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Mechanisms of AM404-induced $[Ca^{2+}]_i$ rise and death in human osteosarcoma cells

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ABSTRACT

The effect of *N*-(4-hydroxyphenyl) arachidonoyl-ethanolamide (AM404), a drug commonly used to inhibit the anandamide transporter, on intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) and viability was studied in human MG63 osteosarcoma cells using the fluorescent dyes fura-2 and WST-1, respectively. AM404 at concentrations $\geq 5\,\mu M$ increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} value of 60 μM. The Ca^{2+} signal was reduced partly by removing extracellular Ca^{2+}. AM404 induced Mn^{2+} quench of fura-2 fluorescence implicating Ca^{2+} influx. The Ca^{2+} influx was sensitive to La^{3+}, Ni^{2+}, nifedipine and verapamil. In Ca^{2+}-free medium, after pretreatment with 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor), AM404-induced $[Ca^{2+}]_i$ rise was abolished; and conversely, AM404 pretreatment totally inhibited thapsigargin-induced $[Ca^{2+}]_i$ rise. Inhibition of phospholipase C with U73122 did not change AM404-induced $[Ca^{2+}]_i$ rise. At concentrations between 10 and 200 μM, AM404 killed cells in a concentration-dependent manner presumably by inducing apoptotic cell death. The cytotoxic effect of 50 μM AM404 was partly reversed by prechelating cytosolic Ca^{2+} with BAPTA/AM. Collectively, in MG63 cells, AM404 induced $[Ca^{2+}]_i$ rise by causing Ca^{2+} release from the endoplasmic reticulum in a phospholipase C-independent manner, and Ca^{2+} influx via L-type Ca^{2+} channels. AM404 caused cytotoxicity which was possibly mediated by apoptosis.

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1. Introduction

N-(4-hydroxyphenyl) arachidonoyl-ethanolamide (AM404) has been widely used as a pharmacological tool to inhibit the transporter of anandamide, an endogenous cannabinoid (Calignano et al., 1997; Kelley and Thayer, 2004; La Rana et al., 2006). However, evidence shows that AM404 possesses other actions. AM404 exerts many in vivo effects such as protection against cerebral ischaemia in gerbils (Zani et al., 2007), decrease in Fos-immunoreactivity in the spinal cord (Borsani et al., 2007), reduction of ethanol self-administration (Cippitelli et al., 2007), modulation of neuropathic and inflammatory pain (La Rana et al., 2006), and potentiation of anandamide hypotension (Calignano et al., 1997). In vitro, AM404 has been shown to inhibit synaptic transmission between rat hippocampal neurons in culture independent of cannabinoid CB1 receptors (Kelley and Thayer, 2004), to inhibit C6 glioma cell proliferation at concentrations used to block the cellular accumulation of anandamide (Jonsson et al., 2003), and to activate vanilloid receptors (Zygmunt et al., 2000).

A regulated rise in $[Ca^{2+}]_i$ is a key signal in all cell types, and can trigger many physio-pathological events (Berridge, 1993, 1997; Bootman et al., 2002); but an unregulated elevation in $[Ca^{2+}]_i$ is often cytotoxic (Annunziato et al., 2003). Thus, it is crucial to explore the effect of an agent on cellular Ca^{2+} signaling in order to understand its in vitro action. We have previously reported that AM404 elevated $[Ca^{2+}]_i$ in canine renal tubular cells, questioning its selectivity as a pharmacological tool for investigating the anandamide transporter (Chen et al., 2001); however, whether AM404 altered cell viability was unknown. In the present study, human MG63 osteosarcoma cells were used to investigate the effect of

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AM404 on $[Ca^{2+}]_i$ and cell viability. MG63 cells have characteristics similar to human osteoblasts and have been used as a model for research on human osteoblasts (Rezzonico et al., 2003). Many endogenous and exogenous reagents can stimulate MG63 cells by inducing a $[Ca^{2+}]_i$ rise, such as 2,2′-dithiodipyridine (Kuo et al., 2003), carvedilol (Liu and Jan, 2004), and desipramine (Jan et al., 2003). The inositol-1,4,5-trisphosphate-sensitive Ca^{2+} store is an important Ca^{2+} store that releases Ca^{2+} into the cytosol when cells are stimulated by endogenous reagents such as histamine (Lee et al., 2001), as well as upon exposure to some exogenous agents. But exogenous agents can release Ca^{2+} from inositol 1,4,5-trisphosphate-insensitive stores (Kuo et al., 2003; Jan et al., 2003).

Using fura-2 as a fluorescent Ca^{2+} indicator, this study shows that AM404 induced a $[Ca^{2+}]_i$ rise in a concentration-dependent manner in MG63 cells. The time course and the concentration–response relationship, the Ca^{2+} sources of the Ca^{2+} signal, and the role of phospholipase C in the signal were investigated. The effect of AM404 on cell viability and its Ca^{2+}-dependence, and the possible role of apoptosis were examined.

2. Materials and methods

2.1. Cell culture

MG63 human osteosarcoma cells obtained from American Type Culture Collection were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. Solutions

Ca^{2+}-containing medium contained (pH 7.4) 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 5 mM glucose. In Ca^{2+}-free medium, Ca^{2+} was substituted with 0.3 mM EGTA. AM404 was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other agents were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter basal $[Ca^{2+}]_i$.

2.3. $[Ca^{2+}]_i$ measurements

Trypsinized cells (10^6/ml) were loaded with 2 μM fura-2/AM for 30 min at 25 °C in culture medium. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25 °C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described (Grynkiewicz et al., 1985). Data are shown as $[Ca^{2+}]_i$ elevation, not the area under the curve. Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$, by recording the excitation signal at the 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-s intervals as described previously (Merritt et al., 1989).

2.4. Cell viability assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Augmentation in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at 10,000 cells/well in culture medium for 24 h in the presence of 0–200 μM AM404. The cell viability detecting reagent 4-3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate (WST-1; 10 μM pure solution) was added to samples after AM404 treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, 10 μM BAPTA/AM was added to cells for 1 h followed by a washout with Ca^{2+}-containing medium. Then cells were incubated in a 37 °C incubator overnight in the presence of 50 μM AM404. Cells were further loaded with 2 μM fura-2/AM for 30 min at room temperature; and were washed again followed by incubation with WST-1 for 30 min at 37 °C. The absorbance of samples (A_{450}) was determined by using enzyme-linked immunosorbent assay reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

2.5. Apoptosis assays: Measurements of subdiploidy nuclei by flow cytometry

After incubation with 0–200 μM AM404 overnight, cells were collected from the media, and were washed with ice-cold Ca^{2+}-containing medium twice and resuspended in 3 ml of 70% ethanol. Then cells were stored at −20 °C. The ethanol-suspended cells were centrifuged for 5 min at 200 × g. Ethanol was decanted thoroughly and the cell pellet was washed with ice-cold Ca^{2+}-containing medium twice, and was then suspended in 1 ml propidium iodide (PI) solution (1% Triton X-100, 20 μg PI, 0.1 mg/ml RNase). The cell pellet was incubated in the dark for 30 min at room temperature. Cell fluorescence was measured in the FACScan flow cytometer (Becton Dickinson immunocytometry systems, San Jose, CA, USA) and the data were analyzed using the MODFIT software.

2.6. Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were from Molecular Probes (Eugene, OR, USA). AM404 was from Biomol International (Plymouth Meeting, PA, USA). The other reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.7. Statistics

Data were reported as means ± S.E.M. of 3–5 experiments, and were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by post-hoc analysis using the Tukey's HSD (honestly significant difference) procedure. A *P*-value less than 0.05 was considered significant.

3. Results

AM404 at concentrations ≥5 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner in Ca^{2+}-containing medium. Fig. 1A shows the typical responses induced by 10, 30, 50 and 100 μM AM404. At a concentration of 1 μM, AM404 had no effect on $[Ca^{2+}]_i$ (=control). The $[Ca^{2+}]_i$ rise induced by 100 μM AM404 reached a net value of 135 ± 2 nM at the time point of 75 s followed by a gradual decay.

Experiments were performed to determine the relative contribution of extracellular Ca^{2+} influx and intracellular Ca^{2+} release in AM404-induced $[Ca^{2+}]_i$ rise. The data show that AM404 also induced $[Ca^{2+}]_i$ rise in Ca^{2+}-free (Ca^{2+} was substituted with 0.3 mM EGTA) medium. Ca^{2+}-free condition was achieved by adding 0.1 ml cell suspension (in 2 mM Ca^{2+}-containing medium) to 0.9 ml Ca^{2+}-free medium in the cuvette right before fura-2 fluorescence was measured. The representative $[Ca^{2+}]_i$ rises induced by 50 and 100 μM AM-404 in Ca^{2+}-free medium are shown in Fig. 1B. Removal of extracellular Ca^{2+} did not alter baseline. AM404 increased $[Ca^{2+}]_i$ by 36 ± 2 nM above baseline at the concentration of 100 μM. The concentration–response curves of AM404-induced $[Ca^{2+}]_i$ rises in both Ca^{2+}-containing medium and Ca^{2+}-free medium are shown in Fig. 1C.

Experiments were performed to exclude the possibility that the smaller AM404-induced response in Ca^{2+}-free medium was caused by 0.3 mM EGTA-induced depletion of intracellular Ca^{2+}. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (Merritt et al., 1989). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} indicates Ca^{2+} influx. Fig. 2 shows that 50 μM AM404 induces an immediate decrease in the 360 nm excitation signal (compared to control). This suggests that AM404-induced $[Ca^{2+}]_i$ rise involved Ca^{2+} influx from extracellular space.

Experiments were performed to distinguish the pathway underlying AM404-induced Ca^{2+} influx. Fig. 3 shows that six inhibitors (La^{3+}, Ni^{2+}, nifedipine, nimodipine, nicardipine and verapamil; all of them are L-type Ca^{2+} channel blockers) of Ca^{2+} entry significantly inhibited 100 μM AM404-induced Ca^{2+} entry.

Previous reports showed that the endoplasmic reticulum was the major Ca^{2+} store in most cell types, including MG63 cells (Lee et

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