Bioscience Reports, Vol. 21, No. 5, October 2001 (© 2002)

Variations in Transmembrane Ca^{2+} Gradient and Apoptosis of Macrophages Induced by Oxidized Low Density Lipoprotein

Xiaoyi Yang,[1] Yuzhong Zhang,[2] Youguo Huang,[3] and Fuyu Yang[3,4]

Received July 10, 2001

While Ca^{2+} has been proposed to be a messenger in OxLDL-induced cell death, few studies have addressed the possibility that it may influence the occurrence of apoptosis and necrosis of macrophages induced by OxLDL in virtue of change of transmembrane Ca^{2+} gradient including that across plasma membrane and intracellular organelle membranes. In this paper, various lipophilic Ca^{2+} fluorescent indicators and specific organelle markers were used to study the relationship between the changes of the transmembrane Ca^{2+} gradients and the OxLDL induced apoptosis of macrophages. Our results showed that following exposure of low dose OxLDL to macrophages, the transmembrane Ca^{2+} gradient across the plasma membrane, as well as the membrane-proximal Ca^{2+} gradient, the transnuclear, and the transmitochondrial membrane Ca^{2+} gradient were all changed significantly. These data suggested that changes in transmembrane Ca^{2+} gradients might be involved in the apoptosis of macrophages induced by OxLDL.

KEY WORDS: Apoptosis; necrosis; macrophage; OxLDL; transmembrane Ca^{2+} gradient; atherosclerosis.

ABBREVIATIONS: LDL, low density lipoprotein; LSCM, Laser Scanning Confocal Microscopy; OxLDL, Oxidized LDL; SR, sarcoplasmic reticulum; TBARS, thiobarbituric acid reactive substances; Tg, Thapsigargin

INTRODUCTION

Calcium ion, primarily as ubiquitous "second messenger", is involved in a wide range of cellular processes. Unlike other signaling molecules such as cAMP, cGMP and IP_3, Ca^{2+} cannot be metabolized nor degraded. Calcium homeostasis is therefore regulated through Ca^{2+} transport systems across the plasma membrane or the membranes of intracellular Ca^{2+} pools [1–3]. It is well known that cytosolic free [Ca^{2+}] in most cells is around 10^{-7}–10^{-6} M, whereas extracellular [Ca^{2+}] is about 10^{-3} M. This results in a 1000–10,000 fold transmembrane Ca^{2+} gradient across the plasma membrane. In fact, there are also intracellular transmembrane Ca^{2+} gradients across

[1]Present address: National Cancer Institute—FCRDC, P.O. Box B, Federick, MD 21702, U.S.A., Fax: 301-846-6187; E-mail: yangx@mail.ncifcrf.gov
[2]Present address: Molecular Probes, Inc., OR 97448, U.S.A.
[3]National Laboratory of Biomacromolecules, Institute of Biophysics, the Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, China. Fax: 86-10-64872026; E-mail: yangfy@sun5.ibp.ac.cn
[4]To whom correspondence should be addressed.

0144-8463/01/1000-0667/0 © 2002 Plenum Publishing Corporation

organelle membranes such as mitochondrial membrane, sarcoplasmic reticular membrane, and nuclear membrane. Recently, it was found that a proper transmembrane Ca^{2+} gradient may play an important role in modulating the conformation and activity of membrane proteins such as SR Ca^{2+}-ATPase, glucose transporter, and those related to signal transduction, e.g., β-adrenergic receptor, stimulatory GTP-binding protein and adenylyl cyclase by mediating the physical state change of the phospholipids [4–8]. However, the correlation of transmembrane Ca^{2+} gradients with cellular functional processes such as cell proliferation, differentiation, apoptosis, and especially their changes in different pathological statuses such as atherosclerosis is still poorly understood.

Within human atherosclerotic plaque, there is no doubt that both macrophages and smooth muscle cell death [9, 10]. It is well known that nearly all the macrophages in the atherosclerotic lesion are bloated within lipid (macrophage derived foam cells). Their life-span is unknown and probably variable, but there is now little doubt their death contributes to the origin and enlargement of the acellular lipid core of the advanced atherosclerotic lesion [11]. Two types of cell death, necrosis and apoptosis, have been discriminated. The mechanism and cell death occurring in atherosclerotic areas may be important, because apoptotic cells are rapidly engulfed and cleared whereas necrotic cell debris may trigger a local inflammatory response [12]. It has been proposed that high dose of OxLDL may be capable of inducing cell toxicity of macrophages by apoptosis or necrosis [12–15]. It is therefore of interest to understand the cellular signal mechanisms regulating the susceptibility of cells to OxLDL cytotoxicity and directing cells toward necrosis or apoptosis. Studies of human peripheral blood mononuclear cells have suggested that this toxicity is in part due to an enhancement of the sphingomyelin/ceramide pathway [16]. Ca^{2+} has been proposed to be a messenger in OxLDL-induced cell death [12]. However, few studies have addressed the possibility that calcium may influence the occurrence of apoptosis and necrosis of macrophages induced by OxLDL by virtue of changing the transmembrane Ca^{2+} gradient including that across the plasma membrane and the intracellular organelle membranes.

In order to investigate whether changes in the transmembrane Ca^{2+} gradients are associated with apoptosis or necrosis of macrophages, we chose macrophages from C57BL/6J, a mouse strain susceptible to atherosclerosis *in vivo* [17, 18], as a model system to confirm that OxLDL could induce the apoptosis of macrophages. Our results showed that exposure of low dose OxLDL to macrophages, the transmembrane Ca^{2+} gradient across the plasma membrane, as well as near-membrane Ca^{2+} gradient, the transnuclear and the transmitochondrial membrane Ca^{2+} gradient were all changed significantly. Taken together, these data suggest that the changes in transmembrane Ca^{2+} gradients may be involved in the occurrence of apoptotic or necrotic pathway of macrophages induced by OxLDL.

MATERIALS AND METHODS

Reagents

Hoechst 33342, Propidium iodine (PI), Mito-Tracker Red, Indo-I/AM, Fluo-3/AM, Fura-Red^{TM} and Calcium Green C_{18} were purchased from Molecular Probes, Inc. (Eugene, OR). Ethidium bromide (EB) and RNase were purchased from Sigma (St. Louis, MO).

Preparation, Modification and Characterization of OxLDL

Human LDL was isolated from fresh serum drawn from healthy, normolipidemic volunteers by sequential density ultracentrifugation in the density range of 1.019–1.063 g/ml using the method described by Havel *et al.* [19]. The isolated LDL was dialyzed to remove EDTA and filtered (0.22 μm membrane). Protein contents were measured by the method of Lowry *et al.* [20]. LDL was stored in the dark in phosphate-buffered saline (PBS) containing 1 mg/ml EDTA under nitrogen at 4°C, and was used within two weeks of the isolation.

LDL oxidation [21] was carried out with copper sulphate (final concentration of 5 μM) at 37°C for 24 hr. The extent of oxidation was estimated as thiobarbituric acid reactive substances (TBARS) by the method of Yagi [22]. Tetramethoxypropane was used as a standard, and results were expressed as nanomoles of malondialdehyde equivalents per milliliter of the diluted LDL solution. The average degree of oxidation corresponded to 17.2 μmol TBARS/g cholesterol.

Cell Culture

Peritoneal macrophages were collected from C57BL/6J mice (body weight, 20 ± 2 g) obtained from the Animal Center, Chinese Academy of Medical Sciences. These peritoneal macrophages were collected with 8 ml of ice-cold phosphate-buffered saline and suspended in RPMI 1640 medium supplemented with 10% heat-inactivated newborn calf serum (Life Technologies, Inc.), 2 mM L-glutamine and penicillin-streptomycin (50 μg/ml and 50 IU/ml respectively). Cell suspensions were dispersed in each well of the appropriate tissue culture plates and incubated for three hours. Non-adherent cells were removed by washing three times with the above medium. More than 98% of adherent cells were judged to be macrophages through Giemsa staining. The macrophage monolayers thus formed were used for following cellular experiments [23].

Flow Cytometry Analysis of Apoptotic and Necrotic Cells [24, 25]

Cells were collected and then incubated with Hoechst 33342 and PI (final concentration 5 μg/ml) at 37°C for 30 min. The incubated cells were centrifuged at 1000 rpm for five minutes. The pellet was washed with PBS. The cell pellet was resuspended with 1 ml PBS. This assay was performed with a flow cytometer (Coulter Epics Flow Cytometer, USA) using 340–360 nm at the excitation wavelength, and 488 nm and 610 nm as the emission wavelengths. Hoechst 33342 is a lipophilic fluorescence probe that permeates the cell membrane and specifically associates with DNA in living cells to emit blue fluorescence. PI normally does not penetrate the cell membrane, and only associates with DNA in the late phase of development of apoptotic cells or necrotic cells when it emits a red fluorescence. Thus, living cells, apoptotic cells and necrosis cells can be distinguished by the fluorescence intensity of the DNA dye associated with them.

DNA Fragmentation Assay

The cells were lysed in 0.5 ml of DNA extraction solution containing 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% N-lauryl sarcosine and 0.5 mg/ml proteinase

K and incubated at 50°C overnight. After phenol extraction, the DNA in the upper aqueous phase was digested with 0.5 mg/ml RNase at 37°C for three hours. DNA electrophoresis was then performed on 2% agarose gel containing 1 μg/ml ethidium bromide, and the DNA fragments were visualized by exposing to UV light and photographed.

Near Membrane Ca^{2+} Measurement by Calcium Green C_{18}

Calcium green C_{18} is lipophilic, Ca^{2+} sensitive long chain fluorescent probe, which emits green fluorescence when excited [26]. This probe attaches itself to the plasma membrane through its C_{18} tail. A strong C_{18} fluorescent intensity is indicative of high amounts of Ca^{2+} at the membrane-proximal region. In experiments utilizing Calcium Green C_{18}, cells were permeabilized and loaded as described by Tanimura *et al.* [27]. Confocal images (256×256 pixels) of Calcium Green C_{18} fluorescence were obtained at 488 nm excitation/514 nm emission using a Laser Scanning Confocal Microscopy System (Bio − Rad). The fluorescence units (counts/s) or the average pixel intensity/cell was determined.

Determination of Mitochondrial Ca^{2+} Concentration

For the determination of mitochondrial Ca^{2+} concentration, cells were loaded with both the mitochondria directed probe MitoTracker Red CMXRos (100 nM) and Ca^{2+} fluorescent probe Indo-1/AM (2 μM) for 15 min at 37°C. After binding with Ca^{2+}, the emission peak of Indo-1/AM (a double wavelength probe) migrates from 485 nm to 405 nm when excited at 351 nm and hence is especially suitable for LSCM to measure the Ca^{2+} variation ratio (F405/F485) with a double wavelength channel. So the concentration of intracellular Ca^{2+} can be determined accurately.

Determination of Nuclear Ca^{2+} Concentration

For the determination of nuclear Ca^{2+} concentration, cells were loaded with Ca^{2+} fluorescent probe Fluo-3/AM (5 μM) for 30 min and Hoechst 33342 (20 μg/ml) for ten minutes at 37°C. After washed, the cells were monitored by LSCM (Bio-Rad) [28–30]. The Hoechst dye was excited at 351 nm and the emission fluorescence monitored at 485 nm was selected by a band-pass filter. The calcium-sensitive dye Fluo-3 dye was excited by the 488 nm and the emission fluorescence monitored at 515 nm was selected by a band-pass filter.

Calibration of Indo-1 and Fluo-3 Signals

The ratiometric probe indo-1 was calibrated according to Herrington *et al.* [31, 32]. Briefly, the fluorescence ratio (R) obtained after subtracting cell autofluorescence (mean fluorescence at 410 and 490 nm of unloaded cells) were compared to ratios obtained using indo-1 pentapotassium salt in buffers of known [Ca^{2+}]. The maximum indo-1 ratio (R_{max}) at the experimental conditions was determined by digitonin lysis (10 μM) in a saturating concentration of external Ca^{2+} (10 mM). The

indo-1 ratio of cells in the presence of the intracellular Ca^{2+} chelator BAPTA was used as the minimum ratio (R_{min}). These values were used to estimate [Ca^{2+}] from the experimental indo-1 ratio by substitution into the standard calibration equation [33] with the use of the stated apparent dissociation factor (K_d) value of 250 nM.

$$[Ca^{2+}] = K_d(R - R_{min})/(R_{max} - R)$$

Fluorescence signals from the single-wavelength indicators Fluo-3 were corrected for autofluorescence observed with nonloaded cells ($< 5\%$ of total signal). The corrected signals (F) were converted to [Ca^{2+}] using the simplified calibration equation:

$$[Ca^{2+}] = K_d(F - F_{free})/(F_{CaSat} - F)$$

Where the K_d determined *in vitro* for Fluo-3 (500 nM) [34] was assumed to apply, and F_{free} and F_{CaSat} were calculated from postexperimental responses of each cell as follows. Cells were sequentially treated with a Mg^{2+}-free saline containing 10 mM $CaCl_2$ and 10 μM ionomycin, then with the same solution in which 2 mM $MnCl_2$ replaced $CaCl_2$.

RESULTS

OxLDL Induced Apoptosis of Macrophages

Although the cytotoxic effect of OxLDL to cultured cells has been reported a long time ago [7, 8], the type of cell death induced by OxLDL has been studied only recently. To confirm whether OxLDL could induce apoptosis of macrophages, mouse peritoneal macrophages from C57BL/6J mouse were incubated in culture medium containing native low-density lipoprotein (LDL) or oxidized LDL (OxLDL). As illustrated in Fig. 1, the characteristic ladder shape of DNA fragmentation (180–200 bp) in agarose gel electrophoresis was appeared in macrophages treated with OxLDL. By contrast, incubation with native LDL or non-lipoprotein medium even for 72 hours had no appreciable effect on DNA fragmentation. These findings indicate that OxLDL is able to induce apoptosis in cultured macrophages.

Association of the Variation of Transmembrane Ca^{2+} Gradient across Plasma Membrane with the Apoptosis of Macrophage Derived Foam Cells Induced by OxLDL

Macrophage derived foam cells in atherosclerotic lesions are largely derived from monocyte-macrophages [35]. We have previously reported macrophages from C57BL/6J mouse incubated with low dosage of OxLDL for 48 hours could load lipids and transform into typical foam cells in culture [36]. Therefore, we focused our attention to investigate whether macrophage derived foam cell formation led progressively to apoptosis or necrosis. Macrophages were induced by low dose of OxLDL for different times. Then, the viable, apoptotic and the necrotic cells were distinguished and monitored by flow cytometry using Hoechst 33342 and PI as fluorescence probes [24, 25]. When the macrophages were incubated with OxLDL

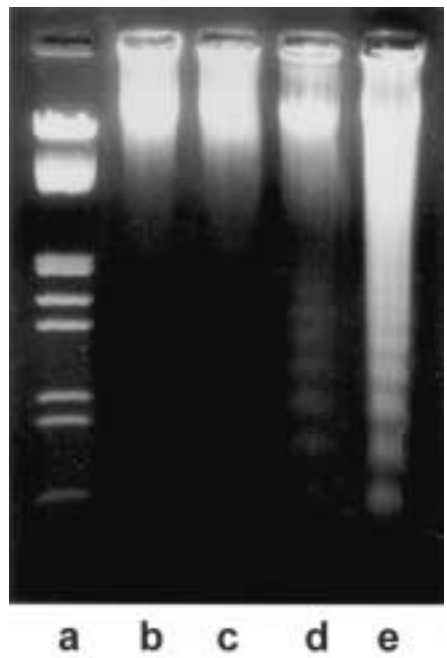

Fig. 1. Agarose gel electrophoresis of DNA extracted from OxLDL-treated macrophages revealed the typical DNA latter pattern. (A), DNA marker; b, no-lipoprotein control; c, LDL treated for 72 hr; d, OxLDL treated for 48 hr; e, OxLDL treated for 72 hr.

(10 μg/ml) for 24 hours, percentages of apoptotic cells were not obviously different from the control (*data not shown*). However, as shown in Table 1, following treatment of OxLDL for 48 hours the percentage of apoptotic cells were significantly increased (32.9%). As previously reported, at this stage the transformation from macrophages to typical foam cells has occurred [8]. After treatment with OxLDL for 72 hours, the percentage of apoptotic cells (43.8%) and the necrotic cells (21.2%) were obviously enhanced while the viable cells decreased dramatically from 86.9 to

Table 1. Correlation Between Changes of Transmembrane Ca^{2+} Gradient Across Plasma Membrane and Apoptosis in OxLDL-treated Macrophages in Culture

	$[Ca^{2+}]_o$ (mM)	$[Ca^{2+}]_i$* (nM)	$[Ca^{2+}]_o/[Ca^{2+}]_i$	Ap (%)	Necrosis (%)
LDL(48 hr)	1	370 ± 16	2702 ± 10	9.5 ± 0.6	2.5 ± 0.5
LDL(72 hr)	1	412 ± 39	2417 ± 12	10.2 ± 0.9	2.9 ± 0.6
OxLDL(48 hr)	1	1050 ± 180	952 ± 21	32.9 ± 1.8	6.1 ± 0.9
OxLDL(72 hr)	1	1160 ± 120	862 ± 25	43.8 ± 3.2	21.2 ± 1.2

*The results were obtained when the cells were loaded with Indo-1/AM in the buffer containing 1 mM $[Ca^{2+}]$. Data is the average of three independent experiments ± S.D.

35.0%. These data suggested that the transformation from macrophages induced by low dose of OxLDL to foam cells led progressively to apoptosis.

To further examine whether the transmembrane Ca^{2+} gradient involves in OxLDL induced macrophage derived foam cell death, we determined the variation of the transmembrane Ca^{2+} gradient across plasma membrane of macrophages treated with OxLDL or LDL using Indo-1/AM as a probe. The extracellular [Ca^{2+}] was 1 mM. Table 1 showed that with the prolongation of the OxLDL treatment, the average concentration of cytosolic free Ca^{2+} in macrophages was markedly increased compared to the LDL treatment. Following treatment with OxLDL for 48 hours, the Ca^{2+} gradient across macrophage plasma membrane was decreased from 2702 ± 10 to 952 ± 12. Concomitantly, some characteristics of apoptotic cells appeared and the apoptotic cells accounted for 32.9% of the total cells. When treated with OxLDL for 72 hours, the Ca^{2+} gradient across the plasma membrane was further decreased to 862 ± 25. Additionally, not only the amount of apoptotic cells increased (43.8%), but the amount of necrotic cells was also increased to 21.2% of the total cells. These data may indicate a link between transmembrane Ca^{2+} gradient and the apoptosis versus necrosis of macrophage derived foam cells induced by OxLDL.

The Change of the Near Membrane Ca^{2+} in the OxLDL-Induced Apoptosis of Macrophages

Increasing evidences suggest that during physiological responses, the [Ca^{2+}] just beneath the cell membranes may be very different from the [Ca^{2+}] in the bulk cytosol. Membrane-associated Ca^{2+} indicators such as C_{18}-Fura-2, FFP-18 and Calcium Green C_{18}, can be used to detect changes in the free Ca^{2+} concentration immediately adjacent to cell membranes, which are obscured in signals recorded using water-soluble, bulk cytosolic fluorescent Ca^{2+} indicators [37–40]. Moreover, in the early stages of apoptosis there are changes occurring on the cell surface. Recently, Lin *et al*. reported [41] that the reactive oxidizing species created near the plasma membrane, rather than at intracellular sites, might be responsible for initiation of apoptosis. Thus, it is interesting to investigate the changes in Ca^{2+} gradient across near plasma membrane in OxLDL-induced macrophage apoptosis.

For this case, the Ca^{2+} concentration near plasma membrane was detected by Calcium Green C_{18}. As shown in Fig. 2, following the treatment with OxLDL for 48 hours, as macrophages transformed into foam cells, fluorescence intensity due to [Ca^{2+}] near the plasma membrane increased 3.46 times compared to the treatment of native LDL. With prolonged OxLDL treatment for 72 hours, the fluorescence intensity increased to 4.6 times. These results clearly demonstrated that when macrophages were exposed to OxLDL, there was not only an obvious change in the Ca^{2+} gradient across the plasma membrane, but also a significant variation of the Ca^{2+} gradient near the membrane.

Changes in the Ca^{2+} Gradient Across the Organelle Membranes During Apoptosis of Macrophage Derived Foam Cells

The maintenance and change of the transmembrane Ca^{2+} gradient, the variation of the spatial Ca^{2+} gradient and the Ca^{2+} gradient across the organelle membranes

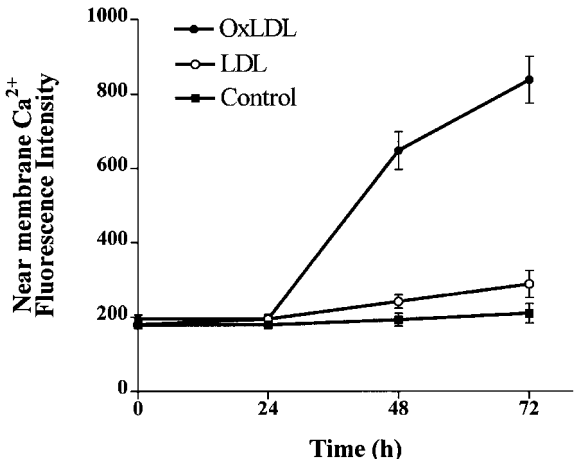

Fig. 2. Quantification of fluorescence intensity of near membrane Ca^{2+} in OxLDL-treated macrophages. Cells were incubated with 10 μg/ml of native LDL or OxLDL or without any lipoproteins for 24 hr, 48 hr, or 72 hr. The Ca^{2+} fluorescence intensity of near membrane Ca^{2+} was detected by Calcium Green C_{18}. The data are expressed as the means ± S.D.

are tightly related events [42]. It is well known that the SR and mitochondria are intracellular calcium pools, and it also shown recently that there are Ca^{2+} permeable barrier and transport systems on the nuclear membrane indicating that the nucleus may also be a kind of [Ca^{2+}] reservoir within the cell [43–46]. Therefore, further knowledge on this aspect is necessary for study of the transformation from macrophages to foam cells and progressively to apoptosis. Since flow cytometry does not provide information on the subcellular distribution of calcium we attempted to determine the change of the Ca^{2+} gradient across the nuclear and mitochondrial membranes using Ca^{2+} indicators of different fluorescent characteristics and specific organelle-directed probes as displayed by means of confocal microscopy.

Increase of the Ca^{2+} Gradient Across the Nuclear Membrane and Direct Visualization of Nuclear Morphology

Single cell imaging with Ca^{2+}-sensitive fluorescent indicators employing conventional or confocal microscopy is widely used for measuring nucleo-cytoplasmic Ca^{2+} gradients [43–47]. These dyes essentially accumulate in the cytosol, yet some of them do have access to the nucleus by diffusion through the nuclear pore complex. In this case, macrophages treated with native LDL or OxLDL were co-loaded with Fluo-3/AM and Hoechst 33342. The intracellular calcium (Fig. 3, right panel) and nuclear morphology (Fig. 3, left panel) were directly visualized on a confocal microscope, which has the capability of exciting both Fluo-3 and the Hoechst (nuclear) dyes. The nuclear morphology of the native LDL-treated macrophages (A) displayed uniformly large, uncondensed, and highly fluorescent. However, the OxLDL-treated

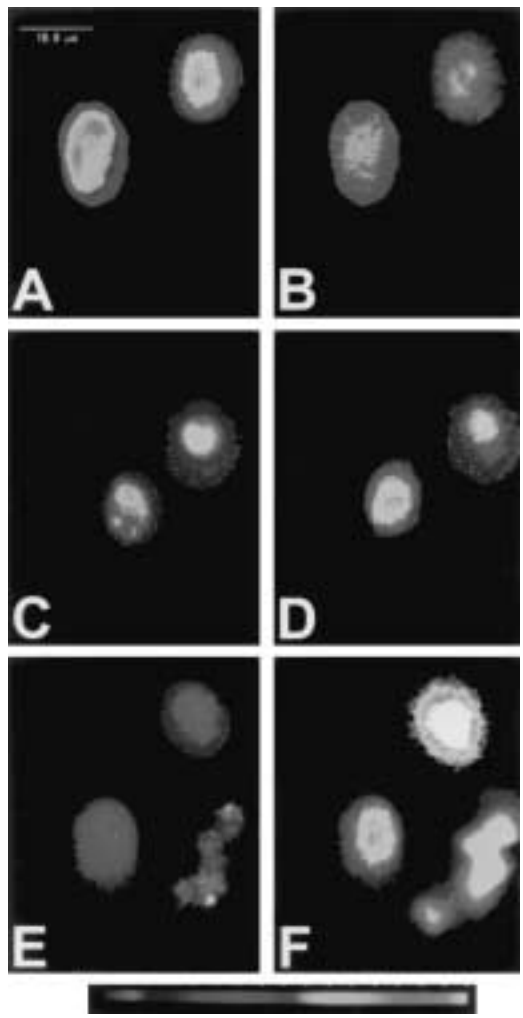

Fig. 3. The typical confocal images of nuclear DNA (left panel) and calcium (right panel) of OxLDL-treated macrophages. Cells were treated with native LDL (A, B), OxLDL for 48 hr (C, D) or OxLDL for 72 hr (E, F). Cells were loaded with Fluo-3/AM and Hoechst 33342 at 37°C. Images were obtained by laser scanning confocal microscopy with dual-channel detection. The lighter the image, the higher the fluorescence intensity.

cells for 48 hours had condensed fluorescent nuclei and intact cell membrane (C). Moreover, the OxLDL-treated cells for 72 hours showed dim fluorescence (E). At the same time point, the Fluo-3 fluorescence (Fig. 3, right panel) was shown much higher in OxLDL-induced cells (D,F) compared to native LDL-treated cells (B). Furthermore, the Ca^{2+} gradient across the nuclear membrane was then measured

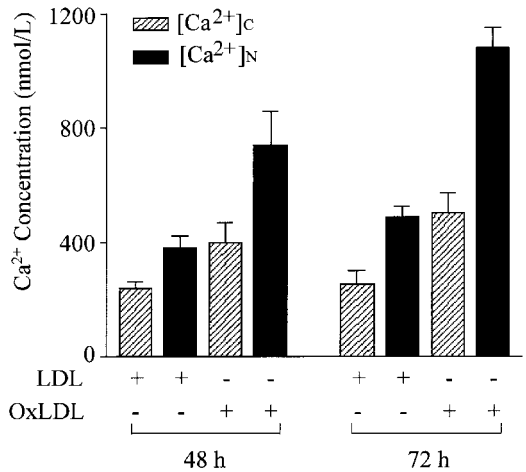

Fig. 4. Changes of transmembrane Ca^{2+} gradient across nuclear membrane in macrophages treated with LDL or OxLDL. Cells were loaded with Fluo-3/AM and Hoechst 33342 at 37°C. The Ca^{2+} concentration of cytoplasmic and nuclear regions was measured by laser scanning confocal microscopy. The data are expressed as the means ± S.D.

and calculated as shown in Fig. 4. With the prolongation of OxLDL treatment, the Ca^{2+} gradient across the nuclear membrane gradually compared to LDL treatment. Interestingly, the decrease in the fluorescence intensity of DNA probed by Hoechst 33342 yielded a better correlation with the increase in trans-nuclear membrane Ca^{2+} gradient.

Decrease of the Ca^{2+} Gradient Across the Mitochondrial Membrane

Mitochondria play an important role in regulating cellular calcium homeostasis as well as in apoptosis [48]. Cells were loaded with Ca^{2+} fluorescent probe Indo-1/AM and MitoTracker Red CMXRos, a mitochondria-specific fluorescent dye, to measure the cytosolic ($[Ca^{2+}]c$) and mitochondrial Ca^{2+} concentration ($[Ca^{2+}]m$) simultaneously. As shown in Fig. 5, the ratios of $[Ca^{2+}]m/[Ca^{2+}]c$, i.e., the Ca^{2+} gradient across the mitochondrial membrane was 2.15 ± 0.11 for the control macrophages, similar to the result reported by Sheu and Jou [49, 50] on heart chamber muscle cell of a new-born mouse. After macrophages treated with OxLDL for 48 hours, both of the cytosolic $[Ca^{2+}]c$ and mitochondria $[Ca^{2+}]m$ increased significantly, but the Ca^{2+} gradient across the mitochondrial membrane ($[Ca^{2+}]m/[Ca^{2+}]c$) was lowered to 1.24 ± 0.11, and it will further be decreased and even disappeared ($[Ca^{2+}]m/[Ca^{2+}]c = 1.08 \pm 0.06$) when macrophages treated with OxLDL for 72 hours. These data indicated that the decreases in the Ca^{2+} gradient across the mitochondrial membrane might be involved in the OxLDL-induced apoptosis of macrophages.

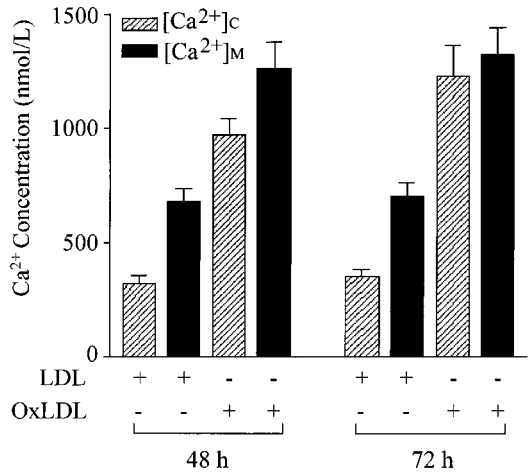

Fig. 5. Changes of transmembrane Ca^{2+} gradient across mitochondrial membrane in macrophages treated with native-LDL or OxLDL. Cells were loaded with indo-1/AM and MitoTracker Red CMXRos at 37°C. The Ca^{2+} concentration of cytoplasmic and mitochondrial regions was measured by laser scanning confocal microscopy. The data are expressed as the means ± S.D.

DISCUSSION

Several laboratories have demonstrated the presence of apoptotic cell death in atherosclerotic plaques. It has been found that concentrations of OxLDL greater than 40 μg/ml appeared to be cytotoxic, as reflected by membrane blebbing, cell detachment, and a decrease in the proportion of cells that excluded trypan blue [52]. Reid [13, 14] reported that a toxic effect could be induced in case of macrophages incubated with a large dose of OxLDL (100 μg/ml). Our results clearly showed that the transformation of macrophage derived foam cells can lead progressively to its apoptosis when macrophages were induced by low dosage of OxLDL (10 μg/ml).

Ca^{2+} has been proposed to be a messenger in OxLDL-induced cell death, however, very little is known about the molecular mechanism by which calcium may influence OxLDL directing macrophage forward apoptosis or necrosis. One general signaling mechanism used to transfer the information delivered by agonists into appropriate intracellular compartments involves the rapid redistribution of ionized calcium throughout the cell, resulting in the transient elevations of the cytosolic free Ca^{2+} concentration. Various physiological stimuli increase $[Ca^{2+}]_i$ transiently and, thereby, induce cellular responses. However, under pathological conditions, changes of $[Ca^{2+}]_i$ are generally more pronounced and sustained. Marked elevations of $[Ca^{2+}]_i$ activate hydrolytic enzymes, lead to exaggerated energy expenditure, impair energy production, initiate cytoskeletal degradation, and ultimately result in cell death. Such Ca^{2+}-induced cytotoxicity may play a major role in several diseases [53]. It was reported in lymphoblastoid cells necrosis and apoptosis induced by OxLDL were subsequent to the rise in calcium, but it was described only as a change in average

intracellular Ca^{2+} concentration [54]. Matsumura *et al.* [55] showed that exposure of mouse macrophages to OxLDL resulted in a transient increase in cytosolic calcium. In the present study, using various Ca^{2+} fluorescent probes of different characteristics, we have found that Ca^{2+} distributed unevenly in macrophages and thus apparent Ca^{2+} concentration differences between the intracellular calcium pools and the cytosol existed. The data presented here demonstrated OxLDL induced macrophage apoptosis as well as alterations in Ca^{2+} gradient including that across plasma membrane, near membrane, and intracellular organelle membranes. Our recent results have shown that significant morphological changes of the macrophage induced by low dose of OxLDL at different times are detected by scanning electron microscopy and transmission electron microscopy (*unpublished results*). So we tentatively suggest that altering the transmembrane Ca^{2+} gradients may play an important role in the initiation of the OxLDL induced macrophage-derived foam cell formation and progressively its apoptosis.

Recently, several pieces of evidence imply that mitochondria, as a regulator, are involved in the process of apoptosis [48]. Mitochondria provide a key amplification step in the apoptotic pathway of many cells by releasing apoptogenic proteins into the cytosol. Mitochondria undergo major changes in membrane integrity before classical signs of apoptosis begin to manifest. Mitochondria in cells undergoing apoptosis under a variety of conditions have been shown to lose membrane potential and to release pro-apoptotic factors. Additional, under certain conditions, mitochondria can sequester calcium. Our results indicated the decrease in Ca^{2+} gradient across the mitochondrial membrane during the OxLDL-induced apoptosis of macrophages. We have also observed that OxLDL resulted in the significant changes in the mitochondrial transmembrane potential (*data not shown*). The anti-apoptotic oncoprotein Bcl-2 acts on mitochondria to stabilize membrane integrity and to prevent the opening of the megachannel. Meilhac *et al.* [12] reported that high Bcl-2 levels in vascular cells may participate in atherogenic processes by shifting the balance from apoptosis toward necrosis (induced by toxic concentrations of OxLDL), which may favor inflammatory events, promote formation or enlargement of the necrotic core of atherosclerotic plaques. An increase in the release of calcium from mitochondria by oxidants stimulates calcium-dependent enzymes such as calcium-dependent proteases, nucleases, and phospholipases, which subsequently trigger the apoptosis of the cells. Borutaite *et al.* [56] found 5 μM free Ca^{2+} might be enough to cause release of cytochrome c from mitochondria and initiate apoptosis in ischaemia-damaged cells. Once the calcium efflux has been triggered, a series of common pathways of apoptosis are initiated, each of which may be sufficient to destroy the cell [48].

It is now accepted that calcium signals do exist in the cell nucleus [57, 58]. Several lines of evidence point to autonomous regulation of nuclear Ca^{2+} by a Ca^{2+}-ATPase on the nuclear membrane. Receptors for Ca^{2+} signaling molecules such as inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetraphosphate have also been located on the nuclear membranes. Also, based on patch-clamp studies, Stehno-Bittel [59] showed IP_3Rs localized on the outer nuclear membrane. However, studies on nuclear Ca^{2+} become relevant only in the light of specific functions. Bolsover *et al.* [60] described that when cells loaded with fluorescent calcium indicators are

stimulated, the amplitude of the resulting fluorescence change is often greater in the nucleus than in the cytosol. Interestingly, the results described here demonstrate that intracellular transmembrane Ca^{2+} gradients across nuclear membrane increased in the apoptosis of macrophage. This also may be indicative of crosstalk between the cytosolic and nuclear Ca^{2+} pools.

Taken together, these results provide new evidence that the variation of transmembrane Ca^{2+} gradient, raising from redistribution of Ca^{2+} pools within the cell, involves in the OxLDL-induced macrophage apoptosis.

ACKNOWLEDGMENTS

This project is financed by the National Nature Science Foundation of China (No. 39730130) and the Chinese Academy of Sciences (KJ951-B1-609).

REFERENCES

1. Carafoli, E. (1987) Intracellular Calcium Homeostasis. *Ann. Rev. Biochem.* **56:**395–433.
2. Mooren, F. C. (1998) Kinne RKH Cellular calcium in health and disease. *Biochim. Biophys. Acta* **1406:**127–151.
3. Brown, E. M. Vassiler, P. M., and Herbert, S. C. (1995) Ca^{2+} ions as extracellular messengers. *Cell* **83:**679–682.
4. Yang, F. Y., Huang, Y. G., and Tu, Y. P. (1995) Transmembrane Ca^{2+} gradient and function of membrane proteins. *Biooscience Rep.* **15:**351–364.
5. Yang, X. Y., Fan, G. F., Huang, Y. G., and Yang, F. Y. (1996) Effect of transmembrane Ca^{2+} gradient on ligand binding of reconstituted β-adrenergic receptors. *Chinese Science Bulletin* **41:**1214–1218.
6. Fan, G. F., Huang, Y. G., Bai, Y. H., and Yang, F. Y. (1994) Effect of transmembrane Ca^{2+} gradient on Gs function. *FEBS Lett.* **357:**13–15.
7. Fan, G. F., Yang, X. Y., Huang, Y. G., and Yang, F. Y. (1996) Effect of transmembrane Ca^{2+} gradient on the coupling of β-adrenergic receptors and adenylyl cyclase. *Bioscience Rep.* **16:**327–341.
8. Yang, X. Y., Fan, G. F., Huang, Y. G., and Yang, F. Y. (1996) Effect of transmembrane Ca^{2+} gradient on ligand binding of reconstituted β-adrenergic receptors. *Chinese Science Bulletin* **41:**1214–1218.
9. Mitchinson, M. J., Hardwick, S. J., and Bennett, M. R. (1996) Cell death in atherosclerotic plaques. *Curr. Opin. Lipidol.* **7:**324–329.
10. Geng, Y. J. (1997) Regulation of programmed cell death or apoptosis in atherosclerosis. *Heart Vessels* **12**(Suppl):76–80.
11. Bjorkerud, S. and Bjorerud, B. (1996) Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am. J. Pathol.* **149:**367–380.
12. Meilhac, O., Escargueil-Blanc, I., Thiers, J. C., Salvayre, R., and Negre-Salvayre, A. (1999) Bcl-2 alters the balance between apoptosis and necrosis, but does not prevent cell death induced by oxidized low density lipoproteins. *FASEB J.* **13:**485–494.
13. Reid, V. C. and Mitchinson, M. J. (1993) Toxicity of oxidized low density lipoprotein towards mouse peritoneal macrophages *in vitro*. *Atherosclerosis* **98:**17–24.
14. Reid, V. C., Hardwick, S. J., and Mitchinson, M. J. (1993) Fragmentation of DNA in P388D1 macrophages exposed to oxidized low-density lipoprotein. *FEBS Lett.* **332:**218–220.
15. Reid, V. C., Mitchinson, M. J., and Skepper, J. N. (1993) Cytotoxicity of oxidized low-density lipoprotein to mouse peritoneal macrophages: an ultrastructural study. *J. Pathol.* **171:**321–328.
16. Kinscherf, R. *et al.* (1998) Apoptosis caused by oxidized LDL is manganese superoxide dismutase and p53 dependent. *FASEB J.* **12:**461–467.

17. Stewart-Phillips, J. L., Lough, J., and Skamene, E. (1988) Genetically determined susceptibility and resistance to diet-induced atherosclerosis in inbred strains of mice. *J. Lab. Clin. Med.* **112:**36–42.
18. Paigen, B., Morrow, A., Brandon, C., Mitchell, D., and Holmes, P. (1985) Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis* **57:**65–73.
19. Havel, R. I., Eder, H. A., and Braigon, J. H. (1955) The distribution and the chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **39:**1345–1363.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.
21. Kosugi, K., Morel, D. W., and DiCorleto, P. E., (1987) Chisholm GM Toxicity of oxidized LDL to cultured fibroblasts is selective for S phase of the cell cycle. *J. Cell Physiol.* **130:**311–320.
22. Yagi, K. (1985) Lipid peroxides and human diseases. *Chem. Phys. Lipids* **45:**337–351.
23. Rees, D., Sloane, T., Jessup, W., and Dean, R. T. (1999) Kritharides L Apolipoprotein A-I stimulates secretion of apolipoprotein E by foam cell macrophages. *J. Biol. Chem.* **274:**27925–27933.
24. Dive, C., Gregory, C. D., Phipps, D. J., Evans, D. L., Milner, A. E., and Wyllie, A. H. (1992) Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim. Biophys. Acta.* **1133:**275–285.
25. Darzynkiewicz, Z., *et al.* (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13:**795–808.
26. Lloyd, Q. P., Kuhn, M. A., and Gay, C. V. (1995) Characterization of calcium translocation across the plasma membrane of primary osteoblasts using a lipophilic calcium-sensitive fluorescent dye, calcium green C_{18}. *J. Biol. Chem.* **270:**22445–22451.
27. Tanimura, A. and Turner, R. J. (1996) Inositol 1,4,5-triphosphate-dependent oscillations of luminal [Ca^{2+}] in permeabilized HSY cells. *J. Biol. Chem.* **271:**30904–30908.
28. Donnadieu, E. and Bourguignon, L. Y. W. (1996) Ca^{2+} signaling in endothelial cells stimulated by bradykinin: Ca^{2+} measurement in the mitochondria and the cytosol by confocal microscopy. *Cell Calcium* **20:**53–61.
29. Yang, X. Y., Yan, K., and Huang, Y. G. (1996) A powerful method for the determination of the spatial and temporal changes of intracellular Ca^{2+} in single macrophages by laser Scanning Confocal Microscopy. *Prog. Biochem. Biophys.* **23:**442–445.
30. Hernandez-Cruz, A., Sala, F., and Adams, P. R. (1990) Subcellular calcium transients visualized by confocal microscopy in a voltage-clamped vertebrate neuron. *Science* **247:**858–862.
31. Herrington, J., Park, Y. B., Babcock, D. F., and Hille, B. (1996) Dominant role of mitochondria in clearance of large Ca^{2+} loads from rat adrenal chromaffin cells. *Neuron* **16:**219–228.
32. Park, Y. B., Herrington, J., Babcock, D. F., and Hille, B. (1996) Ca^{2+} clearance mechanisms in isolated rat adrenal chromaffin cells. *J. Physiol.* **492:**329–346.
33. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indiatrs with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450.
34. Eberhard, M. and Erne, P. (1991) Calcium binding to fluorescent calcium indicators: calcium green, calcium orange and calcium crimson. *Biochem. Biophys. Res. Commun.* **180:**209–215.
35. Ylitalo, R., Jaakkola, O., Lehtolainen, P., and Yla-Herttuala, S. (1999) Metabolism of modified LDL and form cell formation in murine macrophage-like RAW 264 cells. *Life Sci.* **64:**1955–1965.
36. Yang, X. Y., Zhong, Y. Z., Huang, Y. G., and Yang, F. Y. (2000) Changes of transmembrane Ca^{2+} gradient in the formation of macrophage-derived foam cells. *Biosci. Rep.* **20:**1–12.
37. Etter, E. F., Kuhn, M. A., and Fay, F. S. (1994) Detection of changes in near-membrane Ca^{2+} concentration using a novel membrane-associated Ca^{2+} indicator. *J. Biol. Chem.* **269:**10141–10149.
38. Etter, E. F., Minta, A., Poenie, M., and Fay, F. S. (1996) Near-membrane [Ca^{2+}] transients resolved using the Ca^{2+} indicator FFP18. *Proc. Natl. Acad. Sci. USA* **93:**5368–5373.
39. Davies, E. V. and Hallet, M. B. (1996) Near membrane Ca^{2+} changes resulting from store release in neutrophils: detection by FFP-18. *Cell Calcium* **19:**355–362.
40. Tojyo, Y., Tanimura, A., and Matsumoto, Y. (1997) Monitoring of Ca^{2+} release from intracellular stores in permeabilized rat parotid acinar cells using the fluorescent indicators Mag-fura-2 and calcium green C_{18}. *Biochem. Biophys. Res. Commun.* **240:**189–195.
41. Lin, C. P., Lynch, M. C., and Kochevar, I. E. (2000) Reactive oxidizing species produced near the plasma membrane induce apoptosis in bovine aorta endothelial cells. *Exp. Cell Res.* **259:**351–359.

42. Yang, X. Y., Tan, J. M., Huang, Y. G., and Yang, F. Y. (1997) The correlation of transmembrane Ca^{2+} gradients and Ca^{2+} oscillation in single macrophages. *Prog. Natural Sciences* **7:**438–442.
43. Santella, L. and Carafoli, E. (1997) Calcium signaling in the cell nucleus. *FASEB J.* **11:**1091–1109.
44. Himpens, B., De Smedt, H., Droogmans, G., and Casteels, R. (1992). Differences in regulation between the nuclear and cytoplasmic Ca^{2+} in DDT1MF2 smooth muscle cells. *Am. J. Physiol.* **263:**C95–C105.
45. Himpens, B., Missiaen, L., and Casteels, R. (1995) Ca^{2+} homeostasis in vascular smooth muscle. *J. Vasc. Res.* **32:**207–219.
46. Himpens, S., De Smedt, H., and Casteels, R. (1994) Relationship between [Ca^{2+}] changes in nucleus and cytosol. *Cell Calcium.* **16:**239–246.
47. Nicotera, P., Zhivotovsky, B., and Orrenius, S. (1994) Nuclear calcium transport and the role of calcium in apoptosis. *Cell Calcium* **16:**279–288.
48. Kruman, I. I. and Mattson, M. P. (1999) Pivotal role of mitochondrial calcium uptake in neural cell apoptosis and necrosis. *J. Neurochem.* **72:**529–540.
49. Sheu, S. S. and Jou, M. J. (1994) Mirochondrial free Ca^{2+} concentration in living cells. *J. Bioenerg. Biomembr.* **26:**487–493.
50. Jou, M. J. and Sheu, S. S. (1992) Mitochondrial regulation of cytosolic Ca^{2+} in cultured neonatal rat ventricular mytocytes. *Biophys. J.* **61:**A164.
51. Jiang, S., Chow, S. C., Nicotera, P., and Orrenius, S. (1994) Intracellular Ca^{2+} signals activate apoptosis in thymocytes: Studies using the Ca^{2+}-ATPase inhibitor thapsigargin. *Exp. Cell. Res.* **212:**84–92.
52. Martens, J. S., Lougheed, M., Gomez-Munoz, A., and Steinbrecher, U. P. (1999) A modification of apolipoprotein B accounts for most of the induction of macrophage growth by oxidized low density lipoprotein. *J. Biol. Chem.* **274:**10903–10910.
53. Nicotera, P. and Orrenius, S. (1998) The role of calcium in apoptosis. *Cell Calcium* **23:**173–180.
54. Escargueil-Blanc, I., Salvayre, R., and Negre-Salvayre, A. (1994) Necrosis and apoptosis induced by oxidized low density lipoproteins occur through two calcium-dependent pathways in lymphoblastoid cells. *FASEB J.* **8:**1075–1080.
55. Matsumura, T. *et al.* (1997) Two intracellular signaling pathways for activation of protein kinase C are involved in oxidized low-density lipoprotein-induced macrophage growth. *Arterioscler. Thromb. Vasc. Biol.* **17:**3013–3020.
56. Borutaite, V., Morkuniene, R., and Brown, G. C. (1999) Release of cytochrome c from heart mitochondria is induced by high Ca^{2+} and peroxynitrite and is responsible for Ca^{2+}-induced inhibition of substrate oxidation. *Biocheim. Biophys. Acta* **1453:**41–48.
57. Perez-Terzic, C., Pyle, J., Jaconi, M., Stehno-Bittel, L., and Clapham, D. E. (1996) Conformational states of the nuclear pore complex induced by depletion of nuclear Ca^{2+} stores. *Science* **273:**1875–1877.
58. Perez-Terzic, C., Stehno-Bittel, L., and Clapham, D. E. (1997) Nucleoplasmic and cytoplasmic differences in the fluorescence properties of the calcium indicator Fluo-3. *Cell Calcium* **21:**275–282.
59. Stehno-Bittel, L., Luckoff, A., and Clapham, D. E. (1995) Calcium release from the nucleus by InsP3 receptor channels. *Neuron.* **14:**163–167.
60. Bolsover, S. R., Kater, S. B., and Guthrie, P. B. (1996) Spatial gradients of cytosolic calcium concentration in neurones during paradoxical activation by calcium. *Cell Calcium.* **20:**373–379.