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Nifedipine does not induce but rather prevents apoptosis in cardiomyocytes

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Abstract

The potential of Ca^{2+} channel antagonists, particularly nifedipine, to cause apoptotic cell death has been controversial and is of considerable importance for cardiomyocytes as loss of these cells is an important component of the pathophysiology leading to heart failure. To examine the hypothesis that nifedipine induces cell death and modulates calcium-induced apoptosis, cardiomyocytes in culture from embryonic chick hearts, that readily manifest apoptosis, were studied. Apoptosis was evaluated by fluorescent activated cell sorting (FACS) analysis and by quantitative analysis of DNA fragmentation by an enzyme-linked immunosorbent assay (ELISA) specific for histone-associated DNA fragments of mono- and oligonucleosome size. Cell death was evaluated by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Cardiomyocytes were treated with various concentrations of nifedipine over a concentration range relevant to serum concentrations in man. Nifedipine, 1 to 100 μM, did not produce cell death in cardiomyocytes. There was no evidence of apoptosis on FACS analysis of cardiomyocytes stained with fluoresceine diacetate or propidum iodide (PI). Neither was there any evidence of apoptotic nuclei on PI staining of permeabilized cardiomyocytes treated with nifedipine. In contrast, DNA fragmentation consistent with apoptosis was induced in a significant ($P < 0.05$) concentration-dependent manner, by increases in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Importantly, nifedipine reduced DNA fragmentation produced by increased $[Ca^{2+}]_o$. Furthermore, nifedipine blocked calcium-induced cell death as high $[Ca^{2+}]_o$ significantly ($P < 0.05$) reduced cell viability. These data indicate that nifedipine does not induce apoptosis in cardiomyocytes rather apoptosis in cardiomyocytes is under regulatory control by Ca^{2+} and nifedipine can antagonize Ca^{2+}-mediated apoptotic cell death.

Keywords: Apoptosis; Ca^{2+}; Nifedipine; Cardiomyocyte

1. Introduction

Death of cardiomyocytes is fundamental to the process of myocardial infarction and is an important component of the pathophysiology leading to heart failure. Cell necrosis had been considered, until recently, to be the dominant form of myocardial cell death, however, new studies have established that apoptotic cell death has a significant role in myocardial infarction and heart failure in man (Itoh et al., 1995; Narula et al., 1996; Olivetti et al., 1997; Veinot et al., 1997). Apoptosis is a type of cell death in which the cell actively participates in its own destruction utilizing a set of genetic programmed responses and a complex network of enzymes (Wyllie, 1992; MacLellan and Schneider, 1997; Trump et al., 1997). In the search for mechanisms underlying the process of apoptosis, a role for calcium was suggested based on several lines of evidence (for reviews see Dowd, 1995; McConkey and Orrenius, 1996; Nicotera and Orrenius, 1998). First, increased levels of intracellular calcium ($[Ca^{+2}]_i$) may trigger activation of Ca^{+2}-dependent enzymes including Ca^{+2}-dependent kinases and endonucleases culminating in cell death (El Alaoui et al., 1992). Second, the cytotoxicity of various agents leading to apoptosis appears to be mediated through Increases in $[Ca^{+2}]_i$ (Nicotera and Orrenius, 1998). Third, nuclear Ca^{+2} transport and the regulation of nuclear Ca^{+2}-dependent enzymes are involved during apoptosis in many cell types (Nicotera and Orrenius, 1998). Fourth, cell death may be countered by Ca^{+2} chelating agents (Lockshin and Zakeri, 1994). As well, the transfection of genes encoding Ca^{+2} buffering proteins such as calbindin-D28K blocked cell death (Dowd et al., 1992). In contrast, there is considerable data that $[Ca^{2+}]_i$ is not operative in apoptosis. Apoptosis can be produced by factors which are independent of

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calcium, suggesting that calcium may be involved in only a few of the many pathways leading to apoptosis (Alnmeri and Litwak, 1990; Bansal et al., 1990). Reduction of $[Ca^{+2}]_i$ does not consistently reduce apoptosis (Kluck et al., 1994). Furthermore, increases in cytosolic calcium can occur independent of apoptosis (Lennon et al., 1992).

The role of calcium channel blockers particularly nifedipine, in apoptosis has been controversial. It has been suggested that calcium channel blockers use is associated with an increase in risk of cancer because calcium channel blockers antagonize the development of apoptosis, whereby the body can eliminate premalignant cells, but these results were questioned because of the retrospective nature of the their study design (Pahor et al., 1996; Messerli and Grossman, 1998). Nifedipine, in combination with antineoplastic agents, accentuates apoptosis in multidrug-resistance cancer in vitro (Kondo et al., 1995) but this has not been consistently demonstrated in clinical studies (Holmes et al., 1997). Nifedipine has been found to minimize apoptosis in prostate (Kyprianou et al., 1988) but accentuate apoptosis in the thymus (Balakumaran et al., 1996). DeBlois et al. (1997) reported that nifedipine increased apoptosis in aortic media while nifedipine has been shown to protect human endothelial and aortic smooth muscle cells from apoptosis induced by oxidized cholesterol (Ares et al., 1997; Escargueil-Blanc et al., 1997). Nifedipine has been found to limit the potential of some interventions to protect against apoptosis in neurones thereby accentuating apoptosis (Enokido and Hatanaka, 1993) while nifedipine may protect against chemical-induced apoptosis in seminiferous tubules (Li et al., 1997) There is a clear need to determine the effect of nifedipine on cardiomyocyte cell death because of the potential importance of apoptosis in cardiac diseases (Itoh et al., 1995; Narula et al., 1996; MacLellan and Schneider 1997; Olivetti et al., 1997; Veinot et al., 1997). The objective of this study was to examine the hypothesis that nifedipine induces apoptotic cell death in cardiomyocytes and modulates apoptosis produced by other interventions.

2. Materials and methods

2.1. Cell cultures

Chick embryonic ventricular cells were cultured from 7-day chick embryos from white Leghorn eggs as described previously (Rabkin, 1993). The protocol was approved by the University committee on use of animals for research. Myocytes were maintained in culture in medium 818A [73% DBSK (NaCl 116 mM, $MgSO_4$ 0.8 mM, NaH_2PO_4 0.9 mM, dextrose 5, 5 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 26 mM), 20% M199, 2% or 6% fetal calf serum and 1% antibiotic-antimycotic (10,000 μg/ml streptomycin sulfate, 10,000 U/ml penicillin G sodium and 25 μg/ml amphotericin B)] for 72 h prior to the experiment. The proportion of myocytes at this time was at least 90% as verified by the proportion of cells showing spontaneous contraction or displaying muscle specific markers on immunohistologic examination.

2.2. Cell viability: MTT assay

The MTT assay, an index of cell viability and cell growth, is based on the ability of viable cells to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) from a yellow water soluble dye to a dark blue insoluble formazan product (Mosmann, 1983; Loveland et al., 1992). Cardiomyocytes were seeded in multiwell microtitre plates (Falcon #3072, Becton Dickinson, Lincoln Park, NJ, USA) at 20,000 cells per well and incubated at 37°C for 72 h. Then, cardiomyocytes were treated with nifedipine or diluent and returned to the incubator for 24 h. MTT dye, suspended in Delbucco Phosphate Buffered saline (pH 7.35) (Promega, Madison, WI, USA.) was added to each well for the last 4 h of treatment. The reaction was stopped with the addition of solubilization reagent (Promega, Madison, Wi, USA.) and the optical density was determined at 590 nm on a multiwell plate reader (BioRad model #3550, BioRad, Mississauga, Canada). The absorbance or optical density curve of MTT shows that 590 nm is on the linear portion of the curve which extends to 655 nm. Background absorbance of medium in the absence of cells was subtracted. All samples were assayed in duplicate and the mean for each experiment was calculated.

The relationship of the absorbance of MTT to cell number was verified in separate experiments in which 1200 to 37,000 cardiomyocytes were added to different wells of a microtitre plate.

2.3. Fluorescent activated cell sorting (FACS) analysis

Several different cell preparation and staining methods were used followed by FACS analysis. Cardiomyocytes, at 72 h of culture, were exposed to nifedipine. After 24 h, the reaction was stopped by the removal of media followed by exposure to a trypsin (0.01% in DMS8) for 20 min at 37°C to suspend the adherent cells. Shorter durations of trypsin exposure do not remove adherent cells. Trypsinization was stopped by dilution with 818A media containing 6% fetal calf serum. The suspended cardiomyocytes were gently spun down at 1000 g for 5 min) and washed with phosphate buffered saline (PBS — NaCl 137 mM, KCl 3 mM, Na_2HPO_4 4 mM, KH_2PO_4 1.8 mM) to remove the trypsin. Cardiomyocytes were incubated with fluoroscein diacetate (FDA), 20 μM for 30 min at 37°C. The cells were centrifuged at 1000 g for 5 min at 4°C and the supernatant containing FDA was removed. Cells were then resuspended in 0.5 ug/ml propidium iodide (PI), for 30 min at room temperature. Cardiomyocytes, approximately 10,000, were aspirated into the FACS machine (Flow cytometer,

model Epics XL MCL, Coulter Electronics, Burlington, Canada) and examined for fluorescence on separate channels for FDA (FL1) and PI (FL3).

To examine nuclear characteristics based on propidium iodide staining, after cardiomyocytes were exposed to drugs, the reaction was stopped by the removal of media from the cells followed by brief exposure to trypsin (0.01% in DMS8) to suspend the adherent culture. Trypsinization was halted by dilution with 818A media containing 6% fetal calf serum. The suspended cardiomyocytes were gently spun down and washed with PBS. Cells were then permeabilized with 70% ethanol for 30 min at room temperature. Cells were then spun down and the ethanol removed. The resulting permeabilized cells were than stained with PI staining mix (Triton X-100, EDTA, RNAse A, propidium iodide) to visualize the nuclei.

2.4. DNA fragmentation on enzyme-linked immunosorbent assay (ELISA)

DNA fragmentation was quantitated using an assay based on the quantitative sandwich-enzyme immunoassay principle using monoclonal antibodies directed against DNA and histones. This ELISA quantitates histone associated fragments of mono- and oligonucleosome size and has been verified to detect apoptosis in various cell types (Leist et al., 1994; Bonfoco et al., 1995). Cardiomyocytes were grown in petri dishes and treated with drug for predetermined times. After treatment, the medium was removed. Cells were washed with PBS at 4°C, lysed, scaped gently into eppendorf tubes and spun at 20,000 × *g* for 10 min at 4°C. The supernatant was removed and added to wells of a microtitre plate that had been precoated with monoclonal antibodies from mouse (clone H11-4) specific for H1,H2A, H2B, H3 and H4 (Boehringer Mannheim, Laval, Canada). The DNA of the nucleosomes is tightly complexed so that these core histones are protected from endonucleases. The hallmark of apoptosis is endonuclease mediated DNA degradation that in turn exposes these histones. The supernatant was allowed to react with the immobilized primary antibody. After several washings, the secondary antibody, anti-DNA-peroxidase, was added and incubated for 90 min (Boehringer Mannheim, Laval, Canada). The excess antibodies were washed off and the substrate reaction solution (Monolisa®, Sanofi, Marnes La Coquette, France) was added. Absorbance was read at 490 nm against substrate solution as a blank which was subtracted from other wells.

2.5. Materials

All cell culture components were from Gibco/BRL Life Sciences (Burlington, Canada). Nifedipine was from Sigma (St. Louis, USA). Components of the ELISA were from Boerhinger Mannheim (Laval, Canada) and Sanofi (Marnes La Coquette, France). The MTT assay was from Promega (Madison, WI, USA). All chemicals were purchased from Fischer Scientific (Ottawa, Canada). All other chemicals for flow cytometry were from VWR (Mississauga, Canada)

2.6. Data analysis

The data are presented as the mean ± S.E.M. Hypothesis testing used one way analysis of variance. Examination of a pair of groups used Kruskal–Wallis multiple-comparison test. Simple linear least squares analysis was also used. The null hypothesis was rejected if the probability of a Type I error was less than 5% ($P < 0.05$).

3. Results

To examine the potential of nifedipine to induce cell death, cardiomyocytes were treated with nifedipine and cell viability was assessed with the MTT assay. This assay measures cellular metabolic activity and has been found to correlate with the viability of various kinds of cells as it requires viable cells to reduce the MTT dye (Mosmann, 1983; Loveland et al., 1992). The assay was validated in cardiomyocytes and a significant ($P < 0.05$) linear relationship was observed over a range of cell number from 1200 to 37,000 per well between absorbance or optical density and cell number ($r = 0.98$). Based on these data subsequent experiments were performed with 20,000 cardiomyocytes per well. Nifedipine, over a concentration range that is relevant to serum concentrations of this agent in man (Brown et al., 1986; Myers and Raemsch, 1987), namely 0.1 to 100 μM, did not significantly alter cell viability (Fig. 1). There were no trends that suggested a possible dose dependent cardiotoxicity of nifedipine.

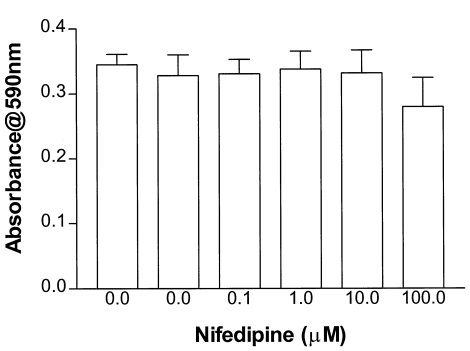

Fig. 1. Cardiomyocyte viability after treatment with nifedipine as assayed by the MTT assay. Cardiomyocytes were seeded in multiwell microtitre at 20,000 cells per well. After 72 h, cells were treated with nifedipine or diluent (control) for 24 h. MTT dye was added to each well for the last 4 h of treatment. The reaction was stopped and the absorbance (optical density) was determined at 590 nm. Background absorbance of medium in the absence of cells was subtracted. The results are presented as the mean ± S.E.M. for control (diluent treatment) ($N = 35$), nifedipine 0.01 μM ($N = 4$), nifedipine 0.1 μM ($N = 18$), nifedipine 1.0 μM ($N = 20$), nifedipine 10 μM ($N = 4$).

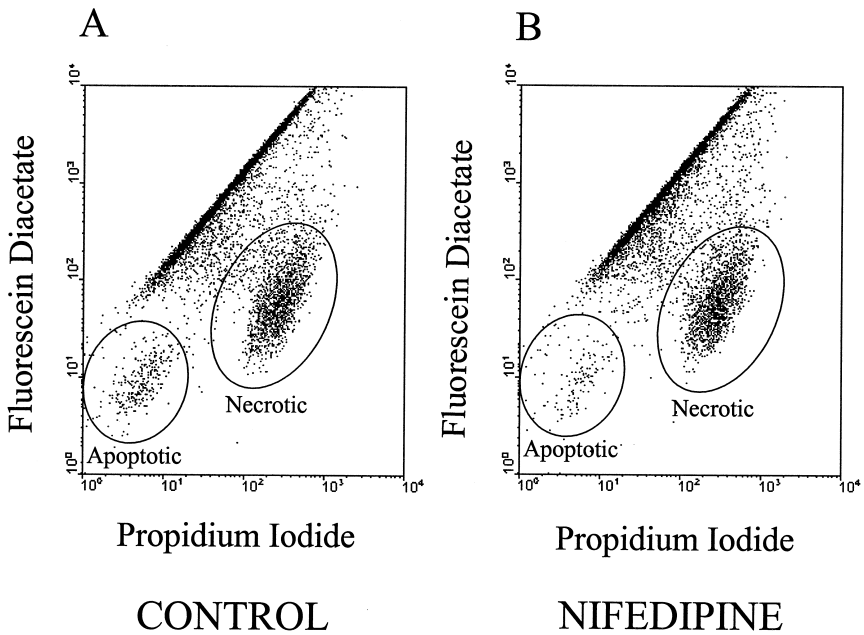

Fig. 2. Cardiomyocyte cell population characterized by dual staining with FDA and PI and examined by FACS analysis. Cardiomyocytes were dually stained with fluorescein diacetate (FDA) (fluorescence intensity shown on the *Y* axis) and propidium iodide (*X* axis) and the population dot plots are shown. An apoptotic population (low FDA, low PI) was observed in cardiomyocytes (circled population) as well as a necrotic population defined as low FDA with higher PI. This shows a representative study of 10,000 control (diluent treated) cells (A) and a representative study of 10,000 cells treated with nifedipine 100 μM.

Other and more sensitive indicators of cell viability, specifically FDA and PI, that rely on different indices of cell function were used next. FDA reflects membrane integrity as this dye readily penetrates the cell and remains in an intracellular location unless the cell is irreversibly damaged and allows the FDA to leak out of the cell (Darzynkiewicz et al., 1977). PI is a fluorochrome that only penetrates cells with damaged membranes but does not penetrate viable cells or cells with an intact sarcolemmal membrane (Darzynkiewicz et al., 1977). This dual staining permits cell to be identified as viable cells by FDA staining and further classifies dead cells by PI staining according to the type of cell death. Dead cells which lack membrane integrity are classified as *necrotic* cells because of the loss of sarcolemmal membranes, while dead cells with little PI staining are in apoptotic cells as they continue to possess membrane integrity (Wyllie, 1992). The majority of the 10,000 cells sampled show FDA uptake with fluorescent values over 10^2 thereby defining the normal population (Fig. 2). Two small populations of cells were identified with lower FDA uptake that were further distinguished by PI fluorescence into a small population with low PI uptake- fluorescence between 10^0 to 10^1, the apoptotic population, and a larger population with PI fluorescence 10^2 to 10^3, the necrotic population. There were no alterations in the cell population characteristics of these cardiomyocytes after nifedipine treatment. The proportion of the population classified as normal or viable cells was $77.5 \pm 1.6\%$ in the control group that was not significantly different from $79.5 \pm 2.2\%$ and $78.1 \pm 1.2\%$ in the group treated with respectively nifedipine 10 μM or 100 μM. Nifedipine, 10 or 100 μM, did not significantly increase the population of apoptotic or necrotic cells (Fig. 3).

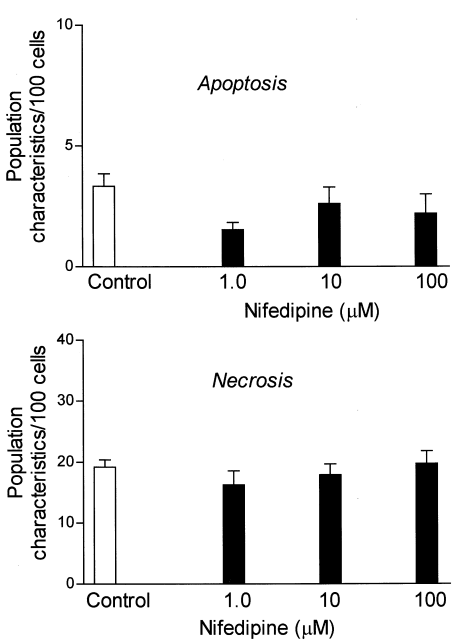

Fig. 3. The effect of nifedipine on the population of apoptotic and necrotic cells. The proportion of the cell population with characteristics of apoptosis (upper panel) or necrosis (lower panel) from FACS analysis of cardiomyocytes treated with nifedipine 1 μM ($N = 5$), 10 μM ($N = 7$) or 100 μM ($N = 3$) are compared to control (diluent treated) cells ($N = 10$) from independent experiments of 10,000 cells each. The data are expressed as the mean ± S.E.M. per 100 cells.

To examine the cellular characteristics of apoptosis, specifically to focus on the nuclear changes of apoptosis, cardiomyocytes were permeabilized and stained with PI. Normal nuclei of permeabilized cells are readily stained by PI, while apoptotic nuclei are poorly stained by PI; a finding attributed to the structural alterations apoptotic nuclear chromatin (Wyllie, 1992; Darzynkiewicz et al., 1977). The majority of permeabilized cardiomyocytes were stained with PI and demonstrated a level of fluorescence greater than 10^2 while a small cell population of apoptotic cells with low PI fluorescence was evident. Nifedipine did not alter the PI staining characteristics of cardiac nuclei (Fig. 4). In contrast, camptothecin, which consistently induces apoptosis (Froelich-Ammon and Osheroff, 1995) increased the population of cells with apoptotic nuclei. There were no significant differences in the proportion of cardiomyocytes with apoptotic nuclei among cardiomyocytes treated with nifedipine compared to control (Fig. 4B).

To investigate the potential of nifedipine to blunt cell death, a calcium-dependent model of cell death was evaluated. Cardiomyocytes that had been pretreated with nifedipine were exposed to a high, 10 mM, concentrations of $[Ca^{2+}]_o$ while osmolarity was maintained by reducing extracellular sodium. $[Ca^{2+}]_o$ of 10 mM produced a significant ($P < 0.05$) decrease in absorbance which translates into a significant loss in cell viability as assayed by the MTT assay (Fig. 5). In the presence of nifedipine, increased $[Ca^{2+}]_o$ did not significantly alter cell viability.

To assess the impact of increased $[Ca^{2+}]_o$ on apoptosis. Cardiomyocytes were maintained in culture while $[Ca^{2+}]_o$ was increased from the usual 1.8 mM to 2.5 or 3.0 or 5.0 or 10 mM with osmolarity being maintained by reducing extracellular sodium. Cell were lysed and the DNA was isolated and the extent of DNA fragmentation was quantitated by means of the ELISA (Fig. 6). Increases in $[Ca^{2+}]_o$ induced a significant ($P < 0.05$) increase in DNA fragmentation, specific for histone-associated DNA fragments

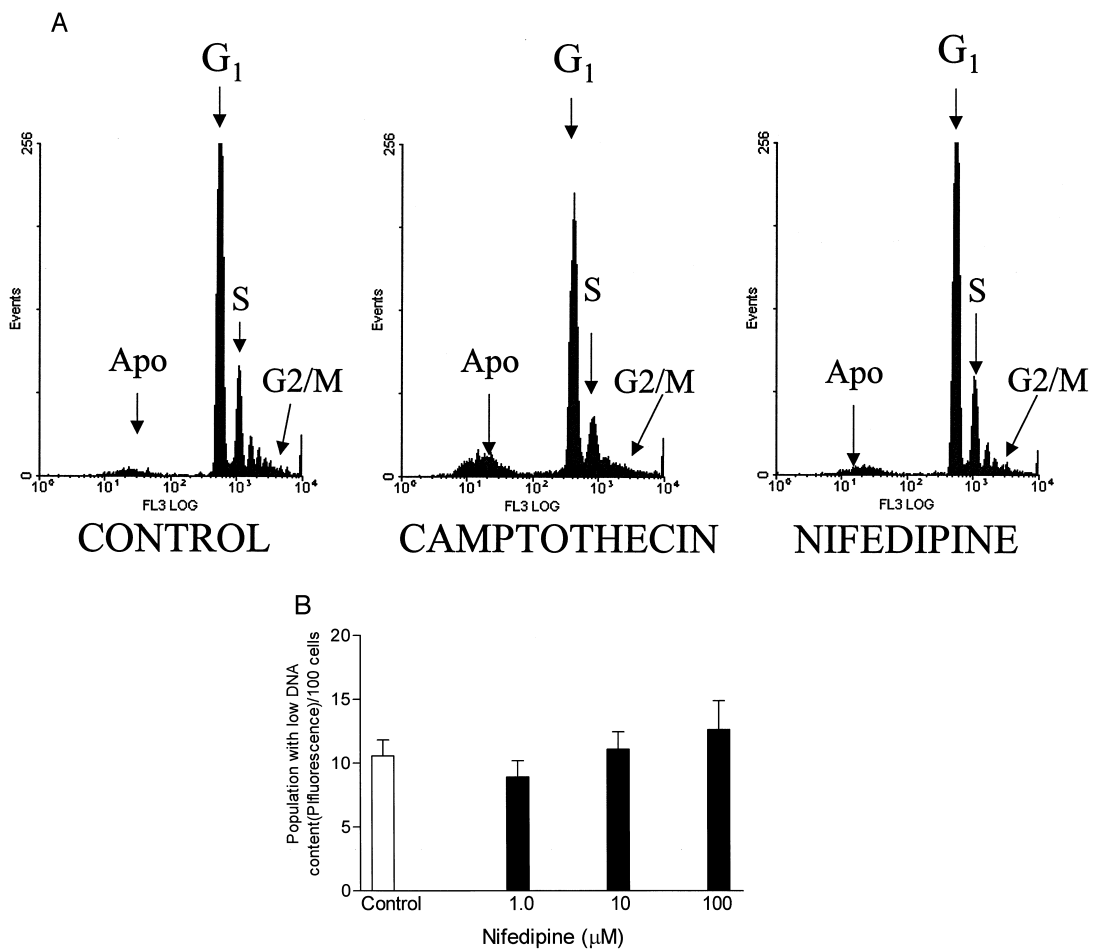

Fig. 4. (A) PI stained DNA histograms of cardiomyocytes. Cardiomyocytes treated with diluent (control), camptothecin (10 μM) or nifedipine, 10 μM, for 24 h were fixed with ethanol and then stained with propidium iodide and assessed by flow cytometry. A representative study of 10,000 cells for each condition is shown. The population of low PI staining is indicative of apoptosis (Apo). (B) The proportions of cardiomyocyte with DNA content consistent with apoptosis. Cardiomyocytes treated with diluent (control) or nifedipine for 24 h were fixed with ethanol and then stained with propidium iodide and assessed by flow cytometry. The proportion of cells with PI staining fluorecence (FL3) less than 10^2 was defined as apoptosis (see Fig. 5a) and is shown. The data is the mean ± S.E.M. for control ($N = 10$), nifedipine 1 μM ($N = 5$), 10 μM ($N = 7$) and 100 μM ($N = 3$) for independent experiments of 10,000 cells each and is expressed per 100 cells.

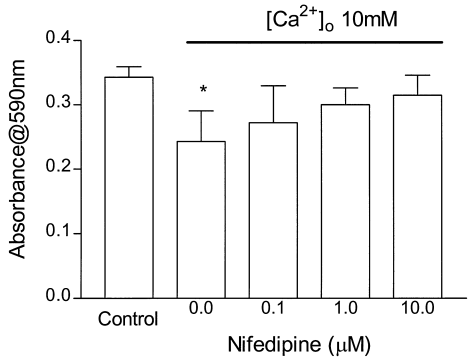

Fig. 5. Cardiomyocyte viability as assayed by the MTT assay after treatment with nifedipine in the presence of high extracellular Ca^{2+} concentrations. Cardiomyocytes were seeded in multiwell microtitre at 20,000 cells per well. After 72 h, cells were treated with nifedipine or diluent (control) in the presence of extracellular calcium concentration of 10 mM for 24 h. MTT dye was added to each well for the last 4 h of treatment. The reaction was stopped and the absorbance (optical density) was determined at 590 nm. Background absorbance of medium in the absence of cells was subtracted. The results are presented as the mean ± S.E.M. Optical density measurements are shown for the groups — calcium 10mM without nifedipine ($N = 7$) or with nifedipine 0.1 ($N = 5$), 1.0 μM ($N = 5$), 10.0 μM ($N = 5$) and the control with extracellular calcium of 1.8 mM ($N = 35$). The control group is repeated from Fig. 1 but includes controls done at the same time as nifedipine groups and it did not differ from the overall mean value. Osmolarity was maintained by altering the sodium chloride in the medium. Data analysis compared each group to control ($^*P < 0.05$).

of mono- and oligonucleosome size characteristic of apoptosis (Leist et al., 1994; Bonfoco et al., 1995). $[Ca^{2+}]_o$ of 10 mM produced a significant ($P < 0.05$) and 2.5-fold increase in the amount of DNA fragmentation. The effect of nifedipine on calcium mediated DNA fragmentation was examined next. The significant increase in DNA fragmentation and apoptosis induced by increasing concentrations of $[Ca^{2+}]_o$ were not observed with nifedipine, 10

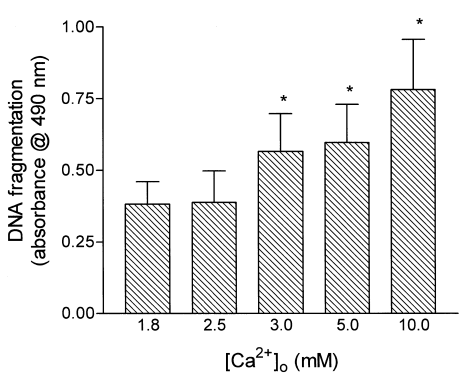

Fig. 6. DNA fragmentation quantitated by an enzyme-linked immunosorbent assay (ELISA). Cardiomyocytes were maintained in multiwell plates for 72 h and then were incubated with different concentrations extracellular calcium ($[Ca^{2+}]_o$). Osmolarity was maintained by altering the sodium chloride in the medium. Each sample was done in duplicate. DNA fragmentation was quantitated by an ELISA with determination of absorbance at 490 nm. The data are the mean ± S.E.M. for $[Ca^{2+}]_o$ of 1.8 mM ($N = 10$), 2.5 mM ($N = 5$), 3 mM ($N = 9$), 5.0 mM ($N = 9$) and 10 mM ($N = 10$). Data analysis compared each group to control ($^*P < 0.05$).

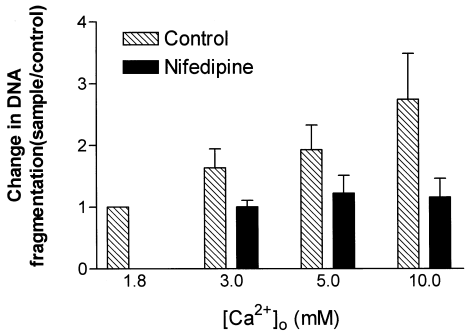

Fig. 7. The effect of nifedipine on apoptosis induced by increases in extracellular Ca^{2+}. Cardiomyocytes were maintained in multiwell plates for 72 h and then were incubated with usual (1.8 mM) or high extracellular calcium ($[Ca^{2+}]_o$) alone or with nifedipine 10 μM for 24 h. Each sample was done in duplicate. DNA fragmentation was quantitated by an enzyme-linked immunosorbent assay (ELISA) with determination of absorbance at 490 nm. The data is expressed as a change from $[Ca^{2+}]_o$ of 1.8 mM for $[Ca^{2+}]_o$ of 3 mM (control $N = 9$ and nifedipine $N = 5$), 5.0 mM (control $N = 9$ and nifedipine $N = 5$) and 10 mM (control $N = 10$ and nifedipine $N = 5$). The data are shown as the mean ± S.E.M.

μM, pretreatment of cardiomyocytes (Fig. 7). Rather the level of DNA fragmentation was no different from control.

4. Discussion

A major finding of this study is its demonstration that nifedipine does not induce apoptotic cell death in cardiomyocytes. We utilized several different approaches to detect cell death and identify apoptosis. FACS analysis has the advantages of specific examination of a large population of individual cells and the assessment of the changes in the proportion of the total cell population displaying characteristics of normal, necrotic and apoptotic cells (Darzynkiewicz et al., 1977; Frey, 1997). Uncharged FDA readily penetrates live cells and because it is a substrate for esterases, ubiquitous enzymes present in all cell types, it is converted to its charged, hydrolysed, fluorescent product. The charged FDA becomes trapped inside the live cell, but can escape from necrotic cells. The dead cell population is identified by a shift of the population to less FDA staining. No increase in the population of dead cells was found after nifedipine treatment. Cross validation of the FACS data was provided by the MTT assay which confirmed that nifedipine did not induce cell death. The MTT assay is based on a different assessment of cell viability namely the ability of viable cells to have sufficient mitochondrial dehydrogenase to reduce MTT (Mosmann, 1983; Loveland et al., 1992). Identification of the type of cell death was achieved using three different approaches for the detection of apoptosis. First, dual staining with PI and FDA, identifies cells with intact or disrupted plasma membrane. In apoptosis, the cell shrinks due to loss of cytoplasmic

volume with condensation of cytoplasmic proteins while the plasma membrane remains intact (Wyllie, 1992; Trump et al., 1997). PI does not penetrate apoptotic cells because of their intact membrane. No increase in the apoptotic cell population was detected in nifedipine treated cardimyocytes using dual staining with FDA and PI. Neither was there an increase in the population of dead cells with disrupted cell membrane that has been classified as necrotic cells. The second approach examined permeabilized cells stained with PI, a dye that readily stains normal nuclei (Darzynkiewicz et al., 1977). The resulting histograms identified a small population of cells that exhibited poorly stained nuclei indicative of apoptosis (Darzynkiewicz et al., 1977; Lin et al., 1997). Again there was no evidence of nifedipine-induced apoptosis. The third approach was an ELISA based method that quantitates histone associated fragments of mono- and oligonucleosome size and has been verified to detect apoptosis in various cell types (Leist et al., 1994; Bonfoco et al., 1995). Taken together these four different methods (FDA/PI staining, reduction of MTT, DNA content analysis and antibodies to mono- and oligonucleosomes) found no evidence of nifedipine-induced cell death and no evidence of nifedipine-induced apoptosis.

The absence of cell death and the absence of apoptosis in nifedipine treated cardiomyocytes, are consistent with the data from human glioblastoma cells in culture (Kondo et al., 1995). In contrast, administration of nifedipine to rats was associated with an increase in apoptotic cells in the thymus and in the aortic media (Balakumaran et al., 1996; DeBlois et al., 1997). Explanations for the differences between our data and others include species differences, the models studied and/or the dosages of nifedipine. Balakumaran et al. (1996) administered nifedipine at 15 mg/kg ip to rats and DeBlois et al. (1997) treated rats with 35 mg/kg per day in the chow. These extremely high doses of nifedipine, by human standards, are necessary because of the rapid metabolism of the drug in the rat. However, they produce high peak and low trough levels of serum nifedipine concentration. This would be anticipated to induce sympathetic activation that may induce apoptosis indirectly through activation of neurohumoral factors (Rabkin and Kong, 1996; Leenen, 1998). Our study used concentrations of nifedipine, 10 and 100 μM, or respectively, 3.5 and 34.6 ng/ml, which are within the range of nifedipine serum concentrations found in patients after acute or chronic nifedipine administration (Brown et al., 1986; Myers and Raemsch, 1987).

Another major finding of this study was that nifedipine reduced apoptosis in cardiomyocytes. This is consistent with its effect to block methoxyacetic acid-induced spermatocyte apoptosis in cultured rat semiferous tubules (Li et al., 1997), apoptosis in prostate cells (Holmes et al., 1997) and oxidized LDL-induced apoptosis in human endothelial cells (Escargueil-Blanc et al., 1997) or human aortic smooth muscle cells (Ares et al., 1997). In contrast, nifedipine antagonized the protective effect of high K^+ to inhibit apoptosis in cerebellar granule cells (Galli et al., 1995) and other neuronal cells (Enokido and Hatanaka, 1993), that was induced by respectively removing extracellular K^+ or high oxygen concentration. Perhaps the adverse effect of nifedipine is relevant to these more complex model of potassium-induced prevention of apoptosis in brain cells produced by certain interventions.

While calcium overload as a mechanism of cardiac cell injury in acute myocardial infarction, reperfusion injury and heart failure is an old hypothesis (Dhalla, 1976; Ver Donck et al., 1993), our demonstration of calcium induced apoptosis in cardiomyocytes is a novel observation. Increases in $[Ca^{2+}]_o$ accentuate myocardial injury during ischemia and reperfusion (Fitzpatrick and Karmazyn, 1984). Our data demonstrate cell death from elevations of $[Ca^{2+}]_o$ and a direct calcium mediated apoptotic cell death in cardiomyocytes. Although other actions of nifedipine might possibly be a factor in our observations, the site of action of nifedipine to prevent apoptosis under these conditions is probably through reductions in calcium entry into the cell as nifedipine blocked the apoptotic effect of increases in $[Ca^{2+}]_o$. Although $[Ca^{2+}]_i$ was not measured in our studies, changes in $[Ca^{2+}]_o$ are readily translated into alterations in $[Ca^{2+}]_i$ (Jimenez et al., 1990; Zaugg et al., 1995). Furthermore, reduction in extracellular calcium often mimics the effects of blockade of calcium channel blockers (Pisani et al., 1998).

The present study used embryonic chick cardiomyocytes. While the origin of the cell type is important in the extrapolation of the data, this cell type has several advantages. First, these cardiac cells in culture permit a clear demonstration of changes of apoptosis (Kong and Rabkin, 1999). Second, components of cell death pathways in this cell type have similarities to humans (Eguchi et al., 1992). Third, intracellular calcium in embryonic chick heart is distributed in three kinetically distinct compartments similar to adult mammals (Prakash et al., 1992).

In summary, we demonstrated that nifedipine does not have a direct effect on cardiomyocytes to induce cardiomyocyte death and specifically apoptosis. In contrast, in a model of cell death induced by elevated extracelluar calcium, nifedipine reduces the extent of apoptosis.

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