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G1 cell cycle arrest by amlodipine, a dihydropyridine Ca^{2+} channel blocker, in human epidermoid carcinoma A431 cells

Junko Yoshida *, *Takaharu Ishibashi*, *Matomo Nishio*

Department of Pharmacology, Kanazawa Medical University, Daigaku 1-1, Uchinada, Ishikawa 920-0293, Japan

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ABSTRACT

We demonstrated previously that amlodipine, a dihydropyridine Ca^{2+} channel blocker, exhibits antitumor effects on human epidermoid carcinoma A431 cells both *in vitro* and *in vivo*, in part through inhibition of capacitative Ca^{2+} entry. In this study, we examined the effects of amlodipine on cell cycle distribution and cell cycle regulatory molecules in A431 cells, since a rise in intracellular Ca^{2+} is required at several points during cell cycle progression. Flow cytometric analysis revealed that treatment with amlodipine (20–30 μM, for 24 h) induced G1 phase cell accumulation. The amlodipine-induced G1 arrest was associated with a decrease in phosphorylation of retinoblastoma protein (pRB), a regulator of G1 to S phase transition, reduction of protein levels of cyclin D1 and cyclin dependent kinase 4 (CDK4), G1 specific cell cycle proteins, and increased expression of $p21^{Waf1/Cip1}$, an inhibitory protein of CDK/cyclin complexes. *In vitro* kinase assay revealed that amlodipine significantly decreased CDK2-, CDK4-, and their partners cyclin E- and cyclin D1-associated kinase activities. The amlodipine-induced reductions in cyclin D1 protein expression and in CDK2 kinase activity were reproduced by a dihydropyridine derivative, nicardipine, having an inhibitory effect on A431 cell growth, but not by nifedipine, lacking the antiproliferative activity. Our results demonstrate that amlodipine caused G1 cell cycle arrest and growth inhibition in A431 cells through induction of $p21^{Waf1/Cip1}$ expression, inhibition of CDK/cyclin-associated kinase activities, and reduced phosphorylation of pRB.

1. Introduction

Ca^{2+} channel blockers have been used for the treatment of hypertension, angina pectoris and ventricular tachyarrhythmias based on their inhibitory effect on Ca^{2+} entry by interacting with plasma membrane voltage-dependent L-type Ca^{2+} channels (L-type VDCCs). In animal models and clinical trials, some Ca^{2+} channel blockers also retard the development of atherosclerosis in part due to their inhibition of smooth muscle cell proliferation [1–4]. The antiproliferative effect of Ca^{2+} channel blockers has been shown also in tumor cell lines, such as HT-39 human breast cancer cells [5], human brain tumor cells [6–9], and LNCaP human prostate cancer cells [10]. We have demonstrated recently that dihydropyridine derivatives, such as amlodipine, nicardipine and nimodipine, inhibited the growth and DNA synthesis of human epidermoid carcinoma A431 cells, whereas verapamil (phenylalkylamine), diltiazem (benzodiazepine), and interestingly, nifedipine (dihydropyridine) did not show such effects [11]. Since intracellular Ca^{2+} is indispensable for the proliferation of various cell types, we examined in our previous study the effect of Ca^{2+} channel blockers on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by single-cell Ca^{2+}-imaging. Fluorometric measurement of $[Ca^{2+}]_i$ revealed that Ca^{2+} channel

* *Corresponding author*. Tel.: +81 76 218 8107; fax: +81 76 286 8191.
E-mail address: yayuyo@kanazawa-med.ac.jp (J. Yoshida).
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blockers with antiproliferative activity specifically blunted Ca^{2+} influx through the store-operated Ca^{2+} channels (SOCs) evoked by thapsigargin, an endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor [11]. In addition, amlodipine, but not nifedipine, attenuated Ca^{2+} responses elicited by stimulation of Gq protein coupled receptors with uridine 5′-triphosphate (UTP) in A431 cells, suggesting that amlodipine also attenuated Ca^{2+} entry through receptor-activated cation channels [12].

In electrophysiological studies, some investigators demonstrated that A431 cell line lacks dihydropyridine-sensitive VDCCs [13], but has plasma membrane Ca^{2+} permeable channels, named I_{SOC} and I_{CRAC} channels instead, both of which are activated by store depletion and stimulation of phospholipase C-coupled receptors [14,15]. The mammalian homologs of the *Drosophila* canonical transient receptor potential (TRPC) channel family have been implicated as molecular candidates for SOCs and receptor-activated cation channels in excitable and non-excitable cells [16–22]. Our reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that A431 cells express mRNAs of TRPC1 and TRPC5 out of seven members of TRPC subfamily, but the cells actually do not express mRNAs for the α and β subunits of L-type VDCC. TRPC1 and TRPC5 are expressed probably in heteromultimers in A431 cells and may function as SOCs and/or receptor-activated cation channels. Thus, we suggested that inhibition of capacitative Ca^{2+} entry through SOCs like TRPCs could be partly involved in the antiproliferative effect of Ca^{2+} channel blockers in A431 cells [23].

To explore the mechanisms underlying the antiproliferative effect of amlodipine further, we investigated here the effect of amlodipine on the cell cycle progression in A431 cells, because a rise in intracellular Ca^{2+} is required at several points during cell cycle progression [20,24]. The results showed that amlodipine induced G1 cell cycle arrest and decreased the phosphorylation level of retinoblastoma protein, essential for the G1 to S phase transition. Then, we examined the effects of amlodipine on the cell cycle regulatory molecules: cyclin D1, cyclin dependent kinase 4 (CDK4), cyclin E, CDK2, $p21^{Waf1/Cip1}$, and their-associated kinase activities. We also examined the effects of intracellular Ca^{2+} modulators, such as thapsigargin [25], an ER Ca^{2+}-ATPase inhibitor, and 2-aminoethoxydiphenyl borate, a SOC inhibitor, to elucidate whether or not these substances emulate the action of amlodipine on the cell cycle regulatory machinery.

2. Materials and methods

2.1. Reagents

Amlodipine vesilate, donated by Sumitomo Pharmaceutical (Ibaraki, Japan), and nifedipine were dissolved in dimethyl sulfoxide (DMSO) each at concentration of 100 mM. Nicardipine was dissolved in distilled water at 10 mM. Thapsigargin and 2-aminoethoxydiphenyl borate (2-APB; Tocris Cookson Inc., Ellisville, MO) were dissolved in DMSO at a concentration of 10 mM and 100 mM, respectively.

The antibodies used for Western blot were mouse monoclonal anti-pRB, rabbit polyclonal anti-phospho pRB, mouse monoclonal anti-cyclin D1, mouse monoclonal anti-CDK4, rabbit polyclonal anti-E2F1 (Cell Signaling Technology, Beverly, MA) and rabbit polyclonal anti-$p21^{Waf1/Cip1}$ (Santa Cruz Biotechnology, Santa Cruz, CA). To confirm equal loading of protein for every immunoblot, mouse monoclonal anti-β-actin antibody (Sigma–Aldrich Inc., St. Louis, MO) was used. Horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) was used as the secondary antibody for Western blot. For immunoprecipitation and *in vitro* kinase assays, we used mouse monoclonal anti-cyclin D1 (Cell Signaling Technology), mouse monoclonal anti-CDK4, mouse monoclonal anti-CDK2, and rabbit polyclonal anti-cyclin E antibodies (Santa Cruz Biotechnology).

2.2. Tumor cell lines

Human epidermoid carcinoma A431 cells were cultured as described previously [11,12,26]. Briefly, the cells were cultured in Dulbecco's modified Eagle medium (DMEM), containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 12.7 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic 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. Flow cytometric analysis of DNA content

The cells (2.5×10^5 cells) were grown in 5 ml of DMEM containing 10% FBS for 24 h and treated either with vehicle or agents for the indicated time. For flow cytometry, cells were trypsinized, pelleted by centrifugation, and resuspended in 0.5 ml of PBS. The same volume of 70% ethanol in PBS was added to the cell suspension and fixed for 30 min at 4 °C. After washing the cells with PBS, cells were resuspended in 1 ml of 2.5 mg/ml RNAse for 1–2 h at room temperature. The pelleted cells were suspended in 0.5–1.0 ml of 50 μg/ml propidium iodide and analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The cell cycle distribution was determined by Modifit cell cycle analysis software (Becton Dickinson Immunocytometry Systems).

2.4. Preparation of total cell lysate and Western blot analysis

The cells (5×10^5 cells) were grown in 10 ml of DMEM containing 10% FBS for 24 h and treated either with vehicle or agents for the indicated time. The cells were lysed in 0.1–0.5 ml of mammalian cell lysis/extraction reagent (CelLytic™-M; Sigma–Aldrich Inc.) containing protease inhibitors (Protease inhibitor cocktail; Sigma–Aldrich Inc.), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_3VO_4. The extract was centrifuged at $12{,}500 \times g$ for 15 min at 4 °C to remove insoluble material. The protein concentration of the supernatant was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). For Western blot analysis, the cell lysate (10–25 μg protein) was denatured in 5× sample buffer (625 mM Tris–HCl, 10% SDS, 25% glycerol,

0.015% bromophenol blue, and 5% 2-mercaptoethanol, pH 6.8) at 95 °C for 5 min, then electrophoretically separated on a polyacrylamide gel (PAG Mini "DAIICH" 4/20 or 15/25: Daiichi Pure Chemical Co., Tokyo, Japan). The protein was transferred to a nitrocellulose membrane, probed with primary antibodies followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Immunoreactive bands were visualized through enhanced chemiluminescence system (Amersham Biosciences Corp., Piscataway, NJ) and laser densitometry (Molecular Dynamics, Sunnyvale, CA). The intensities of bands were analyzed utilizing Molecular Dynamic's ImageQuant Software.

2.5. *Immunoprecipitation and in vitro kinase assays*

Cell lysates containing equal amounts of protein (200–300 μg in each case) were immunoprecipitated with specific anti-CDK2, anti-cyclin E, anti-CDK4 or anti-cyclin D1 antibody at 4 °C for 1 h. Twenty microliter of Protein G PLUS-agarose (Santa Cruz Biotechnology) were added to the immunoprecipitate and rocked on a platform at 4 °C for 1 h or overnight. The immunoprecipitates were washed thrice with radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM ethylene glycol bis (beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP40, and 1% Triton X-100, pH 7.4), and twice with kinase buffer (in mM: 50 HEPES, pH 7.4, 10 $MgCl_2$, 2.5 EDTA, and 1 dithiothreitol [DTT] for CDK2- and cyclin E-associated kinase assays, 50 HEPES, pH 7.4, 10 $MgCl_2$, 2.5 EDTA, 1 DTT, 10 β-glycerophosphate, and 1 NaF for CDK4- and cyclin D1-associated kinase assays). The immunoprecipitate was added with 20 μl kinase reaction buffer (kinase buffer containing 20 μM ATP, 5 μCi of [γ^{32}P]-ATP (Amersham Biosciences Corp.), and 1 μg glutathione S-transferase tagged RB protein (GST-RB: Santa Cruz Biotechnology) or 2 μg histone H1 (Calbiochem, La Jolla, CA), and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 5× sample buffer and heated at 95 °C for 5 min. The samples were separated on a 4–20% polyacrylamide gel (PAG Mini "DAIICH" 4/20: Daiichi Pure Chemical Co.). Gels were dried, exposed to a PhosphorImager screen overnight. Radiolabeled GST-RB and histone H1 bands were analyzed with ImageQuant Software (Molecular Dynamics).

3. Results

3.1. *Amlodipine induces a G1 arrest in cell cycle progression of human epidermoid carcinoma A431 cells*

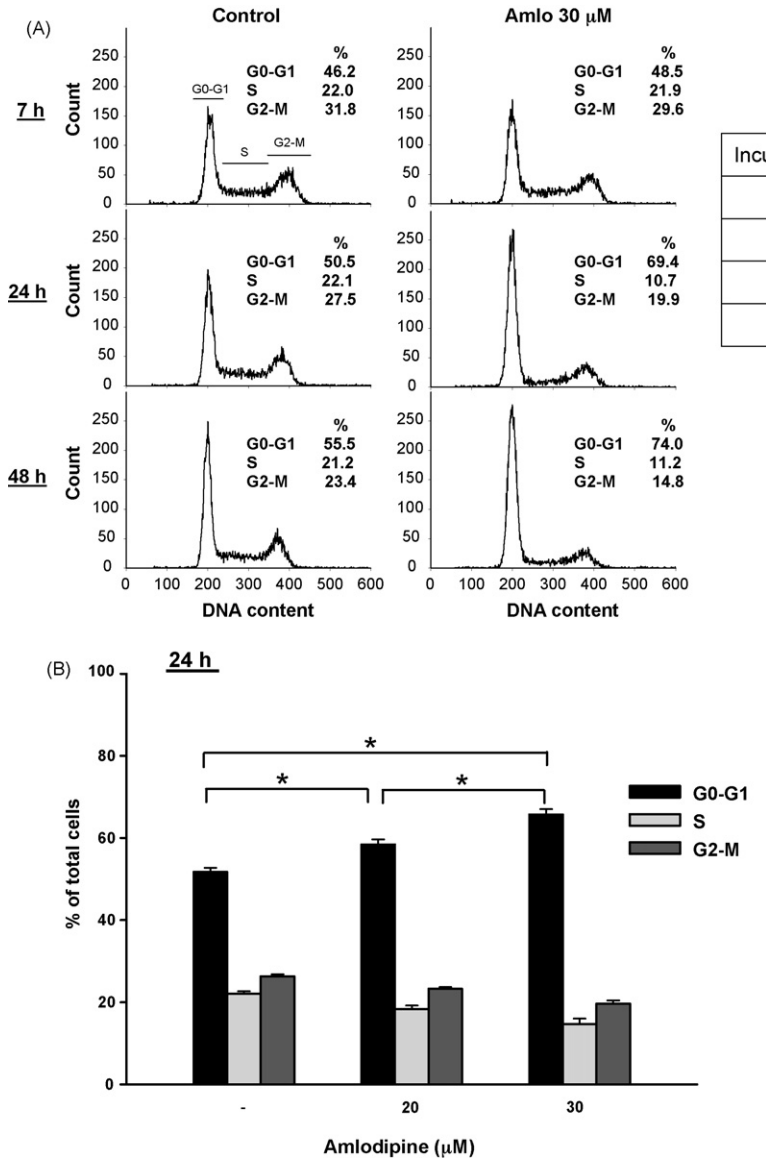
We showed previously that treatment of A431 cells with amlodipine for 48 h inhibited cell growth in a concentration-dependent manner with an IC_{50} value of 25 μM [11]. Since the mean percentages of growth rates at 20 and 30 μM of amlodipine in A431 cells were 70.6 ± 6.4 and 34.8 ± 5.7 (±S.E.M.), respectively, we used in this study mainly 20 and 30 μM of amlodipine concentrations. First, to determine whether the growth inhibitory effect of amlodipine would be cell cycle phase-specific or not, exponentially growing A431 cells were treated with either vehicle or 30 μM of amlodipine for 7 h, 24 h and 48 h and analyzed by flow cytometry. As shown in Fig. 1A, treatment of A431 cells with amlodipine increased G1 phase cell population at 24 h and 48 h, but not at 7 h. The cell numbers at 24 h and 48 h treatment with 30 μM amlodipine were decreased by 89.3% and 38.8% of control, respectively. However, the amlodipine-induced G1 cell cycle arrest at 48 h was relatively similar to that at 24 h. At the 24 h of optimum treatment time, G1 cell cycle arrest by amlodipine was concentration-dependent (Fig. 1B). To assess the effect of amlodipine on the synchronized cells, A431 cells were incubated in FBS-free DMEM for 24 h and then stimulated with 10% FBS in the absence or presence of amlodipine. As shown in Fig. 2A, the A431 cells synchronized at G0 phase progressed into the S phase after 24 h of serum addition. On the other hand, in A431 cells incubated with serum plus 20 μM or 30 μM amlodipine for 24 h, an increase in G1 phase cell population was observed with a slight decrease in the S phase cells. The decrease in cell number by amlodipine treatment (20 μM and 30 μM) was nearly equal (Fig. 2A and B). Thus, the amlodipine-induced G1 cell cycle arrest was confirmed in synchronized A431 cells.

3.2. *Effects of amlodipine on phosphorylation of retinoblastoma protein (pRB), a regulator of G1 to S transition, and other cell cycle regulatory molecules*

Since phosphorylation of retinoblastoma protein (pRB) by G1 specific cyclin dependent kinases (CDKs) is important for the G1 to S phase transition, we examined the effect of amlodipine on the phosphorylation of pRB by Western blot analysis using an antibody that recognizes the pRB phosphorylated at serine 780 residue. The phosphorylated state of pRB was evaluated as the ratio of phosphorylated pRB to unphosphorylated one. Treatment of A431 cells with amlodipine for 22–24 h decreased the phosphorylation of pRB (Fig. 3A), decreased the protein expression of cyclin D1, and increased the expression of $p21^{Waf1/Cip1}$, an inhibitory protein of CDK-cyclin complexes, while the protein level of internal control β-actin remained relatively unchanged (Fig. 3A). $p27^{Kip1}$, a member of CDK-cyclin complex inhibitors, was consistently undetectable in A431 cells (data not shown). Time-course experiments showed that the effects of amlodipine on these molecules were time-dependent (Fig. 3B). Since the reduction in cyclin D1 protein expression by amlodipine was apparent as early as 4 h treatment, we examined the effect of other dihydropyridine Ca^{2+} channel blockers on cyclin D1 protein expression. A similar effect was observed at 4 h treatment of nicardipine, which has an antiproliferative effect on A431 cells; however, such effect could not be observed with nifedipine, a dihydropyridine derivative lacking antiproliferative potency (Fig. 3C). Furthermore, Western blot analysis revealed that amlodipine treatment reduced the protein levels of CDK4 and E2F1, a transcription factor essential for progression through G1 and into the S phase of the cell cycle (Fig. 3D).

3.3. *Effects of amlodipine on cyclin-dependent kinase activities in A431 cells*

Phosphorylation of pRB is regulated by activation of CDKs and their heterodimeric cyclin partners. To elucidate whether the



Cell number (x10^5 cells)

Incubation time (h)	Control	Amlo 30 μM
0	2.5	2.5
7	2.5	2.5
24	2.8	2.5
48	9.8	3.8

Fig. 1 – Effects of amlodipine on cell cycle in A431 cells. Cells were cultured in complete medium and treated with either vehicle (0.03% DMSO) or amlodipine. After the indicated time of incubation, cells were collected, and cellular DNA was stained with propidium iodide and analyzed for cell cycle distribution. 1×10^4 cells were analyzed for each condition. (A) Representative histogram plotting cell count *vs.* DNA content after 7 h, 24 h, and 48 h of incubation of A431 cells with 30 μM of amlodipine (Amlo 30 μM). The percentage of cells present in G0-G1, S, and G2-M phases are shown. The table shows the cell number determined by trypan blue exclusion in the same dishes. (B) Summary of data for amlodipine-induced G1 cell cycle arrest in A431 cells at 24 h treatment. Values are mean ± S.E.M. of four independent experiments. $^*P < 0.05$ by one-way analysis of variance followed by Fisher's PLSD.

amlodipine-induced reduction in the pRB phosphorylation is due to inhibition of CDK-cyclin activities, we examined the effects of amlodipine on CDKs- and their partner cyclin-associated kinase activities. An *in vitro* kinase assay using CDK4 and cyclin D1-immunoprecipitates and GST-RB as a substrate showed that treatment of A431 cells with amlodipine significantly decreased the CDK4- and its partner cyclin D1-associated kinase activities (Fig. 4A). In addition, *in vitro* kinase assay using CDK2 and cyclin E-immunoprecipitates and histone H1 as a substrate revealed that amlodipine treatment resulted in a decrease in CDK2- and its partner cyclin E-associated kinase activities (Fig. 4A). The possibility that the decrease in kinase activity was due to reduced protein expression was ruled out by Western blotting on CDK4-, cyclin D1-, CDK2- and cyclin E-immunoprecipitates; the levels of immunoprecipitated proteins were not difference among vehicle (Control)-, 20 μM amlodipine-, and 30 μM amlodipine-treated cells (data not shown). Similar results to amlodipine (reductions of both cyclin D1- and CDK2-associated kinase activities) were observed for nicardipine (30 μM),

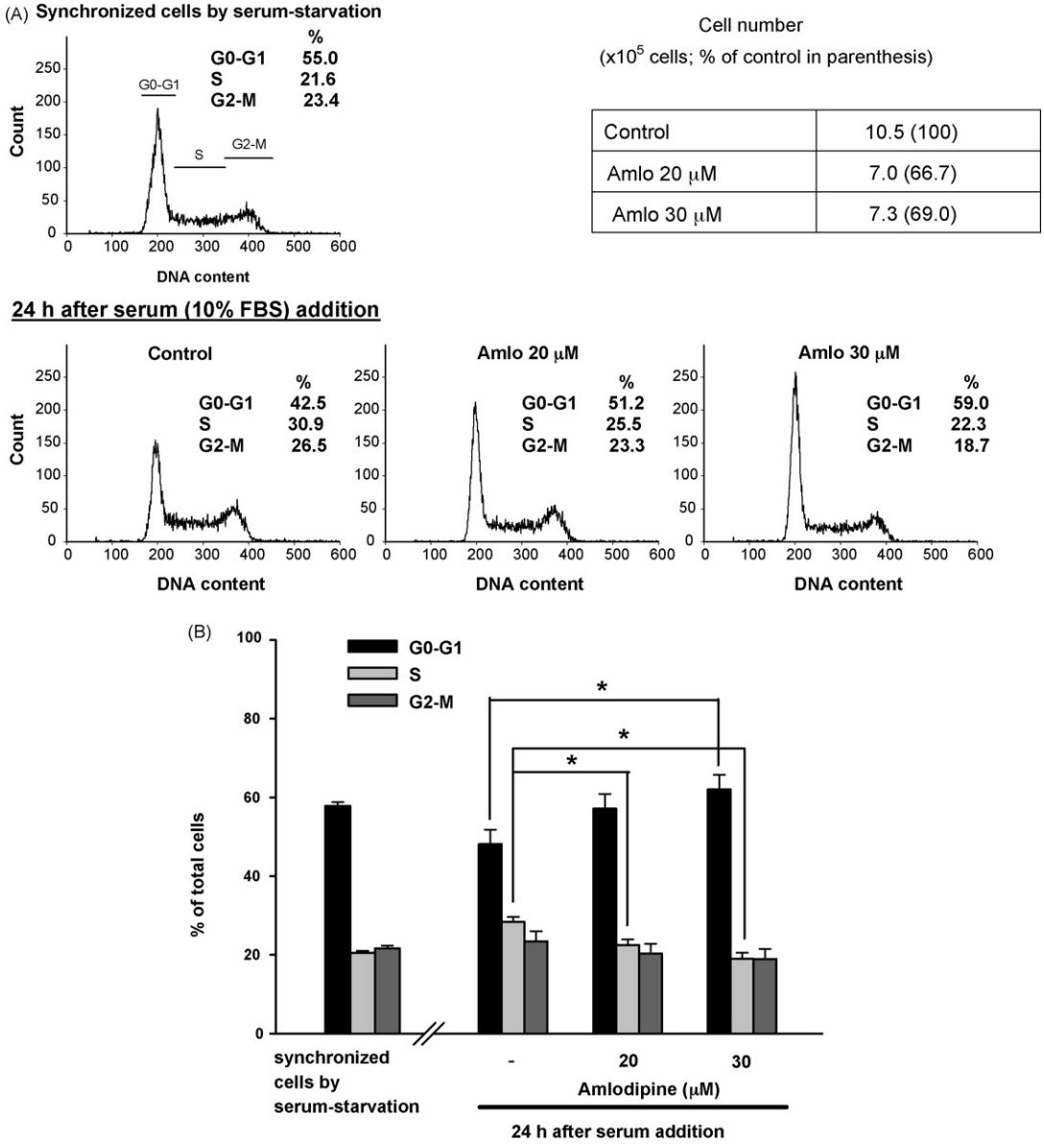


Fig. 2 – Effects of amlodipine on cell cycle in synchronized A431 cells by serum-starvation. Cells were incubated in fetal bovine serum (FBS)-free DMEM for 24 h and stimulated with 10% FBS for 24 h in the absence or presence of amlodipine. (A) Representative histogram plots of cell count *vs.* DNA content after serum-starvation and 24 h incubation with 10% FBS in the absence or presence of amlodipine (Amlo 20 and 30 μM). Numbers represent percentages of cells in G0-G1, S, and G2-M phases. The table shows the cell number at 24 h after serum addition determined by trypan blue exclusion in the same dishes. (B) Quantitative analysis of amlodipine-induced G1 cell cycle arrest in synchronized A431 cells. Values are mean ± S.E.M. of four independent experiments. $^{*}P < 0.05$ by one-way analysis of variance followed by Fisher's PLSD.

an antiproliferative dihydropyridine derivative, but not for nifedipine, a derivative of the same class lacking antiproliferative activity (Fig. 4B). These results were consistent with those by Western blot analysis showing that their effects on the cyclin D1 protein expression correlated with their inhibitory effects on cell growth (Fig. 3C). Taken together, these data suggest that a reduction in cyclin D1 protein expression, as well as inhibition of cyclin D1- and CDK2-associated kinase activities, may be involved in the antiproliferative effect of amlodipine in A431 cells.

3.4. *Effects of thapsigargin, an inhibitor of endoplasmic reticulum (ER) Ca^{2+}-ATPase, and 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of store-operated Ca^{2+} channels, on cell cycle regulatory molecules*

In our reported Ca^{2+} imaging assay in A431 cells, we observed that amlodipine did not only attenuate the capacitative Ca^{2+} entry elicited by thapsigargin, an ER Ca^{2+}-ATPase inhibitor, but also induced Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores [12], i.e., in fluo-3-loaded A431 cells, application of

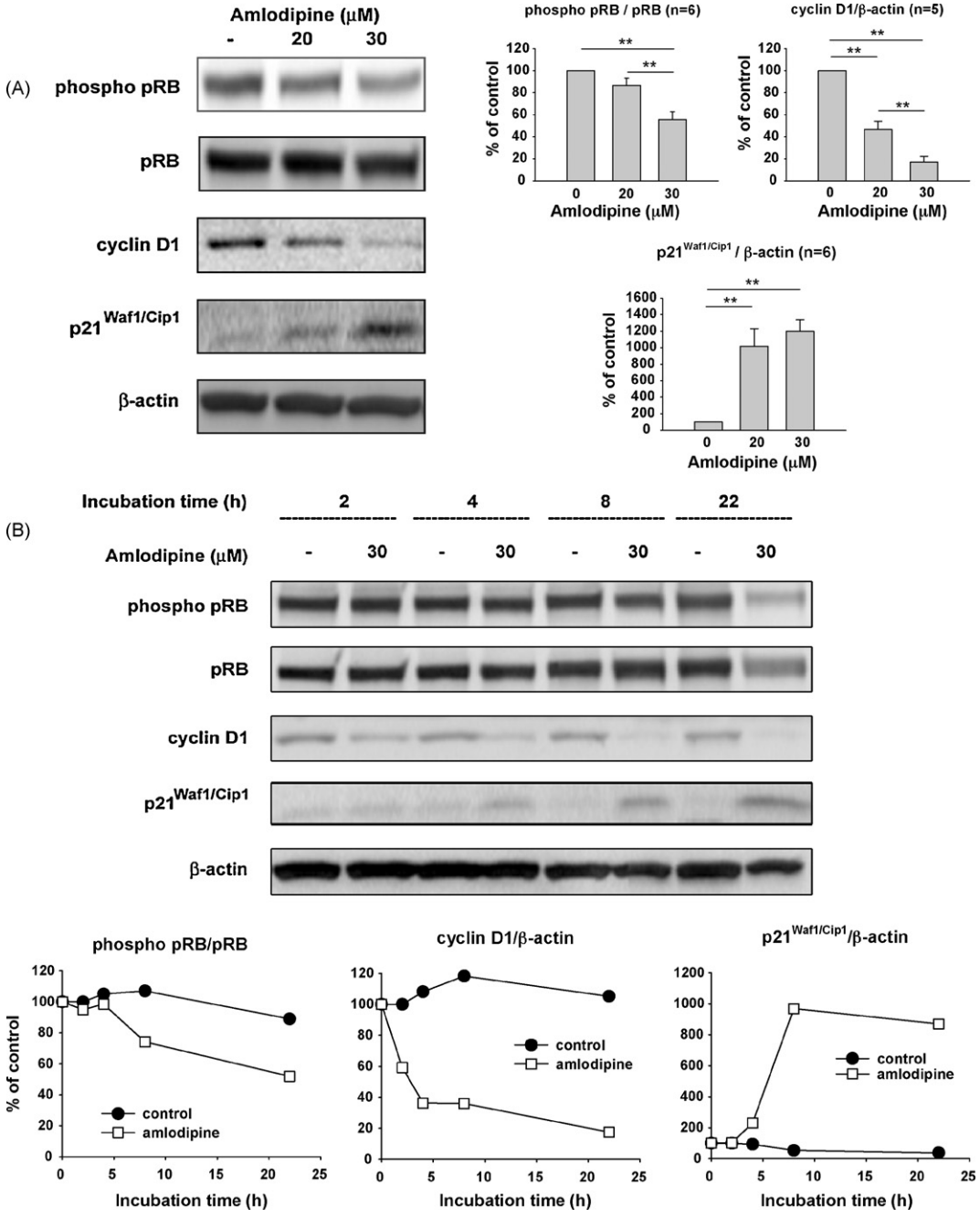


Fig. 3 – Effects of amlodipine on G1 cell cycle regulatory proteins in A431 cells. (A) Left: A431 cells were cultured in complete medium and treated with either vehicle or amlodipine (20 and 30 μM) for 22–24 h. The cell lysates were subjected to SDS-PAGE followed by Western blot analysis with antibodies for phosphorylated and non-phosphorylated form of retinoblastoma protein (phospho pRB and pRB, respectively), cyclin D1 and p21$^{Waf1/Cip1}$. β-actin was used to verify equal gel loading. Right: bars represent the ratio of phosphorylated pRB to pRB, and protein levels of cyclin D1 and p21$^{Waf1/Cip1}$ normalized to β-actin. Values are mean ± S.E.M. of five to six independent experiments. $^{**}P < 0.01$ by one-way analysis of variance followed by Fisher's PLSD. (B) Results of time-course studies of amlodipine-induced inhibition of pRB phosphorylation, reduction in cyclin D1 protein expression, and upregulation of p21$^{Waf1/Cip1}$ in A431 cells. Data are representative results of two independent experiments. (C) Effects of other dihydropyridine Ca^{2+} channel blockers (CCBs) on cyclin D1 protein expression in A431 cells. A431 cells were incubated in complete medium and treated with either 0.03% DMSO (–) or 30 μM amlodipine (Amlo), 30 μM nifedipine (Nife), 0.3% of methanol (MeOH, solvent for the dilution of nifedipine) and 30 μM nicardipine (Nicar) for 4 h, to examine their effects on cyclin D1 protein level. Data are representative results of three to four independent experiments. The graph on the right shows cyclin D1 protein levels normalized to β-actin. (D) Effects of amlodipine treatment (22–24 h) on the protein expression of cyclin dependent kinase 4 (CDK4) and transcription factor E2F1 in A431 cells. Data are representative results of three independent experiments. The graphs on the right show protein levels for CDK4 and E2F1 normalized to β-actin.

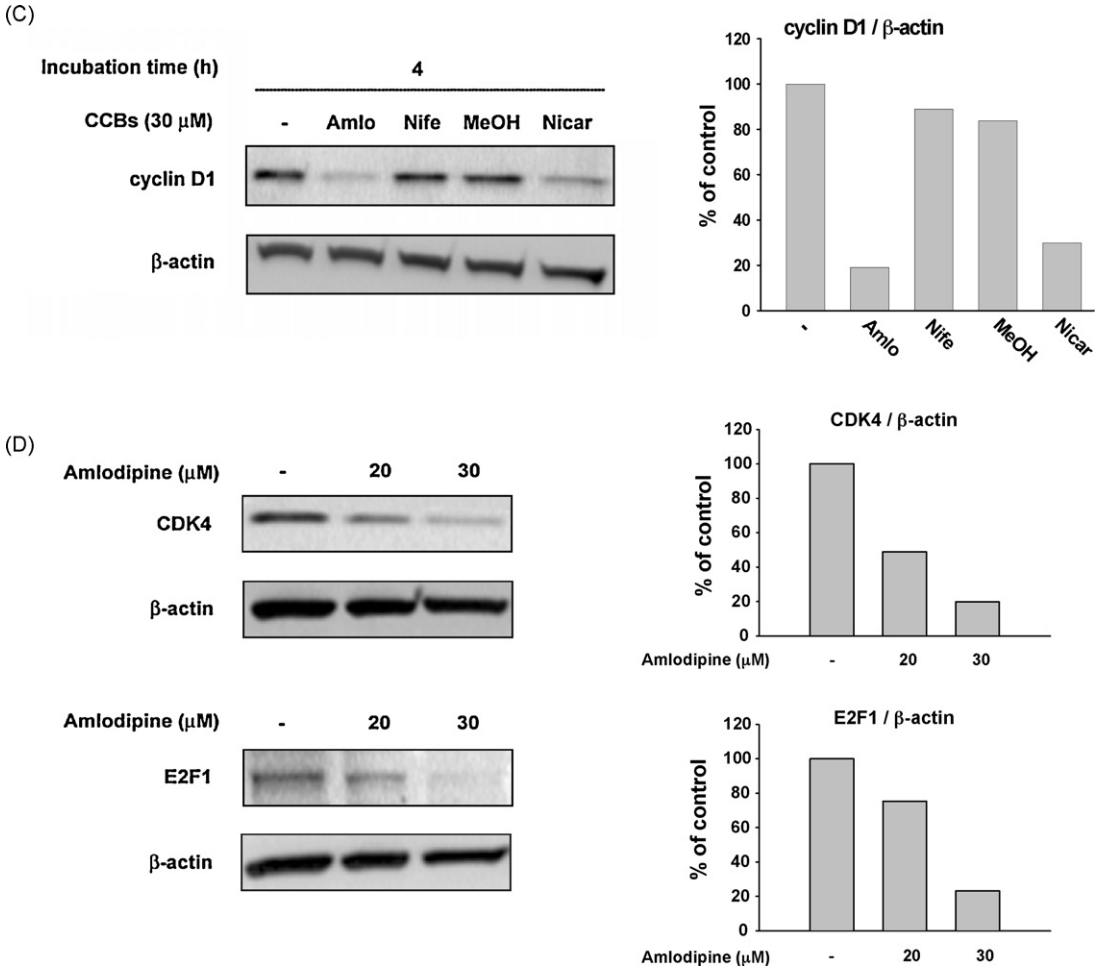


Fig. 3. (Continued).

amlodipine in Ca^{2+}-free medium elicited a transient rise in $[Ca^{2+}]_i$. Thapsigargin applied thereafter caused no rise in $[Ca^{2+}]_i$, and vice versa [12]. Therefore, to assess whether the effects of amlodipine on cell cycle regulatory molecules were due to the Ca^{2+}-releasing effect from thapsigargin-sensitive Ca^{2+} stores or the inhibitory effect on SOCs, we examined the effects of thapsigargin and 2-aminoethoxydiphenyl borate (2-APB), a SOC inhibitor, on the cell cycle machinery. 2-APB at 50 μM has been shown to inhibit effectively the thapsigargin-induced capacitative Ca^{2+} entry in A431 cells [23]. To this end, A431 cells were preincubated with 1 μM thapsigargin or 50 μM 2-APB for 15 min, then incubated for another 24 h with vehicle or amlodipine, and these cells were used in Western blot analysis and *in vitro* kinase assay. Western blot analysis showed that thapsigargin reduced the phosphorylation level of pRB and protein level of cyclin D1, and predominantly upregulated the $p21^{Waf1/Cip1}$ protein expression, while 2-APB showed a modest effect (Fig. 5A). In *in vitro* CDK2 kinase assay using histone H1 as a substrate, thapsigargin showed a considerable reduction in histone H1 phosphorylation, whereas 2-APB decreased the phosphorylation slightly (Fig. 5B). These results imply that the effects of amlodipine on cell cycle regulatory molecules were similar to those of thapsigargin, an ER Ca^{2+}-ATPase inhibitor, rather than those of 2-APB, a SOC inhibitor. That is, the amlodipine-induced effects on cell cycle machinery could not be fully reproduced by inhibiting Ca^{2+} entry with SOC inhibitors, such as 2-APB.

4. Discussion

We have demonstrated that some dihydropyridine Ca^{2+} channel blockers, such as amlodipine, nicardipine and nimodipine, inhibited human epidermoid carcinoma A431 cell growth and DNA synthesis *in vitro* [11], and intraperitoneal injection of amlodipine into nude mice bearing A431 xenografts retarded tumor growth and prolonged the survival of these mice [12]. Several electrophysiological studies [13,14] and our RT-PCR assay [23] indicated that A431 cells do not express L-type VDCCs, which are sensitive to Ca^{2+} channel blockers. Our single cell Ca^{2+} imaging analysis demonstrated that antiproliferative Ca^{2+} channel blockers specifically attenuated Ca^{2+} responses to thapsigargin, an ER Ca^{2+}-ATPase inhibitor, and UTP, a Gq-protein-coupled receptor agonist. It is thus suggested that inhibition of capacitative Ca^{2+} entry through SOCs in A431 cells could be involved in part in the antiproliferative effect of Ca^{2+} channel blockers, such as amlodipine [11,12].

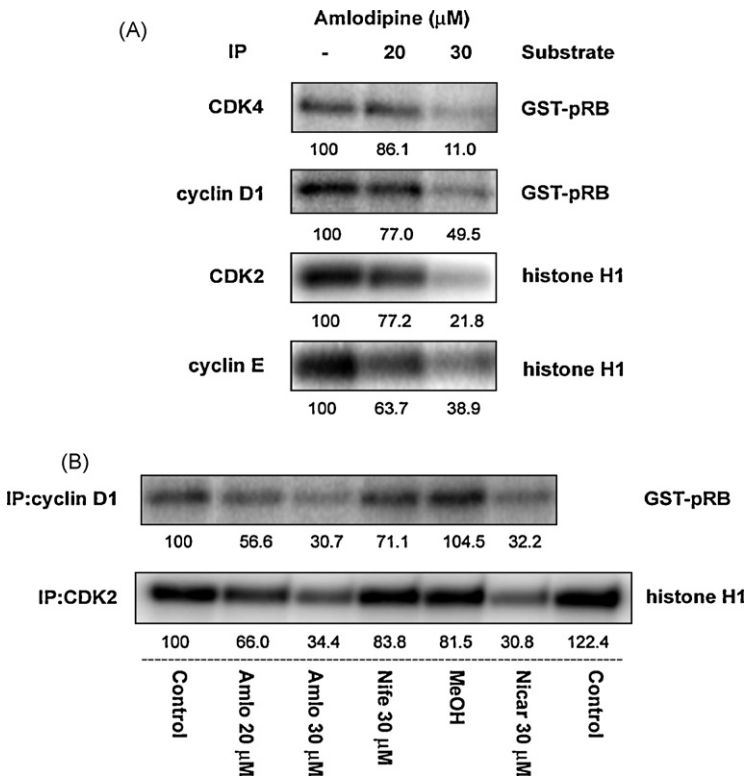


Fig. 4 – Effects of amlodipine on CDK- and cyclin-associated kinase activities. A431 cells were cultured in complete medium and treated with either vehicle (0.03% DMSO) or amlodipine for 24 h. Cell lysate (200–300 μg protein) were immunoprecipitated with antibodies for CDK4, cyclin D1, CDK2 and cyclin E. *In vitro* kinase assays were performed on their immunoprecipitates using GST-RB or histone H1 as a substrate as described in Section 2. (A) Representative scanning data of a PhosphorImager screen (three independent experiments). Kinase activities are expressed as % of the control (vehicle) and the values are shown below each scan. (B) Effects of other dihydropyridine Ca^{2+} channel blockers on cyclin D1-, and CDK2-associated kinase activities. A431 cells were cultured in complete medium and treated with either 0.03% DMSO (Control) or amlodipine (Amlo 20 and 30 μM), nifedipine (Nife 30 μM), 0.3% methanol (MeOH, solvent for the dilution of nifedipine) and nicardipine (Nicar 30 μM) for 24 h. Cyclin D1- and CDK2-associated kinase assays were performed as described in A. Data are representative results of each of two independent experiments. Kinase activities in each treatment (% of the control (vehicle)) are shown below each scan. IP, immunoprecipitation.

Since a rise in intracellular Ca^{2+} is required at several points during cell cycle progression [24], flow cytometric analysis was performed to determine whether amlodipine affects the cell cycle progression or not. Cell cycle analysis revealed that treatment of A431 cells with amlodipine retarded the cell cycle progression at G1 phase in concentration- and time-dependent manners (Figs. 1 and 2). As cells progress through G1 to S phase, cyclin-CDK complexes, such as cyclin D1-CDK4/6 and cyclin E-CDK2 complexes, are sequentially activated and phosphorylate retinoblastoma protein (pRB) on serine and threonine residues [27]. The hyperphosphorylated pRB releases active E2F transcription factors, which, in turn stimulate the transcription of numerous genes whose products are required for the G1 to S transition and S phase progression [28]. There are two families of cyclin-CDK inhibitors, the Cip/Kip and INK4 families, both of which regulate cell cycle progression [29]. Members of the Cip/Kip family, such as $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, bind to cyclin-CDK complexes and inhibit their activities. Then, we examined the effects of amlodipine on some of these cell cycle regulatory molecules by Western blot analysis and *in vitro* kinase assay.

Treatment of A431 cells with amlodipine resulted in reduction of the phosphorylated level of pRB and protein expression of cyclin D1, CDK4 and E2F1 transcription factor. Concomitantly, the protein expression of $p21^{Waf1/Cip1}$, a member of the Cip/Kip family of cyclin-CDK inhibitors, was significantly upregulated (Fig. 3A, B and D). Although $p27^{Kip1}$ is reported to be upregulated in response to antiproliferative signals [30], it was consistently undetectable in A431 cells (data not shown). A reduction in cyclin D1 protein level as early as 4 h incubation was observed by nicardipine, another dihydropyridine derivative, but not by nifedipine, another dihydropyridine lacking antiproliferative activity (Fig. 3C). These results suggest that the reduction in cyclin D1 protein expression correlates with the growth inhibitory effect of Ca^{2+} channel blockers.

In *in vitro* kinase assay using CDK4- and cyclin D1-immunoprecipitates and GST-RB as a substrate, treatment of A431 cells with amlodipine (20 and 30 μM, for 22–24 h) resulted in decreases in the CDK4-, and its partner cyclin D1-associated kinase activities (Fig. 4A). We also determined CDK2- and its partner cyclin E-associated kinase activities

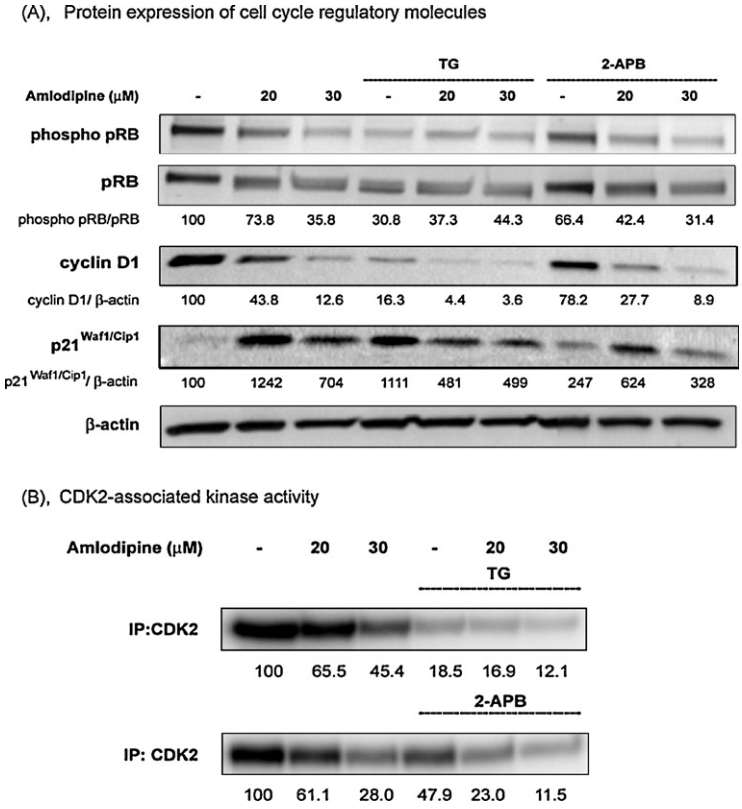


Fig. 5 – Effects of thapsigargin and 2-APB on the G1 cell cycle regulatory molecules in A431 cells. The cells were preincubated with 1 μM thapsigargin (TG) or 50 μM 2-APB for 15 min, then 0.03% DMSO (–) or amlodipine (20 and 30 μM) was added, and the cells were incubated for another 24 h. Cell lysates were used for Western blot analysis (A) and *in vitro* CDK2-associated kinase assay with histone H1 as a substrate (B). Data in A and B are representative results of each of two independent experiments. The ratio of phosphorylated pRB to pRB, and protein levels for cyclin D1 and $p21^{Waf1/Cip1}$ normalized to β-actin band are shown as % of control (vehicle) below each scan in A. CDK2-associated kinase activities (% of the control [vehicle]) are shown below each scan in B. IP, immunoprecipitation.

using their immunoprecipitates and other substrate histone H1. CDK2, but not CDK4, specifically phosphorylates histone H1 as well as pRB [31]. Amlodipine treatment effectively decreased the CDK2- and cyclin E-associated kinase activities in A431 cells (Fig. 4A). Taken together, these results indicate that amlodipine treatment inhibited both cyclinD1-CDK4 and cyclin E-CDK2 kinase activities, which led to a reduction in the level of pRB phosphorylation, thereby G1 cell cycle arrest in A431 cells. In addition, this amlodipine-induced reduction in the cyclin-CDK kinase activities might be due to upregulation of $p21^{Waf1/Cip1}$, a cyclin-CDK inhibitor. Although the ability of $p21^{Waf1/Cip1}$ is known to be induced through tumor suppressor gene p53-dependent and -independent pathways, the amlodipine-induced upregulation of $p21^{Waf1/Cip1}$ in A431 cells appear to be independent of p53, because p53 in A431 cells was shown to be mutant at codon 273 [32]. As far as we know, this is the first study reporting the involvement of $p21^{Waf1/Cip1}$ upregulation in tumor cell growth inhibition by amlodipine, a Ca^{2+} channel blocker.

Recently, amlodipine was reported to exert an antiproliferative effect on human lung vascular smooth muscle cells via $p21^{Waf1/Cip1}$ gene activation [33]. The study demonstrated that amlodipine induced $p21^{Waf1/Cip1}$ promoter activity through the activation of the glucocorticoid receptor and a transcription factor, CCAAT/enhancer binding protein alpha (C/EBPα). Since the role of the negative cell cycle regulator $p21^{Waf1/Cip1}$ has been also demonstrated in atherosclerosis [34,35], it is possible that $p21^{Waf1/Cip1}$ is the key molecule that mediates the anti-atherosclerotic and antitumor actions of Ca^{2+} channel blockers, such as amlodipine.

To explore possible targets for amlodipine, we also examined the effects of thapsigargin and 2-APB, a SOC inhibitor, on the cell cycle regulatory molecules. Based on the protein expression of cell cycle regulatory molecules and CDK2-associated kinase activity, the effects of amlodipine were rather similar to those of thapsigargin than those of 2-APB (Fig. 5A and B). We showed previously that amlodipine resulted in Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores [12]. Is the Ca^{2+}-releasing effect involved in amlodipine-induced effects on cell cycle regulatory molecules? Indeed, Ca^{2+} pool content in ER, as well as cytosolic Ca^{2+}, is a critical factor for cell growth and progression through the cell cycle [36,37]. Legrand et al. [38] were the first group to demonstrate that intracellular Ca^{2+}-ATPases (SERCAs) and Ca^{2+} pool content are closely associated with prostate cancer LNCaP cell growth. When LNCaP cell growth was inhibited by serum deprivation, the Ca^{2+} pool content was reduced and expression of SERCA2b, an isoform of SERCAs, was decreased

[38]. We reported previously that treatment of A431 cells with thapsigargin (1 μM, 48 h) resulted in inhibition of cell growth by up to 50% of the control, due to the depletion of Ca^{2+} stores, and at this dose, thapsigargin tended to augment the antiproliferative effect of amlodipine [11]. However, RT-PCR analysis, performed as described previously [23], using a set of primers for SERCA2b (GenBank accession no. **NM_001681**) showed no detectable changes in mRNA expression of SERCA2b in A431 cells incubated with amlodipine (data not shown). The result indicates at least that the Ca^{2+} releasing effect of amlodipine did not cause a decrease in SERCA2b gene expression, leading to Ca^{2+} store depletion.

Although the exact target(s) of amlodipine is not clear at present, amlodipine may interact with unknown molecules in the thapsigargin-sensitive ER and/or plasma membrane SOCs. It is possible that amlodipine interacts with a molecule involved in Ca^{2+} signaling between Ca^{2+} store depletion and activation of plasma membrane SOCs. Consequently, amlodipine may inhibit capacitative Ca^{2+} entry, resulting in growth inhibition of A431 cells.

In conclusion, our study demonstrated that amlodipine induced $p21^{Waf1/Cip1}$ expression and thereby inhibited CDK/cyclin-associated kinase activities, which led to a reduction in pRB phosphorylation resulting in G1 cell cycle arrest and growth inhibition in A431 cells. Although the direct target of amlodipine is not known at present, amlodipine would be a lead compound for the development of selective inhibitors of store-operated Ca^{2+} influx. Further studies are needed to explore the mechanisms underlying the antiproliferative property of amlodipine. Such studies should provide more definitive data for the development of new antiatherogenic and antitumor drugs acting through inhibition of Ca^{2+} signaling.

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