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Differential effects of Ca^{2+} channel blockers on Ca^{2+} transients and cell cycle progression in vascular smooth muscle cells

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Abstract

We examined the differential effects of Ca^{2+} channel blockers on the elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and G_0/G_1 transition induced by platelet-derived growth factor (PDGF) in rat aortic smooth muscle cells in primary culture. The phase of the cell cycle was determined by an immunocytochemical analysis of cell cycle-specific nuclear antigens. $[Ca^{2+}]_i$ was monitored by fura-2 microfluorometry. The efficacy of Ca^{2+} channel blockers for the inhibition of $[Ca^{2+}]_i$ elevation induced by PDGF ($NiCl_2$ > isradipine > verapamil = diltiazem) did not parallel that for the inhibition of cell cycle progression induced by PDGF (verapamil = diltiazem > $NiCl_2$ > isradipine). In addition, no significant correlation was observed between the extent of $[Ca^{2+}]_i$ elevation and the extent of G_0/G_1 transition. We thus conclude that the inhibitory effects of Ca^{2+} channel blockers on the G_0/G_1 transition induced by PDGF are not simply due to their inhibitory action on the $[Ca^{2+}]_i$ elevations but instead are due to more complex unknown factors. © 1998 Elsevier Science B.V.

Keywords: Ca^{2+} channel blocker; Cell cycle; Atherosclerosis; Ca^{2+}, cytosolic; Primary culture

1. Introduction

Controversy continues to exist regarding the notion that changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) regulate the cell cycle progression in several types of cells. It has been shown that an elevation of $[Ca^{2+}]_i$ was not required for cell proliferation (Casabiell et al., 1993; Hirai et al., 1993; Kobayashi et al., 1994) while, in contrast, several studies have also suggested that an elevation of $[Ca^{2+}]_i$ was necessary to initiate the mitogenic response (Kojima et al., 1988; Mogami and Kojima, 1993). Since Ca^{2+} channel blockers are known to inhibit the $[Ca^{2+}]_i$ elevation in various types of cells (Kanaide et al., 1988; Brocchieri et al., 1996), these drugs have been frequently used to determine the importance of the $[Ca^{2+}]_i$ elevation in cell proliferation. As a result, Ca^{2+} channel blockers have been shown to have an inhibitory effect on the cell proliferation of several types of the cells, including vascular smooth muscle cells (Block et al., 1989; Ko et al., 1992, 1993; Dol et al., 1995).

In all of the studies showing the inhibitory effect of Ca^{2+} channel blockers, cell proliferation was assessed by DNA synthesis, namely [^{3}H]thymidine incorporation into the nucleus (DNA). In vascular smooth muscle cells, since the organic Ca^{2+} channel blockers inhibited the DNA synthesis stimulated by platelet-derived growth factors (PDGF), it was thus concluded that $[Ca^{2+}]_i$ elevation may play an important role in PDGF-induced proliferation of vascular smooth muscle cells (Block et al., 1989; Ko et al., 1992, 1993). Recently, however, it was reported that the inhibitory effects of Ca^{2+} channel blockers on [^{3}H]thymidine incorporation are due to the inhibition of the cellular uptake of [^{3}H]thymidine rather than a direct inhibition of its incorporation into the nuclear DNA (Agrotis et al., 1993; Weir et al., 1993), which thus clearly indicated that careful consideration is therefore required when evaluating the effects of the organic Ca^{2+} channel blockers on DNA synthesis when it is evaluated using the method of [^{3}H]thymidine incorporation.

In the present study, we directly determined the phases of the cell cycle at the single-cell level using a newly developed method of immunocytochemical analysis of cell cycle-specific nuclear antigens (Kobayashi et al., 1994)

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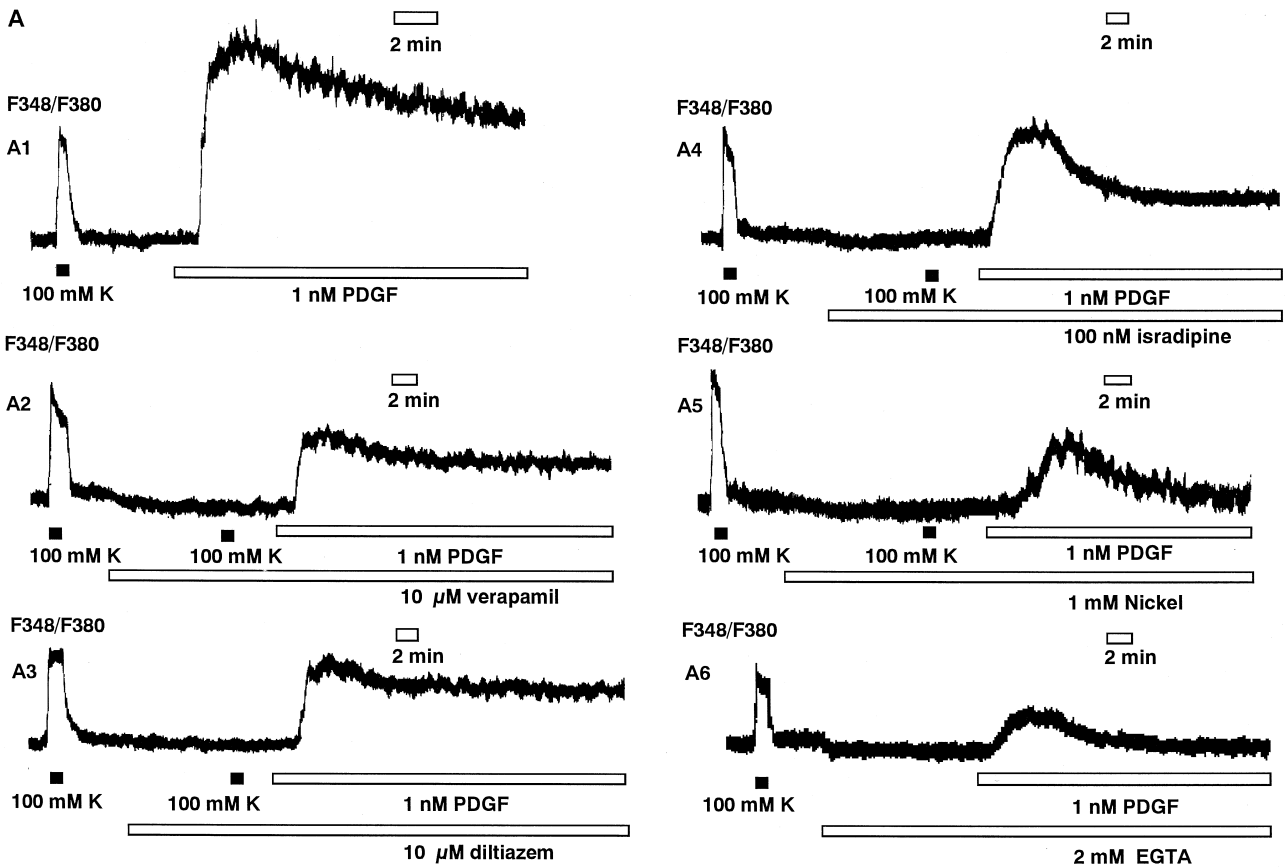
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F348/F380
A1
2 min
100 mM K
1 nM PDGF
F348/F380
A2
2 min
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F348/F380
A3
2 min
100 mM K
100 mM K
1 nM PDGF
10 μM diltiazem
F348/F380
A4
2 min
100 mM K
100 mM K
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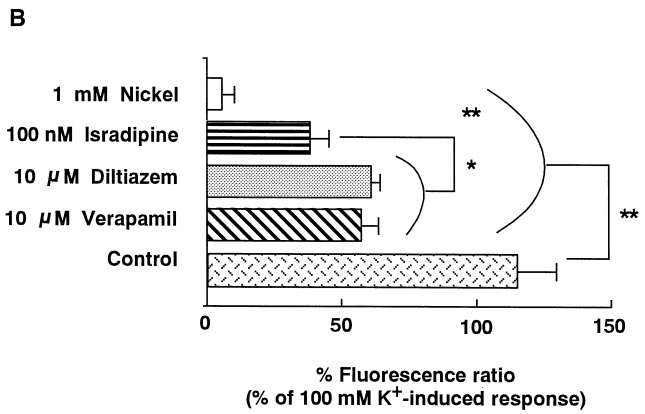
B
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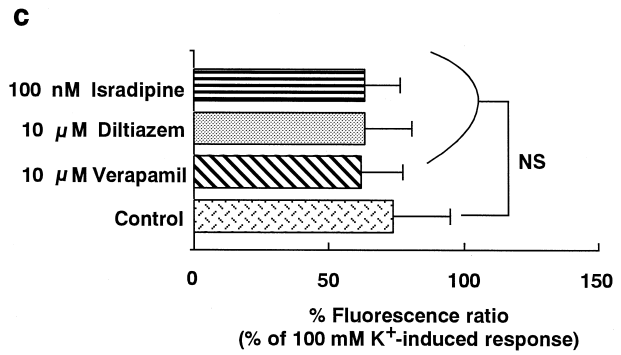
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(% of 100 mM K+-induced response)

and thus investigated the effects of both organic (verapamil, diltiazem and isradipine) and inorganic (nickel) Ca^{2+} channel blockers on the cell cycle progression induced by PDGF in vascular smooth muscle cells. We thus found that the efficacy of Ca^{2+} channel blockers for the inhibition of $[Ca^{2+}]_i$ elevation induced by PDGF ($NiCl_2$ > isradipine > verapamil = diltiazem) did not parallel that for the inhibition of cell cycle progression induced by PDGF (verapamil = diltiazem > $NiCl_2$ > isradipine). Namely, among organic Ca^{2+} channel blockers, isradipine most efficiently blocked the PDGF-induced $[Ca^{2+}]_i$ elevation while, in contrast, it had either no or little inhibitory effect on the progression of the cell cycle. We therefore conclude that the inhibitory effects of the Ca^{2+} channel blockers on the cell proliferation induced by PDGF are not simply due to their inhibitory action on the $[Ca^{2+}]_i$ elevations but instead are also due to more complex unknown factors.

2. Materials and methods

2.1. Primary cell culture

Vascular smooth muscle cells were enzymatically dispersed from the aortic media of male Wistar rats (Yamamoto et al., 1983) and seeded on either ultra-thin cover glass slips (No. 1; Matsunami, Japan) coated with fibronectin (Iwaki, Japan) or chamber slides (No. 4804; Lab-Tek), as previously described (Kobayashi et al., 1994). The primary cultured cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Flow) and antibiotics. The growth medium was changed every 2–3 days. All vascular smooth muscle cells were synchronized in the G_0 phase by culturing in a serum-free medium for 24 h before starting each experiment (Fig. 4A1). Electron microscopic observations and direct immunofluorescence staining of native smooth muscle actin and myosin revealed the cells used in the experiments not to be contaminated with fibroblast or endothelial cells (Yamamoto et al., 1983; Kobayashi et al., 1985; Kanaide et al., 1988). The cells we used contracted in response to norepinephrine (Kanaide et al., 1988) and the $[Ca^{2+}]_i$ level thus became elevated in response to various agonists (Kobayashi et al., 1985; Kanaide et al., 1988). High cell viability (> 99%) was maintained during each experimental procedure, using the Trypan blue exclusion test (Kobayashi et al., 1985, 1994).

2.2. Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were measured using the microfluorometry of fura-2, as described previously (Kobayashi et al., 1994). Briefly, the cells in primary culture on an ultra-thin cover glass were loaded with fura-2 by incubating with 5 μM fura-2/AM (acetoxymethyl ester form of fura-2) in the growth medium for 60 min at 37°C. After stabilization (for at least 30 min) in normal physiological salt solution (normal PSS) at 25°C, fluorescence (500 nm) intensities at alternating (400 Hz) excitation (348 and 380 nm) and the ratio (F348/F380) were then continuously measured using an inverted fluorescent microscope (TMD 56, Nikon, Japan) equipped with a spectrophotometer (CAM 220, Japan Spectroscope Co., Japan). The fluorescence measurements were carried out at 25°C to prevent any leakage and/or sequestration of the dye at a higher temperature. At this temperature there was also no significant leakage of the dye, while the cytosolic concentration of the dye was stable either in the presence or absence of extracellular Ca^{2+} (Kobayashi et al., 1986). No significant difference was seen in the $[Ca^{2+}]_i$ responses between 37 and 25°C, except that the delay in the PDGF-induced Ca^{2+} transients was slightly shorter at 37°C than at 25°C. Since the responses to repeated depolarization with 100 mM K^+ were reproducible up to 90 min, 100 mM K^+-induced $[Ca^{2+}]_i$ transient was used as a reference response in order to normalize the separate measurements. The changes in the fluorescence ratio were expressed as a percentage, assigning the values at normal PSS and in 100 mM K^+ PSS to be 0 and 100%, respectively. The composition of the normal PSS was (in mM): NaCl, 135; KCl, 5; $CaCl_2$,

Fig. 1. Effects of Ca^{2+} channel blockers on the PDGF-induced Ca^{2+} transients. The phase of the cell cycle was synchronized in the G_0 phase by serum deprivation for 24 h (Fig. 4A1) before starting each experiment. The $[Ca^{2+}]_i$ was assessed by changes in the fluorescence ratio (F348/F380) of fura-2. (A1)–(A5) Representative recordings of $[Ca^{2+}]_i$ elevations induced by 100 mM K^+-depolarization and by 1 nM PDGF in the absence (A1, control) and presence of 10 μM verapamil (A2), 10 μM diltiazem (A3), 100 nM isradipine (A4) and 1 mM $NiCl_2$ (A5) in vascular smooth muscle cells in primary culture. (A6) Representative recordings of $[Ca^{2+}]_i$ elevations induced by 1 nM PDGF in the absence of extracellular Ca^{2+} (chelated with 2 mM EGTA). In (A2)–(A5), the Ca^{2+} channel blockers were applied 15 min before and during the application of PDGF. (B) A summary of the repeated experiments as shown in (A1)–(A5). The data were obtained from four to five independent experiments. The data are presented as the mean values (% fluorescence ratio) of the steady-state $[Ca^{2+}]_i$ elevations, 13 min after the application of PDGF. The horizontal bars indicate S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared to the control (without Ca^{2+} channel blockers). (C) A summary of the experiments to show the effects of Ca^{2+} channel blockers on the transient elevation of $[Ca^{2+}]_i$ induced by 1 nM PDGF in the absence of extracellular Ca^{2+}. As shown in (A6), after depolarization of the cells with 100 mM K^+ in normal PSS, the cells were incubated in Ca^{2+}-free PSS for 15 min and subsequently 1 nM PDGF was applied. Ca^{2+} channel blockers were applied 10 min before and during the application of PDGF in the absence (control; area with diagonal dots) and presence of 10 μM verapamil (area with diagonal stripes), 10 μM diltiazem (dotted area) and 100 nM isradipine (horizontally striped area). The data were obtained from four independent experiments. The data are presented as the mean values (% fluorescence ratio) at the peak of the transient $[Ca^{2+}]_i$ elevations induced by PDGF. The horizontal bars indicate S.D. NS, no significant difference ($P > 0.05$).

1; $MgCl_2$, 1; glucose, 5.5 and HEPES, 10 (pH 7.4 at 25°C). For the 100 mM K^+ solution, 100 mM KCl was substituted for 100 mM NaCl, isosmotically.

2.3. Determination of the cell cycle

The phase of the cell cycle of each single cell was determined by an immunocytochemical analysis of the monoclonal antibodies against cell cycle-specific nuclear antigens, as previously described (Kobayashi et al., 1994). Briefly, the cells on each chamber slide were rapidly cryo-fixed in 70% ethanol (−20°C) and then were incubated with the monoclonal antibodies [fluorescein isothiocyanate-conjugated anti-proliferating cell nuclear antigen with 1:20 dilution and *R*-phycoerythrin-conjugated Ki-67 antigen with 1:10 dilution] for 30 min at 30°C. Double labeled immunofluorescent staining was then observed under a fluorescent microscope (Axioskop, Zeiss, Germany) equipped with an objective lens (Plan-Neofluar 40×; Zeiss) and an appropriate filter combination (BP485, FT520, and LP520; Zeiss, Germany). The color pattern of the nucleus produced by double staining results in the following differentiation of the phases of the cell cycle: G_0 phase (no specific nuclear fluorescence); G_1 phase (green); S phase (yellow); G_2 phase (orange) and M phase (red). G_0 cells having no nuclear fluorescence were visualized by staining the nucleus of vascular smooth muscle cells with a DNA staining dye, Hoechst 33258 (0.1 ng/ml) for 3 min and thereafter were observed through another filter combination (BP380, FT395 and LP500-530; Zeiss). The population of the cells at each individual phase of the cell cycle was counted and expressed as a percentage of the total number of cells.

2.4. Drugs and chemicals

Fura-2/AM was purchased from Dojindo (Japan). Verapamil and diltiazem were from Sigma (USA). Isradipine [(±)-PN200-110] was from Research Biochemicals (USA) and $NiCl_2$ from Katayama Chemicals (Japan). The recombinant human BB isomer of PDGF was purchased from Biomedical Technologies (USA). PDGF was dissolved in 5 mM of acetic acid solution, containing 2 mg/ml of bovine serum albumin. The vehicle solution for PDGF had no apparent effect on the $[Ca^{2+}]_i$ levels and phase of the cell cycle (Kobayashi et al., 1994).

2.5. Data analysis

All data are presented as the means ± standard deviation (S.D.). A statistical analysis of the data was made using the unpaired Student's *t*-test and an analysis of variance. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. The effect of Ca^{2+} channel blockers on the $[Ca^{2+}]_i$ elevation induced by PDGF in vascular smooth muscle cells in primary culture

As shown in Fig. 1A1, 1 nM PDGF, with a delay of 1–2 min, induced an initial transient (1st component) and subsequently a lower steady-state (2nd component) elevation of $[Ca^{2+}]_i$ in vascular smooth muscle cells in primary culture which were synchronized in the G_0 phase by serum deprivation for 24 h. The levels of the first and second component were 223.25 ± 23.26% ($n = 25$) and 114.92 ± 14.13% ($n = 25$) (Fig. 1B) of 100 mM K^+-induced elevation of $[Ca^{2+}]_i$, respectively. When the extracellular Ca^{2+} was substituted by 2 mM EGTA in the medium, there was a complete inhibition of the second component, but not the first component, of the $[Ca^{2+}]_i$ elevation induced by PDGF (Fig. 1A6). The level of the transient elevation of $[Ca^{2+}]_i$ in the 2 mM EGTA medium was 73.63 ± 20.84% ($n = 4$) which was significantly smaller than that shown in Fig. 1(A1) ($P < 0.01$). Similar results were obtained when 1 mM $NiCl_2$ was included in the medium to block the Ca^{2+} entry (Fig. 1A5). These results are thus considered to be compatible with previous observations that the first and second components of the PDGF-induced $[Ca^{2+}]_i$ elevation are mainly mediated by the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+}, respectively (Kobayashi et al., 1994).

For experiments with Ca^{2+} channel blockers, we used three representative types of Ca^{2+} channel blockers with different structures, namely verapamil, diltiazem and isradipine, a dihydropyridine. In addition to these organic Ca^{2+} channel blockers, we also used an inorganic Ca^{2+} channel blocker, $NiCl_2$. First, in order to clarify whether Ca^{2+} channel blockers may affect the influx of extracellular Ca^{2+} induced by PDGF, we investigated the effects of Ca^{2+} channel blockers on the PDGF-induced sustained elevations of $[Ca^{2+}]_i$. As shown in Fig. 1(A5) and (B), an inorganic Ca^{2+} channel blocker, $NiCl_2$ (1 mM), completely abolished the sustained elevation (second component) of $[Ca^{2+}]_i$ induced by 1 nM PDGF in vascular smooth muscle cells, without having any significant effect on the first component that was resistant to the removal of extracellular Ca^{2+} (Fig. 1A6). In contrast to the complete inhibition by $NiCl_2$, organic Ca^{2+} channel blockers inhibited partially but significantly ($P < 0.01$) the steady-state elevations of $[Ca^{2+}]_i$ induced by PDGF (Fig. 1A). The sustained $[Ca^{2+}]_i$ elevations (114.92 ± 14.13%) induced by PDGF decreased to 38.12 ± 6.67% ($n = 4$) by 100 nM isradipine, to 61.03 ± 3.07% ($n = 4$) by 10 μM diltiazem and to 57.43 ± 5.85% ($n = 4$) by 10 μM verapamil (Figs. 1 and 2). Such partial inhibitions were not simply due to the insufficient concentrations of these Ca^{2+} channel blockers, because the Ca^{2+} channel blockers, at the concentrations used in the present study, induced a complete

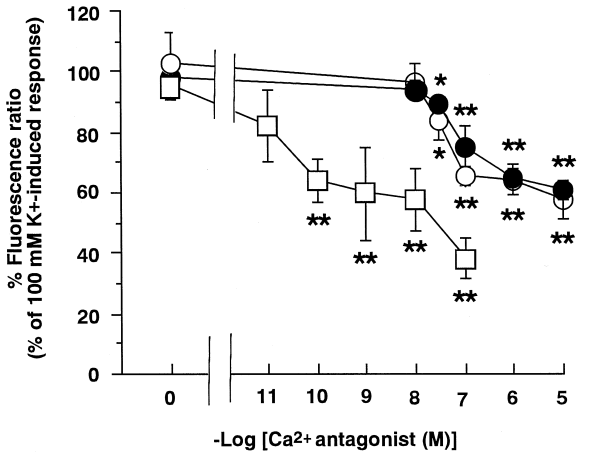

Fig. 2. Concentration-dependent effects of verapamil (○), diltiazem (●) and isradipine (□) on sustained elevation of $[Ca^{2+}]_i$ (2nd component) induced by 1 nM PDGF in vascular smooth muscle cells in primary culture. The cells were synchronized in the G_0 phase by serum deprivation for 24 h. The experiments were carried out according to the protocol shown in Fig. 1. The data are obtained from 6 independent experiments in different cells in separate dishes. The values are the mean ± S.D. (shown by vertical bars). $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared to the respective control.

inhibition of high K^+ depolarization-induced elevation of $[Ca^{2+}]_i$ (Fig. 1). This notion is also supported by our previous finding that these drugs at the above-mentioned concentrations completely abolished the activity of L-type Ca^{2+} channels in the same preparations (rat aorta vascular smooth muscle cells in primary culture) as those used in the present study (Akaike et al., 1989). As shown in Fig. 1B, the inhibitory efficacy of the Ca^{2+} channel blockers at the concentrations studied in the present study was as follows: $NiCl_2$ > isradipine > verapamil = diltiazem.

The level of the first component of $[Ca^{2+}]_i$ elevations (223.25 ± 23.26%) induced by PDGF decreased significantly ($P < 0.01$) to 135.33 ± 4.16% ($n = 4$) by 100 nM isradipine, 108.78 ± 17.34% ($n = 4$) by 10 μM diltiazem, 106.32 ± 12.87% ($n = 4$) by 10 μM verapamil and 65.93 ± 14.12% ($n = 4$) by 1 mM $NiCl_2$ (Fig. 1A). This observations may suggest that these Ca^{2+} channel blockers inhibited Ca^{2+} release as well as Ca^{2+} influx. In order to clarify whether Ca^{2+} channel blockers may also affect the release of the intracellular Ca^{2+} induced by PDGF, we investigated the effects of Ca^{2+} channel blockers on the $[Ca^{2+}]_i$ elevation induced by PDGF in the absence of extracellular Ca^{2+}. In Ca^{2+}-free, 2 mM EGTA PSS, none of these organic Ca^{2+} channel blockers inhibited the transient elevation of $[Ca^{2+}]_i$ induced by 1 nM PDGF, which is mediated by the release of intracellular Ca^{2+} (Fig. 1C).

As shown in Fig. 2, the inhibition of the PDGF-induced sustained elevation of $[Ca^{2+}]_i$ by the organic Ca^{2+} channel blockers depended on the concentration of the drugs. These Ca^{2+} channel blockers, up to the concentrations shown in Figs. 1 and 2, did not affect significantly ($P > 0.05$) the resting levels of $[Ca^{2+}]_i$ in vascular smooth muscle cells (Fig. 1). After 15 min incubation with 10 μM verapamil, 10 μM diltiazem, 100 nM isradipine and 1 mM $NiCl_2$ in the presence of 1 mM $CaCl_2$, the levels of $[Ca^{2+}]_i$ were −0.38 ± 0.86%, −1.82 ± 4.07%, −2.98 ± 4.24% and −2.36 ± 2.62%, respectively (Fig. 1A). Incubation in the 2 mM EGTA containing media significantly reduced the baseline $[Ca^{2+}]_i$ to −12.61 ± 2.23% ($P < 0.01$). However, higher concentrations of isradipine (> 100 nM), verapamil (> 10 μM) and diltiazem (> 10 μM), per se, only

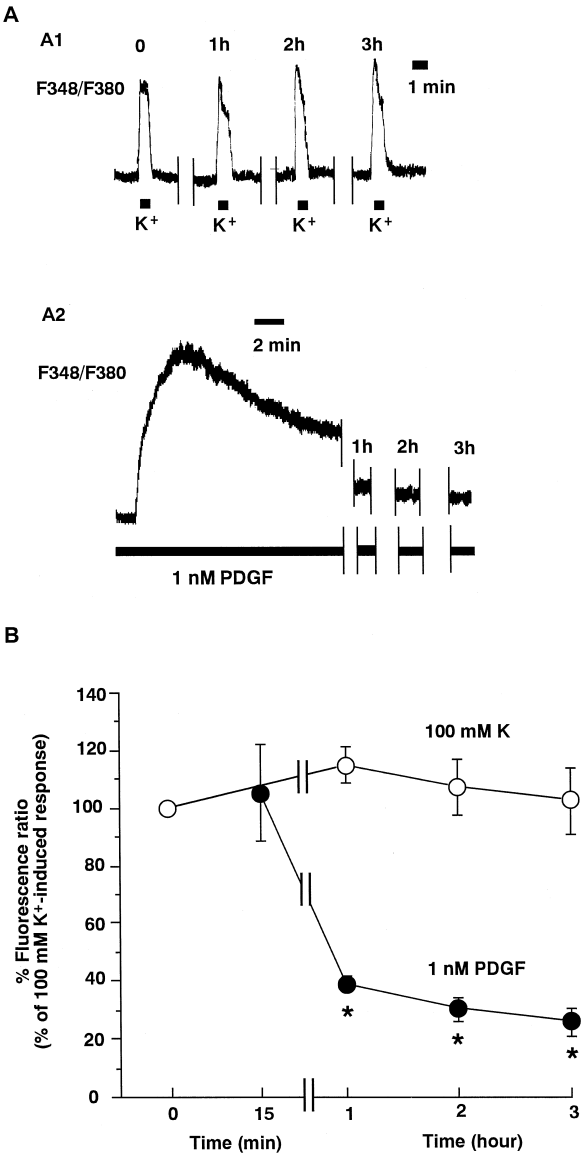

Fig. 3. Time-courses of $[Ca^{2+}]_i$ elevations induced by 100 mM K^+-depolarization and by 1 nM PDGF in the vascular smooth muscle cells in primary culture. (A) Representative recordings of the $[Ca^{2+}]_i$ elevations induced by repetitive applications (every 1 h) of 100 mM K^+-depolarization (A1) and by the continuous application of 1 nM PDGF (A2) for up to 3 h. During the 1 h interval between measurements, the shutter was closed to protect the cells from the UV light and the solution was renewed every 15 min to avoid the evaporation of the solution. (B) Summary of repeated experiments carried out according to the protocol shown in (A). The data indicate the mean ± S.D. (shown by vertical bars) of four experiments. $^{*}P < 0.05$, as compared with the control PDGF response (at 13 min).

caused slight elevations, if at all, in the resting levels of $[Ca^{2+}]_i$, probably due to either the action as Ca^{2+} channel agonist, like Bay K 8644 (a dihydropyridine Ca^{2+} channel agonist), or due to the cytotoxic effects of extremely high concentrations of the drugs. We therefore did not examine the effects of Ca^{2+} channel blockers at higher concentrations, and thus, it was not possible to determine the IC_{50} values in the present study. Nevertheless, it was clearly shown that isradipine did inhibit the PDGF-induced $[Ca^{2+}]_i$ elevations more efficiently than did verapamil and dilti-

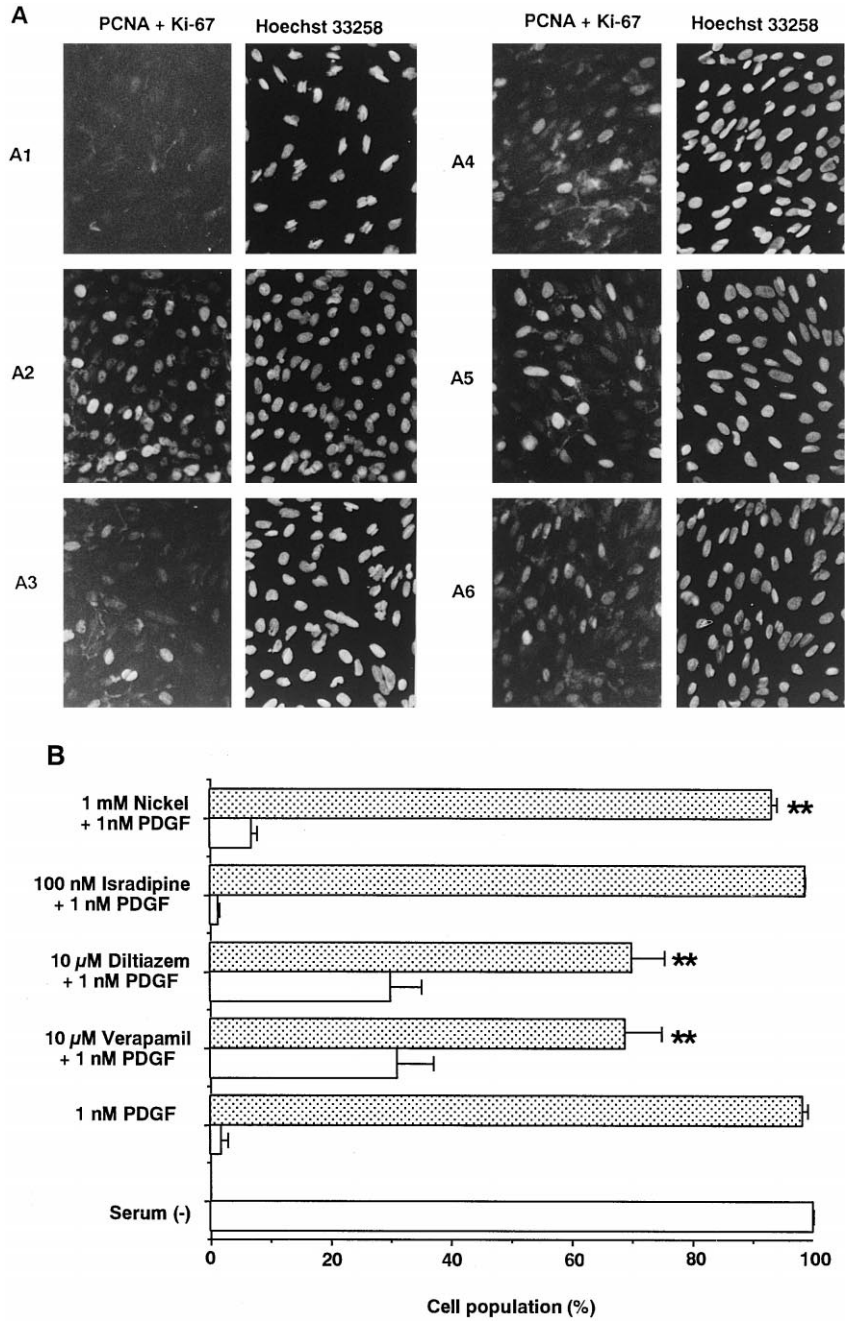

Fig. 4. Effects of Ca^{2+} channel blockers on the G_0/G_1 transition induced by PDGF. (A) Immunofluorescence microphotographs of the vascular smooth muscle cells in primary culture, which were triple-stained with proliferating cell nuclear antigen, Ki-67 (left panel) and Hoechst 33258 (right panel). The serum-deprived cells (A1) were treated with 1 nM PDGF in the absence (A2, control) and presence of 10 μM verapamil (A3), 10 μM diltiazem (A4), 100 nM isradipine (A5) and 1 mM $NiCl_2$ (A6). In A1, the cells had no cell cycle-specific nuclear fluorescence of proliferating cell nuclear antigen + Ki-67 but the nucleus of those cells were clearly identified with the nonspecific nuclear staining with Hoechst 33258 (the G_0 phase). In (A2)–(A6), the nucleus of some cells showed green fluorescence (the G_1 phase). (B) A summary of the repeated experiments carried out as shown in (A). The data were obtained from four to five independent experiments. The data indicate the mean ± S.D. of cell population (%) of each phase of the cell cycle. Only the G_0 phase (open column) and the G_1 phase (dotted column) were depicted and no other phases of the cell cycle were observed. The G_0-synchronized cells were obtained by the serum-deprivation for 24 h. $^{**}P < 0.01$, as compared to the control (1 nM PDGF, but without Ca^{2+} channel blockers).

azem: for example, 10 nM isradipine significantly inhibited the $[Ca^{2+}]_i$ elevations, whereas the same concentration (10 nM) of verapamil and diltiazem had no significant effect (Fig. 2).

In order to estimate the levels of sustained $[Ca^{2+}]_i$ elevations induced by the continuous application of PDGF for a long period of time, we determined the temporal changes in the levels of $[Ca^{2+}]_i$ during the long application of PDGF (up to 3 h), with a 1 h interval when the excitation-light shutter was closed to protect the cells from the UV light. At 1–3 h, the sustained elevations of $[Ca^{2+}]_i$ induced by 1 nM PDGF were markedly attenuated ($P < 0.05$), while the cells were able to consistently elevate the $[Ca^{2+}]_i$ level in response to 100 mM K^+-depolarization (Fig. 3). Up to 3 h, the level of high K^+-induced $[Ca^{2+}]_i$ elevations were maintained ($P > 0.05$). However, after a longer incubation, the response to 100 mM K^+-depolarization also started to decrease. We therefore limited the duration of incubation with PDGF up to 3 h. Even at 3 h, the levels of $[Ca^{2+}]_i$ elevations induced by PDGF were only about 25% of those at 13 min (Fig. 3B). The levels of $[Ca^{2+}]_i$ elevations induced by the application of PDGF for 13 min and 3 h were 105.1 ± 16.82% ($n = 4$) and 26.02 ± 4.86% ($n = 4$) regarding the high K^+-induced $[Ca^{2+}]_i$ elevation, respectively.

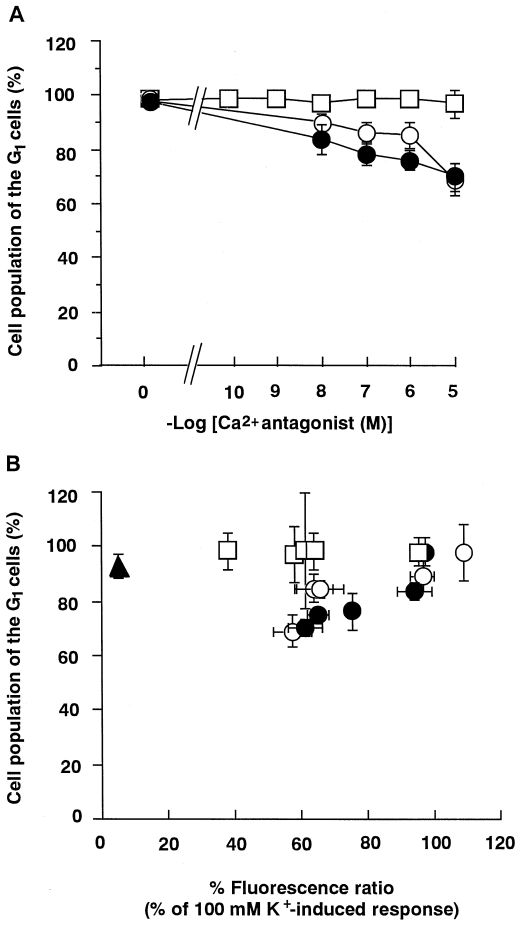

Fig. 5. (A) Concentration-dependent effects of verapamil (○), diltiazem (●) and isradipine (□) on the G_0/G_1 transition induced by PDGF. The cell cycle of vascular smooth muscle cells was synchronized in the G_0 phase by serum deprivation for 24 h before starting the experiment. The data indicate the mean ± S.D. of the cell population (%) of the G_1 cells. The data were obtained from three to five independent experiments. (B) Effects of verapamil (○), diltiazem (●), isradipine (□) and $NiCl_2$ (▲) on the relationship between the Ca^{2+} transients and the G_0/G_1 transition, as induced by 1 nM PDGF. The data were reconstructed from the results shown in Fig. 2 and Fig. 5(A) ($n = 4$–5). The two parameters were measured separately from the different cells in the different dishes, but from the same primary culture of the same animal. The data indicate the mean ± S.D. (as shown by the vertical and horizontal bars).

3.2. Effects of Ca^{2+} channel blockers on the G_0/G_1 transition induced by PDGF in vascular smooth muscle cells in primary culture

The serum-deprivation of vascular smooth muscle cells in primary culture for 24 h resulted in a complete (100%) synchronization of the cell cycle in the G_0 phase (100%) (Fig. 4A1). PDGF (1 nM) stimulated the entry of G_0 cells into G_1 phase after 24 h (Fig. 4A2). We thus found that 98.19 ± 1.03% ($n = 5$) (Fig. 4B) of cells were in the G_1 phase (green fluorescence), but not in either the S phase (yellow fluorescence) or the M phase (red fluorescence) even after a prolonged incubation (48 h) with 1 nM PDGF. When the cells were incubated with 10 μM verapamil (Fig. 4A3), 10 μM diltiazem (Fig. 4A4), 100 nM isradipine (Fig. 4A5) and 1 mM nickel (Fig. 4A6) 30 min before and during stimulation with 1 nM PDGF for 24 h, the population of the G_1 cells were 68.88 ± 6.07% ($n = 5$), 70.09 ± 5.20% ($n = 5$), 98.57 ± 0.129% ($n = 5$) and 93.05 ± 0.89% ($n = 5$), respectively (Fig. 4B). The inhibition of the PDGF-induced G_0/G_1 transition by verapamil, diltiazem and nickel were significant ($P < 0.01$), whereas the effect of isradipine was insignificant ($P > 0.05$). The inhibitory efficacy was as follows: verapamil = diltiazem > $NiCl_2$ > isradipine. It was also observed that verapamil and diltiazem concentration-dependently inhibited the PDGF-induced G_0/G_1 transition with a similar efficacy (Fig. 5A). Isradipine (up to 10 μM) had no significant inhibitory effect on the cell cycle progression.

Fig. 5B shows the relationship between the extent of $[Ca^{2+}]_i$ elevations and the extent of the G_0/G_1 transition induced by 1 nM PDGF with or without treatment using various concentrations of Ca^{2+} channel blockers. There is no significant linear relationship was observed between these two parameters, with a very low correlation coefficient ($r = 0.068$, $P = 0.8038$).

4. Discussion

It has been well documented that Ca^{2+} channel blockers inhibit DNA synthesis in vascular smooth muscle cells, as assessed by thymidine incorporation (Block et al., 1989; Ko et al., 1992; Ko et al., 1993). Considering the inhibitory effect of Ca^{2+} channel blockers on the $[Ca^{2+}]_i$ levels, these results may thus suggest the important role of

$[Ca^{2+}]_i$ elevations in the cell proliferation of vascular smooth muscle cells. However, recent reports that Ca^{2+} channel blockers inhibited the cellular uptake of thymidine rather than its incorporation into the nuclear DNA (Agrotis et al., 1993; Weir et al., 1993) allowed us to investigate the effects of Ca^{2+} channel blockers on the cell cycle progression, using a direct determination of the phases of the cell cycle by an immunocytochemical analysis of the cell cycle (Kobayashi et al., 1994). In the present study, Ca^{2+} channel blockers inhibited not only $[Ca^{2+}]_i$ elevation (Figs. 1 and 2), but also the cell cycle progression induced by PDGF (Figs. 4 and 5A). One of the novel findings of the present study, however, is that the extent of the inhibition of the $[Ca^{2+}]_i$ elevation by Ca^{2+} channel blockers did not parallel the extent of inhibition of the cell cycle progression by Ca^{2+} channel blockers (Fig. 5B). The order of the efficacy of the inhibition of the $[Ca^{2+}]_i$ elevation and of the G_0/G_1 transition induced by the Ca^{2+} channel blockers at the concentrations determined in the present study was different: $NiCl_2$ > isradipine > verapamil = diltiazem for the $[Ca^{2+}]_i$ elevation (Figs. 1 and 2) and verapamil = diltiazem > $NiCl_2$ > isradipine for the cell cycle progression (Figs. 4 and 5A). In particular, 100 nM isradipine inhibited the $[Ca^{2+}]_i$ elevation induced by PDGF by about 60% (Figs. 1 and 2), but did not affect the PDGF-induced G_0/G_1 transition (Figs. 4 and 5A). These results suggest that the inhibitory effects of Ca^{2+} channel blockers on the cell proliferation may not be simply due to its blocking action on the $[Ca^{2+}]_i$ elevations. We previously reported that PDGF can stimulate the G_0/G_1 transition of vascular smooth muscle cells when the PDGF-induced $[Ca^{2+}]_i$ elevations were completely blocked by the combined treatment of ryanodine and $NiCl_2$ (Kobayashi et al., 1994) and that prominent elevations of $[Ca^{2+}]_i$ induced by endothelin-1 and by angiotensin II are not associated with the G_0/G_1 transition in vascular smooth muscle cells in primary culture (Jahan et al., 1996). These findings are considered to be compatible with the notion that the $[Ca^{2+}]_i$ elevation may play little role in the G_0/G_1 transition in vascular smooth muscle cells. This notion is also supported by the findings of the present study in which the levels of the sustained $[Ca^{2+}]_i$ elevations induced by PDGF attenuated rapidly and markedly within 1–3 h (Fig. 3).

The Ca^{2+} channel blockers selectively inhibited the influx of extracellular Ca^{2+}, but not the release of intracellular Ca^{2+}, as induced by PDGF (Fig. 1). Our previous study demonstrated that Ca^{2+} channel blockers (verapamil and diltiazem) at high concentrations inhibited an increase in $[Ca^{2+}]_i$ induced by an agonist, norepinephrine, but not by caffeine (Kanaide et al., 1988). The inhibitory effect of Ca^{2+} channel blockers on the Ca^{2+} release induced by agonist, but not by growth factor, may thus be explained by either the action of Ca^{2+} channel blockers as receptor antagonists (Matsumoto et al., 1989), or by the inhibitory action of Ca^{2+} channel blockers on the G-proteins that activate phospholipase C and thereby mediate the release of intracellular Ca^{2+} (Kobayashi et al., 1991). It is well known that PDGF stimulates phospholipase C-γ-1 to produce inositol trisphosphate without the activation of G-proteins (Williams, 1989; Ma et al., 1996), whereas the agonists such as norepinephrine stimulates phospholipase C-β through the activation of trimeric G-proteins (Labelle and Polyak, 1996). In contrast to the selective inhibition by Ca^{2+} channel blockers of agonist-induced Ca^{2+} release, but not of PDGF-induced one, tyrosine kinase blockers selectively inhibited the Ca^{2+} release induced by PDGF, but not by agonists (Kobayashi et al., 1994).

In the present study, we demonstrated for the first time that Ca^{2+} channel blockers inhibit the G_0/G_1 transition (Figs. 4 and 5A). In all of the previous studies, the effects of Ca^{2+} channel blockers on cell proliferation have been assessed by DNA synthesis, cell count, or flowcytometry. In these studies, the G_0 phase and the G_1 phase were not clearly distinguished. In the present study, however, using an immunocytochemical analysis, we could clearly distinguish these two phases of the cell cycle, and also show the inhibitory effects of Ca^{2+} channel blockers on the G_0/G_1 transition induced by PDGF (Figs. 4 and 5A). Since the G_0/G_1 transition is the initial step of the cell proliferation of vascular smooth muscle cells, our results are thus considered to be compatible with the findings of previous human studies which showed that Ca^{2+} channel blockers prevent the development of atherosclerosis (Lichtlen et al., 1990; Waters et al., 1990).

No molecular mechanisms of inhibitory action of Ca^{2+} channel blockers on cell proliferation were determined in the present study. Several possible mechanisms, however, could be proposed including: (1) the inhibition of protein kinase C. It has been reported that verapamil inhibits the activity of protein kinase C as a phospholipid-interacting drug (Mori et al., 1980). Block et al. (1993) reported that Ca^{2+} channel blockers (manidipine, verapamil and diltiazem) inhibited the PDGF-induced translocation of delta and epsilon isoforms of protein kinase C, whose activities are known to be independent of Ca^{2+}. Therefore, Ca^{2+} channel blockers may inhibit the activity of such Ca^{2+}-independent protein kinase C and block the cell cycle progression in $[Ca^{2+}]_i$-independent manner. (2) The inhibition of the expression of protooncogenes. It has been reported that Ca^{2+} channel blockers, including verapamil and diltiazem, inhibited the PDGF-induced transcription of c-*fos* and c-*jun* genes (Block et al., 1993). In addition, it has also been reported that Ca^{2+} channel blockers block cyclic AMP phosphodiesterase (Sakamoto et al., 1978), which may regulate the gene expression. As a result, Ca^{2+} channel blockers may thus attenuate the expression of cell cycle-related genes, including protooncogenes, through the inhibition of phosphodiesterase.

In summary, based on the above findings we conclude that: (1) the Ca^{2+} channel blockers inhibited both the cell cycle progression from the G_0 phase to the G_1 phase and the sustained elevation of $[Ca^{2+}]_i$, as induced by PDGF

and (2) this antiproliferative effect of Ca^{2+} channel blockers is not simply related to their inhibitory action on the $[Ca^{2+}]_i$ elevation but instead is due to more complex unknown factors.

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