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A Phospholipase C-Dependent Intracellular Ca^{2+} Release Pathway Mediates the Capsaicin-Induced Apoptosis in HepG2 Human Hepatoma Cells

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The effect of capsaicin on apoptotic cell death was investigated in HepG2 human hepatoma cells. Capsaicin induced apoptosis in time- and dose-dependent manners. Capsaicin induced a rapid and sustained increase in intracellular Ca^{2+} concentration, and BAPTA, an intracellular Ca^{2+} chelator, significantly inhibited capsaicin-induced apoptosis. The capsaicin-induced increase in the intracellular Ca^{2+} and apoptosis were not significantly affected by the extracellular Ca^{2+} chelation with EGTA, whereas blockers of intracellular Ca^{2+} release (dantrolene) and phospholipase C inhibitors, U-73122 and manoalide, profoundly reduced the capsaicin effects. Interestingly, treatment with the vanilloid receptor antagonist, capsazepine, did not inhibit either the increased capsaicin-induced Ca^{2+} or apoptosis. Collectively, these results suggest that the capsaicin-induced apoptosis in the HepG2 cells may result from the activation of a PLC-dependent intracellular Ca^{2+} release pathway, and it is further suggested that capsaicin may be valuable for the therapeutic intervention of human hepatomas.

Key words: Apoptosis, Ca^{2+} signal, Capsaicin, HepG2 cell

INTRODUCTION

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), a pungent ingredient in a variety of red peppers of the genus *Capsicum*, is well known for interacting at primary sensory neurons exerting its characteristic actions, including excitation, desensitization and neurotoxicity (Holzer, 1991). These actions of capsaicin appear to be mediated by the stimulation of specific vanilloid receptors (VRs) (Szallasi and Blumberg, 1999), which may have therapeutic value, particularly relieving pain (Dray and Urban, 1996). In addition, capsaicin interacts with several targets other than neuronal VRs, such as inhibition of K^+ channels (Dubois, 1982; Kuenzi and Dale, 1996), altered membrane fluidity (Aranda *et al.*, 1995), formation of so-called pseudochannels (Feigin *et al.*, 1995), and inhibition of tyrosyl-tRNA synthetase (Cochereau *et al.*, 1996).

There have been conflicting reports in animal studies of the effects of capsaicin on carcinogenesis. Capsaicin itself was mutagenic (Lawson and Gannett, 1989; Azizan and Blevins, 1995) and promoted tumor formation (Agrawal *et al.*, 1986; Toth *et al.*, 1984), whereas it had protecting effects on the mutagenesis and tumorigenesis induced by various carcinogens (Surh, 1999). *In vitro* studies have also shown capsaicin to inhibit the growth of various immortalized or malignant cell lines (Morre *et al.*, 1995; Morre *et al.*, 1996; Takahata *et al.*, 1999). The anti-proliferative effects of capsaicin in transformed cells have been attributed not to activation of the VRs, but to the inhibition of plasma membrane NADH oxidase (PMNO) (Morre *et al.*, 1995; Morre *et al.*, 1996; Takahata *et al.*, 1999) which is highly expressed in the transformed cells, but absent in normal cells (Morre *et al.*, 1995). In addition, capsaicin has also been shown to induce apoptosis in transformed cells (Wolvetang *et al.*, 1996; Macho *et al.*, 1998; Macho *et al.*, 1999), but not in their normal counterparts (Kang *et al.*, 2003; Morre *et al.*, 1995; Wolvetang *et al.*, 1996; Zhang *et al.*, 2003). Intracellular Ca^{2+} and reactive oxygen species (ROS) have been suggested as the signaling molecules associated with the apoptotic effects of capsaicin in cancer cells (Wolvetang *et al.*, 1996;

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Macho *et al.*, 1999). However, the source of the increased intracellular Ca^{2+} and the exact mechanism of the apoptotic action of capsaicin have not been completely unraveled.

Apoptosis is a highly organized cell death process, which is characterized by early and prominent condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of proteases and endonucleases, enzymatic cleavage of the DNA into oligonucleosomal fragments and segmentation of the cells into membrane-bound apoptotic bodies (Kidd, 1998). Apoptosis has been recognized to play an important role in the maintenance of tissue homeostasis by the selective elimination of excessive cells (Nayfield *et al.*, 1991); Genetic changes resulting in loss of apoptosis or derangement of apoptosis-signaling pathways, are likely to be critical components of carcinogenesis (Kastan *et al.*, 1998; Schulte-Hermann *et al.*, 1997). Additionally, the induction of apoptosis of cancer cells is recognized as a valuable tool for cancer treatment (Kornblau, 1998).

Thus, the purposes of this study were to investigate (i) whether capsaicin induces apoptosis in the HepG2 cells, (ii) whether an intracellular Ca^{2+} signal is involved in the mechanism of capsaicin-induced apoptosis, and (iii) a possible intracellular Ca^{2+} source and the role of this pathway as a mediator of capsaicin-induced apoptosis.

MATERIALS AND METHODS

Materials

The HepG2 human hepatoblastoma cell line was purchased from the American Type Culture Collection (Rockville, MA). The powdered Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, sodium pyruvate, capsaicin, capsazepine, manoalide, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5,-dione (U-73122), ethylene glycol-bis-(aminoethylether)-ethane-*N,N,N,N*-tetraacetic acid (EGTA), propidium iodide (PI), ribonuclease A and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). The bis-(*o*-aminophenoxy)-ethane-*N,N,N',N*-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) and 1-(2,5-carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethane-*N,N,N',N*-tetraacetoxy-lmethyl ester (Fura-2/AM) were purchased from Molecular Probes, Inc. (Eugene, OR). The fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The BAPTA/AM and Fura-2/AM were prepared as stock solutions in dimethyl sulfoxide (DMSO), and then diluted with aqueous medium to the final desired concentrations. The stock solutions of drugs were sterilized by filtration through 0.2 ìm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

HepG2 cells were grown in a MEM supplemented with 10% FBS, 200,000 IU/L penicillin, 200 mg/L of streptomycin and 1 mM sodium pyruvate, at 37 °C in a humidified incubator under a 5% CO_2/95% air mixture. The culture medium was replaced every other day. After attaining confluence, the cells were subcultured, following trypsinization treatment.

Flow cytometry assays

For flow cytometry analysis, HepG2 cells were collected and washed twice with PBS buffer, pH 7.4. After fixing in 80% ethanol for 30 min, the cells were washed twice and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 mg/L PI and 50 mg/L ribonuclease A, for DNA staining. The cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using the WinBryte software (BIO-RAD, Hercules, CA) to determine the percentage of nuclei with hypodiploid contents indicative of apoptosis (Bombeli *et al.*, 1997).

The normal lipid organization of the plasma membrane is altered soon after the initiation of apoptosis. Thus, Annexin-V binding was also employed as an indicator of apoptosis (Vermes *et al.*, 1995) to demonstrate the loss of phospholipid asymmetry and the presence of phosphatidylserine on the outer layer of the plasma membrane. The Annexin-V binding was analyzed using a commercial kit (Boehringer Mannheim Biochemicals, Mannheim, Germany). The cells were washed in cold PBS, and then resuspended in binding buffer. An aliquot of the cell suspension (500 μL) was exposed to Annexin-V-FLUOS. The cells were gently vortexed, incubated at room temperature for 20 min in the dark and then analyzed by FACScan within 1 h of staining.

Intracellular Ca^{2+} measurement

Aliquots of the HepG2 cells were washed in EBSS, 5 μM Fura-2/AM added and the cells incubated for 30 min at 37 °C. Unloaded Fura-2/AM was removed by centrifugation at 150 × *g* for 3 min. Cells were resuspended at a density of 2×10^9 cells/L in Krebs-Ringer buffer (KRB), containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose and 2.5 mM probenecid (pH 7.4). Fura-2-loaded cells were maintained at 25°C for 90 min before the fluorescence measurement. For each experiment, a 0.5 mL aliquot of Fura-2-loaded cells was equilibrated to 37 °C in a stirred quartz cuvette. The fluorescence emission (510 nm) was monitored with excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experiment, the fluorescence maximum and minimum values at

each excitation wavelength were obtained by first lysing cells with 20 mg/L digitonin for the maximum fluorescence value and then adding 10 mM EGTA for the minimum. After obtaining these maxima and minima values, the 340:380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using the software, F-4500 Intracellular Cation Measurement System, provided by Hitachi.

Data analysis

All experiments were performed four times. The data were expressed as means ± standard error of the mean (SEM) and the results analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's tests for individual comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

Induction of apoptosis by capsaicin in HepG2 cells

The effect of capsaicin on apoptotic cell death of HepG2 cells was examined using two independent methods. The capsaicin-induced apoptosis was identified using Annexin-V binding assay to detect the loss of phospholipid asymmetry and the presence of phosphatidylserine on the outer layer of the plasma membrane, which occurs at an early stage of apoptosis (Vermes *et al.*, 1995), as shown in Fig. 1A. Capsaicin was also found to induce DNA fragmentation, a hallmark of apoptosis (Kidd, 1998), in a time-dependent manner, as studied by flow cytometry for determining the hypodiploid DNA content stained with PI (Milner *et al.*, 1998), as depicted in Figs. 1B and 1C. These results clearly demonstrate that capsaicin induced apoptotic cell death in the HepG2 cells.

Role of intracellular Ca^{2+} signal in the capsaicin-induced apoptosis

Since the intracellular Ca^{2+} signal appears to be commonly involved in the mechanism of apoptosis (McConkey and Orrenius, 1997), whether capsaicin alters the intracel-

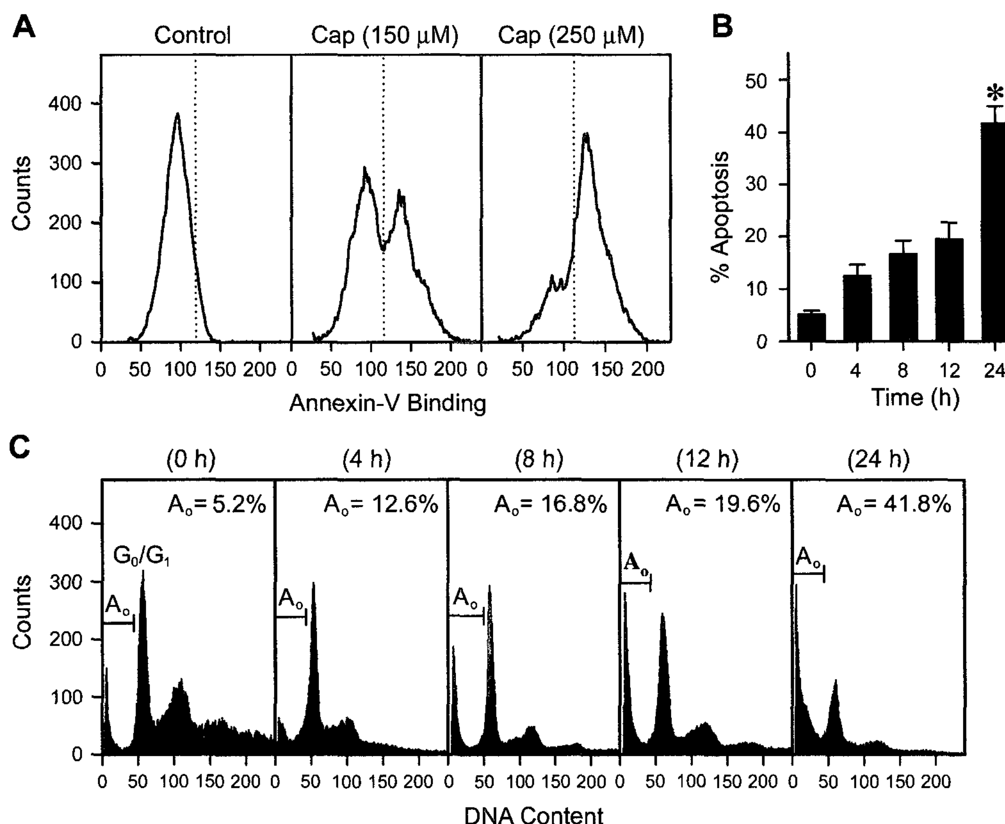

Fig. 1. Capsaicin induces apoptosis in concentration- and time-dependent manners in HepG2 human hepatoma cells. In the experiments of (A), the cells were incubated in the absence (control) or presence of capsaicin (Cap) for 24 h. Cells were stained with Annexin-V-FLUOS and analyzed by flow cytometry. Note that in the presence of Cap there is a shift in the Annexin-V-FLUOUS fluorescence. This is due to the binding of Annexin-V to the membrane phospholipids of cells undergoing apoptosis. The results are representative of four experiments. In the experiments of (B and C), the cells were treated with 250 μM Cap at each designated time. The number of apoptotic cells was measured by flow cytometry as described in the text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. In the graphs (B), the data represent the mean values of four replications, with the bars indicating the SEM. *p<0.05 compared to control.

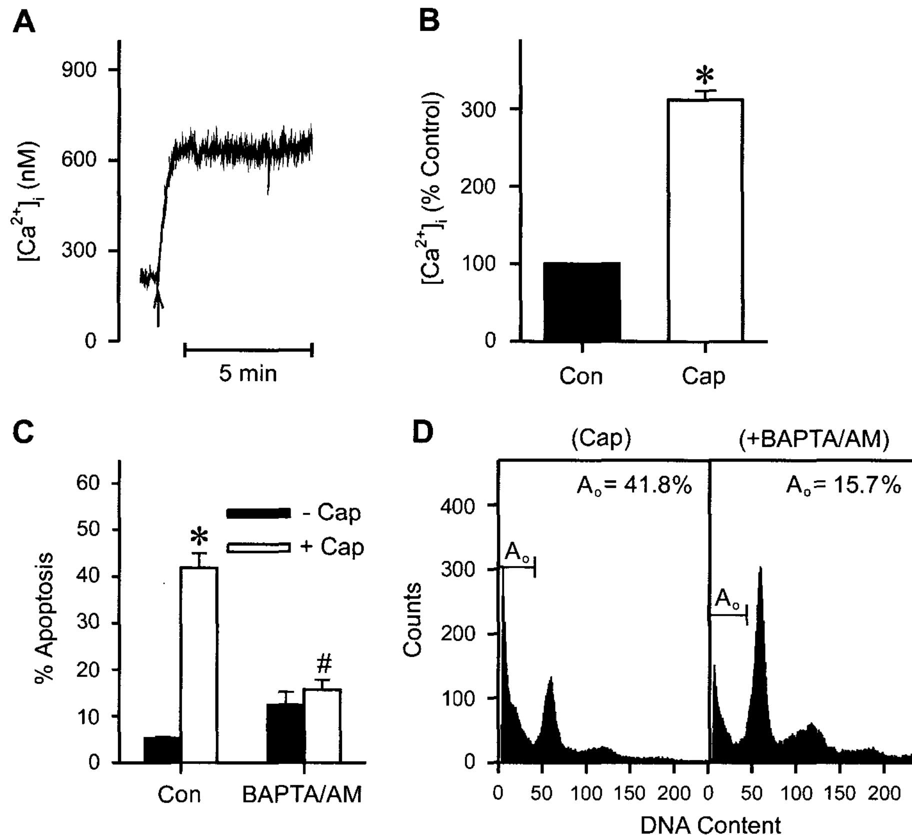

Fig. 2. Capsaicin-induced apoptosis is dependent on the intracellular Ca^{2+} in HepG2 human hepatoma cells. The intracellular Ca^{2+} concentration was assessed by the Fura-2 fluorescence technique, and the data (A) represent the intracellular Ca^{2+} changes with time. The arrow shows the time point for the addition of capsaicin (Cap; 250 μM). The data (B) are expressed as the percent change of the control conditions where the cells were incubated with Cap-free medium. (C and D) The number of apoptotic cells was measured by flow cytometry. Cells were treated for 24 h with capsaicin (Cap; 250 μM). BAPTA/AM (1 μM) was added to the cells 4 h before Cap treatment. In the graphs (C), the data represent the mean values of four replications, with the bars indicating the SEM. $^{*}p<0.05$ compared to control. $^{\#}p<0.05$ compared to Cap alone.

lular Ca^{2+} concentration was examined using the Fura-2 fluorescence technique (Grynkiewicz *et al.*, 1985). Capsaicin, at the concentration inducing apoptosis (250 μM), rapidly increased the intracellular Ca^{2+} concentration, as shown in Figs. 2A and 2B. Treatment with BAPTA/AM (1 μM), an intracellular Ca^{2+} chelator, significantly suppressed the capsaicin-induced apoptosis as shown in Figs. 2C and 2D. These results suggest that capsaicin-induced apoptosis may be due to increased intracellular Ca^{2+}.

To determine the source of the capsaicin-induced intracellular Ca^{2+} increase, the intracellular Ca^{2+} concentration was measured using a nominal Ca^{2+}-free medium containing 1 mM EGTA. This experimental protocol can effectively reduce the extracellular free Ca^{2+} concentration, and thus, blunt available Ca^{2+} influx. Under these conditions the cellular Ca^{2+} response to capsaicin was not significantly altered as illustrated in Fig. 4. Treatment with dantrolene, an inhibitor of intracellular Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (Ehrlich *et al.*, 1994), completely inhibited the Ca^{2+}-increasing effect of capsaicin. These results indicate that the increased intracellular Ca^{2+} concentration caused by capsaicin may be due to Ca^{2+} release from IP_3-dependent internal stores. Since phospholipase C (PLC) seems to act as an enzyme to liberate IP_3 from the plasma membrane (Hughes and Putney, 1990), the role of PLC was further examined in these actions of capsaicin. Treatment with U-73122, a specific PLC inhibitor (Jin *et al.*, 1994), or manoalide, a non-specific inhibitor of PLC and phospholipase A_2 (Bennett *et al.*, 1987), significantly inhibited the capsaicin-induced intracellular Ca^{2+} release, as illustrated in Fig. 4. Moreover, treatment with these inhibitors (dantrolene, U-73122, manoalide) also significantly prevented capsaicin-induced apoptosis, whereas EGTA had no significant effect, as depicted in Fig. 5. These results strongly support that PLC may mediate capsaicin-induced intracellular Ca^{2+} release through the liberation of IP_3, ultimately resulting in the induction of apoptosis.

Roles of vanilloid receptors (VRs) in the capsaicin-induced intracellular Ca^{2+} increase and apoptosis

Since the various actions of capsaicin appear to be

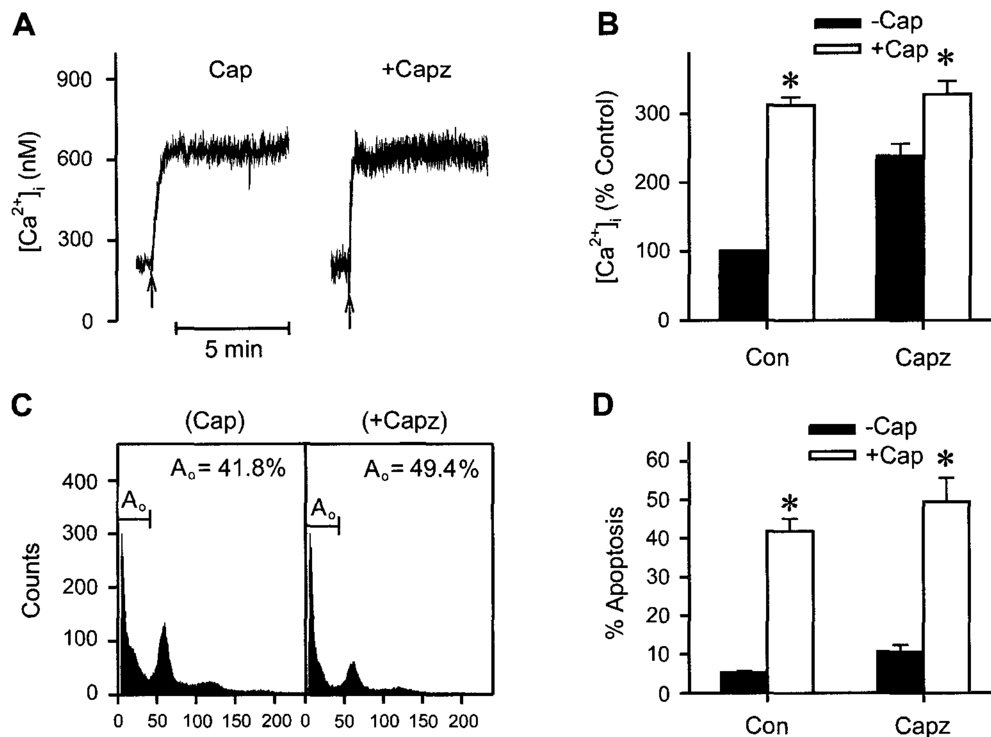

Fig. 3. The effects of capsazepine, a vanilloid receptor (VR) antagonist, on the capsaicin-induced intracellular Ca^{2+} increase and apoptosis in HepG2 human hepatoma cells. The intracellular Ca^{2+} concentration was measured by the Fura-2 fluorescence technique, and the data (A) represent the intracellular Ca^{2+} changes with time. The arrows show the time points for the addition of capsaicin (Cap; 250 μM). Capsazepine (Capz; 25 μM) was added 5 min before Cap application. The data (B) are expressed as a percent change of the control conditions where the cells were incubated with Cap-free medium. (C and D) The number of apoptotic cells was measured by flow cytometry. Cells were treated for 24 h with capsaicin (Cap; 250 μM). Capz (25 μM) was added to the cells 2 h before the Cap addition (250 μM). In the graphs (D) the data represent the mean values of four replications, with the bars indicating the SEM. *p<0.05 compared to control.

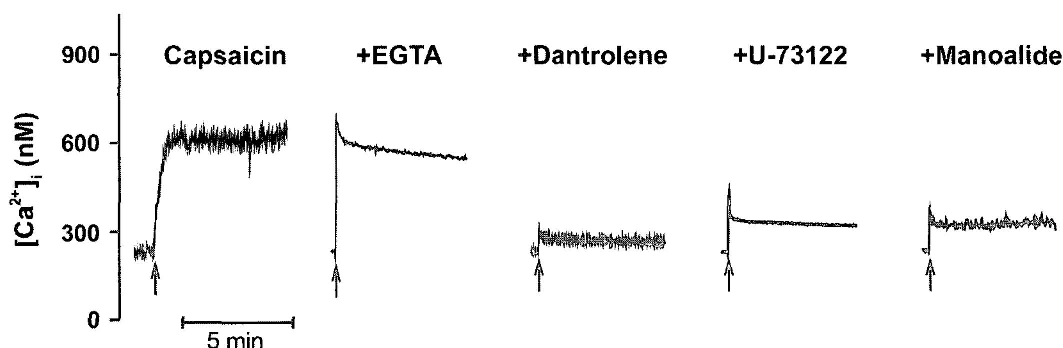

Fig. 4. The effects of various Ca^{2+} inhibitors on the capsaicin-induced intracellular Ca^{2+} increase in HepG2 human hepatoma cells. The intracellular Ca^{2+} concentration was assessed by the Fura-2 fluorescence technique, and the data represent the intracellular Ca^{2+} changes with time. The arrows show the time points for the addition of capsaicin (250 μM). In these experiments an intracellular Ca^{2+} release blocker (25 μM dantrolene) and PLC inhibitors (10 μM U-73122 and 2 μM manoalide) were added to the cells 5 min before Cap treatment. In the experiment with EGTA, an extracellular Ca^{2+} chelator, nominal Ca^{2+}-free medium, containing 1 mM EGTA, was used.

mediated by the stimulation of specific vanilloid receptors (VRs) (Szallasi and Blumberg, 1999), their possible role in the capsaicin-induced increase in intracellular Ca^{2+} concentration was investigated. Capsazepine, a VR antagonist, did not significantly affect the capsaicin effect, but rather, capsazepine alone increased the intracellular Ca^{2+} concentration as much as capsaicin, as shown in Figs. 3A and 3B. These results suggest that capsaicin increased the intracellular Ca^{2+} level independently of the activation of VRs in HepG2 cells. Furthermore, treatment with capsazepine did not significantly alter capsaicin-induced apoptosis (Figs. 3C and 3D), suggesting that VRs may

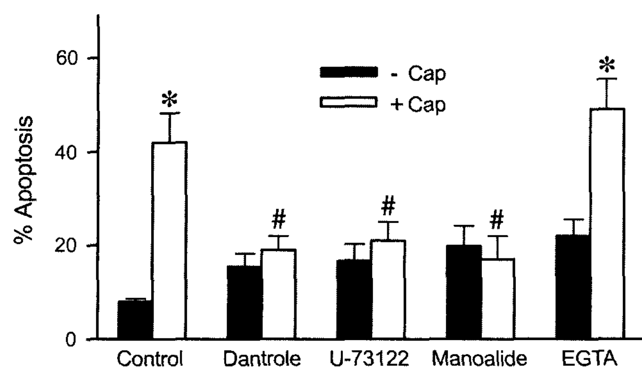

Fig. 5. The effects of various Ca^{2+} inhibitors on the capsaicin-induced apoptosis in HepG2 human hepatoma cells. In the experiments, the cells were treated for 24 h with capsaicin (Cap; 250 μM). The number of apoptotic cells was measured by flow cytometry. In these experiments an extracellular Ca^{2+} chelator (1 mM EGTA), an intracellular Ca^{2+} release blocker (25 μM dantrolene) and PLC inhibitors (10 μM U-73122 and 2 μM manoalide) were added 4 h before Cap application. The data represent the mean values of four replications, with the bars indicating the SEM. *$p<0.05$ compared to control. #$p<0.05$ compared to Cap alone.

not play an essential role in the mechanism of capsaicin-induced apoptosis in HepG2 cells.

DISCUSSION

Capsaicin has been reported to induce apoptosis in various cancer cells (Wolvetang *et al.*, 1996; Macho *et al.*, 1998; Macho *et al.*, 1999). Consistently, the results of the present study also demonstrated that capsaicin induced apoptotic cell death in HepG2 human hepatoma cells, as evaluated by two independent methods; the detection of phosphatidylserine translocation through the Annexin-V binding assay (Fig. 1A) and measurement of the hypodiploid DNA contents through flow cytometry (Figs. 1B and 1C).

Since intracellular Ca^{2+} has been shown to act as a common mediator of chemical-induced cell death (Harman and Maxwell, 1995), and also to act as a signal transducer in the mechanism of apoptosis (Distelhorst and Dubyak, 1998; McConkey and Orrenius, 1996), whether an intracellular Ca^{2+} signal is involved in the observed capsaicin-induced apoptosis of the HepG2 cells was also investigated. Indeed, capsaicin increased the intracellular Ca^{2+} concentration (Fig. 2A and 2B), and the treatment with BAPTA/AM, an intracellular Ca^{2+} chelator, significantly suppressed capsaicin-induced apoptosis (Fig. 2C and 2D). This implied that capsaicin induced apoptosis through a mechanism of disturbance of the intracellular Ca^{2+} homeostasis.

Capsaicin appeared to elevate the intracellular Ca^{2+} level through internal Ca^{2+} release, since this action of capsaicin was not altered by reducing the extracellular Ca^{2+} concentration within a nominal Ca^{2+}-free medium containing 1 mM EGTA, but was completely inhibited by the intracellular Ca^{2+} release blocker, dantrolene (Fig. 4). Significant inhibition of the capsaicin-induced apoptosis by the Ca^{2+} release blocker (Fig. 5) implies that Ca^{2+} release from internal stores may act as a major mediator for the capsaicin-induced apoptotic cell death in HepG2 cells.

Members of the PLC family of enzymes hydrolyze phosphatidylinositol-(4,5)-bisphosphate (PIP_2), generating IP_3 and diacylglycerol (Hughes and Putney, 1990). The PLC inhibitors, U-73122 or manoalide, significantly prevented the capsaicin-induced elevation of intracellular Ca^{2+} (Fig. 4), indicating that PLC may mediate the capsaicin-induced intracellular Ca^{2+} mobilization. In addition, the significant inhibition of capsaicin-induced apoptosis by these PLC inhibitors (Fig. 5), suggests that PLC activation may be necessary for this apoptotic process. However, the exact mechanism by which capsaicin activates the PLC is unknown. This issue remains to be determined in future studies.

Although it is not clearly understood, the mechanism of apoptosis induced by capsaicin appears to not be related with its well-known stimulatory effects on VRs, similarly to in the sensory neurons (Szallasi and Blumberg, 1999). Consistently with these previous reports, the VRs were not involved in the apoptotic activity of capsaicin in HepG2 cells, since capsazepine, a specific VR antagonist, did not significantly alter the capsaicin-induced apoptosis (Fig. 3C and 3D). Capsaicin induces Ca^{2+} influx in sensory neurons (Szallasi and Blumberg, 1999) and some transformed cells (Macho *et al.*, 1999) through activation of VRs. In HepG2 cells, capsaicin also increased the intracellular Ca^{2+} (Figs. 2A and 2B), but its effect was not blocked, but augmented by capsazepine (Fig. 3A and 3B), suggesting that the capsaicin-induced intracellular Ca^{2+} increase may also be via a non-VR mechanism. Another possibility is that the cells may not possess the VRs, on which the information is not currently available.

In this study, the downstream mechanism of Ca^{2+} increase linked to the final apoptosis induction was not determined, so this mechanism remains to be studied. There is however, an accumulating body of evidence that implies that an intracellular Ca^{2+} signal plays an important role in the mechanism of apoptosis (McConkey and Orrenius, 1996). Two of the targets for the elevation of the intracellular Ca^{2+} concentration are the activation of Ca^{2+}-dependent protein kinases and phosphatases. Activation of calcineurin, a Ca^{2+}/calmodulin-dependent protein serine /threonine phosphatase, due to an intracellular Ca^{2+} increase (Bonnefoy-Berard *et al.*, 1994) has been shown to induce apoptosis by regulating the activity of the transcription factor, NF-AT (nuclear factor of activated T

cells) (Shibasaki *et al.*, 1997). Direct activation of the Ca^{2+}-dependent neutral proteinase, calpain, may represent another target for the intracellular Ca^{2+} action in apoptosis. Calpain is rapidly activated in apoptotic cells (Squier *et al.*, 1994), and the specific inhibitors of calpain block apoptosis in many different types of cells (Jordan *et al.*, 1997; Squier *et al.*, 1997). Increased intracellular Ca^{2+} activates Ca^{2+}/Mg^{2+}-dependent endonuclease (Cohen and Duke, 1984), resulting in DNA fragmentation, which is the most characteristic biochemical feature of apoptosis (Wyllie *et al.*, 1984). Ca^{2+}-dependent transglutaminase which catalyzes the post-translational coupling of amines to proteins and the crosslinking of proteins, also appears to be a target for Ca^{2+} action. The enzyme is highly activated in apoptotic cells (Fesus *et al.*, 1987), and the overexpression of this enzyme triggers apoptotic cell death (Melino *et al.*, 1994).

In conclusion, capsaicin was shown to induce apoptosis in HepG2 human hepatoma cells. Activation of the PLC, and the subsequent release of intracellular Ca^{2+} from IP_3-sensitive stores may be essentially involved in the mechanism of this action of capsaicin. These results further suggest that capsaicin may be a good candidate as a therapeutic drug against human hepatomas.

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