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Carbonyl stress: malondialdehyde induces damage on rat hippocampal neurons by disturbance of Ca^{2+} homeostasis

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Abstract The objective of this study was to investigate the influences of carbonyl stress induced by malondialdehyde (MDA), a typical intermediate of lipid peroxidation, on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) alterations in cultured hippocampal neurons of rat. The microphotographic study clearly demonstrated that the hippocampal neurons became gradually damaged following exposure to different concentrations of MDA. Further study indicated that the plasma membrane Ca^{2+}-ATPase (PMCA) activity was inhibited by MDA in a concentration- and time-dependent manner. The supplementation of 100 μM MDA was found to cause a notable early phase increase of $[Ca^{2+}]_i$ in hippocampal neuron cultures followed by a more pronounced late-phase elevation of $[Ca^{2+}]_i$. Such effect of MDA was prevented by the addition of nimodipine, an inhibitor of L-type calcium channel or by an extracellular Ca^{2+} chelator EGTA. The identification of the calcium signalling pathways were studied by applying U73122, an inhibitor of PL-C, and H-89, an inhibitor of protein kinase A (PKA), showing the involvement of PL-C/IP3 pathway but not the PKA/cAMP pathway. These results suggested that MDA-related carbonyl stress caused damages of rat hippocampal neurons by triggering Ca^{2+} influx and influencing Ca^{2+} homeostasis in cultured neurons, and also MDA may act as a signalling molecule regulating Ca^{2+} release from intracellular stores.

Keywords MDA · Hippocampal neurons · $[Ca^{2+}]_i$ · PMCA · VOCC

Abbreviations

$[Ca^{2+}]_i$	intracellular free Ca^{2+} concentration
cAMP	cyclic adenosine monophosphate
ER	endoplasmic reticulum
EGTA	ethylene glycol bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid
H-89	*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride

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HEPES	*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid)
4-HNE	4-hydroxynonenal
IP3	inositol-1,4,5-trisphophate
MDA	malondialdehyde
PL-C	phospholipase C
PIP2	phosphatidylinositol-4,5-bisphophate
cAMP/PKA	cyclic adenosine monophosphate/protein kinase A
PMCA	plasma membrane Ca^{2+}-ATPase
PUFA	polyunstaturated fatty acid
TMP	1, 1, 3, 3-tetramethoxypropane
U73122	1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1H-pyrrole-2,5-dione
VOCC	voltage operation calcium channel

Introduction

Calcium ion as an important secondary messenger plays pivotal roles in a diversity of cellular functions, such as cytoskeletal contraction, neuronal synaptic plasticity, motility, inflammation, cell adhesion, cell cycle regulation, cell growth, and even cell death. Ca^{2+} is also involved in numerous signalling processes like the regulation of hormones, neurotransmitters, and membrane excitability (Altin and Bygrave 1998; DeLorenzo et al. 1979; Fujita 2002). After activated by the related signal transduction, the Ca^{2+} state must be recovered so that cells can return to a resting level waiting to respond to a new stimulus. The mechanisms of Ca^{2+} restoring to resting levels include either Ca^{2+} sequestration into intracellular organelles such as endoplasmic reticulum (ER) and mitochondria, or Ca^{2+} extrusion through the plasma membrane by Ca^{2+}-ATPase or Na^{+}/Ca^{2+} exchanger (see Fig. 1 for a schematic overview).

Sustained intracellular Ca^{2+} dyshomeostasis was reported to be the major events of cell necrosis or apoptosis associated with brain aging (Verkhratsky and Toescu 1998). It was first proposed by Khachaturian (1984) that the high cytosolic calcium concentration activates phospholipases, leading to cell swelling, membrane degradation and reducing ATP synthesis. An ER calcium hypothesis of cell degeneration, however, was put forward based on the assumption that neuronal cell death was triggered by a depletion of ER calcium stores which may therefore result in increment of plasma Ca^{2+} (Paschen and Doutheil 1999; Paschen 2000, 2001). Numerous studies indicated that oxidative stress (Vergun et al. 2001), hypoxia/ischemic reperfusion

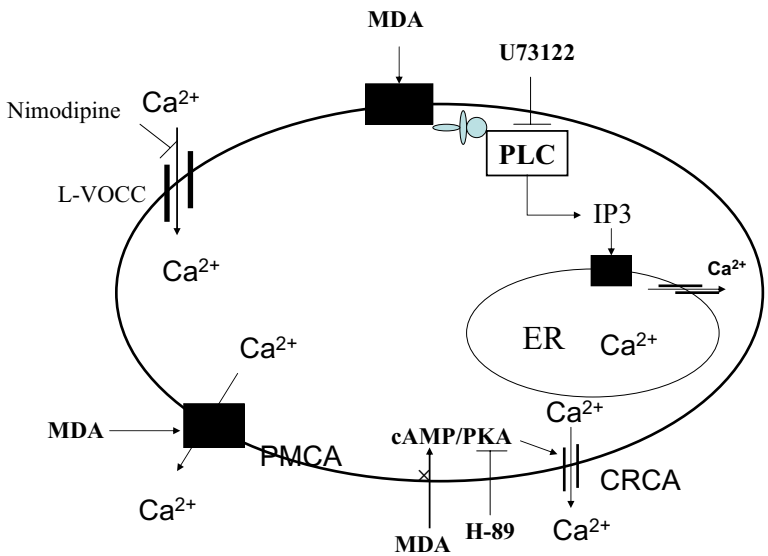

Fig. 1 Scheme of various calcium channels and signalling pathways related with this investigation system. *CRAC* Ca^{2+} release-activated Ca^{2+} channel; *IP3* inositol-1,4,5-trisphophate; *MDA* malondialdehyde; *PKA* protein kinase A; *PL-C* phospholipase C; *PMCA* plasma membrane Ca^{2+}-ATPase; *L-VOCC* L-type voltage operation Ca^{2+} channel. —×▶, no effect; *arrow*, activating effect; *inverted T*, inhibitory effect

(Bandali et al. 2004; Buyukuysal 2004; Saini et al. 2005), biological toxins, and metal irons (Valko et al. 2005) may impair cellular Ca^{2+} homeostasis leading to persistent elevation of cytosolic Ca^{2+} level. At the resting state, intracellular Ca^{2+} was maintained at certain low levels by different regulators, including Ca^{2+} binding proteins, the ER Ca^{2+}-ATPase, the Na^{+}/Ca^{2+} exchanger and the plasma membrane Ca^{2+}-ATPase. The opening of the Ca^{2+}-channels in the cytoplasmic membrane and in the ER membrane (also see Fig. 1) were considered to be responsible for disruptions and damages of Ca^{2+} homeostasis and cellular functions.

There are increasing evidences that a variety of unsaturated aldehydes was generated endogenously during oxidative stress thus resulting in damages in cells or tissues, such as in the hippocampal area of Alzheimer's patients (Markesbery and Lovell 1998; Pryor and Stanley 1975). Several bio-toxic aldehydes, representatively, acrolein, 4-hydroxynonenal (4-HNE), and malondialdehyde (MDA), have been well studied. Such unsaturated carbonyls are electrophiles which may react with sulfhydryl group (cysteine) and nucleophilic amino groups, e.g. ε-amino-group of lysine, guanido group of arginine, and imidazolyl group of histidine in proteins (Esterbauer et al. 1991). The cytotoxicity and genotoxicity of 4-hydroxyalkyenals, such as their deleterious effects on cell proliferation and gene expression, have been widely investigated (Esterbauer et al. 1991; O'Brien et al. 2005; Uchida and Stadtman 1992, 1993). Many studies indicated that 4-HNE enhanced oxidative stress, modulated adenylate cyclase activity, stimulated phospholipase C (PL-C) and regulated calcium channels and Ca^{2+}-ATPases activity, leading to remarkable elevation of intracellular Ca^{2+} concentration (Crifo et al. 2005).

MDA is formed in the process of oxidative degradation of polyunsaturated fatty acid (PUFA) with more than two methylene-interrupted double bonds, such as linolenic acid (18:3) and arachidonic acid (20:4). Possible mechanisms for the formation of MDA from those PUFA were proposed by some scientific workers (Esterbauer et al. 1991; Yin 1996). In vivo, MDA exhibited ubiquitous reactivity with various biomolecules including proteins, nucleic acids, and phospholipids, and quickly reduced cellular glutathione level (Esterbauer et al. 1991; Yin 1996). However, MDA (or thiobarbituric acid reactive substances, TBARS) was used generally as an experimental or diagnostic index of peroxidative tissue injury. Studies on whether MDA can affect the cytosolic Ca^{2+} concentration or regulate Ca^{2+} signalling pathways have not been documented in the literature. In this work, we present, for the first time, the MDA-induced effects on neuronal calcium channels and the possible signalling mechanisms resulting in elevation of $[Ca^{2+}]_i$ in cultured hippocampal neurons.

Materials and methods

Chemicals

N-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), ethylene glycol bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), soybean trypsin inhibitor, Fura-2/AM, thapsigargin, nimodipine, H-89 (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), methacholine (Acetyl- β-methylcholine chloride), and U73122 (1-(6-{[17β-3-methoxyestra-1,3,5 (10)-trien-17-yl] amino} hexyl)-1 H-pyrrole-2,5-dione) were purchased from Sigma (St Louis, USA). Neuronal culture medium and associated reagents were purchased from Invitrogen (NY, USA). All other chemicals were of analytical grade and were purchased from Sangon (Shanghai, China). MDA was made from 1,1,3,3-tetramethoxypropane (TMP) which was obtained from Fluka Chemie AG (Buchs, Switzerland). A fresh MDA stock solution (100 mM) was prepared by hydrolyzing TMP, which was modified according to a method described by Kikugawa et al. (1981). Thus, 0.845 ml (0.5 mM) TMP was mixed with 2 ml 1.0 N HCl, and shaken at 40°C for about 2.5 min. After the TMP was fully hydrolyzed, the pH was adjusted to 7.4 with 6.0 N NaOH, and the stock solution was finally made up to 50 ml with 0.1 M sodium phosphate buffer (pH 7.4).

All experimental procedures and materials were carried out according to regulations on the care and treatment of laboratory animals claimed by the Chinese Academy of Science and all the animal procedures had approved by our institutional ethics committees

Hippocampal neuron cultures

Neurons were isolated from the hippocampus of Sprague–Dawley (SD) rat pups 2–4 days postnatal. The preparation and culture of the hippocampal neurons were following a modified protocol as described by Brewer et al. (1993). The animals were dissected after

head knock–shock, and the brains were taken out quickly on an ice plate. The cerebella were then removed and the hippocampi were carefully isolated. The tissues were minced and incubated for 15 min at 37°C with 0.03% trypsin in HBSS at 37°C for 15 min. Hippocampal cells with glia were then pre-cultured at a density of $3–7\times10^5$ cm^{-1} on coverslips coated with 0.1% (*w*/*v*) poly-L-lysine in the Neurobasal medium supplemented with 5% fetal calf serum, 0.5 mM glutamine, 2% B-27, 50 U/ml penicillin, 50 μg/ml streptomycin, and 25 μM glutamate. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Four hours after pre-culture, the medium was replaced with serum-free Neurobasal-supplemented with 0.5 mM glutamine, 2% B-27, 50 U/ml penicillin, and 50 μg/ml streptomycin. The neurons were then cultured at the same condition, and one-third of the medium was replaced twice a week. The cultured neurons were harvested and used 10 days afterwards for different assays.

Determination of hippocampus synaptic Ca^{2+}-ATPase activity

Hippocampus was isolated from the rat cerebellum; the remainder of the rat hippocampus was homogenized for the preparation the synaptic plasma membrane as previously described by Michaelis et al. (Michaelis et al. 1983; Zaidi et al. 1998). Simply, the rat hippocampus free from the cerebrum were homogenated in a buffer (10 mM Tris–HCl, 1.2 mM $MgCl_2$, 1.0 mM EGTA, 1.0 mg/ml soybean trypsin inhibitor and 0.32 M sucrose, pH7.4), and the homogenate was centrifuged twice (1,000 ×*g*/10 min/4°C) to remove the residues and the supernatant was centrifuged at 30,000 *g*/4°C for 30 min. Then, the pellets were collected for hypotonic treatment to obtain the synaptic plasma membrane (synaptic membrane). The synaptic membrane materials were stored in 10 mM Tris, 10 μM $MgCl_2$, 0.30 M sucrose, pH7.4, in small aliquots at −70°C. Protein measurement was done by the Lowry method (Kit from Pierce, IL, USA). The protein recoveries and the enrichment of the plasma membrane were monitored by measuring Na^+/K^+-ATPase following the preparation instructions as described by Michaelis and coworkers (Michaelis et al. 1983; Zaidi et al. 1998). The activity of plasma membrane Ca^{2+}-ATPase (PMCA) was determined as reported by Atkinson et al. (1973), at 37°C in an assay system in a total volume of 500 μl: 25 mM Tris–HCl, pH 7.4, 50 mM KCl, 1.0 mM $MgCl_2$, 20 μg of membrane protein, 0.10 mM ouabain, 4 μg/ml oligomycin. After a 5-min pre-incubation at 37°C, the reaction was started by the addition of 1.0 mM ATP, continued for 30 min, and stopped by the addition of 1.0 ml of a solution containing 2% ammonium molybdate in 1.8 M H_2SO_4 and 5% (*v*/*v*) of polyoxyethylene ether (W-1). The yellow color was read immediately at 390 nm. The PCMA activity was expressed as nanomoles of inorganic phosphate liberated per milligram of membrane protein per hour (nmol pi/mg/h).

Determination of the intracellular free Ca^{2+}concentration

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in neurons was measured using the fluorescent indicator dye Fura-2/AM. Briefly, the cultured hippocampal neurons ($3–6\times10^5$ cells/ml) were collected and re-suspended to load 5 μM Fura-2/AM in a modified Krebs medium (containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM Mg_2SO_4, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, and 20 mM HEPES, pH 7.4) for 30 min at 37°C in a 50-ml glass bottle, and continuous fluxing of 5% CO_2, 95% air. Cells were then washed, re-suspended in the same medium and further incubated at 37°C to allow complete de-esterification of Fura-2/AM. Fluorescence was measured in a quartz cell with a computer-assisted Perkin-Elmer LS-50 fluorimeter. Excitation wavelength was positioned at 340 and 380 nm alternatively; emission wavelength was set at 510 nm. $[Ca^{2+}]_i$ was calculated as indicated in the report of Gutierrez-Martin Y et al. (Carini et al. 1996; Gutierrez-Martin et al. 2005) with the equation:

$$\left[Ca^{2+}\right]_i = K_d \times \{(R - R_{min})/(R_{max} - R)\} \times \beta$$

Where R was the measured fluorescence ratio (340/380) and calibration values for R_{max} and R_{min} were obtained by permeabilization for Ca^{2+}-bound of cultures with digitonin (10^{-5} g/ml) followed by addition of 5 mM EGTA (for Ca^{2+}-free) with Tris to adjust the final pH (at 7.4). A value of 224 nM was used for K_d, the dissociation constant of the Fura-2:Ca^{2+} complex. β was the ratio of baseline fluorescence (380 nm) under Ca^{2+}-free and -bound conditions.

Statistical analysis

All data were reported as means of a few replicates±SD after different experiments, data analyzed with Microal Origin 6.0 biological statistical software. Statistical analyses were carried out using ANOVA followed by Dunnett-*t* test, *P*-values of significance were denoted as the following: **P*<0.05; ***P*<0.01.

Results

The photomicrograph of morphological alterations in cultured hippocampal neurons affected by different concentrations of MDA

The deleterious effects of MDA on hippocampal neurons are shown in Fig. 2. After hippocampal neurons were

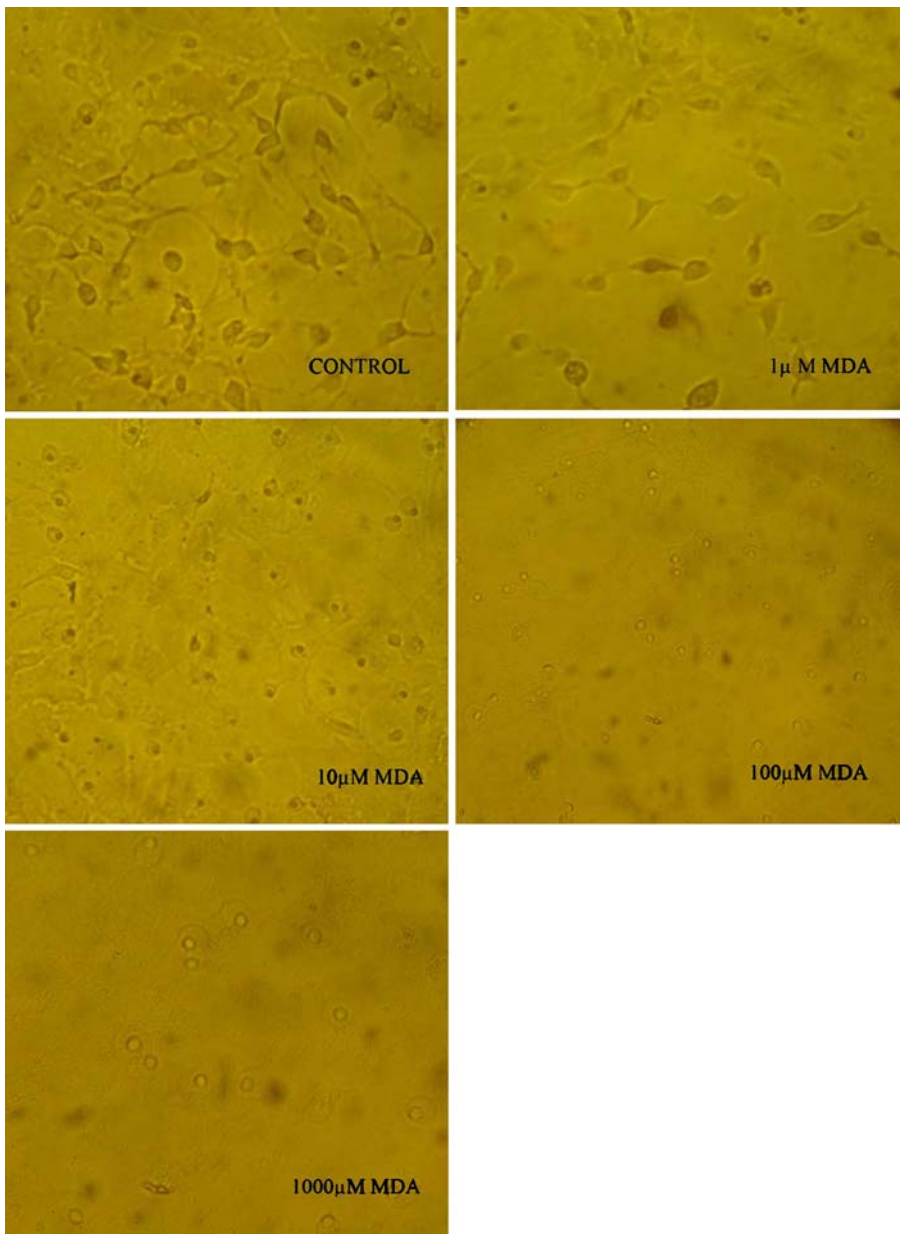

Fig. 2 The morphological alterations of hippocampal neuron cultures were affected by different concentration of MDA for 3 h (photomicrographs ×300). Compared to the control, 1 μM MDA induced the cytons to be swollen and the axons became shortened. Ten micromolar of MDA induced the nucleus to undergo pycnosis. One hundred micromolar and 1,000 μM MDA brought about a malformed cell shape, but there were no significant differences in cell morphological alterations except for the size of neurons enlarged in 1,000 μM MDA concentration

cultured for 10 days, the neurons were exposed to 1.0 μM, 10 μM, 100 μM and 1,000 μM MDA for 3 h, a series of photomicrographs were then taken. Compared to the control, the hippocampal cytons became progressively swollen, and the neuronal synapses and axons became diminutive and shortened. The nuclei of cultures underwent pycnosis and the verge of neurons became unclear when MDA concentration was 10 μM. As the concentration reached up to 100 μM and 1,000 μM, the hippocampal neurons displayed much more severe damage with the increase of MDA concentration, and the neurons were almost completely collapsed.

The effects of MDA on plasma membrane Ca^{2+}-ATPase activity (PMCA)

Effects of MDA on PMCA are shown in Fig. 3. Before determination of the PMCA activity, the addition of 1.0 μM of thapsigarigin (a selective inhibitor of ER Ca^{2+}-ATPase) into the system had only a slight effect, which indicated that the preparations had negligible contamination of ER membrane proteins (data not shown). The synaptic membrane proteins of hippocampal neurons were exposed to 1.0, 10, 100, and 1,000 μM MDA, and the PMCA activity was estimated at 0, 5, 10, 15, 20, 25, and 30 min, respectively. The inhibition of PMCA activity was found to be intensified with the increase of MDA concentrations and time. When the MDA concentrations were at 1.0 μM and 10 μM, they induced only minute inhibitions on PMCA activity within 30 min. When MDA concentration was elevated to more than 100 μM, the activity of PMCA was found strikingly reduced (Fig. 3.).

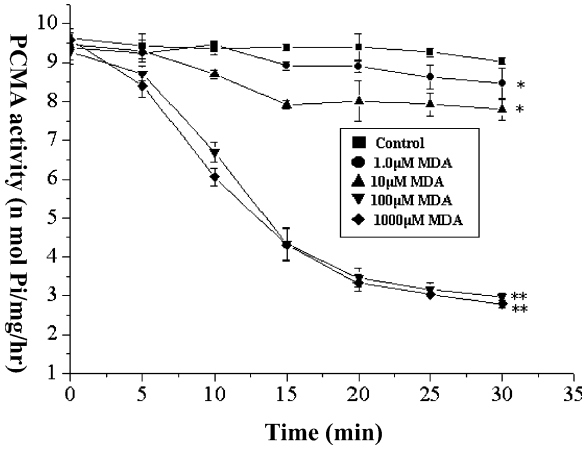

Fig. 3 Inhibitory effects of MDA on plasma membrane Ca^{2+}-ATPase activity (PMCA). The data presented are means of six measurements±SD. $^{**}P<0.01$ vs. control (0 μM of MDA, in PBS, pH7.4), $^{*}P<0.05$ vs. control

The effects of MDA on intracellular free Ca^{2+} concentration

The cytosolic free Ca^{2+} concentration was measured following the Fura-2/AM loading (30 min) on the cultured hippocampal neurons (Fig. 4). The hippocampal neurons cultured in the presence of 1.0, 10, 100,

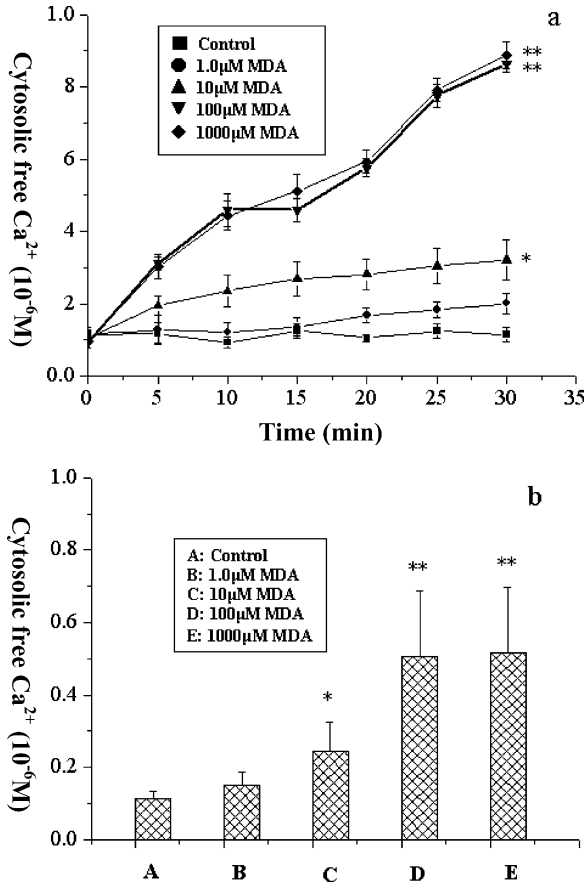

Fig. 4 **a** and **b** Different concentrations of MDA-induced alteration of cytosolic free $[Ca^{2+}]_i$ in hippocampal neuronal cultures. **a** Time course of cytosolic free $[Ca^{2+}]_i$ alteration. There were significant differences among 10 min, 15 min, and 20 min at 100 μM MDA concentration (F=37.884, $P<0.05$). From 10 to 15 min, there was no difference (P=0.861) in cytosolic free $[Ca^{2+}]_i$ alteration, but from 15 to 20 min, there was significant difference ($P<0.05$). **b** Cytosolic free $[Ca^{2+}]_i$ after incubated with different concentrations of MDA for 30 min. The data presented are means of seven measurements±SD. $^{**}P<0.01$ vs. control (0 μM of MDA, in PBS, pH7.4) or 1 μM of MDA, $^{*}P<0.05$ vs. control or 1 μM of MDA

and 1,000 μM MDA resulted in a concentration- and time-dependent increase of $[Ca^{2+}]_i$. As seen in Fig. 4a, 1.0 μM MDA caused no change of $[Ca^{2+}]_i$, while at 10 μM, slight but non-variable elevation of $[Ca^{2+}]_i$ was seen. When MDA concentrations reached up to 100 μM, an early slow elevation and late rapid increase of $[Ca^{2+}]_i$ elevation were observed and there were significant differences in cytosolic $[Ca^{2+}]_i$ at the time point between 10 to 15 min and 15 to 20 min. The results suggested that MDA (not less than 10 μM) induced $[Ca^{2+}]_i$ elevation in two phases: an early progressive elevation phase within the first 15 min (early phase), followed by a rapid increase phase from 15 to 30 min (late phase). It was presumed that the different phases of $[Ca^{2+}]_i$ increase might result from two different Ca^{2+} origins: the early phase was a release of Ca^{2+} from intracellular compartments and the late phase was an influx of Ca^{2+} from the extracellular space. These results were in agreement with previously published data as 4-HNE triggered calcium elevation in isolated rat hepatocytes (Carini et al. 1996).

The effects of nimodipine and EGTA on intracellular free Ca^{2+} concentration

The plasma membrane's L-type VOCC (voltage operation calcium channel) blocker, nimodipine, and the extracellular calcium chelator EGTA were used to determine their effects on the two different phases of cytosolic $[Ca^{2+}]_i$ alteration in cultured hippocampal neurons under MDA stress. The experimental results are given in Fig. 5 (also see Fig. 1 for viewing Ca^{2+} channel scheme).

In the presence of 100 μM MDA, 3 μM nimodipine caused inhibition of the MDA-induced late-phase elevation of $[Ca^{2+}]_i$, whereas the early phase elevation was almost not effected (Figs. 5a and 4a), indicating that the late-phase elevation of $[Ca^{2+}]_i$ was a result of the L-type calcium channel opening on cellular membrane. Moreover, the excess amount of Ca^{2+} chelator, 5 mM EGTA in combination with 100 μM MDA resulted in only a transient and diminutive Ca^{2+} elevation, which might attributed to the release of a finite intracellular Ca^{2+}-store. After that, the Ca^{2+} in the cytoplasm was "extracted out" because of the chelation by EGTA and the $[Ca^{2+}]_i$ decreased to normal level (Fig. 5b).

In sum, as compared with adding 100 μM MDA alone, 3 μM nimodipine together with 100 μM MDA,

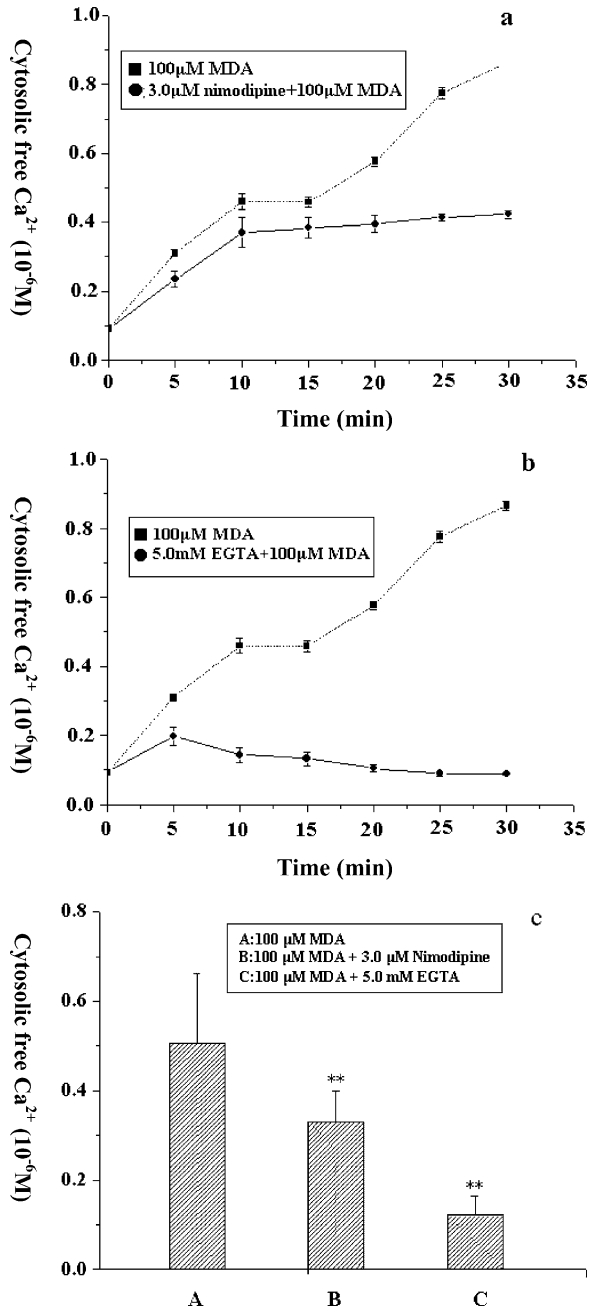

Fig. 5 **a**–**c** Inhibitory effect of nimodipine and EGTA on MDA-induced $[Ca^{2+}]_i$ increase in hippocampal neuronal cultures. The *dashed line*, as a control, was taken from Fig. 4a (100 μM MDA trace). **a** Three micromolar nimodipine in combination with 100 μM MDA inhibited the MDA-induced late-phase $[Ca^{2+}]_i$ elevation; **b** 5.0 mM EGTA in combination with 100 μM MDA inhibited the MDA-induced early phase and late-phase $[Ca^{2+}]_i$ elevation; **c** the total $[Ca^{2+}]_i$ was determined under the treatment of: 100 μM MDA, 100 μM MDA+3.0 μM nimodipine, and 100 μM MDA+5.0 mM EGTA. The data presented are means of seven measurements ± SD. $^{**}P<0.01$ vs. control (100 μM MDA)

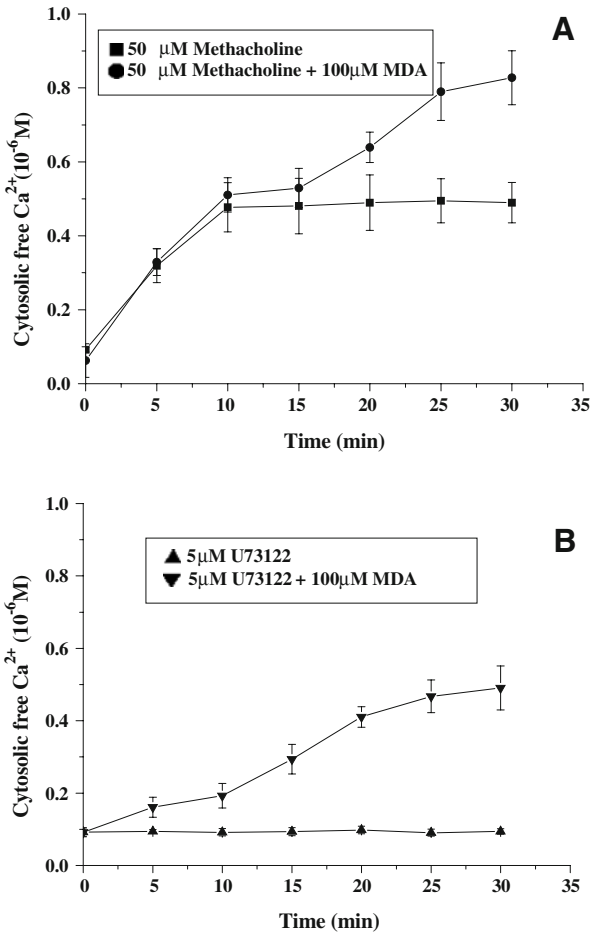

Fig. 6 **a** and **b** Effects of methacholine and U73122 on MDA-induced $[Ca^{2+}]_i$ increase in hippocampal neuron cultures. **a** Fifty micromolar methacholine resulted alteration of $[Ca^{2+}]_i$ in the presence or absence of 100 μM MDA; **b** 5.0 μM U73122 resulted alteration of $[Ca^{2+}]_i$ in the presence or absence of 100 μM MDA. The data presented are means of four measurements±SD

and 5 mM EGTA in combination with 100 μM MDA in the system (30 min treatment) decreased the $[Ca^{2+}]_i$ to 34.76%, and 76.02% respectively (Fig. 5c).

The effects of MDA on calcium signalling pathways

In order to probe into the mechanisms of calcium signalling pathway under MDA stress, an agonist, methacholine, which is believed to mobilize Ca^{2+} through the IP3 pathway(Putney et al. 1986), and a phospholipase C inhibitor, U73122, were applied to the system and the experimental results are shown in Fig. 6 (also see Fig. 1 for viewing calcium signalling pathways).

Treatment of cultured hippocampal neurons with 50 μM methacholine for 30 min caused an early phase elevation of $[Ca^{2+}]_i$ which was basically identical to the MDA effect in this phase (Figs. 4a and 6a). In addition, when the system was treated by 100 μM MDA together with 50 μM methacholine, the elevation of $[Ca^{2+}]_i$ (in both early and late phases) appeared to be the same as the MDA effect (Figs. 4a and 6a). Combining above observations (Figs. 4a, 5b and 6a), we could infer that early phase elevation of $[Ca^{2+}]_i$ was from intracellular Ca^{2+}-stores.

Incubation of hippocampal neurons, however, with only 5 μM U73122, resulted in an almost complete inhibition of the early phase increase of $[Ca^{2+}]_i$ (Fig. 6b). The MDA-induced late-phase effect appeared not interfered by U73122 supplementation. These data suggested that the MDA-initiated early phase elevation of $[Ca^{2+}]_i$ was responsible for activating the phospholipase C activity and subsequent formation of IP3, which then activated the IP3-sensitive calcium channel in the ER membrane, ensuing release of calcium ions into intracellular compartment.

It was reported that PKA may phosphorylate and activate several types of Ca^{2+} channels including Ca^{2+}

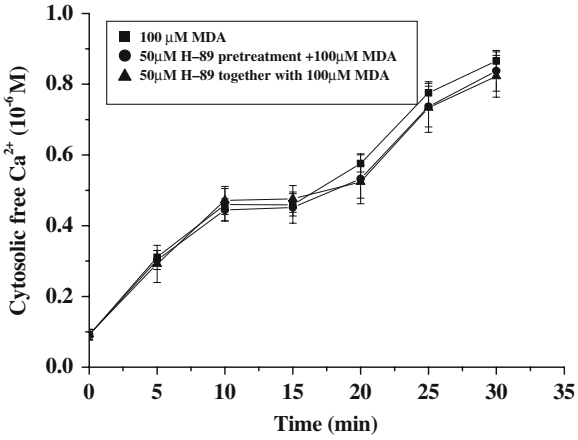

Fig. 7 Effects of cAMP/PKA inhibitor H-89 on MDA-induced $[Ca^{2+}]_i$ increase in hippocampal neuron cultures. Whether H-89 pretreated the system or together with 100 μM MDA, it could not effect on the alteration of $[Ca^{2+}]_i$ induced by 100 μM MDA. The data presented are means of four measurements±SD

release-activated Ca^{2+} channel (Hahn et al. 2000; Wu et al. 1999), resulting to the elevation of $[Ca^{2+}]_i$. In order to examine whether cAMP/PKA signalling pathways were involved in the regulation of mobilizing intracellular Ca^{2+} under the condition of MDA stress, H-89, a selective PKA inhibitor was used (Fig. 7). H-89, 50 μM, either pretreated alone (for 30 min) or applied together with 100 μM MDA, did not significantly interfere the MDA-induced increase of $[Ca^{2+}]_i$. These suggested that MDA was not involved in the cAMP/PKA signalling pathway to mobilize intracellular Ca^{2+}.

Discussion

Many researches reported that reactive oxygen species could inhibit the activity of Ca^{2+}-ATPase, Na^+/K^+-ATPase in the model systems employing erythrocytes (Hahn et al. 2000; Wu et al. 1999) and neurons (Bogdanova et al. 2003; Petrushanko et al. 2006). The "Ca^{2+} hypothesis of brain aging" was supported by considerable evidence that showed age-related changes in Ca^{2+}-dependent cellular damages (Campbell et al. 1996; Mattson and Magnus 2006; Moyer et al. 1992; Porter et al. 1997). Other studies demonstrated that histidine (Aldini et al. 2002; Slatter et al. 2004), arginine (Oe et al. 2003), lysine (Ishii et al. 2006) as well as a variety of neuron transmitters, such as dopamine and histamine (Li et al. 2005), may react with MDA, 4-HNE or other lipid peroxidation intermediates to form pyrimidine ring or various crosslinking complexes. In the present study, the shapes and fine structures of hippocampal neurons were found damaged with the increase of MDA concentration. Although the deterioration mechanisms were unclear, the activity of PMCA being gradually inhibited with the increase of MDA concentration implied that MDA reacting with various key amino residues in the proteins (such as histamine, lysine, and arginine which presented active centers of PMCA) should be responsible for the impairment effects.

The heterogeneity of the hippocampal neuron population was also observed in the study and appeared to be less effective in the system. Considering the common mechanisms of carbonyl toxicity, our study at this level was focused on investigating the general impact of MDA on $[Ca^{2+}]_i$ of hippocampal neurons rather than to discriminate its effect on different subpopulations. Further studies are needed to distinguish carbonyl stress on different neuron types in the future, also considering the reactivity of toxic carbonyls to different neuron transmitters.

The quantitative study of the $[Ca^{2+}]_i$ increase in cultured hippocampal neurons under MDA stress indicated that nimodipine, a cytoplasmic membrane L-type VOCC blocker, and an extracellular calcium chelator, EGTA, can substantially prohibit the MDA-induced alteration of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ alteration of Fig. 5a indicated that MDA-induced opening of the L-type calcium channel, caused calcium influx (late phase), which was verified by nimodipine's specialized inhibitory effect on $[Ca^{2+}]_i$ increase.

Our experimental results also clearly demonstrated that the mechanisms for activating Ca^{2+} entry into cytoplasmic space cause to elevate $[Ca^{2+}]_i$ by MDA stress via two pathways: the one (early phase) was Ca^{2+} released from intracellular calcium store, which was through activating phospholipase C signalling pathway forming IP3 (Fig. 1, upper right pathway); the other (late phase) was Ca^{2+} influx from extracellular spaces, which was responsible for inhibiting PMCA activity and opening membrane calcium channels, such as L-type VOCC (Fig. 1, upper left channel). Also, these observations presented that Ca^{2+} released from calcium store was earlier than Ca^{2+} influx from extracellular space, which may imply that the signalling pathway regulating calcium release from different stores associated with calcium homeostasis was more sensitive to

MDA stress than calcium channels in cytoplasmic membrane in cultured hippocampal neurons.

Rossi et al. (1990, 2001) have reported similar signalling transduction effects of some other carbonylic products of lipid peroxidation including a group of 4-hydroxyalkenals, such as 4-hydroxyhexenal, 4-hydroxyoctenal and 4-HNE, in HL-60 and hepatic cell. Their experimental results suggested that 4-hydroxyalkenals, in the range of 10^{-9} up to 10^{-6} M were able to stimulate the activity of phospholipase C, which then converted phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphophate (IP3) and diacylalycerol, to mobilize Ca^{2+}. Another research also showed that 4-HNE triggered Ca^{2+} influx in isolated rat hepatocytes by activating PL-C/IP3 signalling pathway. In the present study, we have investigated the signalling mechanism following MDA stress. Our data suggested that, in the presence of 100 μM MDA, U73122 inhibited the elevation of $[Ca^{2+}]_i$ in the early phase, suggesting that PL-C/IP3 signalling pathway was involved in mobilizing Ca^{2+}. But H-89, an inhibitor of PKA, did not effect the change of $[Ca^{2+}]_i$, indicating that cAMP/PKA signalling pathway was not engaged in triggering Ca^{2+} events under MDA stress (Fig. 1, down side).

Compared with 4-HNE studies, the effective MDA concentration in these system was relatively high (100 μM) which was in the range of pathological conditions. The requirement of the supra-physiological concentration of MDA may be partly due to a quick metabolization of MDA by intracellular enzymes and biological "consumption" of various free thiol and amino groups in the biomaterials in the model system (data not shown).

In summary, our study clearly demonstrated that disturbances of Ca^{2+} homeostasis under carbonyl stress in cultured hippocampal neurons induced inhibition of synaptic PMCA and induced the opening of various types of calcium channels, thus ensuing the increase of $[Ca^{2+}]_i$ in different patterns and mechanisms. Our results also suggested that MDA may act as a signalling molecule, hence, regulating Ca^{2+} release from intracellular stores, causing damages to rat hippocampal neurons by triggering Ca^{2+} influx and influencing Ca^{2+} homeostasis in cultured neurons.

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