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Antitumor effects of amlodipine, a Ca^{2+} channel blocker, on human epidermoid carcinoma A431 cells in vitro and in vivo

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Abstract

Amlodipine, a dihydropyridine Ca^{2+} channel blocker, is reported to inhibit proliferation of human epidermoid carcinoma A431 cells, and specifically attenuates Ca^{2+} responses evoked by thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPases. In this study, we further examined the possible mechanism of the antiproliferative action of amlodipine and its antitumor effect on A431 xenografts in nude mice. Amlodipine reduced BrdU incorporation into nucleic acids in serum-starved A431 cells, and the reduction was diminished by uridine 5′-triphosphate (UTP), a phospholipase C (PLC)-linked agonist. Fluorometric measurement of intracellular free Ca^{2+} concentration revealed that amlodipine blunted the UTP-induced Ca^{2+} release from the internal Ca^{2+} stores and consequently Ca^{2+} influx through Ca^{2+}-permeable channels on the plasma membrane. Although amlodipine alone caused Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores, such an effect was not reproduced by other dihydropyridine Ca^{2+} channel blockers, including nicardipine and nimodipine, despite their antiproliferative effects in the cells. Daily intraperitoneal administration of amlodipine (10 mg/kg) for 20 days into mice bearing A431 xenografts retarded tumor growth and prolonged the survival of mice. Our results suggest a potential antitumor action for amlodipine in vitro and in vivo, which may be in part mediated by inhibiting Ca^{2+} influx evoked by the passive depletion of internal Ca^{2+} stores and by PLC-linked agonist stimulation.

Keywords: Amlodipine; Proliferation; Epidermoid carcinoma A431 cell, human; Uridine 5′-triphosphate; Intracellular Ca^{2+}; A431 xenograft

1. Introduction

Ca^{2+} channel blockers have been used therapeutically to control hypertension, angina pectoris and ventricular tachyarrhythmias through their inhibitory effect on Ca^{2+} entry via an interaction with voltage-dependent L-type Ca^{2+} channels on plasma membrane. However, in addition to their blockade of L-type Ca^{2+} channels, Ca^{2+} channel blockers also interact with many other cellular structures (Zernig, 1990) resulting in ancillary effects in both vascular and non-vascular tissues. For example, Ca^{2+} channel blockers have been reported to retard development of atherosclerosis in part due to their inhibitory effect on proliferation of smooth muscle cells (Schachter, 1997a,b; Stepien et al., 1998, 2002; Stepien and Marche, 2000; Waters and Lesperance, 1994). Furthermore, antiproliferative effects in a variety of neoplastic cell lines have been demonstrated by verapamil (Jensen et al., 1995, 2000; Jensen and Wurster, 2001; Lee et al., 1994; Rybalchenko et al., 2001; Taylor and Simpson, 1992), diltiazem (Jensen et al., 1995, 2000; Jensen and Wurster, 2001; Lee et al., 1994; Taylor and Simpson, 1992), nifedipine (Jensen et al., 1995, 2000; Jensen and Wurster, 2001), and amlodipine (Taylor and Simpson, 1992), although the underlying mechanism of action remains obscure.

In elucidating the possible mechanism of action, we have recently reported that dihydropyridine derivatives, such as amlodipine, nicardipine and nimodipine, inhibited the growth and DNA synthesis of human epidermoid carcinoma A431 cells with IC_{50} values of 20–30 μM, whereas phenylalkylamine verapamil, benzodiazepine diltiazem, and interestingly, a dihydropyridine, nifedipine, did not show such effects (Yoshida et al., 2003). The growth inhibitory effect of amlodipine was not affected by an L-type Ca^{2+} channel opener, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl] pyridine-3-carboxylic acid methyl ester ((±)-Bay K8644), and was augmented by the coaddition of thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-

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ATPases, into cultures. Fluorometric measurement of intracellular free Ca^{2+} concentration revealed that Ca^{2+} channel blockers, which have an antiproliferative effect, specifically blunted the thapsigargin-induced Ca^{2+} release from internal Ca^{2+} stores and the ensuing Ca^{2+} influx through the store-operated Ca^{2+} channels (Yoshida et al., 2003). These results suggested that Ca^{2+} channel blockers, including amlodipine, inhibit the growth of A431 cells by interfering with the intracellular Ca^{2+} regulating system responsible for internal Ca^{2+} stores, but not L-type Ca^{2+} channels.

In the present study, we examined the mechanism underlying the antiproliferative action of amlodipine by fluorometric measurement of intracellular free Ca^{2+} concentration and by BrdU incorporation assay. In addition, since little is known about the antitumor effect of amlodipine in vivo (Taylor and Simpson, 1992), we performed experiments to examine whether or not amlodipine could exert an antitumor effect on A431 xenografts in nude mice.

2. Materials and methods

2.1. Reagents

Amlodipine, donated from Sumitomo Pharmaceutical (Ibaraki, Japan), and nifedipine were dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution of 100 mM. Nimodipine and nicardipine were dissolved in methanol and distilled water at a concentration of 10 mM, respectively. Thapsigargin (Sigma, St. Louis, MO) was dissolved in DMSO at 10 mM. Uridine 5′-triphosphate and U73122 (Sigma) were dissolved in distilled water (100 mM) and ethanol (1.4 mM), respectively.

2.2. Tumor cell lines

Human epidermoid carcinoma A431 cells (Fabricant et al., 1977) were kindly supplied by Professor Katsuzo Nishikawa (Second Department of Biochemistry, Kanazawa Medical University) and were cultured as described previously (Yoshida et al., 2001). Briefly, the cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 12.7 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.12% sodium bicarbonate, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37 °C in humidified air containing 5% CO_2. Cells were seeded at a density of 3×10^5/plate in 10 cm diameter plastic culture dishes and passaged every 3–4 days.

2.3. Measurement of DNA synthesis

Incorporation of 5-bromo-2′-deoxyuridine (BrdU) was determined to evaluate DNA synthesis by a proliferation kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the instructions provided by the manufacturer. The cells (5×10^3 cells/0.1 ml) were plated in 96-well plates in DMEM containing 10% fetal bovine serum and cultured for 16–24 h and were incubated in 200 μl serum-free DMEM for 1 day. Test agents or solvents were added and incubated for 2 days. The cells were incubated with BrdU (10 μM) for another 2 h, fixed and the incorporated BrdU was labeled with a peroxidase-conjugated anti-BrdU antibody before addition of a peroxidase substrate. The absorbance of the wells was measured on a microplate reader with a test wavelength of 405 nm and a reference wavelength of 490 nm. The % incorporation of BrdU was calculated as $100 \times [(T-B)/(C-B)]$, where T and C are the absorbance values in wells containing test agents and in wells containing solvents, respectively, and B is the value in wells containing medium alone. Values shown are the mean ± S.E.M. of independent experiments each performed in triplicate.

2.4. Measurement of intracellular free Ca^{2+} concentrations

For the microscopic fluorometric measurement of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), A431 cells were plated on poly-D-lysine coated glass bottom dishes (MatTek, Ashland, USA). They were grown in DMEM containing 10% fetal bovine serum and used 1–2 days later. The cells were washed twice with a HEPES-buffered physiological saline solution (HBSS) (in mM: 130 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, and 2 $CaCl_2$, pH adjusted to 7.4 with NaOH) and incubated for 30 min in the dark at room temperature (25 ± 2 °C) in a solution supplemented with 3 μM acetoxymethyl ester of fura-2 or fluo-3 (fura-2/AM or fluo-3/AM: Dojindo Laboratories, Kumamoto, Japan) containing 0.005% Cremophore EL (Sigma). Cells were washed, post-incubated in HBSS for 30 min and the dishes were placed on the stage of a Nikon inverted microscope (ECLIPSE TE 300, Nikon, Tokyo) equipped with a Nikon x40 S-fluor objective. Fluorescence images of the cells were recorded and analyzed with a video image analysis system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan). The agents were applied by continuous perfusion (30 ml/h) with a peristaltic pump or sequentially applied to the cells containing 2 ml of recording medium in a volume of 20–100 μl. Image pairs were captured at 10-s intervals. Fura-2 fluorescence was monitored at an emission wavelength of 510 nm by exciting fura-2 alternatively at 340 and 380 nm. Fluo-3 fluorescence was monitored at an emission wavelength of 527 nm by exciting fluo-3 at 480 nm. The 340 nm to 380 nm fluorescence ratio (F340/F380) and the 480 nm to 480 nm at the first image fluorescence ratio (F480/$F480_0$) were used to indicate the changes in $[Ca^{2+}]_i$.

2.5. Tumor xenograft studies

Male Balb/c-nu Slc athymic nude mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan), and housed at Experimental Animal Center of Division of Core

Facility, Kanazawa Medical University. Animal care and experiments were conducted according to the Institutional Animal Care and Use Guidelines. Cultured A431 cells were trypsinized, resuspended in serum-free DMEM, and cell suspensions (5×10^5 cells/0.2 ml) were injected s.c. into 6-week-old nude mice weighing 17–22 g. Three days after tumor inoculation, tumor-bearing mice were randomized to either a control (vehicle) or treated group. Amlodipine for this experiment was dissolved in DMSO to prepare a stock solution (100 mg/ml DMSO) and diluted to 1:100 in phosphate-buffered saline (PBS), sterilized with 0.2-μm filters on the day of administration. For vehicle treatment, 100% DMSO was diluted 1:100 in PBS and sterilized by filtration. Amlodipine (10 mg/kg) or vehicle was administered intraperitoneally (i.p.) once daily for consecutive days. Signs of toxicity and survival of mice were monitored daily. Tumor size was measured by caliper at 2–3-day intervals. The tumor size (cm^3) was calculated by the formula, $\pi/6 \times L \times W \times W$, where L is longest diameter (cm), and W is shortest diameter (cm). Differences in tumor size and survival days between the control and the treated groups were analyzed using the Mann–Whitney U non-parametric statistical test and the Kaplan–Meier method followed by the log rank test, respectively. Differences were considered statistically significant when $P < 0.05$.

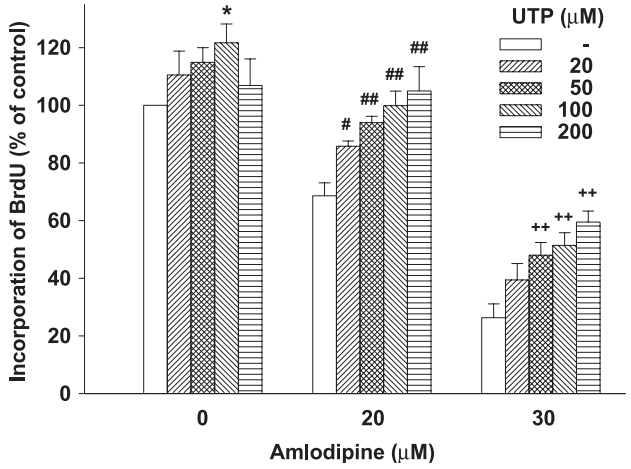

Fig. 1. Effect of UTP on amlodipine-induced inhibition of DNA synthesis in A431 cells. Uridine 5′-triphosphate (UTP, 20–200 μM) was added to serum-starved A431 cells in the absence or presence of amlodipine (20 or 30 μM), incubated for 48 h, and then labeled with BrdU for 2 h. The BrdU incorporated into nucleic acids of A431 cells was determined as described in Materials and methods. Values are the mean ± S.E.M. of four independent experiments. $^{*}P < 0.05$ versus control cultures (solvent alone). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus cultures with 20 μM amlodipine alone. $^{++}P < 0.01$ versus cultures with 30 μM amlodipine alone. Differences in each group were analyzed by one-way analysis of variance followed by Fisher's PLSD.

3. Results

3.1. Effect of UTP on the amlodipine-induced inhibition of DNA synthesis

To examine the effect of uridine 5′-triphosphate (UTP) on DNA synthesis, serum-starved A431 cells were incubated with UTP (20–200 μM) for 48 h, and labeled with the nucleic acid precursor BrdU for 2 h. Any concentration of UTP except for 100 μM (121.7% of the control) did not significantly affect incorporation of BrdU into the cells (Fig. 1). On the other hand, amlodipine at concentrations of 20 and 30 μM reduced BrdU incorporation to 68.6% and 26.3% of the control, respectively (Fig. 1). When UTP was added to the medium containing amlodipine, the amlodipine-induced reduction of BrdU incorporation was partially diminished by UTP in a concentration-dependent manner (Fig. 1). As UTP is a phospholipase C (PLC)-linked agonist, which enhances inositol 1,4,5-triphosphate (IP_3)-induced Ca^{2+} release and stimulates Ca^{2+} entry across the plasma membrane (Gonzalez et al., 1989), the antiproliferative effect of amlodipine may involve intracellular Ca^{2+} mobilization evoked by IP_3.

3.2. Effect of amlodipine on UTP-induced Ca^{2+} responses

As shown in Fig. 2, perfusion of fura-2-loaded A431 cells with 100 μM UTP resulted in an increase in $[Ca^{2+}]_i$ in the absence or presence of external Ca^{2+} (2 mM), corresponding to IP_3-mediated Ca^{2+} release from internal Ca^{2+} stores and the capacitative Ca^{2+} influx across the plasma membrane, respectively. To examine the effect of amlodipine on UTP-induced Ca^{2+} responses, A431 cells were preincubated with 30 μM amlodipine in DMEM containing 10% fetal bovine serum for 1 h at 37 °C. Preincubation with amlodipine significantly attenuated the UTP-induced increases in $[Ca^{2+}]_i$, both in the absence and presence of extracellular Ca^{2+} (Fig. 2(A) and (B)). Incubation of the cells with solvent (0.03% DMSO) alone had a negligible effect on the response to UTP (data not shown).

In our previous study, we also used a visible wavelength excitable indicator, fluo-3, to rule out the possible spectral interference of amlodipine with fura-2. Similarly, in this study, we examined the effect of amlodipine on the UTP-evoked Ca^{2+} responses using fluo-3-AM. Test agents were sequentially applied to fluo-3-loaded cells as indicated. $CaCl_2$ was added to the medium to initiate capacitative Ca^{2+} entry. Fig. 3(A) shows that application of 100 μM UTP in Ca^{2+}-free HBSS evoked Ca^{2+} release from intracellular Ca^{2+} stores followed by Ca^{2+} entry upon readdition of Ca^{2+} to the medium. In the cells preincubated with 30 μM amlodipine for 1 h at 37 °C, the $[Ca^{2+}]_i$ increases evoked by UTP and Ca^{2+}-readdition were lower than those in control cells (Fig. 3(B)). Thus, there is no spectral interference of amlodipine with fura-2 in the present study. Furthermore, preincubation with the putative non-selective P_2-purinoceptor antagonist suramin (10 μM) or PLC inhibitor U73122 (10 μM) inhibited the UTP-induced Ca^{2+} responses modestly or completely, respectively (Fig. 3(C) and (D)), indi-

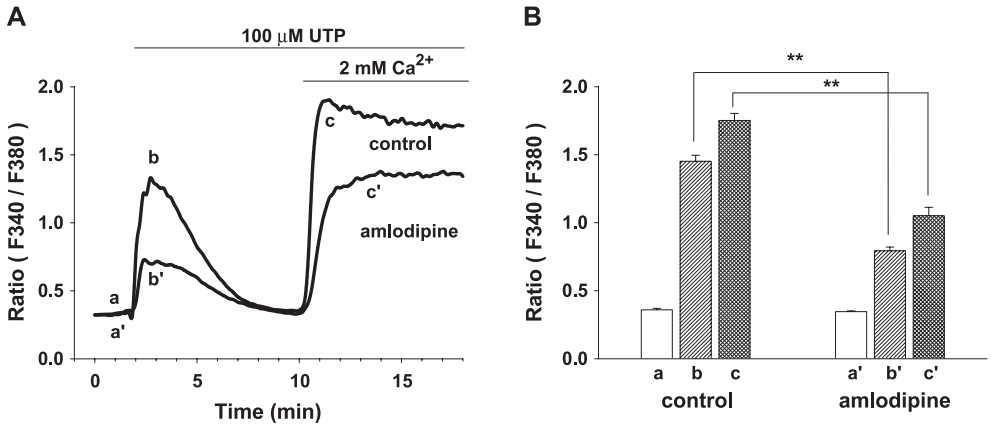

Fig. 2. Effect of amlodipine on UTP-induced Ca^{2+} responses in fura-2-loaded cells. (A) A431 cells were preincubated with 30 μM amlodipine or vehicle (control) for 1 h at 37 °C. These cells were loaded with fura-2 and perfused with 100 μM UTP in the absence or presence of external 2 mM Ca^{2+}. Each trace shown is the mean value of 7 cells in a field. Ratio (F340/F380); 340 to 380 nm fluorescence ratio. a: basal $[Ca^{2+}]_i$. b: maximum rise in $[Ca^{2+}]_i$ induced by UTP in Ca^{2+}-free HBSS. c: maximum rise in $[Ca^{2+}]_i$ by addition of external Ca^{2+}(2 mM). a', b' and c': values in the cells preincubated with amlodipine. (B) Summarized data. Values are the mean ± S.E.M. of 28 cells from four independent cultures, each of them measured on a field of seven single cells (**$P<0.01$ by the unpaired Student's *t*-test).

cating that the UTP-induced Ca^{2+} responses are mediated via stimulation of P_2-purinoceptor and PLC activation.

3.3. Direct effect of amlodipine on intracellular Ca^{2+} stores

We then investigated whether amlodipine interacts directly with intracellular Ca^{2+} stores in fluo-3-loaded cells. Application of amlodipine in Ca^{2+}-free HBSS evoked a transient rise in $[Ca^{2+}]_i$. Thapsigargin applied thereafter caused no rise in $[Ca^{2+}]_i$, and vice versa (Fig. 4(A) and (B)), suggesting that amlodipine induced Ca^{2+} release from the thapsigargin-sensitive Ca^{2+} stores. When amlodipine was applied after UTP, an additive rise in $[Ca^{2+}]_i$ was observed, while UTP after amlodipine did not cause any

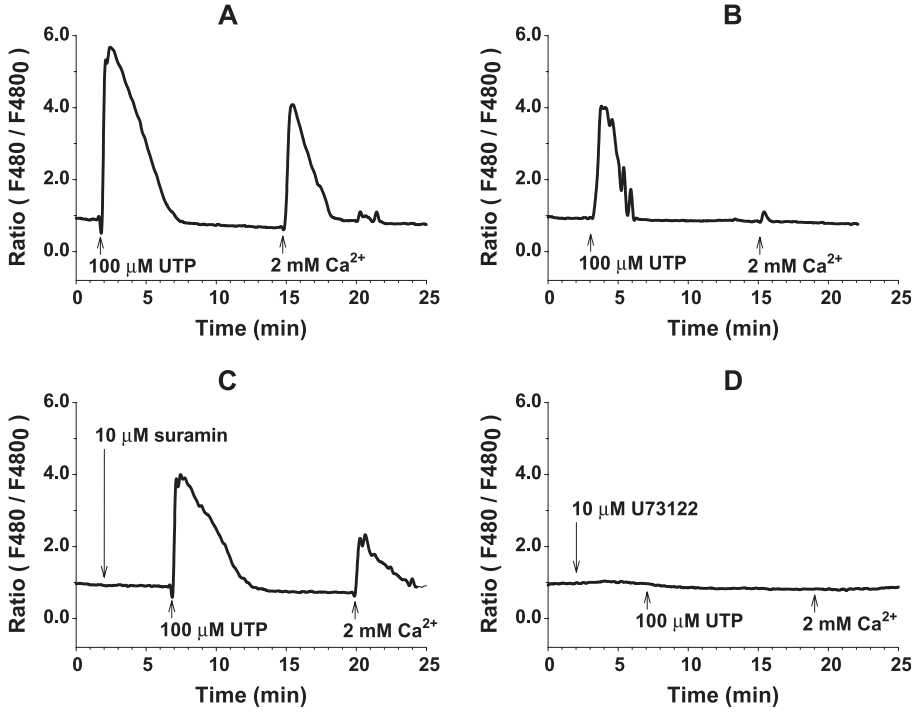

Fig. 3. Effect of amlodipine on the Ca^{2+} responses induced by sequential application of UTP and external Ca^{2+} in fluo-3-loaded cells. (A) Control cells in Ca^{2+}-free HBSS were sequentially administered with 100 μM UTP and $CaCl_2$ (final concentration of 2 mM) to initiate capacitative Ca^{2+} entry. (B) The cells preincubated with 30 μM amlodipine for 1 h at 37 °C were exposed to UTP and 2 mM Ca^{2+} as described above. (C) and (D) Fluo-3-loaded cells were preincubated with P_2-purinoceptor antagonist suramin (10 μM) or PLC inhibitor U73122 (10 μM) for 5 min, and then exposed to UTP and 2 mM Ca^{2+}. Ratio ($F480/F480_0$); 480 to 480 nm at the first image fluorescence ratio. The data shown are representative records from four experiments. Each trace is the mean value of seven cells in a field.

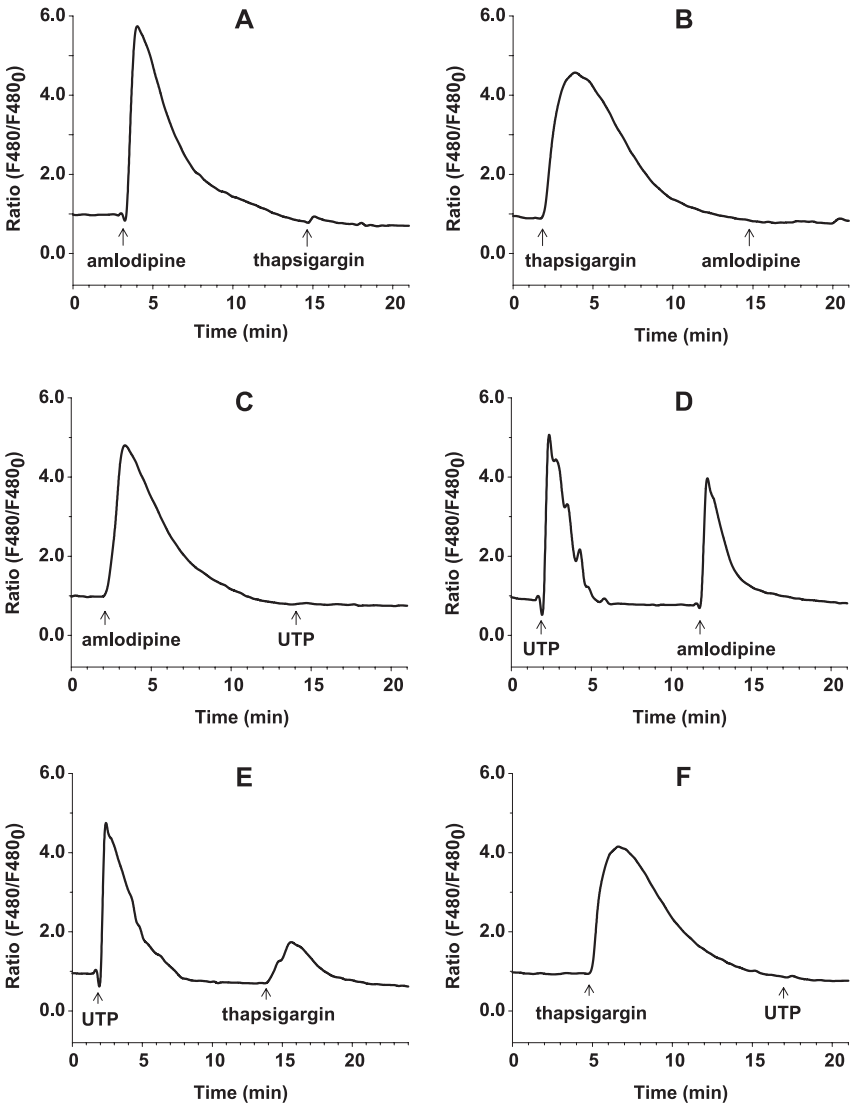

Fig. 4. Effect of amlodipine on $[Ca^{2+}]_i$ of A431 cells in Ca^{2+}-free HBSS. Amlodipine (30 μM), thapsigargin (1 μM) and UTP (100 μM) were sequentially applied to the fluo-3-loaded cells in Ca^{2+}-free HBSS as indicated. Data shown are representative records from four experiments. Each trace is the mean value of seven cells in a field. Ratio (F480/F480$_0$); 480 to 480 nm at the first image fluorescence ratio.

increase in $[Ca^{2+}]_i$. This indicates that amlodipine caused Ca^{2+} release from both IP_3-sensitive and IP_3-insensitive Ca^{2+} stores (Fig. 4(C) and (D)). As is shown in Fig. 4(E) and (F), thapsigargin subsequently evoked a rise in $[Ca^{2+}]_i$ by UTP, while UTP after thapsigargin did not, indicating that thapsigargin induced Ca^{2+} release from both IP_3-sensitive and IP_3-insensitive Ca^{2+} stores. In contrast, no $[Ca^{2+}]_i$ increases were induced by nicardipine or nimodipine (Fig. 5(A) and (B)), both of which have an antiproliferative effect on A431 cells and also blunted the thapsigargin-induced Ca^{2+} release from internal Ca^{2+} stores and the ensuing Ca^{2+} influx (Yoshida et al., 2003). In addition, the Ca^{2+}-releasing effect of amlodipine was evoked with as low as 3 μM (Fig. 5(C)).

Although the presence of functional ryanodine receptors and caffeine-sensitive Ca^{2+} stores in A431 cells have been demonstrated (Mozhayeva and Kiselyov, 1998), no detectable changes in $[Ca^{2+}]_i$ were evoked by caffeine (5–20 mM) or ryanodine (10 μM) when applied alone in Ca^{2+}-free HBSS under our experimental conditions (data not shown).

3.4. Effect of amlodipine on Ca^{2+}-ATPases of endoplasmic reticulum compared with the effect of thapsigargin

We next performed another set of experiments to test whether amlodipine could inhibit Ca^{2+}-ATPases of endoplasmic reticulum, similar to thapsigargin. As shown in Fig. 6(A), application of thapsigargin in Ca^{2+}-free HBSS induced a transient rise in $[Ca^{2+}]_i$ followed by another rise in $[Ca^{2+}]_i$ upon Ca^{2+}-addition. This indicates that since thapsigargin irreversibly inhibits Ca^{2+}-ATPases and depletes Ca^{2+} stores, Ca^{2+}-readdition after thapsigargin caused a rise in $[Ca^{2+}]_i$ due to the activation of the Ca^{2+} influx

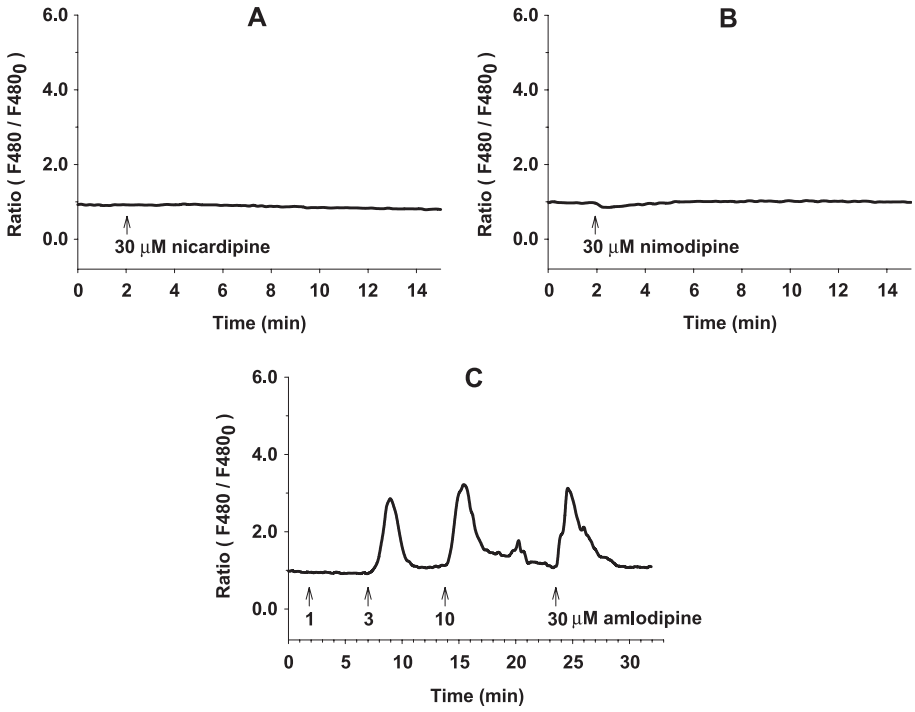

Fig. 5. Changes in $[Ca^{2+}]_i$ induced by nicardipine, nimodipine and amlodipine in Ca^{2+}-free HBSS. Nicardipine (30 μM) (A) and nimodipine (30 μM) (B) were applied to the fluo-3-loaded cells in Ca^{2+}-free HBSS. (C) Amlodipine was applied cumulatively into Ca^{2+}-free HBSS to make final concentrations of 1, 3, 10 and 30 μM. Data shown are representative records out of three experiments. Each trace is the mean value of seven cells in a field.

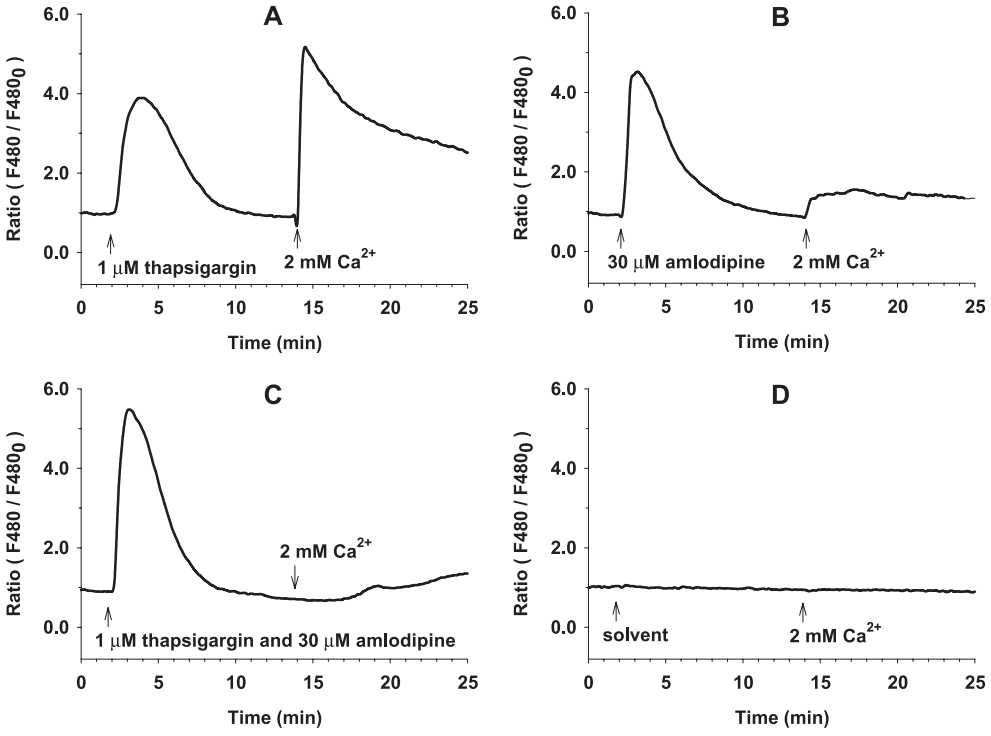

Fig. 6. Amlodipine inhibits the store-operated Ca^{2+}influx evoked by thapsigargin. (A) Fluo-3-loaded cells in Ca^{2+}-free HBSS were treated with 1 μM thapsigargin and then $CaCl_2$ (final concentration 2 mM) was added to activate store-operated Ca^{2+} entry. (B, D) Amlodipine or vehicle and 2 mM Ca^{2+} were applied as described above. (C) Thapsigargin and amlodipine were simultaneously applied and then 2 mM Ca^{2+} was added.

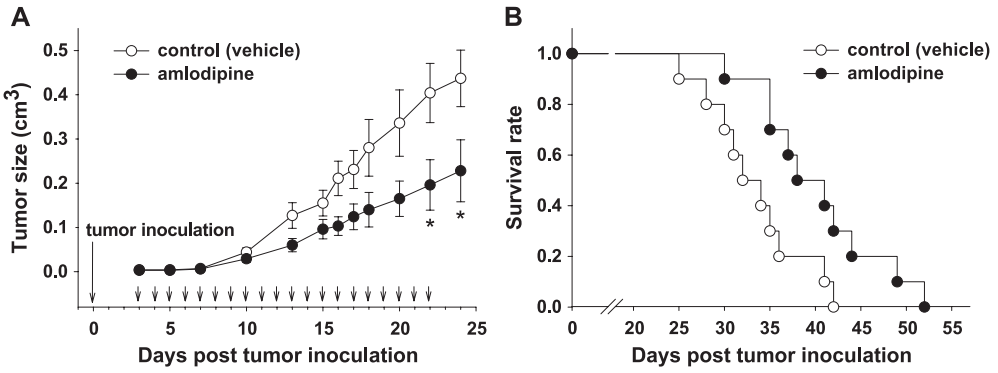

Fig. 7. Effect of amlodipine on A431 xenografts in nude mice. A431 cells were suspended in serum-free DMEM and inoculated s.c. in nude mice. From the third day after inoculation, vehicle (control) or amlodipine (10 mg/kg) was administered i.p. once daily for 20 consecutive days (indicated by short arrows). (A) Changes in mean tumor size (± S.E.M.) for 10 mice. *$P<0.05$ versus the control group by Mann–Whitney U non-parametric statistical test. (B) Survival rate after tumor inoculation. A significant lifespan-prolonging effect ($P<0.02$) was observed after amlodipine administration compared with vehicle administration by the Kaplan–Meier method followed by the log rank test.

pathway. However, addition of Ca^{2+} after application of amlodipine caused a slight rise in $[Ca^{2+}]_i$ (Fig. 6(B)). Further, when thapsigargin was applied in combination with amlodipine, Ca^{2+} influx upon Ca^{2+}-readdition was diminished, indicating that amlodipine strongly attenuated the store-operated Ca^{2+} influx evoked by thapsigargin (Fig. 6(C)). Application of vehicle and Ca^{2+}-readdition caused no changes in $[Ca^{2+}]_i$ (Fig. 6(D)).

3.5. Antitumor effect of amlodipine on A431 xenografts

Preliminary studies indicated that p.o. or i.p. administration of amlodipine at 3 mg/kg once daily for 10 days had no significant effect on tumor growth and survival of mice. The i.p. administration of amlodipine at 10 mg/kg for 10 days retarded tumor growth compared with the control group. At 19 days post-tumor inoculation, the mean tumor size (± S.E.M.) of the amlodipine-treated group (0.298 ± 0.072 cm^3, $n=8$) was significantly ($P<0.05$) smaller than that of the control (0.567 ± 0.086 cm^3, $n=14$). In the follow-up experiments, daily i.p. administration of amlodipine at 10 mg/kg for up to 20 days (Day 3–Day 22) caused a significant retardation of tumor growth (Fig. 7(A)) and prolonged the survival of tumor-bearing mice ($P<0.02$) (Fig. 7(B)). Although there was no significant difference in mouse body weight between the control (vehicle) and amlodipine-treated groups (data not shown), stretching responses as a sign of pain were observed in 30–70% of mice injected with amlodipine, immediately after injection from Day 17 to Day 22.

4. Discussion

Intracellular Ca^{2+} is indispensable for cell proliferation of various types of cells. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is controlled by Ca^{2+} entry pathways in the plasma membrane and Ca^{2+} release from internal Ca^{2+} stores localized in the sarco(endo)plasmic reticulum (Clapham, 1995; Putney et al., 2001; Tsien and Tsien, 1990). A431 cells were demonstrated to lack the classical voltage-activated Ca^{2+} channels found in nerves and muscles (Moolenaar et al., 1986). Consistent with this report, we previously observed no rise in $[Ca^{2+}]_i$ by increasing the concentration of K^+ in the bath solution to 80 mM (data not shown).

Electrophysiological characterization of endogenous channels in A431 cells identified Ca^{2+}-permeable channels activated by depletion of Ca^{2+} stores, which somewhat resemble the Ca^{2+} release-activated Ca^{2+} channel current (I_{CRAC}) in mast cells and the depletion-activated current (I_{DAC}) in lymphocytes (Lückhoff and Clapham, 1994). More recently, Kaznacheyeva et al. (2000) demonstrated that plasma membrane Ca^{2+} channels in A431 cells functionally coupled to IP_3 receptor-phosphatidylinositol 4,5-bisphosphate complexes, and that depletion of intracellular Ca^{2+} stores and phospholipase C (PLC) activation stimulate the same Ca^{2+} channels, named I_{CRACL} (I_{CRAC}-like) channels (Kaznacheyeva et al., 2001).

In previous studies, we demonstrated that amlodipine blunted the Ca^{2+} release from intracellular Ca^{2+} stores and the consequent Ca^{2+} influx by store-depression with cyclopiazonic acid or thapsigargin, Ca^{2+}-ATPase inhibitors of intracellular Ca^{2+} stores (Yoshida et al., 2003). These findings indicated that amlodipine at least attenuated the capacitative Ca^{2+} entry via Ca^{2+}-permeable channels on the plasma membrane.

In the present study, we examined the involvement of PLC-dependent mechanisms in the antiproliferative effect of amlodipine. First, we tested the effect of amlodipine on intracellular Ca^{2+} mobilization induced by a PLC-linked agonist, uridine 5′-triphosphate (UTP). In A431 cells, UTP has been shown to evoke IP_3-induced Ca^{2+} release and to stimulate Ca^{2+} entry across the plasma membrane (Gonza-

lez et al., 1989). We confirmed the UTP-induced Ca^{2+} responses both in fura-2- and fluo-3-loaded cells, that is, UTP evokes Ca^{2+} release from intracellular Ca^{2+} stores and subsequent Ca^{2+} influx upon an addition of external Ca^{2+} (Figs. 2 and 3(A)). Rise in $[Ca^{2+}]_i$ upon Ca^{2+}-application to fura-2-loaded cells by continuous perfusion was persistent (Fig. 2(A)), while the rise in $[Ca^{2+}]_i$ by bolus application of 2 mM Ca^{2+} to fluo-3-loaded cells was transient (Fig. 3(A)). The sustained rise in $[Ca^{2+}]_i$ in fura-2-loaded cells may partly reflect Ca^{2+} influx due to shear stress generated by continuous perfusion. In fluo-3-loaded cells, both the putative non-selective P_2-purinoceptor antagonist suramin and PLC inhibitor U73122 inhibited the UTP-induced Ca^{2+} responses (Fig. 3(C) and (D)), indicating that these Ca^{2+} responses are mediated by P_2-purinoceptor stimulation and PLC activation. Under those conditions, pretreatment of the cells with amlodipine significantly depressed the Ca^{2+} responses to UTP (Figs. 2 and 3(B)).

We also examined the effect of UTP on the growth of A431 cells by BrdU incorporation assay. Certain nucleotides, such as ATP and UTP, have been shown to stimulate DNA synthesis in various types of cells (Albert et al., 1997; Gao et al., 1999; Moll et al., 2002; Tu et al., 2000; Wilden et al., 1998). In A431 cells, ATP and adenosine showed synergistic stimulation of DNA synthesis, although each agonist alone displayed very little mitogenic activity (Huang et al., 1989). In our experimental conditions, only 100 μM UTP slightly increased BrdU incorporation into nucleic acids of serum-starved A431 cells, and the inhibition of BrdU incorporation by amlodipine was partially diminished by UTP in a concentration-dependent manner (Fig. 1). Thus, our previous and present results of $[Ca^{2+}]_i$ measurement and BrdU incorporation assay clearly indicated that both thapsigargin- and UTP-induced Ca^{2+} responses were attenuated by amlodipine, which may be involved in the antiproliferative action of amlodipine.

The above findings that the UTP-induced rise in $[Ca^{2+}]_i$ in Ca^{2+}-free HBSS was diminished in cells preincubated with amlodipine suggest that the Ca^{2+} content in intracellular Ca^{2+} stores may decrease in response to incubation of the cells with amlodipine. We then examined whether amlodipine interacts directly with intracellular Ca^{2+} stores in fluo-3-loaded cells. Fig. 4(A) and (B) shows that amlodipine evoked a transient Ca^{2+} release from thapsigargin-sensitive internal Ca^{2+} stores in A431 cells. On the other hand, nicardipine and nimodipine, despite having an inhibitory effect on A431 cell growth, did not evoke such an effect (Fig. 5(A) and (B)). These findings suggest that the antiproliferative effect of amlodipine may be mediated by inhibition of Ca^{2+} influx rather than Ca^{2+} release from intracellular Ca^{2+} stores. In addition, since capacitative Ca^{2+} entry is believed to be required for refilling Ca^{2+} to the emptied sarcoplasmic reticulum (Doi et al., 2000; Putney et al., 2001), the primary inhibitory effect on the Ca^{2+} influx pathway may account for our findings that amlodipine, nicardipine and nimodipine diminished the thapsigargin-induced Ca^{2+} release from internal Ca^{2+} stores (Yoshida et al., 2003).

With respect to the Ca^{2+}-releasing effect of amlodipine, we performed another set of experiments to examine whether amlodipine could inhibit the intracellular Ca^{2+}-ATPases, like thapsigargin. Thapsigargin has been shown to inhibit the growth of DDT1MF-2 cells by depleting Ca^{2+} stores (Short et al., 1993). Thapsigargin irreversibly inhibited endoplasmic reticulum Ca^{2+}-ATPases, resulting in Ca^{2+} release from internal Ca^{2+} stores and store-operated Ca^{2+} influx upon readdition of Ca^{2+} (Fig. 6(A)). When thapsigargin was applied in the presence of amlodipine, the rise in $[Ca^{2+}]_i$ upon Ca^{2+}-readdition was diminished (Fig. 6(C)). This indicates that amlodipine strongly inhibited Ca^{2+} entry induced by store-depletion with thapsigargin. Although the possibility that amlodipine could inhibit the endoplasmic reticulum Ca^{2+}-ATPases like thapsigargin cannot be excluded, the agent may interact simultaneously with intracellular Ca^{2+} stores and store-operated Ca^{2+} channels on the plasma membrane. The functional significance of the Ca^{2+}-releasing action specifically induced by amlodipine is not clear at present. To explore molecular mechanisms underlying the interference with intracellular Ca^{2+} mobilization by amlodipine, electrophysiological studies will be required. Very recently, Gusev et al. (2003) described the presence of two types of Ca^{2+} channels in A431 cells, I_{SOC} (I_{min}/I_{CRACL}) channels and I_{CRAC} channels, by their patch-clamp studies. Both channels were activated by store depletion and by a PLC-linked agonist, but had a number of differences in functional properties (Gusev et al., 2003). Amlodipine may affect Ca^{2+} currents through these Ca^{2+} channels.

In this study, we also performed antitumor experiments with amlodipine in athymic nude mice, because little is

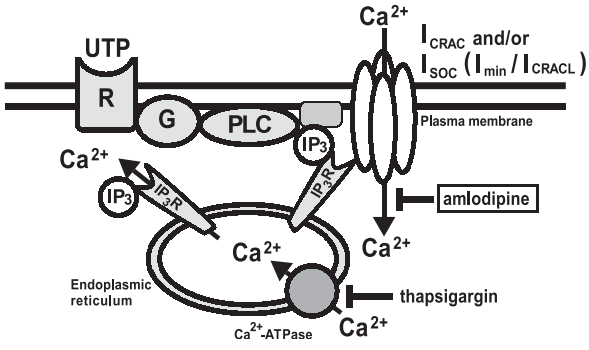

Fig. 8. A suggested model of the antiproliferative effect of amlodipine in vitro. UTP, uridine 5′-triphosphate; R, P_2-purinoceptor ; G, Gq protein; PLC, phospholipase Cβ; SOC, store-operated Ca^{2+} entry; I_{CRAC}, Ca^{2+} release activated channel; I_{CRACL}, I_{CRAC}-like; IP_3, inositol 1,4,5-triphosphate; IP_3R, IP_3 receptor; Symbol ⊢, inhibition. The model drawing is adapted from the reports by Kaznacheyeva et al. (2001) and Gusev et al. (2003).

known about the antitumor effect of this agent in vivo (Taylor and Simpson, 1992). In one report, oral administration of amlodipine (0.35 mg/day dissolved in drinking water) for 10 days inhibited HT-39 breast tumor growth in athymic mice without any effect on body weight or gross organ morphology (Taylor and Simpson, 1992). The final concentration of amlodipine received was estimated to be approximately 7.3 mg/kg/day according to the measurement of water consumption (Taylor and Simpson, 1992). We examined the effect of amlodipine at 3 and 10 mg/kg by p.o. or i.p. administration into athymic nude mice. Administration of amlodipine at 10 mg/kg once daily i.p. for up to 20 days (Day 3–Day 22) caused significant retardation of tumor growth (Fig. 7(A)) and prolonged survival time (Fig. 7(B)). During the experiment, we observed stretching responses as a pain response in some mice injected with amlodipine on Day 17–Day 22. The reason for this is not known, but these pain responses might be explained by an increase in kinin generation with chronic amlodipine administration (Xu et al., 2002). With respect to the vasorelaxant effect of amlodipine, amlodipine was shown to inhibit serum angiotensin converting-enzyme activity, resulting in increases in nitrite production through the mediation of bradykinin B_2 receptors (Xu et al., 2002). It is plausible that repeated injection of amlodipine may increase local bradykinin concentration. Further this observation implies that the nitrite production by amlodipine might be partly responsible for the antitumor effect of amlodipine in athymic nude mice. However, at least the blocking effect of amlodipine on L-type Ca^{2+} channels would restrict its clinical use in cancer therapy. Specific structural characteristics have been indicated for amlodipine (Mason et al., 1999; Zhang et al., 2002) and we have observed no substantial antiproliferative effect of a dihydropyridine derivative, nifedipine, although it chemically belongs to the same class as amlodipine. These findings may be helpful to explore novel antitumor agents that have modulating effects on intracellular Ca^{2+} homeostasis without possessing the ability to block L-type Ca^{2+} channels.

In conclusion, our previous and present results indicate that amlodipine attenuated both thapsigargin-induced and UTP-induced Ca^{2+} influx (Fig. 8), which may be responsible for the antiproliferative effect of amlodipine in A431 cells. Further, a significant retardation of tumor growth and lifespan-prolonging effect of amlodipine were demonstrated in A431 xenografts in athymic nude mice.

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