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Vasopressin Stimulates Na-dependent Phosphate Transport and Calcification in Rat Aortic Smooth Muscle Cells

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Abstract. We investigated the effect of arginine vasopressin (AVP) on inorganic phosphate (Pi) transport in A-10 rat aortic vascular smooth muscle cells (VSMCs). AVP time- and dose-dependently stimulated Na-dependent Pi transport in A-10 cells. This stimulatory effect of AVP on Pi transport was markedly suppressed by V1 receptor antagonist. A protein kinase C (PKC) inhibitor calphostin C partially suppressed the stimulatory effect of AVP. The selective inhibitors of c-Jun-NH_2-terminal mitogen-activated protein (MAP) kinase (Jun kinase) attenuated AVP-induced Pi transport, but Erk kinase or p38 MAP kinase inhibitors did not. Wortmannin, a phosphatidylinositol (PI) 3-kinase inhibitor, suppressed AVP-induced Pi transport. Rapamycin, a selective inhibitor of S_6 kinase, reduced this effect of AVP, while Akt kinase inhibitor did not. The combination of inhibitors for PKC, Jun kinase and PI 3-kinase completely suppressed the AVP-enhanced Pi transport. Furthermore, AVP rescued the VSMC from high phosphate-induced cell death and enhanced mineralization of these cells. In summary, these results suggest that AVP stimulates both Na-dependent Pi transport and mineralization in VSMCs. The mechanism is mediated by the activation of multiple signaling pathways including PKC, PI 3-kinase, S_6 kinase and Jun kinase.

Key words: Vascular smooth muscle cells, Inorganic phosphate transport, Arginine vasopressin
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VASCULAR calcification is often encountered in the development of atherosclerotic lesions [1]. Until recently, vascular calcification was considered to be a passive, degenerative, end-stage process of vascular disease. However, a recent study by Jono *et al.* [2] suggests that aortic vascular smooth muscle cell (VSMC) acquires the phenotype of osteoblast-like cells, and that the expression of inorganic phosphate transporter (PiT) on VSMC is important in the process of vascular calcification. The enhancement of Pi transport in bone-forming cells is essential for the mineralization of skeletal tissues [3], and several growth factors and hormones have been reported to stimulate both proliferation and Pi transport [4–7]. As for VSMCs, it is nowadays well accepted that the proliferation of VSMC plays a crucial role in the pathogenesis of hypertension and atherosclerosis, and is a key event during the formation of intimal hyperplasia after artery injury [1, 8]. After vascular injury, VSMC at the artery media is activated and migrates to the intima, where the cells proliferate and secrete extracellular matrix components [1]. We have recently reported that platelet-derived growth factor (PDGF) stimulates Pi transport activity through the expression of type III Na-dependent PiT, which is considered to be essential for the mineralization of bone tissues [3, 9], in rat A-10 aortic VSMC [10]. However, the precise mechanism of the enhancement of Pi transport activity by extracellular stimuli in VSMC has yet to be fully elucidated.

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Arginine vasopressin (AVP) plays a major role in the regulation of body fluid volume, and exerts potent vasoconstrictor action [11]. *In vitro*, AVP has been reported to stimulate the proliferation of VSMCs [12, 13]. There are at least three subtypes of heterotrimeric GTP-binding protein-coupled receptors (GPCRs) which bind to AVP, V1a, V1b and V2 [14]. V1a receptor is located mainly in the central nervous system and cardiovascular organs including VSMC, while V1b and V2 are located in the central nervous system and kidney, respectively. As for intracellular signaling mechanisms, it has been reported that AVP mobilizes intracellular Ca^{2+} and stimulates protein kinase C (PKC) through the activation of phospholipase (PL) C and PLD in VSMC [15–17], which is involved in the mechanism of AVP-induced proliferation of these cells [18]. Other important signaling pathways to mediate the mitogenic and differentiation processes are tyrosine kinases. It has been reported that AVP also stimulated tyrosine kinases in VSMC [19], and that AVP-stimulated cell proliferation was mediated by the activation of extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase and S_6 kinase in glomerular mesangial cells [20]. In the present study, we investigated the effect of AVP on Pi transport system in rat A-10 VSMCs to gain more insight into the mechanism of vascular calcification.

Materials and Methods

Chemicals

Cell culture reagents were purchased from ICN Biochemicals, Inc. (Costa Mesa, CA, USA). Fetal calf serum (FCS), glutamine, antibiotics and trypsin/EDTA were from Gibco Life Technologies Ltd. (Paisley, UK). AVP and [Pmp^1, $Tyr(Me)^2$]-Arg^8-vasopressin (V1 receptor antagonist) were purchased from Peptide Institute, Inc., (Minoh, Japan). Calphostin C, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB203580), anthra(i,g-cd)pyrazol-6(2H)one (SP600125), wortmannin, 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor), and rapamycin were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Apoptosis *in situ* detection kit was from Wako Chemicals Co. (Osaka, Japan). $H_3[^{32}P]O_4$ was from DuPont de Nemours (Brussels, Belgium). $^{45}CaCl_2$ was from Amersham Pharmacia Biotech (Little Chalfont, UK). All other chemicals were from standard laboratory suppliers and were of the highest purity commercially available.

Cell culture

Cloned aortic smooth muscle A-10 cells derived from embryonic rat were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Subcultures were obtained twice a week by using 0.25% trypsin in Ca^{2+}- and Mg^{2+}- free Eagle's salt solution that contained 0.02% EDTA. They were seeded at 10,000 cells/cm^2 and cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air. As for the experiments of Ca acquisition, the cells were seeded on type I-collagen precoated plates.

Influence of AVP on Pi transport activity

The effect of AVP on Pi transport was analyzed in confluent A-10 cells cultured in DMEM containing 2% FCS for 24 h. Unless specified, they were then incubated for 6 h in the same culture medium with either various doses of AVP or its vehicle. Following cells treatment with agents, Pi transport activity was determined in Earle's buffered salt solution (EBSS) containing 0.1 μM labeled $H_3[^{32}P]O_4$ as previously described [4]. Before the transport assay, the cell layer was rinsed three times with EBSS without radioactive or cold substrate. The transport measurement started after adding 0.3 ml of EBSS containing the labeled substrate (1 μCi/ml). After 10 min incubation, the uptake solution was aspirated, and the cell layer was rinsed three times with 0.3 ml of ice-cold substrate free EBSS. At the end of the experiment, cells were solubilized with 0.25 ml of 0.2 N sodium hydroxide and the radioactivity contained in 200 μl aliquot was counted by using a standard liquid scintillation technique. Finally, the radioactivity in each well was adjusted by DNA content of the cells treated with same protocol as Pi transport activity was measured. Preliminary experiments indicated that Na-independent component of Pi transport in A-10 cells was less than 10% of total uptake of Pi determined in the presence of 143 mM choline chloride

and this component was not influenced by AVP (data not shown). Therefore this sodium-independent component was neglected in this study.

Alizarin red staining

Confluent A-10 cells (day 8) were treated with 0.1 μM of AVP or its vehicle every other day in DMEM containing 10% FCS and 1.6 mM NaH_2PO_4. The final concentration of extracellular Pi was 2.5 mM. At day 14, mineralized nodules were determined by alizarin red staining.

Effect of AVP on the Ca deposition on A-10 cells

Confluent A-10 cells were cultured in DMEM containing 10% FCS and 1.6 mM NaH_2PO_4 for indicated periods. The final concentration of extracellular Pi was 2.5 mM. The medium was exchanged with or without 0.1 μM AVP every other day. At day 8 and 14, Ca deposition was determined in assay buffer (5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$ and 1 mM $CaCl_2$ with 1 μCi/ml $^{45}CaCl_2$). After 24 h incubation, the solution was aspirated. Before the assay, the cell layer was rinsed four times with 0.3 ml of assay buffer without radioactive substrate. At the end of the experiment, ^{45}Ca accumulation in extracellular matrices was examined as previously described [21, 22].

TUNEL assay

Confluent A-10 cells were cultured in DMEM containing 10% FCS and 1.6 mM NaH_2PO_4. The final concentration of extracellular Pi was 2.5 mM. The medium was exchanged with or without 0.1 μM AVP every other day. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUDP nick end-labeling (TUNEL) staining using an Apoptosis In Situ Detection kit (WAKO Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacture's instructions.

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). A two-sided unpaired Student's *t*-test or analysis of variance (ANOVA) for multiple comparisons was used for statistical analysis. A difference between experimental groups was considered to be significant when the *P* value was <5%.

Results

AVP dose-dependently enhanced Na-dependent Pi transport in A-10 cells. A significant effect was detected at 0.01 nM, and maximal stimulation was reached at a dose of 10 nM (Fig. 1A). The effect was time-dependent and was detected significantly after 3 h incubation (Fig. 1B). A maximal response was reached after 6 h. On the contrary, AVP did not affect [^3H] alanine transport in A-10 cells (data not shown).

We next explored the intracellular signaling mechanisms involved in the AVP-induced stimulation of Pi transport in A-10 VSMC. The pretreatment with V1 receptor antagonist (0.1 μM) [23] suppressed AVP (0.1 μM)-induced enhancement of Pi transport (vehicle, 204 ± 7; AVP, 294 ± 3; V1 antagonist, 225 ± 4; V1 antagonist + AVP, 222 ± 11 pmol/10 min/μgDNA, mean ± SEM). Calphostin C, a potent inhibitor for PKC [24], partially suppressed AVP-induced Pi transport in A-10 cells (Table 1). MAP kinases are known as key enzymes in controlling mitogenic and differentiation processes induced by growth factors [25]. In this study, AVP-induced Pi transport was not influenced by either U0126, a specific inhibitor of ERK kinase (MEK) [26], or SB203580, a selective p38 MAP kinase inhibitor [27] in A-10 cells (Fig. 2). On the contrary, SP600125, which selectively inhibits c-Jun-NH2-terminal kinases (Jun kinase) at 20 μM [28], suppressed the enhancement of Pi transport in these cells (Fig. 2). The phosphatidylinositol (PI) 3-kinase pathway is another important intracellular signaling mechanism previously shown to influence the stimulation of solute transport activities in response to activated PDGF receptors in A-10 VSMC [10]. Wortmannin, a specific inhibitor of PI 3-kinase [29], significantly reduced the change in Pi transport induced by AVP in A-10 cells (Table 2). Furthermore, rapamycin, a selective inhibitor of S_6 kinase [30], reduced the stimulatory effect of AVP on Pi transport in the range between 0.01 and 100 nM, while Akt inhibitor had no effect (Fig. 3). The pretreatment with a combination of three inhibitors, calphostin C, SP600125 and wortmannin, completely suppressed AVP-induced Pi transport (Table 3). A-10 VSMC cultured in high phosphate (2.5 mM) DMEM

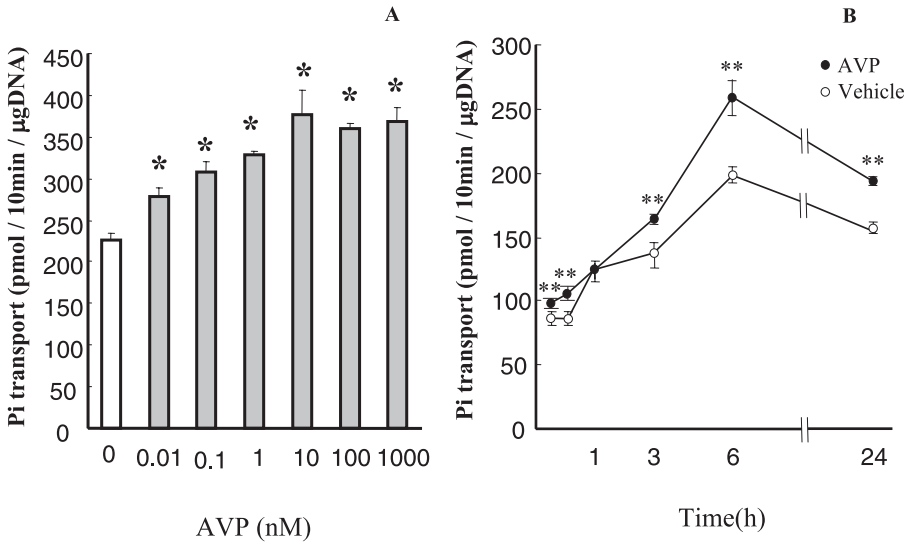

Fig. 1. Characteristics of Pi transport stimulation incubated by arginine vasopressin (AVP) in A-10 cells. (A) Dose-dependent effect of AVP on Pi transport. Confluent A-10 cells (Day 8) were cultured in DMEM containing 2% FCS for 24 h, then incubated for 6 h with various doses of AVP. (B) Time-dependent effect of AVP on Pi transport. Confluent A-10 cells (Day 8) were cultured in DMEM containing 2% FCS for 24 h, then incubated with either 0.1 μM of AVP (●) or its vehicle (○) for the indicated periods. Pi uptake was determined in EBSS containing 0.1 μM $H_3[^{32}P]O_4$. Each value represents the mean ± SEM of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. The Na-independent component of Pi transport was less than 10% of the total uptake of Pi, and was not influenced by AVP (data not shown).
**p* <0.05 compared with vehicle (ANOVA, Fisher's test)
***p*<0.05 compared with vehicle (unpaired Student's t-test)

Table 1. Effect of calphostin C, a protein kinase C (PKC) inhibitor, on Pi transport stimulation induced by arginine vasopressin (AVP) in A-10 cells

	Pi transport (pmol/10 min/μgDNA)
Vehicle	204 ± 4.8
AVP	286.4 ± 0.12
Calphostin C	170.6 ± 4.3
Calphostin C + AVP	198.1 ± 6.8*

Confluent A-10 cells (day 8) were cultured for 24 h in DMEM containing 2% FCS. They were pretreated with calphostin C (0.5 μM) for 1 h, and then incubated with AVP (0.1 μM) or their vehicle for 6 h. Pi uptake was determined in EBSS containing 0.1 μM $H_3[^{32}P]O_4$. Each value represents the mean ± SEM of three independent cell preparations. Similar results were obtained with two additional cell preparations.
**p*<0.0001 compared with AVP alone (ANOVA, Scheffe's test)

Table 2. Effects of the inhibitor of phosphatidylinositol (PI) 3-kinase on Pi transport stimulation induced by arginine vasopressin (AVP) in A-10 cells

	Pi transport (pmol/10 min/μgDNA)
Vehicle	220.7 ± 7.3
AVP	296 ± 4.2
Wortmannin	212.9 ± 32
Wortmannin + AVP	233.8 ± 4*

Confluent A-10 cells (day 8) were cultured in DMEM containing 2% FCS for 24 h. They were pretreated with 10 μM of wortmannin, or their vehicle for 1 h, and then incubated with either 0.1 μM AVP or its vehicle for additional 6 h. Pi uptake was determined in EBSS containing 0.1 μM $H_3[^{32}P]O_4$. Each value represents the mean ± SEM of three independent cell preparations. Similar results were obtained with two additional cell preparations.
**p*<0.05 compared with the value of AVP alone (ANOVA, Scheffe's test)

for 6 days showed cell death, but the treatment with 0.1 μM AVP rescued the cells and enhanced the mineralization of these cells (Fig. 4A, B). TUNEL staining analysis showed that 2.5 mM high phosphate induced apoptosis in A-10 cells (Fig. 4C), and this apoptosis was rescued by the treatment of 0.1 μM AVP (Fig. 4D). We also found that AVP enhanced ^{45}Ca accumulation according to the duration of the culture (Fig. 5).

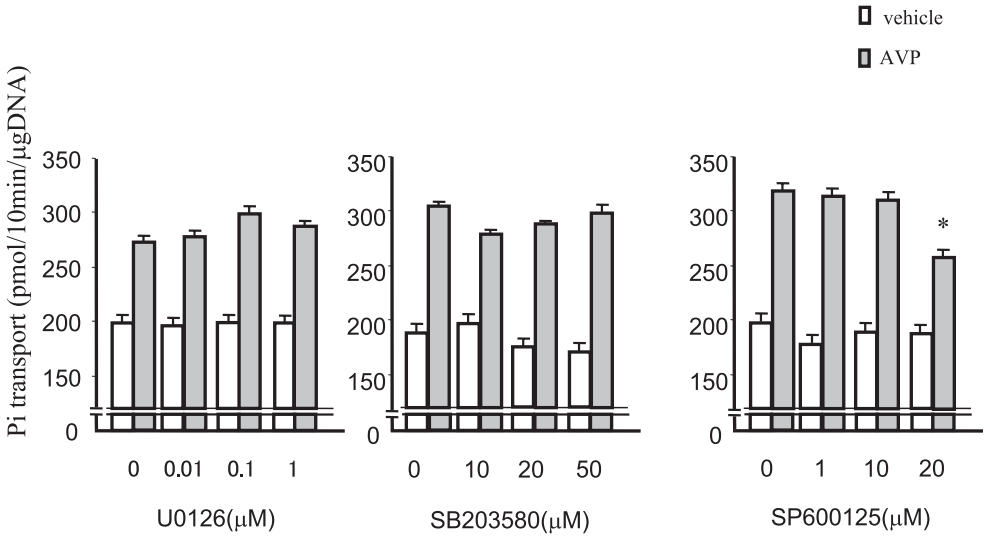

Fig. 2. Effects of MAP kinase inhibitors, U0126, a specific inhibitor of ERK kinase (MEK), SB203580, a selective p38 MAP kinase inhibitor, and SP600125, a selective inhibitor of Jun kinase, on Pi transport induced by arginine vasopressin (AVP) in A-10 cells. Confluent cells were cultured in DMEM containing 2% FCS for 24 h, then pretreated with various doses of U0126, SB203580, SP600125 or their vehicle for 1 h, and then incubated with either 0.1 μM AVP or its vehicle for additional 6 h. Pi uptake was determined in EBSS containing 0.1 μM $H_3[^{32}P]O_4$. Each value represents the mean ± SEM of three independent cell preparations. Similar results were obtained with two additional cell preparations.
*p<0.05 compared with the value of AVP alone (ANOVA, Scheffe's test)

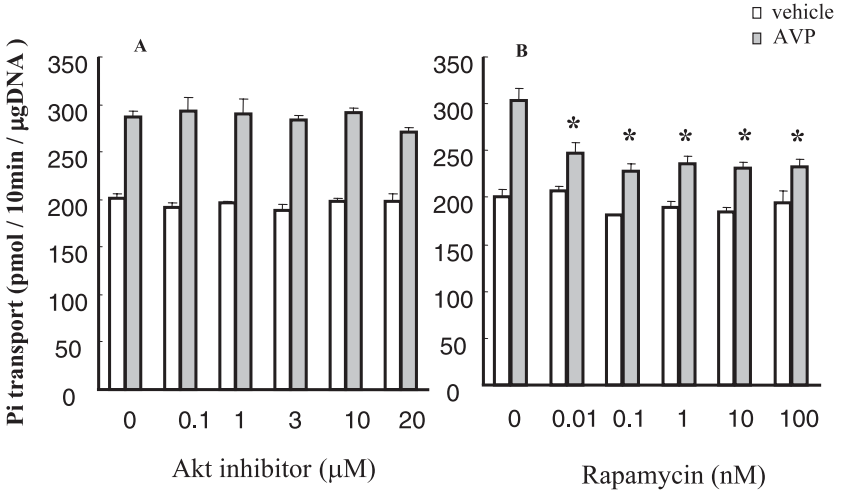

Fig. 3. Effects of Akt inhibitor (A) or rapamycin (B), on Pi transport stimulation induced by arginine vasopressin (AVP) in A-10 cells. Confluent A-10 cells (Day 8) were cultured in DMEM containing 2% FCS for 24 h, then pretreated with various doses of Akt inhibitor (A), rapamycin (B) or their vehicle for 1 h, and then incubated with either 0.1 μM AVP or its vehicle for additional 6 h. Pi uptake was determined in EBSS containing 0.1 μM $H_3[^{32}P]O_4$. Each value represents the mean ± SEM of three independent cell preparations. Similar results were obtained with two additional cell preparations.
*p<0.05 compared with the value of AVP alone (ANOVA, Scheffe's test)

Discussion

The results of the present study indicate that AVP stimulates Na-dependent Pi transport activity in rat A-10 VSMC. A significant increase of Pi transport was found just after 3 h stimulation by AVP. The stimulative effect of AVP was sustained for up to 24 h, suggesting that AVP augments Pi transport during

Table 3. Combined effect of calphostin C, wortmannin and SP600125 on Pi transport stimulation by arginine vasopressin (AVP) in A-10 cells

	% of control
Control	100
AVP alone	158.8 ± 0.7*
AVP + calphostin C	123.2 ± 0.1*
AVP + wortmannin	112.3 ± 0.1*
AVP + SP600125	125.6 ± 0.2*
AVP + calphostin C + wortmannin + SP600125	104.1 ± 0.3

Confluent A-10 cells (day 8) were cultured for 24 h in DMEM containing 2% FCS. They were pretreated with calphostin C (0.5 μM), wortmannin (10 μM), SP600125 (20 μM) or their combination for 1 h, and then incubated with AVP (0.1 μM) or its vehicle for 6 h. Pi uptake was determined in EBSS containing 0.1 μM $H_3[^{32}P]O_4$. Each value represents the mean ± SEM of three independent cell preparations. Similar results were obtained with two additional cell preparations.
*$p<0.0001$ compared with control (ANOVA, Scheffe's test)

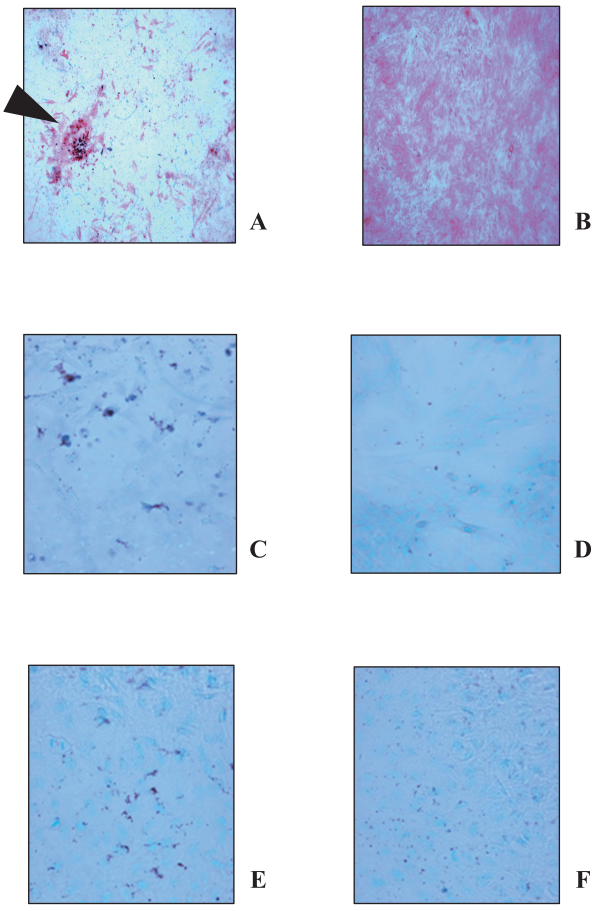

Fig. 4. Effect of arginine vasopressin (AVP) on cell survival and mineralization in A-10 cells. Confluent A-10 cells (Day 8) were cultured in DMEM containing 10% FCS including 2.5 mM phosphate for an additional 6 days. The medium was exchanged with or without 0.1 μM AVP every other day. The Ca deposition was evaluated by alizarin red staining on day 14 (A, B). Some cells were detached from the plate and the other cells died showing the condensation of nucleus (arrow) in vehicle-treated cells (A). On the other hand, AVP-treated cells at day 14 survived and showed enhanced Ca deposition in high phosphate media (B). At the same time, apoptosis of A-10 cells were evaluated by TUNEL assay (C, D, E, F). High phosphate medium-induced apoptosis of A-10 cells (C) was rescued by the treatment with AVP (D). Positive and negative controls of TUNEL assay were shown in E and F, respectively.

proliferation of VSMC. Next, we examined the intracellular signalling mechanism involved in AVP-induced stimulation of Na-dependent Pi transport in A-10 cells. AVP has been reported to show its vasoconstrictive effect through V1a receptor in cardiovascular organs including VSMC [14]. We here showed that V1 receptor antagonist attenuated the effect of AVP on Pi transport, suggesting the involvement of V1 receptor, possibly V1a, in this mechanism in VSMC.

The fact that AVP plays a protective role and supports cell survival of A-10 VSMC suggests that proliferative and/or anti-apoptotic signalling pathways are involved in the mechanism of AVP-induced Na-dependent Pi transport in these cells. As AVP has been reported to stimulate proliferation through PKC activation in VSMC [18], we examined whether PKC mediates the stimulation of Pi transport by AVP in A-10 cells. We here showed that PKC inhibitor, calphostin C, partially but significantly attenuated AVP-enhanced Pi transport in these cells, suggesting, at least in part, the involvement of PKC in the mechanism of AVP-induced enhancement of Pi transport in VSMC. Among the downstream effectors of growth factor receptor tyrosine kinases, MAP kinases are well known to be major pathways for the regulation of mitogenic and differentiation processes, including AVP-induced proliferation of VSMC, and it has been reported that AVP stimulates the activation of MAP kinases in A-10 cells [25, 31]. Three structurally related MAPK pathways have been characterized in mammalian cells. The ERKs are activated downstream of cell membrane receptors and regulate cell proliferation and differentiation [32]. Jun kinase and the p38 MAPK pathways are generally activated by treatment of cells with inflammatory cytokines or by environmental stress leading to apoptosis and have therefore been named stress-

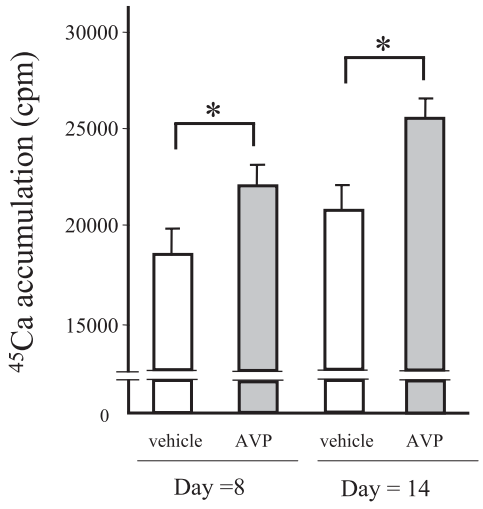

Fig. 5. The enhancement of Ca accumulation by arginine vasopressin (AVP) in A-10 cells. Confluent A-10 cells (Day 8) were cultured in DMEM containing 10% FCS including 2.5 mM phosphate with or without 0.1 μM AVP every the other day. On day 8 and 14, ^{45}Ca accumulation for 24 h was detected by the method described in Materials and Methods. Each value represents the mean ± SEM of three independent cell preparations. Similar results were obtained with two additional cell preparations.
*p<0.05 compared with vehicle (ANOVA, Scheffe's test)

activated protein kinases [33]. However, accumulating evidence suggests that Jun kinase also shows anti-apoptotic effect and contributes to multiple cellular processes including cell proliferation, differentiation, and cell transformation [34]. We have recently shown that Jun kinase is involved in bone morphogenetic protein-2-induced activation of both Pi transport activity and mineralization in osteoblastic cells [35]. The results obtained in this study using specific inhibitors of MEK, p38 and Jun MAP kinases suggest that not ERK or p38 MAP kinase pathways, but Jun kinase is involved in the regulation of Pi transport in response to AVP in VSMC. Another intracellular signalling pathway to transmit survival-promoting signals is PI 3-kinase-dependent pathway. PI 3-kinase is important in particular for cell migration, actin reorganization, and prevention of cell death of apoptosis [36]. We have recently shown that PI 3-kinase and S_6 kinase are involved in the mechanism of PDGF-induced enhancement of Pi transport in A-10 cells [10]. In the present study, we showed that wortmannin, a selective PI 3-kinase inhibitor, significantly decreased Pi transport stimulation induced by AVP in A-10 cells. The activation of PI 3-kinase phosphorylates PI to D_3-phosphorylated PI, such as PI (3,4,5)P_3 [37]. D_3-phosphorylated PI directly binds to pleckstrin-rich homology domain of several signalling molecules such as Akt and PI-dependent kinase-1, which results in the activation of S_6 kinase. It has recently been reported that AVP-induced HSP27 phosphorylation is mediated by PI 3-kinase/Akt in A-10 VSMC [38]. In the present study, we showed that rapamycin, a selective inhibitor of S_6 kinase, reduced the stimulative effect of AVP on Pi transport in A-10 cells. On the other hand, Akt kinase inhibitor had no effect on either basal or AVP-induced Pi transport in A-10 cells. These findings suggest the PI 3-kinase and S_6 kinase also play important roles in AVP-induced enhancement of Pi transport in VSMC. Finally, we showed that the combination of the inhibitors of PKC, Jun kinase and PI 3-kinase completely suppressed AVP-induced Pi transport, suggesting their major role in this mechanism in VSMC.

Phosphate uptake at the cellular membrane is essential to maintain the cell activity because phosphate has to be supplied for ATP synthesis. Generally, the extracellular signal to stimulate cellular proliferation also induces the enhancement of phosphate uptake, that is, Pi transport activity. Growth factors are known to stimulate the proliferation through the activation of receptor tyrosine kinases in ubiquitous cells including VSMC. We have already shown that PDGF-BB, a potent stimulator of the proliferation of VSMC, enhances Pi transport activity through the increase of newly-synthesized type III NaPit, Pit-1, in A-10 VSMC [10]. In this study, we showed that AVP, a vasoactive GPCR agonist, also induces Na-dependent Pi transport activity during the proliferation of VSMC as well as growth factors. This enhancement of Pi transport activity is suggested to contribute to the augmentation of cellular activity and proliferation of VSMC. On the contrary, accumulating evidence suggests that phosphate overload from the extracellular milieu would be stressful for the cells, because phosphate uptake itself might induce the formation of apoptosome, resulting in the apoptosis of the cells [39]. In the present study, we found that high phosphate (2.5 mM) induces apoptotic cell death of A-10 VSMC. However, AVP, which stimulated the proliferation of these cells in normal media (data not shown), rescued the cells from the cell death induced by high phosphate. Furthermore, we here showed that AVP enhanced the Ca deposition on A-10 VSMC in high phosphate media. Ca deposition in ex-

tracellular matrix is considered to be regulated by Pi transport activity in matrix vesicles, and VSMC has been reported to produce matrix vesicles at the atherosclerotic tissues [40]. Jono *et al.* [2] have reported that high phosphate in extracellular milieu also induces the expression of Pit-1 Pi transporter in VSMC and its phenotypic change to osteoblastic cell. The magnitude of the enhancement of Pi transport by AVP in this study was more than 50%, which we have shown to be enough to enhance the calcification of extracellular matrix in bone-forming cells *in vitro* [35]. These results suggest that, as well as growth factors, GPCR-binding humoral factors including AVP, which shows proliferative and/or anti-apoptotic effect on VSMC, may in turn induce calcification of artery through the enhancement of Pi transport activity of VSMC.

VSMC of the arterial media contributes to the functional and structural alteration of the arterial wall in pathophysiological process through its contractility, secretion of proteins, proliferation and migration [1]. Vasoactive humoral factors such as endothelin, angiotensin and AVP act as both systemic and local factors affecting the vascular tone and the response to the vascular injury [13]. Among them, AVP is known to be a potent vasoconstrictor that stimulates the proliferation of VSMC [12, 13, 18]. While three receptor subtypes exist for AVP (V1a, V1b and V2), V1a receptor has been reported to play the dominant role in cardiovascular system and behavior [41, 42]. It has recently been reported that V1a–/– mice showed lower blood pressure through the reduction of blood volume and the impairment of the baroreceptor reflex [42]. On the other hand, V1b–/– mice have been reported to show mildly impaired social recognition [43], but so far little information is provided on cardiovascular disorder in V1b–/– mice. These findings suggest the important role of V1a receptor in maintaining resting blood pressure not by its direct vasoconstriction but by regulating neural and hormonal actions of AVP. In this study, we showed the possible involvement of V1 receptor in the mechanism of AVP-induced enhancement of Pi transport in VSMC. Because of the striking effect of AVP on body fluid retention and hyponatremia, we cannot maintain a high concentration of AVP for long duration *in vivo*. In addition, it has been reported that overexpression of AVP in mouse results in the down-regulation of AVP receptor, and blunted the effect of AVP *in vivo* [44]. Although we here showed that AVP is a potent effector for both proliferation and calcification in VSMC, it seems to be difficult to prove the involvement of AVP on the progression of atherosclerosis under physiological condition *in vivo*. However, the elevation of serum AVP level is known to be induced by cigarette smoking, which is associated with hyperlipidemia and atherogenic changes of arteries [45]. It has also been suggested that AVP-induced hypertension causes the altered response of aorta connective tissue to vascular injury [46]. These findings as a whole suggest that elevated serum AVP level may take part in the progress of atherogenic change of arterial wall including arterial calcification under certain pathological conditions. Further investigations are required.

In conclusion, the results of the present study indicate that AVP stimulates Na-dependent Pi transport and mineralization in rat A-10 VSMCs. The mechanism is mediated by the activation of multiple signaling pathways including PKC, PI 3-kinase, S_6 kinase and Jun kinase.

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References

1. Lusis AJ (2000) Atherosclerosis. *Nature* 407: 233–241.
2. Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Mori K, Morii H, Giachelli CM (2000) Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res* 87: e10–17.
3. Caverzasio J, Montessuit C, Bonjour JP (1996) Functional role of Pi transport in osteogenic cells. *News Physiol Sci* 11: 119–125.
4. Suzuki A, Palmer G, Bonjour JP, Caverzasio J (2000) Stimulation of sodium-dependent phosphate transport and signaling mechanisms induced by basic fibroblast growth factor in MC3T3-E1 osteoblast-like cells. *J*

Bone Miner Res 15: 95–102.
5. Suzuki A, Palmer G, Bonjour JP, Caverzasio J (2001) Stimulation of sodium-dependent inorganic phosphate transport by activation of Gi/o-protein-coupled receptors by epinephrine in MC3T3-E1 osteoblast-like cells. *Bone* 28: 589–594.
6. Masukawa H, Miura Y, Sato I, Oiso Y, Suzuki A (2001) Stimulatory effect of endothelin-1 on Na-dependent phosphate transport and its signaling mechanism in osteoblast-like cells. *J Cell Biochem* 83: 47–55.
7. Sato I, Suzuki A, Kakita A, Ono Y, Miura Y, Itoh M, Oiso Y (2003) Stimulatory effect of prostaglandin $F_{2\alpha}$ on Na-dependent phosphate transport in osteoblast-like cells. *Prostaglandins Leukot Essent Fatty Acids* 68: 311–315.
8. Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362: 801–809.
9. Palmer G, Guicheux J, Bonjour JP, Caverzasio J (2000) Transforming growth factor-β stimulates inorganic phosphate transport and expression of the type III phosphate transporter Glvr-1 in chondrogenic ATDC5 cells. *Endocrinology* 141: 2236–2243.
10. Kakita A, Suzuki A, Nishiwaki K, Ono Y, Kotake M, Ariyoshi Y, Miura Y, Itoh M, Oiso Y (2004) Stimulation of Na-dependent phosphate transport by platelet-derived growth factor in rat aortic smooth muscle cells. *Atherosclerosis* 174: 17–24.
11. Share L (1988) Role of vasopressin in cardiovascular regulation. *Physiol Rev* 68: 1248–1284.
12. Altura BM, Altura BT (1977) Vascular smooth muscle and neurohypophyseal hormones. *Fed Proc* 36: 1853–1860.
13. Campbell-Boswell M, Robertson AL Jr (1981) Effects of angiotensin II and vasopressin on human smooth muscle cells in vitro. *Exp Mol Pathol* 35: 265–276.
14. Jard S, Barberis C, Audigier S, Tribollet E (1987) Neurohypophyseal hormone receptor systems in brain and periphery. *Prog Brain Res* 72: 173–187.
15. Miwa M, Kozawa O, Suzuki A, Watanabe Y, Shinoda J, Oiso Y (1995) Vasopressin activates phospholipase D through pertussis toxin-insensitive GTP-binding protein in aortic smooth muscle cells: function of Ca^{2+}/calmodulin. *Biochem Cell Biol* 73: 191–199.
16. Watanabe Y, Tokuda H, Suzuki A, Shinoda J, Kotoyori J, Ito Y, Oiso Y, Kozawa O (1995) Glucocorticoid amplifies vasopressin-induced phosphoinositide hydrolysis in aortic smooth muscle cells. *J Cell Biochem* 57: 522–529.
17. Nemenoff RA (1998) Vasopressin signaling pathways in vascular smooth muscle. *Front Biosci* 3: d194–d207.
18. Caramelo C, Okada K, Tsai P, Schrier RW (1989) Phorbol esters and arginine vasopressin in vascular smooth muscle cell activation. *Am J Physiol* 256: F875–881.
19. Gonzalez CB, Reyes CE, Figueroa CD, Barra V, Troncoso S (1999) Vasopressin stimulates tyrosine phosphorylation by activation of PKC in the rat smooth muscle cell line, A-10. *Cel Biol Int* 23: 195–201.
20. Ghosh PM, Mikhailova M, Bedolla R, Kreisberg JI (2001) Arginine vasopressin stimulates mesangial cell proliferation by activating the epidermal growth factor receptor. *Am J Physiol Renal Physiol* 280: F972–979.
21. Warshawsky H, Moor G (1967) A technique for the fixation and decalcification of rat incisors for electron microscopy. *J Histochem Cytochem* 15: 542–549.
22. Kozawa O, Takatsuki K, Tokuda H, Miwa M, Kotake K, Yoneda M, Oiso Y, Saito H (1990) Insulin-like growth factor-I stimulates ^{45}Ca-accumulation in cultures of osteoblast-like cells. *J Bone Miner Metab* 8: 161–164.
23. Kruszynski M, Lammek B, Manning M, Seto J, Haldar J, Sawyer WH (1980) [1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid), 2-(*O*-methyl)tyrosine]arginine-vasopressin and [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)]arginine-vasopressin, two highly potent antagonists of the vasopressor response to arginine-vasopressin. *J Med Chem* 23, 364–368.
24. Kobayashi E, Nakano H, Morimoto M, Tamaoki T (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* 159: 548–553.
25. Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathway: regulation and physiological functions. *Endocr Rev* 22: 153–183.
26. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle RL, Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273: 18623–18632.
27. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 364: 229–233.
28. Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G, Caverzasio J (2003) Activation of p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. *J Bone Miner Res* 18: 2060–2068.
29. Nakamura I, Takahashi N, Sasaki T, Tanaka S, Udagawa N, Murakami H, Kimura K, Kabuyama Y, Kurokawa T, Suda T, Fukui Y (1995) Wortmannin, a specific inhibitor of phosphatidylinositol-3 kinase, blocks osteoclastic bone resorption. *FEBS Lett* 361: 79–84.
30. Terada N, Franklin RA, Lucas JJ, Blenis J, Gelfand

EW (1993) Failure of rapamycin to block proliferation once resting cells have entered the cell cycle despite inactivation of $p70S_6$ kinase. *J Biol Chem* 268: 12062–12068.

31. Ito T, Kozawa O, Tanabe K, Niwa M, Matsuno H, Sakai N, Ito H, Kato K, Uematsu T (2000) p38 MAP kinase is required for vasopressin-stimulated HSP27 induction in aortic smooth muscle cells. *Hypertension* 35: 673–678.
32. Cobb MH (1999) MAP kinase pathways. *Prog Biophys Mol Biol* 71: 479–500.
33. Tibbles LA, Woodgett JR (1999) The stress-activated protein kinase pathways. *Cell Mol Life Sci* 55: 1230–1254.
34. Ip YT, Davis RJ (1998) Signal transduction by the c-jun N-terminal kinase (JNK)-from inflammation to development. *Curr Opin Cell Biol* 10: 205–219.
35. Suzuki A, Ghayor C, Guicheux J, Magne D, Quillard S, Kakita A, Ono Y, Miura Y, Oiso Y, Itoh M, Caverzasio J (2006) Enhanced expression of the inorganic phosphate transporter Pit-1 is involved in BMP-2-induced matrix mineralization in osteoblast-like cells. *J Bone Miner Res* 21: 674–683.
36. Bai H, Pollman MJ, Inishi Y, Gibbons GH (1999) Regulation of vascular smooth muscle cell apoptosis. Modulation of bad by a phosphatidylinositol 3-kinase-dependent pathway. *Circ Res* 85: 229–237.
37. Chan TO, Rittenhouse SE, Tsichlis PN (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinases activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 68: 965–1014.
38. Suga H, Nakajima K, Shu E, Kanno Y, Hirade K, Ishisaki A, Matsuno H, Tanabe K, Takai S, Akamatsu S, Kato K, Oiso Y, Kozawa O (2005) Possible involvement of phosphatidylinositol 3-kinase/Akt signal pathway in vasopressin-induced HSP27 phosphorylation in aortic smooth muscle A10 cells. *Arch Biochem Biophys* 438: 137–145.
39. Schafer ZT, Kornbluth S (2006) The apoptosome: Physiological, developmental, and pathological modes of regulation. *Dev Cell* 10: 549–561.
40. Kim KM (1976) Calcification of matrix vesicles in human aortic valve and aortic media. *Fed Proc* 35: 156–162.
41. Bielsky IF, Hu SB, Szegda KL, Westphal H, Young LJ (2004) Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. *Neuropsychopharmacology* 29: 483–493.
42. Koshimizu T, Nasa Y, Tanoue A, Oikawa R, Kawahara Y, Kiyono Y, Adachi T, Tanaka T, Kuwaki T, Mori T, Takeo S, Okamura H, Tsujimoto G (2006) V1a vasopressin receptors maintain normal blood pressure by regulating circulating blood volume and baroreflex sensitivity. *PNAS* 103: 7807–7812.
43. Wersinger SR, Ginns EL, O'Carroll A-M, Lolait SJ, Young WS III (2002) Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. *Mol Psychiatry* 7: 975–984.
44. Tachikawa K, Yokoi H, Nagasaki H, Arima H, Murase T, Sugimura Y, Miura Y, Hirabayashi M, Oiso Y (2003) Altered cardiovascular regulation in arginine vasopressin overexpressing transgenic rat. *Am J Physiol Endocrinol Metab* 285: E1161–1166.
45. Krupski WC (1991) The peripheral vascular consequences of smoking. *Ann Vasc Surg* 5: 291–304.
46. Drobnik J, Dabrowski R, Szczepanowska A, Giernat L, Lorenc J (2000) Response of aorta connective tissue matrix to injury caused by vasopressin-induced hypertension or hypercholesterolemia. *J Physiol Pharmacol* 51: 521–533.