dis. 16, 189–193

61. Horn, J., de Haan, R. J., Vermeulen, M., and Limburg, M. (2001) Stroke 32, 461–465

62. Hoffmeister, F., Kazda, S., and Krause, H. P. (1979) Acta Neurol. Scand. 60, 358–359

63. Rada, J. M., Carceller, F., Diez-Tejedor, E., and Avendaño, C. (1995) Stroke 26, 1888–1892

64. Maroto, R., de la Fuente, M. T., Artalejo, A. R., Abad, F., López, M. G., García-Sanchez, J., and Garcia, A. G. (1994) Eur. J. Pharmacol. 270, 301–309

65. Maroto, R., de la Fuente, M. T., Zapater, P., Abad, F., Esquerro, E., and García, A. G. (1996) Brain Res. 714, 209–214

66. Murphy, T. H., Welley, P. F., and Baraban, J. M. (1991) Neuron 7, 625–635

67. Blank, W. K., and Kirshner, H. S. (1977) Brain Res. 123, 115–124

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Mariá F. Cano-Abad, Mercedes Villarroya, Antonio G. García, Nelson H. Gabilan and Manuela G. López

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