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Antiproliferative effect of Ca^{2+} channel blockers on human epidermoid carcinoma A431 cells

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Abstract

The effects of Ca^{2+} channel blockers on the proliferation of human epidermoid carcinoma A431 cells were investigated by microtiter tetrazolium (MTT) proliferation assay and bromodeoxyuridine (BrdU) incorporation assay. Dihydropyridine derivatives, such as amlodipine, nicardipine, and nimodipine inhibited A431 cell growth and the incorporation of BrdU into cells with IC_{50} values of 20–30 μM, while verapamil, diltiazem and dihydropyridine nifedipine inhibited neither the cell growth nor BrdU incorporation at the same concentration. Though extracellular Ca^{2+} is indispensable to the cell growth, an L-type Ca^{2+} channel agonist, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]pyridine-3-carboxylic acid methyl ester (200 nM), did not affect the antiproliferative action of amlodipine. Thapsigargin, an inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum, inhibited itself the growth of A431 cells and also showed a synergistic effect with the antiproliferative action of amlodipine. In the fluorimetric measurement of intracellular free Ca^{2+} concentration in fura-2 or fluo-3 loaded A431 cells, amlodipine blunted the thapsigargin- or cyclopiazonic acid-induced Ca^{2+} release from endoplasmic reticulum and the ensuing Ca^{2+} influx through Ca^{2+}-permeable channels. The effect on the thapsigargin-induced Ca^{2+} responses could be reproduced by nicardipine and nimodipine but not by nifedipine or verapamil, lacking antiproliferative potency. These findings suggest that the intracellular Ca^{2+} control system responsible for thapsigargin- and cyclopiazonic acid-sensitive endoplasmic reticulum, but not L-type Ca^{2+} channels, may be modulated by amlodipine, which results in the inhibition of A431 cell growth.
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Keywords: Ca^{2+} channel blocker; Proliferation; Human epidermoid carcinoma A431 cell; Intracellular Ca^{2+}; Fura-2; Fluo-3; Amlodipine

1. Introduction

Intracellular Ca^{2+} signalings play an important role in muscle cell contraction, secretion of hormones, cellular differentiation and proliferation, and apoptosis of various types of cells. The intracellular Ca^{2+} concentration 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 and Bird, 1993; Putney et al., 2001; Tsien and Tsien, 1990). Ca^{2+} channel blockers have been used therapeutically to control hypertension, angina, and ventricular tachyarrythmia due to their inhibitory effect on Ca^{2+} entry via an interaction with the α1 subunit of the voltage-dependent L-type Ca^{2+} channel on the plasma membrane. In addition to their blockade of the L-type Ca^{2+} channel, Ca^{2+} channel blockers at high concentrations have been shown to interact with many other cellular structures, opening new therapeutic approaches, such as reversal of drug resistance to chemotherapy in tumor patients or protection of ischemic tissue (Zernig, 1990). The antiproliferative effects of several kinds of Ca^{2+} channel blockers on vascular smooth muscle cells and a variety of neoplastic cell lines have been demonstrated recently. In animal models and clinical trials, Ca^{2+} channel blockers have been shown to retard the development of atherosclerosis in part due to their inhibition of smooth muscle cell proliferation (Schachter, 1997a,b; Waters and Lesperance, 1994). Rat and human vascular smooth muscle cell proliferation has been demonstrated to be inhibited by amlodipine, suggesting the protective effect of amlodipine on atherosclerosis and post-angioplasty restenosis (Marche and Stepien, 2000; Stepien et al., 1998, 2002; Stepien and Marche, 2000). In tumor cell lines, verapamil, nifedipine and diltiazem have been shown to inhibit the growth of human brain tumor cells in vitro (Jensen et al., 1995, 2000; Lee et al., 1994). Further, verapamil and diltiazem inhibited

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the growth of subcutaneous xenograft meningiomas in nude mice (Jensen and Wurster, 2001). In LNCaP human prostate cancer cells, verapamil was shown to inhibit the cell proliferation through a K^+ channel block (Rybalchenko et al., 2001). Oral administration of amlodipine, diltiazem or verapamil were demonstrated to inhibit HT-39 human breast cancer cell proliferation in the athymic mouse without any effect on mouse body weight or gross organ morphology (Taylor and Simpson, 1992).

Although interference with agonist or growth factor-induced intracellular Ca^{2+} mobilization has been demonstrated to involve in the antiproliferative action of Ca^{2+} channel blockers, the precise mechanisms of the action of these drugs in proliferation have not been elucidated. Here, we attempted to investigate whether Ca^{2+} channel blockers inhibit the proliferation of human epidermoid carcinoma A431 cells, for which the overexpression of epidermal growth factor receptors and the mitogenic signaling pathways have been well documented. Microtiter tetrazolium (MTT) proliferation assay showed that the growth of A431 cells was inhibited by some Ca^{2+} channel blockers, including amlodipine. We further investigated the mechanism of the antiproliferative effect of amlodipine on A431 cells and report evidence of interactions with targets other than L-type Ca^{2+} channels.

2. Materials and methods

2.1. Reagents

Amlodipine, donated from SUMITOMO Pharmaceutical (Ibaraki, Japan), and nifedipine were dissolved in dimethylsulfoxide (DMSO) to make a stock solution of 100 mM. Nimodipine was dissolved in methanol at a concentration of 10 mM. Nicardipine, verapamil and diltiazem were dissolved in distilled water at a concentration of 10 mM. Thapsigargin and cyclopiazonic acid (Sigma, Oregon, USA) were dissolved in DMSO at 10 and 30 mM, respectively. 1, 4-Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl] pyridine-3-carboxylic acid methyl ester ((±)-Bay K8644; Sigma) was dissolved in ethanol at 10 mM.

2.2. Tumor cell lines

The human epidermoid carcinoma A431 cells were kindly supplied by Professor Katsuzo Nishikawa (Second Department of Biochemistry of Kanazawa Medical University) and were cultured as previously described (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 passed every 3–4 days.

2.3. Proliferation assay

Cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay (Chemicon International, Temecula, CA). The cells ($2–3 \times 10^3$ cells/0.1 ml) were dispensed within 96-well culture plates and incubated at 37 °C for 24 h in a CO_2 incubator. Test agents or solvents were added to the wells (Day 0) and further incubated for 2 days. The MTT assay was performed on Day 0 and Day 2 according to the manufacturer's instructions. To perform the assay, 10 μl of the MTT solution was added to each well and incubated for 4 h. The formazan crystals generated by cellular reduction of the MTT reagent were dissolved in 0.1 ml of color development solution (isopropanol with 0.04 N HCl). The absorbance of the wells was measured on a microplate reader 3550 (Bio-Rad Laboratories, CA, USA) with a test wavelength of 550 nm and a reference wavelength of 655 nm. Growth rate in treated cultures was expressed as a percentage of increase in absorbance of control cultures between Day 0 and Day 2, using the following equation: Growth rate (% of control) = $100 \times [T(\text{Day } 2) - C(\text{Day } 0)/C(\text{Day } 2) - C(\text{Day } 0)]$ where C(Day 0) and C(Day 2) are the absorbance values determined on Day 0 and Day 2 in the wells containing solvents, respectively. T(Day 2) is the absorbance determined on Day 2 in the wells containing test agents. Values are the mean ± S.E. of independent experiments each performed in triplicate.

2.4. Measurement of DNA synthesis

Incorporation of 5-bromo-2′-deoxyuridine (BrdU) was determined to evaluate DNA synthesis by a proliferation kit (Boehringer Mannheim, Mannheim, Germany) according

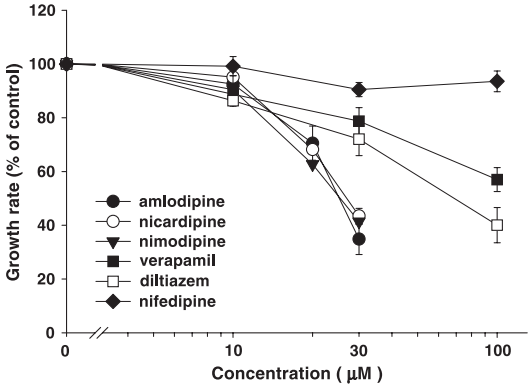

Fig. 1. Effects of Ca^{2+} channel blockers on the growth of A431 cells. A431 cells were plated and incubated for 24 h, then Ca^{2+} channel blockers or solvents were added (Day 0), and cells were further incubated for 2 days. Growth rate (%) was determined by MTT assay as described in Materials and methods. The values are the mean ± S.E. of three independent experiments.

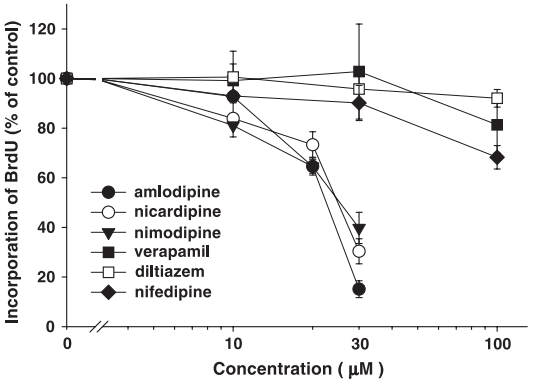

Fig. 2. Effects of Ca^{2+} channel blockers on DNA synthesis of A431 cells. A431 cells were plated and incubated for 16–24 h, then incubated with Ca^{2+} channel blockers or solvents for 18 h, and after labeled with BrdU for 2 h. The incorporated BrdU into nucleic acids of A431 cells was determined as described in Materials and methods. The values are the mean ± S.E. of three independent experiments.

to the manufacturer's instructions. The cells (3×10^3 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 with test agents or solvents for the indicated period. BrdU (10 μM) was added to the culture and further incubated for 2 h. Cells were 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 percent 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 are the mean ± S.E. of independent experiments each performed in triplicate.

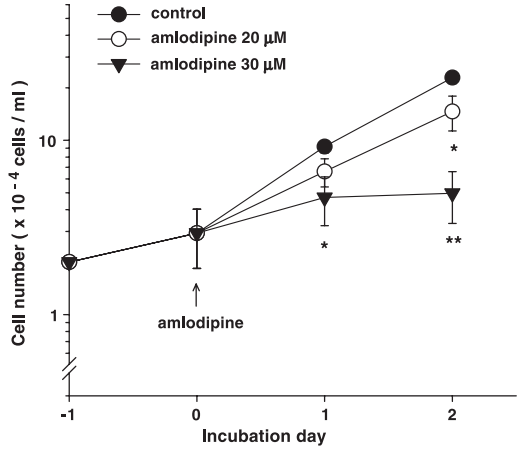

Fig. 3. The time course of the growth inhibitory effect of amlodipine on A431 cells. A431 cells were plated (Day-1) and amlodipine was added to the cultures on Day 0. The values are the mean ± S.E. of three independent experiments (*$P<0.05$, **$P<0.01$ versus each day of control cultures (solvent alone) by one-way ANOVA followed by Fisher's PLSD).

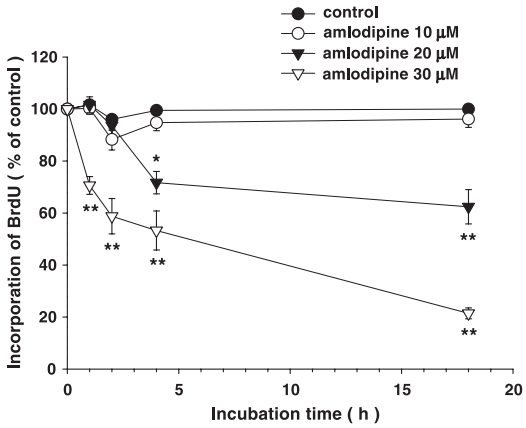

Fig. 4. The time course of the inhibitory effect of amlodipine on DNA synthesis of A431 cells. A431 cells were preincubated in the presence of amlodipine or solvent for 1, 2, 4 and 18 h and then labeled with BrdU for 2 h. The values are the mean ± S.E. of three independent experiments (*$P<0.05$, **$P<0.01$ versus each time of control cultures (solvent alone) by one-way ANOVA followed by Fisher's PLSD).

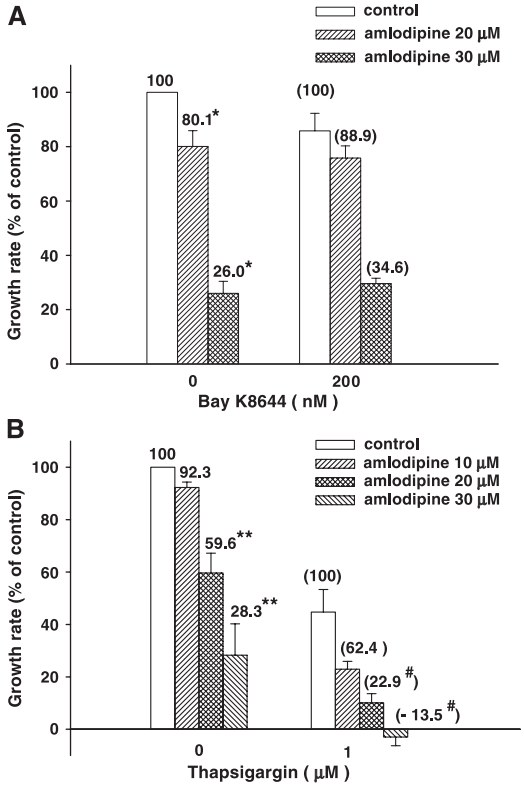

Fig. 5. Effects of (±)-Bay K8644 (A) and thapsigargin (B) on the growth inhibitory effect of amlodipine. A431 cells were plated and incubated for 24 h. Amlodipine and (±)-Bay K8644 (200 nM) or thapsigargin (1 μM) were added on Day 0. The cells were further incubated for 2 days. Growth rate (%) was determined by MTT assay as described in Materials and methods. The values are the mean ± S.E. of four (in A) or three (in B) independent experiments (*$P<0.05$, **$P<0.01$ versus control cultures (solvent alone) by one-way ANOVA followed by Fisher's PLSD). Growth rate in parenthesis is expressed as a percentage of (±)-Bay K8644 or thapsigargin alone ($^{\#}P<0.05$ versus each concentration of amlodipine alone by paired Student's t-test).

2.5. Measurement of cytoplasmic Ca^{2+} concentrations

For the microscopic fluorimetric 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 day 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 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) (Dojindo Laboratories, Kumamoto, Japan) and 0.005% Cremophore EL (Sigma). Cells were washed, postincubated in HBSS for 30 min and the dishes were placed on the stage of a Nikon inverted microscope (Nikon ECLIPSE TE 300) equipped with a Nikon ×40 S-fluor objective. The dishes containing the cells and 2 ml of HBSS were continuously perfused with the solution (30 ml/h) by a peristaltic pump. Fluorescence images of the cells were recorded and analyzed with a video image analysis system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan). Fura-2 fluorescence was monitored at an emission wavelength of 510 nm by exciting fura-2 alternatively at 340 and 380 nm. Image pairs were captured at 5–20 s intervals depending on the Ca^{2+} responses. The 340 to 380 nm fluorescence ratio (F340/F380) was used to indicate the changes in $[Ca^{2+}]_i$.

In some experiment, the cells were loaded with 3 μM acetoxymethyl ester of fluo-3 (fluo-3/AM) (Dojindo Laboratories) with the same procedure for fura-2/AM. Fluo-3 fluorescence was monitored at an emission wavelength of 527 nm by exciting fluo-3 at 480 nm. The 480 to 480 nm at 0 time fluorescence ratio ($F480/F480_0$) was used to indicate the changes in $[Ca^{2+}]_i$.

3. Results

3.1. Effects of Ca^{2+} channel blockers on the proliferation of A431 cells

Dihydropyridine derivatives, such as amlodipine, nicardipine and nimodipine inhibited the growth of A431

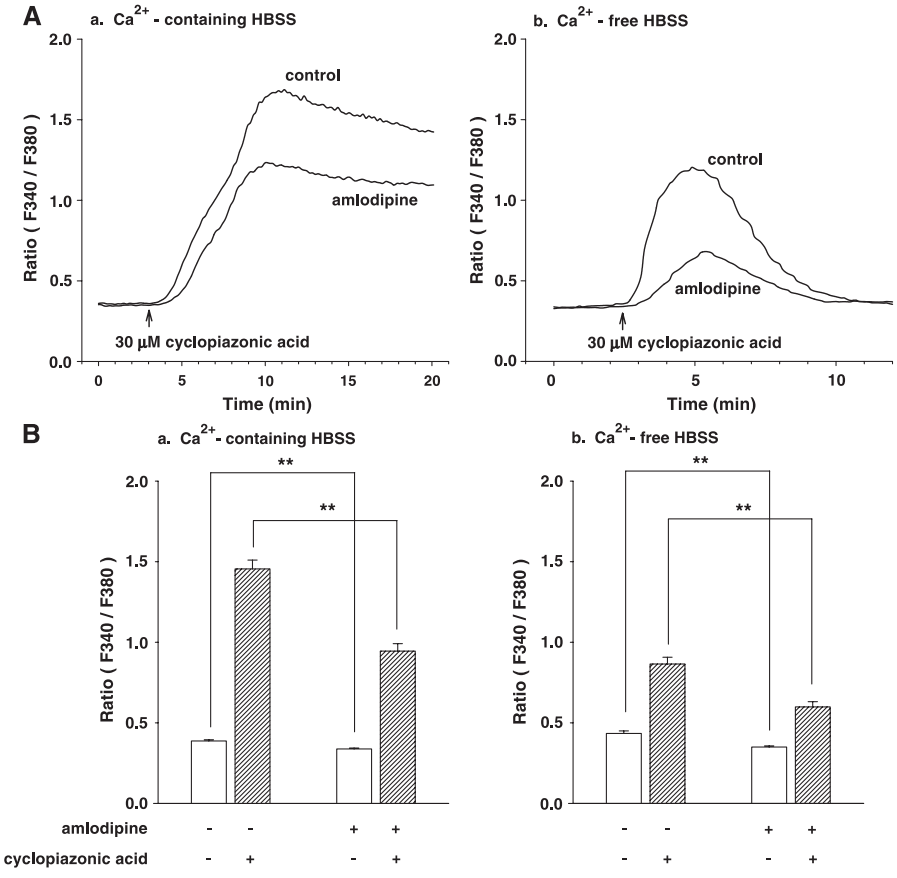

Fig. 6. Representative records of the time course of $[Ca^{2+}]_i$ changes in response to cyclopiazonic acid (30 μM). Control cells or the cells preincubated with 30 μM amlodipine for 1 h at 37 °C were loaded with fura-2, and exposed to 30 μM cyclopiazonic acid in HBSS (Aa) and in Ca^{2+}-free HBSS (Ab). Each trace shown is the mean value of seven cells in a field. Ratio (F340/F380); 340 to 380 nm fluorescence ratio. (B) Summarized data showing the amplitudes of cyclopiazonic acid-induced increases in $[Ca^{2+}]_i$. The values are the mean ± S.E. of 28 cells from four independent cultures, each of them measured on a field of 7 single cells (**$P < 0.01$ by unpaired Student's t-test).

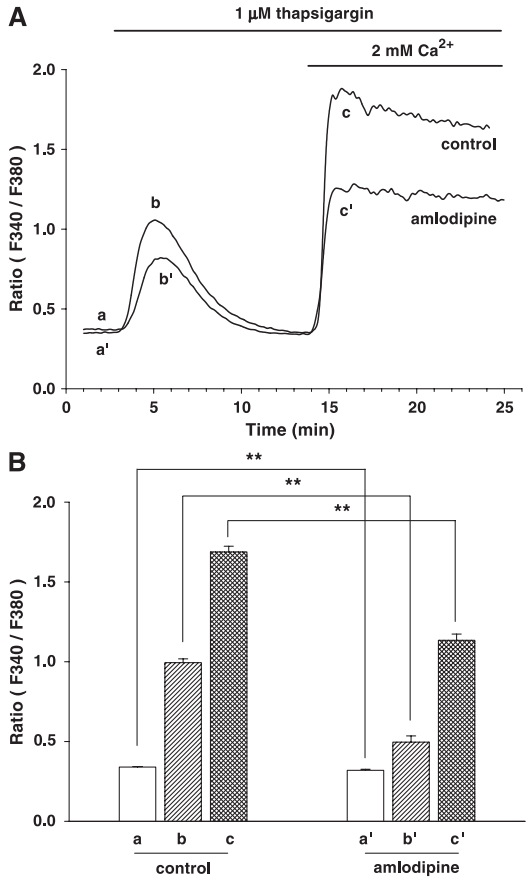

Fig. 7. (A) Representative records of the time course of $[Ca^{2+}]_i$ changes in response to thapsigargin (1 μM). Control cells or the cells preincubated with 30 μM amlodipine for 1 h at 37 °C were loaded with fura-2 and exposed to 1 μM thapsigargin in the absence or presence of external 2 mM Ca^{2+}. Each trace shown is the mean value of seven cells in a field. (B) Summarized data showing the amplitudes of thapsigargin-induced increases in $[Ca^{2+}]_i$. The values are the mean ± S.E. of 28 cells from four independent cultures, each of them measured on a field of 7 single cells (**$P<0.01$ by unpaired Student's *t*-test).

cells. The IC_{50} values were calculated according to the sigmoidal Hill equation: $E=E_{max}\times C^b/(C^b+IC_{50}^b)$, where E and E_{max} represent the growth rate (% of control) and its maximum, respectively, and C and b represent the drug concentration and the Hill coefficient, respectively (SigmaPlot™, ver. 8; Jandel Scientific, California, USA). The IC_{50} values were 25 μM for amlodipine, 27 μM for nicardipine and 26 μM for nimodipine. However, verapamil, a phenylalkylamine, and diltiazem, a benzodiazepine, showed less antiproliferative effect with IC_{50} values of >100 and 64 μM, respectively. Interestingly, nifedipine, a dihydropyridine derivative, did not inhibit the cell growth (IC_{50}; >100 μM) (Fig. 1). The antiproliferative effect of amlodipine, nicardipine and nimodipine was not due to cytotoxicity because the percentage of viable cells determined by trypan blue exclusion was the same (>97.5%) for drug-treated and vehicle-treated cells.

3.2. Effects of Ca^{2+} channel blockers on the nucleic acid synthesis of A431 cells

A431 cells were preincubated for 18 h with Ca^{2+} channel blockers and labeled with the nucleic acid precursor BrdU for 2 h. The BrdU incorporated into nucleic acids during the 2-h incubation was determined as described in Material and methods. Similar to the results obtained from the MTT proliferation assay, amlodipine, nicardipine and nimodipine inhibited the incorporation of BrdU into the nucleic acids. The IC_{50} values calculated by the sigmoidal Hill equation were 23 μM for amlodipine, 26 μM for nicardipine and 25 μM for nimodipine, whereas neither verapamil, diltiazem nor nifedipine inhibited the BrdU incorporation at a concentration of 20–100 μM as shown in Fig. 2.

3.3. Time course of antiproliferative effect of amlodipine on A431 cells

The time course of the antiproliferative action of amlodipine, having a considerable inhibitory effect (Figs. 1 and 2), was examined by MTT assay and BrdU incorporation assay. For determination of cell numbers, standard curves were made with known cell numbers by MTT assay. Fig. 3 shows that amlodipine inhibited the A431 cell growth both dose- and time-dependently. For an assay of time dependency of the inhibitory effect on BrdU incorporation, A431 cells were preincubated with amlodipine or solvent for 1, 2, 4 and 18 h and then labeled with BrdU for 2 h. Fig. 4 shows that the inhibitory effect of amlodipine on DNA synthesis was also time-dependent. Amlodipine, at a concentration of 30 μM, significantly reduced the incorporation of BrdU into nucleic acids during the first 1-h preincubation. The inhib-

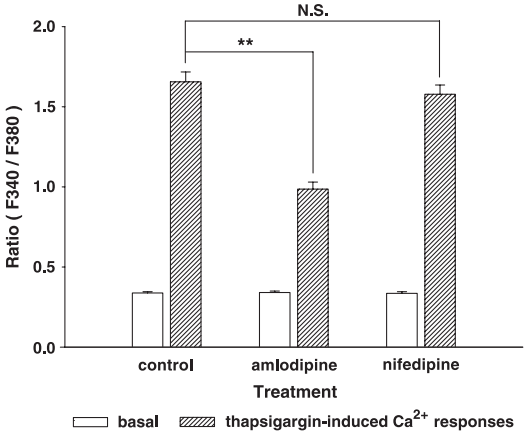

Fig. 8. Effects of amlodipine and nifedipine on the thapsigargin-induced $[Ca^{2+}]_i$ changes. Fura-2 loaded A431 cells were incubated with amlodipine or nifedipine for 10 min at room temperature, washed five times with Ca^{2+}-containing HBSS and exposed to 1 μM thapsigargin in the presence of external 2 mM Ca^{2+}. The values are the mean ± S.E. of 21–28 cells from three to four independent cultures, each of them measured on a field of 7 single cells (**$P<0.01$ by unpaired Student's *t*-test, N.S., not significant).

itory effect of amlodipine at a concentration of 20 μM was apparent after a 4-h preincubation.

3.4. Effects of a Ca^{2+} channel agonist and depletion of intracellular Ca^{2+} store on the antiproliferative effect of amlodipine

Since amlodipine inhibits Ca^{2+} entry via an interaction of the plasma membrane L-type Ca^{2+} channels, we examined whether or not the Ca^{2+} channel agonist (±)-Bay K8644 could attenuate the effect of amlodipine by MTT assay. The result, shown in Fig. 5A, indicates that (±)-Bay K8644 (200 nM) did not affect the inhibitory effect of amlodipine on A431 cell growth. On the other hand, the addition of thapsigargin (1 μM), an agent known to deplete the intracellular Ca^{2+} stores by inhibiting Ca^{2+}-ATPases of the sarco(endo)plasmic reticulum (Thastrup et al., 1990), to cultures not only inhibited the growth of A431 cells but also tended to exert a synergistic effect on the growth inhibitory action of amlodipine (Fig. 5B). These results demonstrated the antiproliferative action of amlodipine was not due to the blockade of L-type Ca^{2+} channels and could be augmented by depleting the intracellular Ca^{2+} stores.

3.5. Effect of amlodipine on intracellular free Ca^{2+} concentration

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was examined in fura-2 loaded A431 cells. Perfusion of A431 cells with 30 μM cyclopiazonic acid, another Ca^{2+}-ATPase inhibitor, in the presence of external 2 mM Ca^{2+}, caused a slow rise in $[Ca^{2+}]_i$ levels that plateaued within 6–7 min followed by a sustained level (Fig. 6Aa). To test the effect of amlodipine on the cyclopiazonic acid-induced Ca^{2+} response, A431 cells were preincubated in the presence of 30 μM amlodipine in DMEM containing 10% fetal bovine serum for 1 h at 37 °C, since under this condition an incorporation of BrdU into nucleic acids of A431 cells was significantly inhibited (Fig. 4). The cells were then washed and loaded with fura-2 with the same procedure performed in the control cells. Preincubation of the cells with solvent (0.03% DMSO) alone had a negligible effect

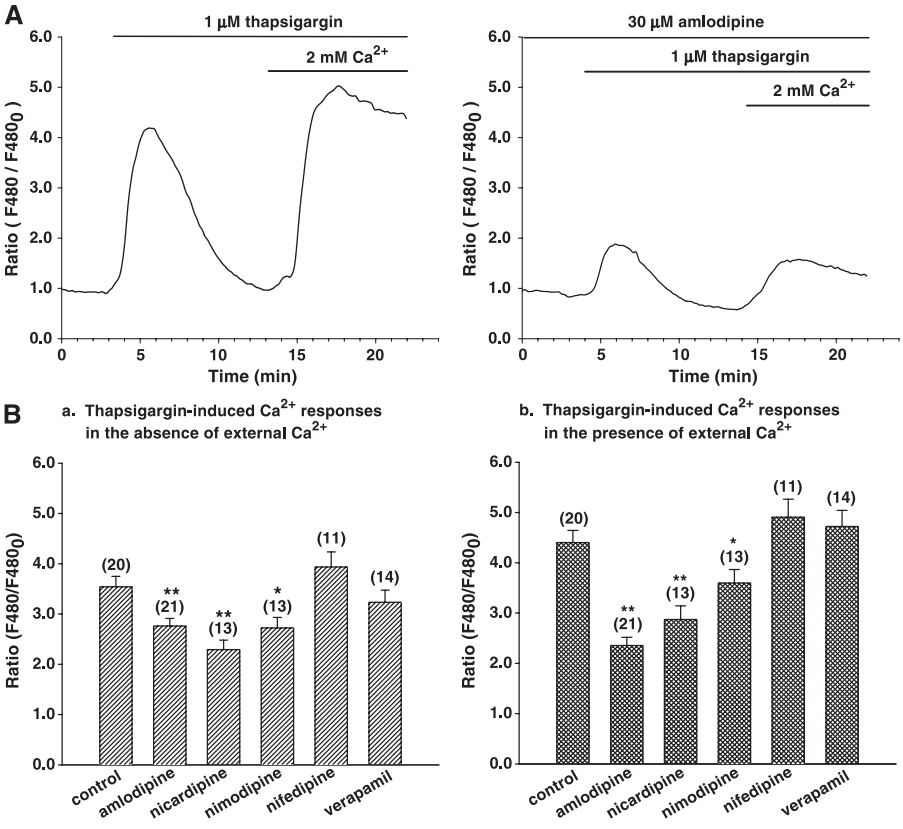

Fig. 9. (A) $[Ca^{2+}]_i$ changes to thapsigargin in the fluo-3 loaded cells in the absence or presence of Ca^{2+} channel blockers. To test the effect of amlodipine, fluo-3 loaded cells were preincubated for 20 min in Ca^{2+}-free HBSS containing 30 μM amlodipine and then exposed to 1 μM thapsigargin in the presence of amlodipine. Each trace shown is the mean value of six to seven cells in a field. Ratio ($F480/F480_0$); 480 to 480 nm at 0 time fluorescence ratio. (B) Summarized data showing the effects of Ca^{2+} channel blockers on thapsigargin-induced increases in $[Ca^{2+}]_i$ in the absence (a) or presence of external Ca^{2+} (b). Fluo-3 loaded cells were preincubated with Ca^{2+} channel blockers (each of 30 μM) as the same procedure to amlodipine. The values are the mean ± S.E. of 11–21 cells (shown in parenthesis) from two to three independent cultures, each of them measured on a field of 5–7 single cells (*$P<0.05$, **$P<0.01$ versus control by unpaired Student's *t*-test).

on the Ca^{2+} response to cyclopiazonic acid (data not shown). In A431 cells preincubated with amlodipine, basal $[Ca^{2+}]_i$ was slightly lower than that in the control cells and the cyclopiazonic acid-induced rise in $[Ca^{2+}]_i$ was significantly inhibited (Fig. 6Aa,Ba). When A431 cells were exposed to 30 μM cyclopiazonic acid in Ca^{2+}-free HBSS, $[Ca^{2+}]_i$ rose transiently and then decreased to the basal level. Preincubation of the cells with amlodipine blunted the cyclopiazonic acid-induced rise in $[Ca^{2+}]_i$ in Ca^{2+}-free HBSS (Fig. 6Ab,Bb).

We also tested the effect of thapsigargin on the Ca^{2+} mobilization in the presence or absence of external Ca^{2+}. A431 cells were exposed to 1 μM thapsigargin in Ca^{2+}-free HBSS for 10–12 min and then switched to Ca^{2+}-containing HBSS medium. Thapsigargin rose $[Ca^{2+}]_i$ in control cells as shown in Fig. 7A. In the cells preincubated with amlodipine for 1 h, the thapsigargin-induced rises in $[Ca^{2+}]_i$ were significantly inhibited irrespective of the presence of external Ca^{2+} (Fig. 7A,B).

Taylor and Simpson (1992) showed that 3 and 10 μM amlodipine lowered $[Ca^{2+}]_i$ of the indo-1 loaded HT-39 human breast cancer cells and suggested that the modulation of $[Ca^{2+}]_i$ by amlodipine resulted in an inhibition of HT-39 cell proliferation. Thus, to examine the direct effect of amlodipine on $[Ca^{2+}]_i$, the fura-2 loaded A431 cells were exposed to amlodipine. However, as pointed out by Taylor and Simpson (1992), we observed that amlodipine emitted fluorescence at the excitation and emission wavelengths for fura-2, i.e., addition of amlodipine to the fura-2 loaded cells apparently lowered the fluorescence ratio (F340/F380) from the basal value 0.38 ($n=21$) to 0.27 ($n=21$) because the fluorescence intensity of amlodipine is higher at 380 nm excitation wavelength than at 340 nm excitation wavelength. The ratio due to the amlodipine was returned to basal level by washing thoroughly with Ca^{2+}–HBSS. Then, in another set of experiments, the fura-2 loaded cells were preincubated with test agents for 10 min at room temperature, washed five times with Ca^{2+}–HBSS and exposed to 1 μM thapsigargin in the presence of external 2 mM Ca^{2+}. Under this condition, the thapsigargin-induced rise in $[Ca^{2+}]_i$ was also diminished in amlodipine-treated cells. However, nifedipine (30 μM), a dihydropyridine without antiproliferative effect, had no effect on Ca^{2+} response to 1 μM thapsigargin (Fig. 8).

To rule out the possibility of spectral interference of amlodipine with fura-2, we performed the Ca^{2+} experiments using a visible wavelength excitable indicator fluo-3. Fluo-3 loaded cells were exposed to 1 μM thapsigargin in the presence or absence of 30 μM amlodipine. As shown in Fig. 9A, amlodipine diminished the thapsigargin-induced Ca^{2+} responses. The effect could be reproduced with nicardipine or nimodipine (each of 30 μM), which is a potent antiproliferative agent, but could not be reproduced with nifedipine or verapamil (each of 30 μM), which lack an antiproliferative effect (Fig. 9B), indicating that the effects of Ca^{2+} channel blockers on the thapsigargin-induced Ca^{2+} responses correlate with their effects on the cell growth.

In addition, no increase in $[Ca^{2+}]_i$ was observed by increasing the bath $[K^+]$ from 2.5 to 80 mM, indicating that these cells do not express functional L-type Ca^{2+} channels (data not shown).

4. Discussion

A431 cell proliferation was inhibited by dihydropyridine derivatives, such as amlodipine, nicardipine and nimodipine but not nifedipine, while it was less sensitive to verapamil or diltiazem as assessed by MTT proliferation assay (Fig. 1). The effects of these Ca^{2+} channel blockers were confirmed by a BrdU incorporation assay, that is, amlodipine, nicardipine and nimodipine, but not nifedipine, inhibited BrdU incorporation into nucleic acids of the cells during an 18-h incubation period, whereas verapamil or diltiazem did not (Fig. 2). Among these effective dihydropyridine derivatives, amlodipine was studied further, since little is known about the agent on the tumor cell growth inhibitory effect (Taylor and Simpson, 1992). Amlodipine showed an inhibitory effect on cell growth and on nucleic acid synthesis in a dose- and time-dependent manner (Figs. 3 and 4). Though Ca^{2+} channel blockers including amlodipine have been shown to block the L-type Ca^{2+} channel of the plasma membrane, the L-type Ca^{2+} channel agonist (±)-Bay K8644 (200 nM) could not attenuate the effect of amlodipine (Fig. 5A). In the measurement of $[Ca^{2+}]_i$ in fura-2 loaded A431 cells, we observed no rise in $[Ca^{2+}]_i$ by increasing the bath $[K^+]$ to 80 mM (data not shown). These findings are consistent with the report by Moolenaar et al. (1986) demonstrating that A431 cells lack classical voltage-activated Ca^{2+} channels found in nerves and muscles. Thus, these results suggest that amlodipine may act through a mechanism other than the plasma membrane L-type Ca^{2+} channel antagonism, although a sustained Ca^{2+} influx through plasma membrane Ca^{2+} channels is required for A431 cell growth. Since Ca^{2+} influx processes through store-operated Ca^{2+} channels (Parekh and Penner, 1997), named as capacitative Ca^{2+} entry (Putney and Bird, 1993; Putney et al., 2001) are known, the effect of thapsigargin, an inhibitor of Ca^{2+}-ATPase of endoplasmic reticulum, on A431 cell growth was investigated. Thapsigargin itself inhibited the growth of A431 cells and exerted a synergistic effect on the antiproliferative action of amlodipine (Fig. 5B). It has been shown that the intracellular Ca^{2+} pool content is linked to the control of cell growth (Magnier-Gaubil et al., 1996; Short et al., 1993). The irreversible Ca^{2+}-ATPase inhibitor thapsigargin was shown to inhibit the DNA synthesis and proliferation of DDT_1MF-2 smooth muscle cells, suggesting that the depletion of Ca^{2+} from the endoplasmic reticulum eliminates the source of Ca^{2+} signals mediated through inositol phospholipid breakdown, required for cell growth activation (Short et al., 1993). Our

result suggests that the Ca^{2+} maintained within the endoplasmic reticulum is required for A431 cell growth and that thapsigargin-sensitive Ca^{2+} stores may be involved in the action of amlodipine.

In the measurement of $[Ca^{2+}]_i$ in fura-2 loaded A431 cells, thapsigargin or another Ca^{2+}-ATPase inhibitor of endoplasmic reticulum, cyclopiazonic acid, increased $[Ca^{2+}]_i$ in the presence and the absence of external Ca^{2+}. The rise in $[Ca^{2+}]_i$ by these agents was assumed to be due to Ca^{2+} release from the endoplasmic reticulum and the ensuing capacitative Ca^{2+} entry through store-operated Ca^{2+} channels. In the cells preincubated with amlodipine for 1 h, basal $[Ca^{2+}]_i$ was slightly but significantly low as compared to the control cells, and the thapsigargin or cyclopiazonic acid-induced rise in $[Ca^{2+}]_i$ was diminished (Figs. 6 and 7). Further, measurement of $[Ca^{2+}]_i$ in fluo-3 loaded cells demonstrated that amlodipine, nicardipine and nimodipine, but not nifedipine or verapamil inhibited the thapsigargin-evoked Ca^{2+} responses related to their antiproliferative potency (Fig. 9A,B).

Thus, it was demonstrated that amlodipine specifically inhibited the thapsigargin-evoked Ca^{2+} release from the internal Ca^{2+} stores and the consequential store-operated Ca^{2+} entry. The exact molecular targets of amlodipine in modulating Ca^{2+}-mobilization resulting in the A431 cell growth inhibition are not clear at present. Amlodipine may directly or indirectly modulate thapsigargin and cyclopiazonic acid-sensitive Ca^{2+} stores or/and store-operated Ca^{2+} entry. Since mitochondria have the potential to regulate intracellular Ca^{2+} signaling (Hoth et al., 2000) and it was demonstrated that human tumor cell proliferation was suppressed through mitochondrial targeting (Holmuhamedov et al., 2002), the possibility that amlodipine affects functional mitochondria cannot be ruled out.

In the electrophysiological characterization of the endogenous channels in A431 cells, Lückhoff and Clapham (1994) first showed that these cells have Ca^{2+} permeable channels activated by depletion of Ca^{2+} stores, having some characteristics similar to the Ca^{2+} release-activated Ca^{2+} channel current (I_{CRAC}) in mast cells and the depletion activated current (I_{DAC}) in lymphocytes. The low-conductance high-selective inositol 1,4,5-trisphosphate (IP_3) activated Ca^{2+} channels were demonstrated in the plasma membrane of A431 cells (Kiselyov et al., 1997). Furthermore, the channels are suggested to be I_{CRAC}-like Ca^{2+} channels coupled to an IP_3-responsive protein (Kiselyov et al., 1999). Recently, Kaznacheyeva et al. (2000) demonstrated that plasma membrane Ca^{2+} channels in A431 cells are functionally coupled to IP_3 receptor-phosphatidylinositol 4,5-bisphosphate complexes and that activation of Ca^{2+} entry in A431 cells by store depletion and phospholipase C-dependent mechanisms converge on I_{CRAC}-like Ca^{2+} channels (Kaznacheyeva et al., 2001). The cyclopiazonic acid- or thapsigargin-induced rise in $[Ca^{2+}]_i$ in the presence of external Ca^{2+} observed in the present study may be carried out through these I_{CRAC}-like Ca^{2+} channels. Additional experiments to examine the effect of amlodipine on uridine triphosphate- and epidermal growth factor-induced rises in $[Ca^{2+}]_i$, both of which are known to result from IP_3, and the consequential release of Ca^{2+} from intracellular stores (Mozhayeva and Kiselyov, 1998; Hepler et al., 1987), will serve as a clue to explore the precise mechanism by which amlodipine, nicardipine and nimodipine inhibit A431 cell growth.

The reasons why nifedipine, a dihydropyridine, did not exert antiproliferative effects are not known. However, some specific structural characteristics of effective dihydropyridine derivatives may account for differences in antiproliferative action. This may be helpful to explore novel antitumor agents that have modulating effects on intracellular Ca^{2+} homeostasis.

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