［原著］Elevation of intracellular CAMP inhibited cell proliferation and migration induced by platelet-derived growth factor in cultured rat vascular smooth muscle cells

Elevation of intracellular cAMP inhibited cell proliferation and migration induced by platelet-derived growth factor in cultured rat vascular smooth muscle cells

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ABSTRACT

We investigated whether elevation of intracellular cyclic nucleotides (cAMP and cGMP) inhibit cell proliferation and migration of cultured embryonic rat aortic vascular smooth muscle (VSM) cells (A7r5). After treatment with drugs for up to 120 h, cell proliferation and migration were measured by adding WST-1 reagent and by counting migrated cells through the pores of cell culture insert (8-μm pore size), respectively. Cell proliferation was significantly inhibited by dibutyryl cAMP (db-cAMP) in a time- and concentration-dependent manner (0.03 to 3 mM), whereas dibutyryl cGMP (db-cGMP, 3 mM) had no effect. Olprinone hydrochloride (OPN, 1 mM), type 3 phosphodiesterase inhibitor, inhibited cell proliferation, and synergistically inhibited in the presence of db-cAMP (0.3 mM). Cell migration was significantly inhibited by treatment with db-cAMP (3 mM), but not by db-cGMP (3 mM). Combination of db-cAMP (0.3 to 3 mM) with OPN (1 mM) or forskolin (FSK) (0.1 mM) significantly inhibited cell migration after 72-h treatment. Platelet-derived growth factor (PDGF)-BB significantly stimulated cell proliferation and migration in serum-free culture condition. Treatment of the cells with db-cAMP, OPN or FSK significantly inhibited the stimulatory effect of PDGF-BB on proliferation and migration. Thus, elevation of cAMP inhibited serum stimulated and PDGF-BB induced cell proliferation and migration of A7r5 cells. *Ryukyu Med. J., 27(3,4) 93~103, 2008*

Key words: cAMP, cGMP, olprinone hydrochloride, cell proliferation, migration, platelet-derived growth factor, vascular smooth muscle cells

INTRODUCTION

Ability of vascular smooth muscle (VSM) cells in response to vascular injury is cell proliferation and migration from the media into intima, which results in the formation of neointimal hyperplasia[1], thereby leading to progression of atherosclerosis or restenosis after angioplasty. Platelet-derived growth factor (PDGF), a heparin-binding epidermal growth factor (HB-EGF) and endothelin (ET) etc. stimulate VSM cell proliferation and migration by binding to their specific receptors and activate various intracellular signal transduction systems including cyclic nucleotides, $[Ca^{2+}]_i$, and mitogen-activated protein (MAP) kinase[2-4].

PDGF is composed of a dimer of two chains termed the A and B chains. PDGF-BB is a B chain homodimer protein and binds to both PDGF-Rα and -Rβ receptors with a similar affinity[5]. The PDGF receptors are known to associate with non-receptor type of tyrosine kinases (e.g., Src), phospholipase C (PLC)γ, phosphoinositide 3-kinase (PI3K), the adaptor protein of Grb2, and the Src homology-containing phosphatase 2 (Shp-2)[6,7]. VSM cells produce PDGF that stimulates cell proliferation and migration by binding to PDGF-Rα or PDGF-Rβ

receptors expressed in various cell types including VSM cells, fibroblast, osteoblast etc[8,9]. Stimulation of PDGF production by other growth factors including ET-1, fibroblast growth factor (FGF), and interleukin (IL)-6 is a main autocrine mechanism of VSM proliferation[10]. In addition, PDGF-BB expression in vascular endothelium was increased in response to the reduced blood flow, thereby stimulating growth and migration of VSM cells by a paracrine mechanism. In fact, PDGF-BB mRNA was highly expressed in luminal VSM cells after balloon injury[11].

Intracellular levels of cyclic nucleotides are dynamically regulated by the balance of activities between cyclic nucleotide phosphodiesterases (PDEs) and adenyl cyclases (ACs). PDE hydrolyzes intracellular cAMP and cGMP, thereby decreasing intracellular levels of cAMP and cGMP. In VSM cells, type 3 and 4 of PDEs are mainly involved in regulation of intracellular cyclic nucleotides[12]. Cyclic nucleotides have been reported to inhibit VSM cell proliferation and migration[13-15]. It has been reported that elevation of intracellular cAMP by type 3 PDE inhibitors (quazione, trequinsin, and cilostamide) inhibited cell growth and migration[16,17]. Olprinone hydrochloride (olprinone), a type 3 PDE inhibitor, was developed as a drug having an inotropic effect[18]. In addition, olprinone has been reported to have a relaxing effect on VSM cells[19]. As compared to other type 3 PDE inhibitors, olprinone more specifically act to VSM cells than to tracheal smooth muscle cells[20].

In the present study, we investigated which cyclic nucleotides, cAMP or cGMP plays an important role in cell proliferation and migration by employing embryonic aortic VSM cell line (A7r5). In addition, we examined whether olprinone inhibits PDGF-stimulated cell proliferation and migration in A7r5 cells. Since olprinone effect was thought to be mainly mediated by cAMP/PKA, we compared the effects of olprinone on cell proliferation and migration with membrane permeable cAMP and cGMP analogues, dibutyryl cAMP (db-cAMP) and dibutyryl cGMP (db-cGMP) and an adenyl cyclase activator, forskolin (FSK) under normal culture medium condition containing 10% fetal bovine serum (FBS). We also examined the effects of olprinone, db-cAMP and FSK on PDGF-BB-induced cell proliferation and migration. The changes in intracellular cAMP levels in PDGF-BB-stimulated migration and proliferation were also examined.

MATERIALS AND METHODS

I. Drugs and reagents

Olprinone hydrochloride (olprinone) was provided by Eisai Pharmaceutical Co. (Tokyo, Japan). db-cAMP, db-cGMP, and FSK were purchased from BIOMOL International L.P. (Plymouth Meeting, PA). Human PDGF-BB was purchased from PeproTech (Rocky Hill, NJ). Cell Proliferation Reagent WST-1 (WST-1) was purchased from Roche Diagnostics GmbH (Penzberg, Germany). Calcein-AM was purchased from DOJINDO Laboratories (Kumamoto, Japan).

II. Cell culture

The clonal cell line A7r5 was purchased from Dainippon Sumitomo Pharma Co. (Osaka, Japan) and the cells were grown in Dulbecco's modified eagle medium (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.3 mg/ml of L-glutamine, 0.1 μg/ml of pyruvic acid, 100 μg/ml of penicillin, 100 μg/ml of streptomycin and supplemented with 10% FBS (Sanko Junyaku, Tokyo, Japan). The cells were cultured at 37℃ in a humidified water-jacketed incubator (Astec Inc., Fukuoka, Japan) under 5% CO_2 and 95% air atmosphere.

III. Cell proliferation assay

To examine the effects of various reagents on cell proliferation, the dispersed 3.0×10^3 cells by trypsin-EDTA solution were plated into each well of 96 well-micro plate and were cultured for up to 120 h in 100 μl of 10% FBS-DMEM in the absence or presence of db-cAMP, db-cGMP, olprinone or FSK. In the case of testing the effect of these drugs on cell proliferation stimulated by PDGF-BB, serum-free DMEM (SFM) was used to exclude proliferative effects by other growth factors in FBS. Five microlitter of the WST-1 reagent was added to each well. After 1 h incubation with the WST-1 reagent, the absorbance at the wavelength of 415 nm (reference wavelength of 550 nm) was measured by an MTP-300 micro plate reader (Corona Electric, Ibaragi, Japan). The blank value was the absorbance of the culture medium without cells.

IV. Migration assay

To test the effect of db-cAMP (3 mM), olprinone

(1 mM) and FSK (0.1 mM) on cell migration, the cultured VSM cells (in 6-well plate) were cultured for 48 h in the presence or absence of those reagents in 10% FBS-DMEM. In the case of testing the effect of these drugs on cell migration stimulated by PDGF-BB (2 nM), serum-free DMEM was used to exclude effects of cell motility by other growth factors in FBS. After the incubation period, the cells were trypsinized, suspended in 10% FBS-DMEM, and plated onto the 8-μm FALCON® HTS FluoroBlok™ Inserts (Becton Dickinson and Company, NJ, USA) at a density of 1.0×10^4 cells/insert. In the lower wells of 24-well plate, 500 μl of 10% FBS-DMEM were added. After incubation for 23 h at 37℃, a fluorescent dye, calcein-AM was added to each insert at the final concentration of 2 μg/ml and further incubated for another 1 h to label the cells. The VSM cells on the inserts were fixed by 10% formalin-PBS. The membranes were removed and mounted on slide glasses with cover slips. The VSM cells that had migrated to the lower side of the membrane were counted under the microscope (magnification:× 400) with the B-2E/C fluorescence filter. The migration activity is expressed as averaged number of migrated cells in five different high power fields (HPF).

V. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from approximately 1×10^6 cultured cells using PURESCRIPT® RNA Isolation Kit (Gentra System, Minneapolis, MN, USA). After reverse transcription with moloney murine leukemia virus reverse transcriptase (8 unit/-ml) using an oligo $(dT)_{15}$ primer for 3 h at 37℃, the first-stranded cDNA templates were amplified by 0.2 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA) in 20 μl of 10 mM Tris buffer (pH 9.0) containing 50 mM KCl, 0.2 mM of dNTPs, 2.5 mM $MgCl_2$, and 1 μM of primers. The primers used for targeting rat cDNAs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), type 3A PDE, type 3B PDE, and type 4D PDE were used as previously reported[21]. The annealing temperature and the number of PCR cycles for comparative PCR were also performed accordingly[21]. The PCR products were separated on 1.7% agarose gel containing ethidium bromide with 44.5 mM Tris-borate/1 mM EDTA electrophoresis buffers (pH 7.5).

VI. Measurement of intracellular cAMP

Intracellular cAMP content was measured using the Tropix cAMP-Screen chemiluminescent ELISA system (Applied Biosystems Japan, Tokyo, Japan) following manufactures' manuscript. In brief, the cultured cells (ca. 1.0×10^5 cells/well) on 24-well plate were lysed by adding 100 μl of assay/lysis buffer, mixed well and incubated at 37℃ for 30 min. Sixty μl of serially diluted standard cAMP solutions and the cell lysates were applied into monoclonal anti-cAMP-coated 96-well plate and 30 μl of diluted cAMP-alkaline phosphatase conjugates were added to each well. After incubation at room temperature for 1 h with gentle shaking, the solutions were removed from the wells and washed. A 100 μl of CSPD®/Sapphire RTU substrate/enhancer solution™ was added and incubated at room temperature for 30 min. The intensities of the chemiluminescent signals were measured in a Fluoroskan Ascent FL™ (Thermo Electro, MA, USA). To determine the effect of db-cAMP, olprinone, FSK, and PDGF-BB on intracellular cAMP concentration, cell lysates were prepared at 0 and 72 h after treatment. Since cell proliferation was changed by the reagents, intracellular cAMP was varied with the number of cells at the end of experiments. Thus, the cells at the same density were cultured in another 24-well plate to count the cell number concomitantly with measurement of cAMP. Intracellular cAMP concentrations were indicated by dividing them by the number of cells.

VII. Data analysis

All data represent mean ± standard error (SE). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by a multiple comparison test using the contrast method, one-way ANOVA followed by Fisher's PLSD multiple comparison test or Student's unpaired *t*-test using Stat View v. 5.0 software (SAS Institute, Cary, NC) and SUPER ANOVA (Abacus Concepts, Berkeley, CA). $P < 0.05$ was defined as a statistical significance.

RESULTS

I. Effect of membrane permeable cyclic nucleotides on cell proliferation

A7r5 cells proliferated with a doubling time of ca. 48 h in 10% FBS-DMEM without db-cAMP

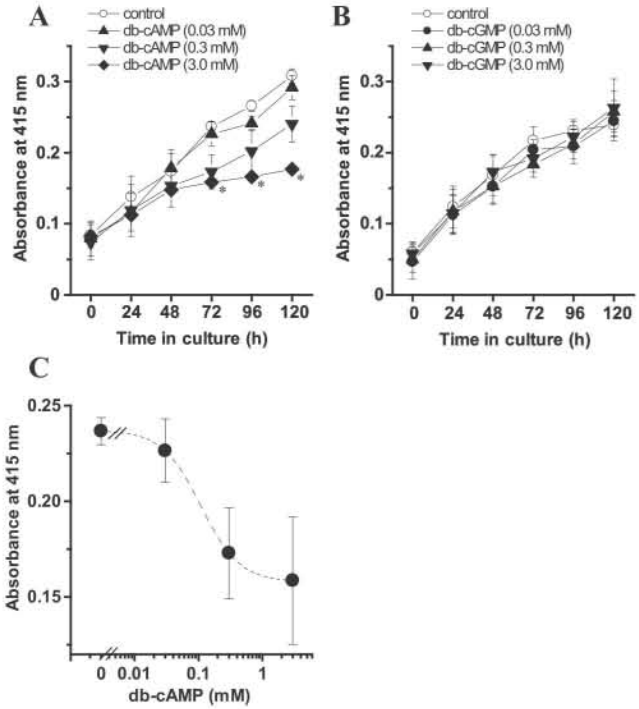

Fig. 1 Effect of cyclic nucleotides on basal cell proliferation of A7r5.
The cells were cultured for indicated times (from 0 to 120 h) with different concentrations of db-cAMP (A) and db-cGMP (B) in 10% FBS-DMEM. Absorbance at 415 nm was measured after 1-h addition of WST-1. A: Open circles, control (0 mM); upper triangles, db-cAMP (0.03 mM); down triangles, db-cAMP (0.3 mM); and diamonds, db-cAMP (3 mM). B: Open circles, control (0 mM); upper triangles, db-cGMP (0.03 mM); down triangles, db-cGMP (0.3 mM); and diamonds, db-cGMP (3 mM). C: Concentration-dependent curve for db-cAMP inhibition of cell proliferation, which was fitted by the logistic function to calculate IC_{50} and Hill coefficient. Data represent means ± SE from three independent experiments. Statistically significant differences between means were tested using two-way ANOVA, followed by post hoc multiple comparison test. * indicates $P<0.05$, as compared with control.

or db-cGMP (Figs. 1A and 1B). Two-way ANOVA indicated that db-cAMP (3 mM) significantly inhibited cell growth in a time- and concentration-dependent manner ($P<0.05$) but db-cGMP did not (Figs. 1A and 1B). In addition, inhibition of cell proliferation was exerted by 3 mM of db-cAMP as early as after 72-h treatment. To make concentration-response curve of db-cAMP inhibition of cell proliferation, absorbance (A_{415}) measured after 72 h-treatment with db-cAMP was plotted (Fig. 1C). Data points were fitted with the logistic equation:

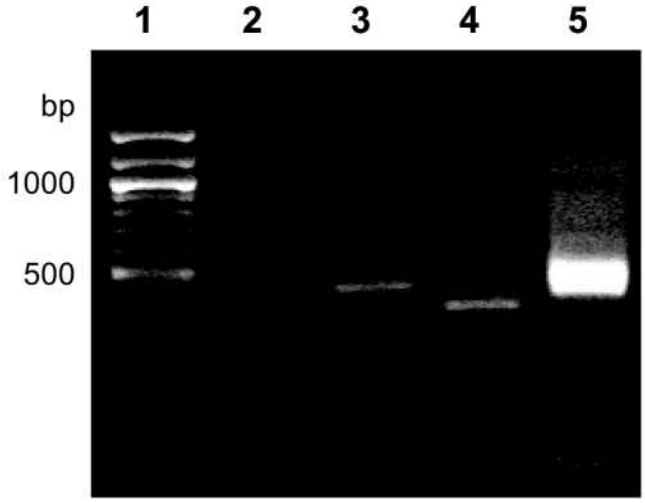

Fig. 2 Basal mRNA expression of type 3 and 4 phoshodiesterases in A7r5 cells.
Lane 1, DNA ladder marker; lane 2, type 3A PDE; lane 3, type 3B PDE; lane 4, type 4 PDE; lane5, GAPDH.

A_{415} (db-cAMP)/A_{415} (control) = {A_{415} (max) - A_{415} (min)}/{1+([db-cAMP]/IC_{50})^{nH}} + A_{415} (min), where A_{415} (control) is the absorbance after 72-h treatment with 10% FBS-DMEM alone, A_{415} (db-cAMP) is the absorbance after 72-h treatment with db-cAMP at the different concentrations from 0.03 to 3 mM, A_{415} (max) is the value at 0.03 mM, A_{415} (min) is the value at 3 mM, IC_{50} is the concentration of db-cAMP required to decrease the A_{415} to half, and n_H is the Hill coefficient. The IC_{50} value was 0.11 mM, and the n_H value was 1.44 (Fig. 1C).

II. Expression of phosphodiesterase mRNA

Basal mRNA expressions of PDE3A, 3B, and 4D were examined in A7r5 cells. Type 3B and 4D PDE and GAPDH mRNA expressions were detected at the expected positions of 480 bp, 370 bp and 440 bp, respectively. Type 3A PDE mRNA expression was not detected in A7r5 cells (Fig. 2).

III. Measurement of intracellular cAMP

As shown in Figure 3A, the standard curve for estimation of cAMP concentration showed that cAMP concentration ranging from 10^{-2} pmol/μl to 10 pmol/μl could be measured in the chemiluminescent ELISA system. Relative Light Unit (RLU) was decreased with the increase in cAMP concentration with an IC_{50} of 0.125 pmol/μl. In addition, to test whether db-cAMP can be measured in the ELISA system, serially diluted db-cAMP solutions from 50

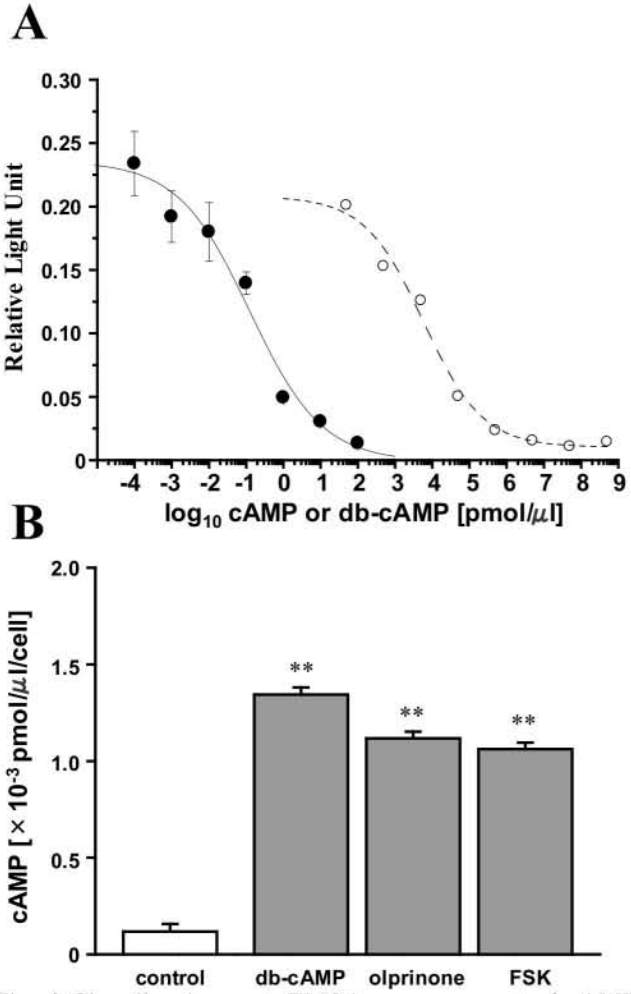

Fig. 3 Chemiluminescent ELISA measurement of cAMP in A7r5 cells

A: Standard curve for chemiluminescent ELISA measurement of cAMP (●) and db-cAMP (○). B: Summarized bar graphs of the intracellular cAMP concentrations in A7r5 cells after treatment without drugs (open bar, control) or with drugs (gray bars) including db-cAMP (3 mM), olprinone (1 mM), and FSK (0.1 mM). Data represents means ± SE from four independent experiments. $^{**}P<0.01$ when compared with control by one-way ANOVA followed by post hoc multiple comparison.

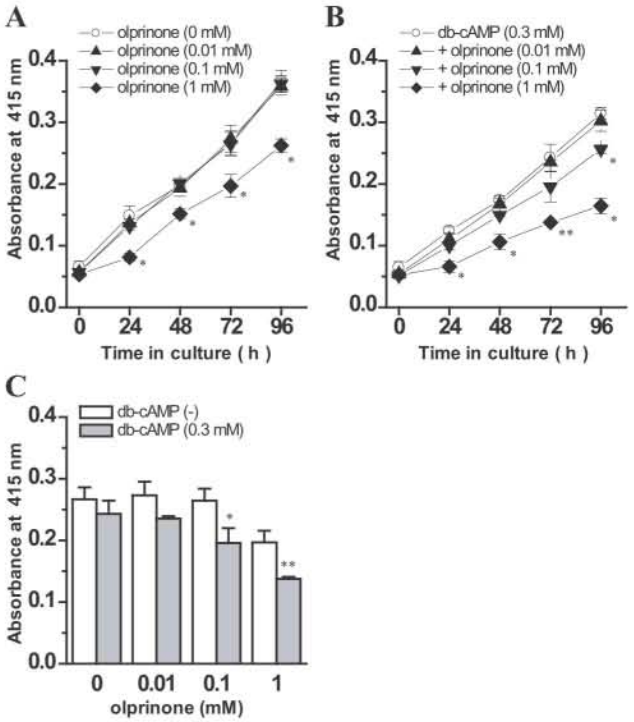

Fig. 4 Synergistic inhibition of cell proliferation by olprinone and db-cAMP

Proliferation of A7r5 cells were measured at 0, 24, 48, 72, and 96 h after treatment with different concentrations of olprinone (from 0 to 1 mM) (A) or with different concentrations of olprinone (from 0 to 1 mM) plus db-cAMP (0.3 mM) (B). C: The bar graph shows the concentration-dependent inhibition of cell proliferation by olprinone alone (open bars) and by olprinone plus db-cAMP, which are summarized from the data at 72 h in panels A and B. Data represents means ± SE from four independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ when compared with the same concentration of olprinone alone by Student's unpaired t-test.

pmol/μl to 50 mmol/μl were measured. RLU was decreased with the increase in db-cAMP concentration with an IC_{50} of 5.82 nmol/μl. Thus, cross-reactivity specification of anti-cAMP against db-cAMP was 0.0021%. To test whether endogenous cAMP was elevated by olprinone and FSK, cell lysates were prepared at 72 h after treatment of A7r5 cells with olprinone (1 mM) or FSK (0.1 mM). As can be seen, cAMP concentration was significantly increased by olprinone and FSK as compared with that by control (no treatment) ($P<0.01$ by one-way ANOVA, Fig. 3B). Although cAMP concentration seemed to be significantly increased by treatment with db-cAMP (3 mM), this should be caused by intracellularly-introduced db-cAMP. When db-cAMP was measured by standard curve made by db-cAMP, the predicted concentration of intracellularly-introduced db-cAMP was 63.9 μmol/μl/cell.

IV. Effect of olprinone on cell proliferation

Two-way ANOVA indicated that olprinone significantly inhibited cell proliferation at the concentration of 1 mM ($P<0.05$), but did not at the concentrations of 0.01 and 0.1 mM (Fig. 4A). In addition, inhibition of cell proliferation was exerted by olprinone after 24-h treatment, which was

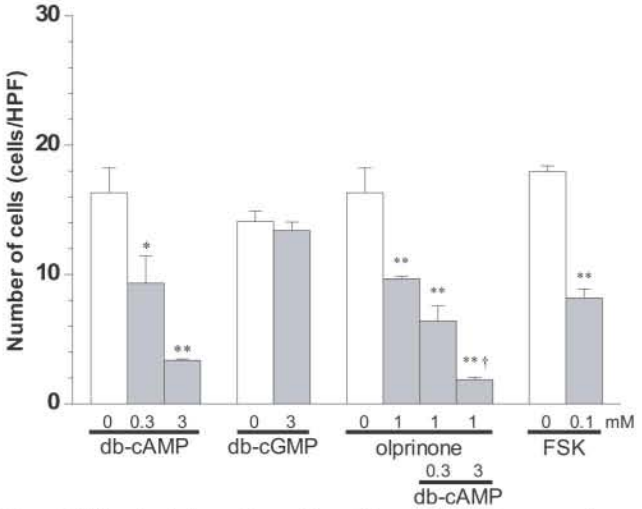

Fig. 5 Effect of elevation of cyclic nucleotides on migration

A7r5 cells were incubated for 72 h in the absence (open bars) or presence (gray bars) of different concentrations of db-cAMP, db-cGMP, olprinone (or plus db-cAMP), and FSK. During the last 24 h, the cells were transferred to cell culture insert to allow them to migrate. Data represent means± SE from 3 to 4 independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ when compared with 0 mM and $^{\dagger}P<0.05$ when compared with olprinone alone by one-way ANOVA followed by post hoc multiple comparison.

earlier than that by db-cAMP (0.3 mM and 3 mM). To test the effect of combination of olprinone and db-cAMP, the cells were treated with three different concentrations of olprinone (0.03, 0.3 and 3 mM) in the presence of db-cAMP (0.3 mM). The inhibition of cell proliferation by olprinone was significantly enhanced (Fig. 4B). The bar graph showing concentration-response relationships made from the data at 72 h indicated that olprinone synergistically inhibited cell proliferation with a statistical significant at the concentrations of 0.1 mM ($P<0.05$) and 1 mM ($P<0.01$) of olprinone in the presence of db-cAMP (0.3 mM), as compared to the respective concentrations of olprinone alone (Fig. 4C).

V. Effect of db-cAMP, db-cGMP, olprinone, and forskolin on cell migration

The mean number of cells migrated through 8-μm pore of membrane were ranged from 14.1 to 17.9 cells/HPF in 10% FBS-DMEM (open bars in Fig. 5). Treatment of the cells with db-cAMP for 72 h concentration-dependently inhibited cell migration with a statistical significance at the concentrations of 0.3 mM ($P<0.05$) and 3 mM ($P<0.01$), whereas

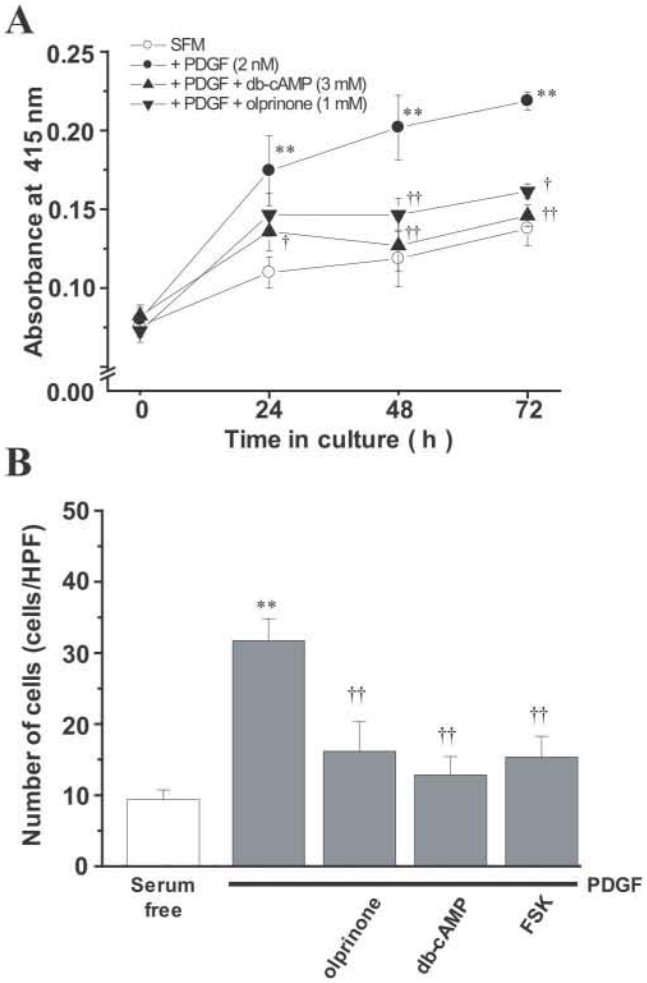

Fig. 6 Effect of elevation of cAMP on PDGF-BB-induced cell proliferation and migration.

A: A7r5 cells were cultured in serum-free DMEM (SFM) or in SFM containing PDGF-BB (2 nM) without or with db-cAMP and olprinone for 0, 24, 48, and 72 h. Absorbance at 415 nm was measured at 1 h after addition of WST-1. Open circles, SFM; closed circles, PDGF-BB (2 nM); upper triangles, PDGF-BB (2 nM) plus db-cAMP (3 mM); and down triangles, PDGF-BB (2 nM) plus olprinone (1 mM). B: A7r5 cells were incubated for 72 h in SFM (open bar) or in the presence (gray bars) of PDGF-BB alone (2 nM), PDGF-BB (2 nM) plus olprinone (1 mM), PDGF-BB (2 nM) plus db-cAMP (3 mM), or PDGF-BB (2 nM) plus FSK (0.1 mM). During the last 24 h, the cells were transferred to cell culture insert to allow them to migrate. Data represent means± SE from 3 to 4 independent experiments. $^{**}P<0.05$ when compared with SFM and $^{\dagger\dagger}P<0.01$ when compared with PDGF-BB alone by one-way ANOVA followed by post hoc multiple comparison.

treatment with db-cGMP (3 mM) had no effect (Fig. 5). Treatment of the cells with olprinone and FSK for 72 h significantly inhibited cell migration at the concentrations of 1 mM and 0.1 mM, respectively with statistical significances ($P<0.01$ for each, Fig. 5). Furthermore, olprinone inhibition of cell migration was further inhibited in the presence of db-cAMP at the concentration of 3 mM as compared with the inhibition by olprinone alone (Fig. 5)

VI. Effect of db-cAMP, olprinone and FSK on PDGF-BB induced cell proliferation and migration

Cell proliferation rate of 0.021 (A_{415}/h) in SFM was as about half as the rate of 0.043 (A_{415}/h) in 10% FBS-DMEM (Fig. 1A and Fig. 6A). PDGF-BB significantly stimulated cell proliferation with the rate of 0.039 (A_{415}/h) in SFM at the concentration of 2 nM at 24, 48, and 72 h ($P<0.01$, Fig. 6A). Two-way ANOVA indicated that PDGF-BB stimulation of cell proliferation was significantly inhibited by db-cAMP and olprinone at the concentrations of 3 mM and 1 mM, respectively with statistical significances at 24, 48, and 72 h (Fig. 6A).

Migration measured after a 72-h culture in SFM showed a reduction to half as compared to that in 10% FBS-DMEM (Fig. 5 and Fig. 6B). PDGF-BB-treatment at the concentration of 2 nM resulted in 3.4-fold increase in migration as compared to that in SFM (31.7 ± 3.1 vs. 9.4 ± 1.4 cells/HPF, $P<0.01$) (Fig. 6B). This PDGF-BB stimulation of migration was significantly inhibited by db-cAMP (12.8 ± 2.6), olprinone (16.2 ± 4.1) and FSK (15.4 ± 2.9) after 72-h treatment with these reagents as compared to the PDGF-BB treatment ($P<0.01$ for each, Fig. 6B).

DISCUSSION

In the present study, we have found that db-cAMP but not db-cGMP inhibited cell proliferation and migration under both the basal culture condition (10% FBS-DMEM) and PDGF-BB-stimulated condition (serum-free DMEM) when using embryonic rat aortic VSM cell line of A7r5. Elevation of intracellular cAMP by olprinone and FSK inhibited cell proliferation and migration under both the serum-stimulated and the PDGF-BB-stimulated conditions.

Cyclic nucleotides, cAMP and cGMP, are involved in cell growth and differentiation[2]. Many reports suggested that cells can be made quiescent by serum depletion with the induction of elevated cAMP level[2]. Treatment of various types of cell with db-cAMP leads growth inhibition by blocking them in G_1 or G_2 phases of cell cycles[22,23]. It has been reported that cAMP inhibited cyclin-dependent kinase 2 and 4, which resulted in growth arrest of cells at the G_1 phase[24-26]. In addition, it was demonstrated by Klemm *et al* that cAMP response element-binding protein (CREB) could function as a molecular determinant of smooth muscle cell proliferation[27]. Our data indicated that treatment of the cells with db-cAMP and olprinone inhibited cell proliferation, as expected. However, the time course of inhibition of basal cell proliferation by db-cAMP was delayed as compared with that by olprinone. Olprinone inhibited cell proliferation after 24-h treatment, whereas db-cAMP inhibited after 72-h treatment. This difference might be caused by non-specific effect of olprinone on L-type Ca^{2+} channel current. That is, in our preliminary data, nifedipine (50 μM) inhibited cell proliferation after 24-h treatment, and olprinone reduced L-type Ca^{2+} channel current by 27.3% in A7r5 cells. Therefore, olprinone probably exerted inhibitory action on cell proliferation by elevation of cAMP and/or by inhibition of L-type Ca^{2+} channel current.

On the other hand, role of cGMP in cell proliferation is controversial. Elevation of cGMP by prostaglandin (PG) $F_{2\alpha}$ or insulin activated the cells to synthesize DNA[28], whereas no activation was observed by elevation of cGMP using 3T3 cells[29]. In VSM cells, it is apparent that endothelium-derived nitric oxide (NO) inhibits cell proliferation of VSM cells at least in part by elevation of cGMP[13]. In addition, cGMP-treatment of cultured rat VSM cells has been shown to inhibit cell proliferation through activation of vasodilator-stimulated phosphoprotein (VASP)[14]. Since our data demonstrated that db-cGMP failed to inhibit cell proliferation, the expression of protein kinase G (PKG) or VASP might be reduced in A7r5 cells.

Culture of A7r5 cells in 10% FBS-DMEM allowed the cells to migrate, whereas migration was suppressed by serum depletion. Thus, migration can be induced by growth factors, hormones, and cytokines contained in serum. Several studies suggest that elevation of intracellular cyclic nucleotides could be regarded as important signaling molecules for control of migration. IL-6 produced by VSM cells contributes to cell migration induced by tumor

necrosis factor (TNF)-α and elevation of cAMP inhibited TNF-α-induced release of IL-6, and migration of VSM cells[30]. Insulin inhibited VSM migration by inhibiting calmodulin-dependent kinase (CaMK)-II activity via stimulation of cGMP production[31]. PGE_2, salmeterol, and a type 4 PDE inhibitor, cilomolast, inhibit basal airway smooth muscle (ASM) cells and pulmonary VSM (PVSM) migration by elevating cAMP[32]. We have found that treatment of the cell with db-cAMP, olprinone and FSK inhibited migration, but not with db-GMP. Olprinone (1 mM) and FSK (0.1 mM) inhibited both basal and PDGF-induced migration to the same degree. Since elevation of cAMP was almost the same level when the cells were treated with the two drugs, inhibitory effect of the drugs on migration is suggested to be mediated through elevation of cAMP. Although it is unclear why db-cGMP had no effect on migration, the expression of PKG or CaMK-II might be reduced in A7r5 cells.

PDGF-BB activates multiple signaling pathways in VSM cells, including SHP-2, Src, PLCγ, Ras, PKA, PI3K, and MAP kinases. Ras, Src, and c-Jun participate in PDGF-induced VSM proliferation[33-36] and PI3K is involved in PDGF-induced VSM migration[37]. PDGF-BB is also known to induce plasminogen activator inhibitor type-1 (PAI-1) in VSM cells[38] and PAI-1 has been shown to be a potent regulator of vascular cell migration *in vitro*[39]. It is not clarified how elevation of cAMP by db-cAMP, olprinone, and FSK inhibit PDGF-induced cell proliferation and migration in cultured VSM cells. Marienfeld *et al* demonstrated that PDGF-BB induced cell proliferation can be inhibited by a cAMP analog, whereas activation of the endogenous PKG had no effect on cardiac fibroblast cell growth, indicating that the suppression of MAP kinase phosphorylation plays the major role in inhibition of cardiac fibroblast proliferation by cAMP[40]. Edin *et al* also reported that NIH3T3 fibroblasts expressing a dominant negative PKA subunit show diminished migration in response to serum or growth factors[41]. PGE_2 and cilomolast also inhibited PDGF-stimulated migration of ASM and PVSM cells[32]. VSM cell migration was inhibited to a similar extent by an anti-platelet drug, trapidil[42]. Taken together, PDGF-induced cell proliferation and migration might be inhibited through activation of a cAMP/PKA pathway in A7r5 cells.

Inflammatory cytokines including TNF-α and IL-1 promote VSM cell proliferation and migration, thereby contributing to the development of atherosclerosis[10,43]. It has been reported that inflammatory cytokines activate nuclear factor-κB (NF-κB) in monocyte/macrophages, vascular endothelial cells and VSM cells, which regulate the transactivation of many genes involved in the development of atherosclerosis[44]. Since elevation of cAMP is known to inhibit the activation of NF-κB, cAMP would be a possible therapeutic strategy for prevention of atherosclerosis.

In conclusion, elevation of cAMP by db-cAMP, olprinone, and FSK inhibited basal and PDGF-BB-induced cell proliferation and migration in A7r5 cells. Interestingly, inhibitory effect of olprinone on cell proliferation was exerted earlier than that of db-cAMP, which suggests that olprinone might have a pleiotropic action besides PDE inhibition. Although the precise mechanism whereby cAMP inhibits the stimulatory effect of PDGF-BB in A7r5 cells remains unkonwn, further clarification of the inhibitory mechanism of cAMP on the PDGF-BB effects would bring us to develop a new strategy to inhibition of cytokine-induced VSM cell proliferation. We previously demonstrated that cilostazol, another type 3 PDE inhibitor, reduced vascular endothelial dysfunction of diabetic rats[21]. Thus, the cAMP/PKA pathway may be important in the integrated treatment of vascular diseases such as atherosclerosis and vascular restenosis due to angioplasty.

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