Effects of Serum Amyloid A and Lysophosphatidylcholine on Intracellular Calcium Concentration in Human Coronary Artery Smooth Muscle Cells

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SUMMARY

Serum amyloid A (SAA), an acute-phase protein, and lysophosphatidylcholine (LPC), an oxidized LDL component, contribute to the physiological processes of atherosclerosis and cardiovascular disease. However, the effects of SAA/LPC on human coronary artery smooth muscle cells (hCASMCs) have not been fully investigated. Therefore, we examined the effects of SAA/LPC on Ca2+/Mg2+ mobilization and its underlying mechanisms in hCASMCs. Intracellular Ca2+/Mg2+ concentration ([Ca2+]i/Mg2+) was measured with fura-2 AM/mag-fura-2 AM. Conventional RT-PCR analysis was also performed. Both SAA and LPC increased [Ca2+]i by Ca2+ entry. The SAA-induced Ca2+ entry was inhibited by Gd3+, SKF96365, and 2-aminophosphindolphene borate (2-APB), a nonselective transient receptor potential (TRP) channel blocker, but not nifedipine. The LPC-induced Ca2+ entry was blocked by Gd3+, but not nifedipine, SKF96365 and 2-APB. U-73122 and PTX prevented the activation of SAA-, but not LPC-induced Ca2+ influx. LPC, but not SAA, increased [Mg2+]i as well as [Ca2+]i. The RT-PCR analysis revealed the expression of TRPC1/4, TRPV1/2/4, and TRPM7/8 mRNA. These results suggest that SAA/LPC activate Ca2+ influx in hCASMCs; SAA activates it via PTX-sensitive G-protein, PLC and TRPC pathways, while LPC activates it independently of these pathways, where TRPM7 may be partly involved. Thus, TRP protein appears to be a target molecule of Ca2+ signaling in hCASMCs elicited by SAA/LPC, which may play roles in coronary muscle dysfunction under pathophysiological and inflammatory conditions such as atherosclerosis. (Int Heart J 2011; 52: 185-193)

Key words: Serum amyloid A, Human coronary arterial smooth muscle cells, Lysophosphatidylcholine, Transient receptor potential, Fura-2 AM, GTP-binding protein, TRPM7

Serum amyloid A (SAA) proteins are a family of apolipoproteins associated with high-density lipoproteins (HDL) in plasma. Its structure has four homologous, alpha-helical proteins encoded by the genes located on chromosome 11 in humans. It consists of SAA1/SAA2, acute phase proteins, and SAA4, which is expressed constitutively. SAA3 is not expressed in human.

These proteins are produced predominantly by the liver in response to inflammation, but they are also synthesized in endothelial cells, smooth muscle cells and macrophages at the atherosclerotic lesion. The plasma concentration of SAA is normally below 0.08 μM, however, during acute inflammation, IL-1, IL-6 and TNF induce secretion of SAA, resulting in an increase in the plasma concentration to more than 40 μM. Furthermore, the production of SAA by smooth muscle cells and other nonhepatic cells suggests that SAA is not only a sensitive marker of acute inflammatory and atherosclerotic state but contributes to the progression of the inflammation and atherosclerosis. SAA levels have also been reported to be elevated in patients with atherosclerosis diseases, diabetes and obesity.

Johnson, et al (2004) reported that the SAA level is associated with the severity of coronary disease including a number of coronary lesions and diseased coronary branches. SAA has several biological functions in the vascular system. It causes endothelial dysfunction of porcine coronary arteries and human coronary endothelial cells by producing reactive oxygen species, and decreasing eNOS expression. It also enhances smooth muscle cell migration. These functions may play a role in remodeling of the vessel under the various conditions including atherosclerosis.

On the other hand, lysophosphatidylcholine (LPC, 1-acyl-sn-glycero-3-phosphocholine), also called lyssolecithin, is a major phospholipid component of oxidized low-density lipoprotein and is a bioactive proinflammatory lipid. It plays an important role in atherosclerosis, and acute and chronic inflammation. LPC is a class of chemical compound derived from phosphatidylcholine (PC) of lipoprotein or from cell membrane-derived PC by hydrolyzing PC. The hydrolysis is induced by the enzymatic action of phospholipase A2 (PLA2) and lecithin-cholesterol acyl-transferase (LCAT), which is secreted from the liver and transfers a fatty acid from PC to cho-
lsterol. In plasma, most of the circulating LPC is reversibly bound to albumin, erythrocytes and lipoproteins. Albumin acts as a reservoir for LPC, effectively controlling LPC bioavailability. Concentrations of LPC in vivo are in the range of 5-180 μM and vary in different tissues and body fluids. However, increased concentrations of LPC in blood have been reported in several diseases, such as atherosclerosis, diabetes, hyperlipidemia, and obesity. LCAT activity levels have also been reported to be increased depending on the severity of coronary atherosclerosis and the changes in the activity of this enzyme are appropriately reflected by an increase in LPC concentration in plasma. Thus, atherosclerotic arteries are chronically exposed to high concentrations of LPC as compared with normal arteries. LPC-mediated biological effects are diverse on numerous types of cells, including coronary artery cells. LPC impairs endothelium-dependent relaxing factor-mediated vasodilatation and inhibits nitric oxide production. It has been also reported to increase reactive oxygen species (ROS) generation, and induce up-regulation of cytokines (IL-1 has been also reported to increase reactive oxygen species (1995). TRPC5 could be activated by lysophospholipids in HEK293 cells.

TRPV1 channel activity is required for LPC-induced [Ca\(^{2+}\)]\(_i\) influx in normal arteries. In vivo, LPC activates Ca\(^{2+}\) influx pathways in hCASMCs; SAA activates via a PTX-sensitive G-protein and PLC that is carried by TRPC channels, while LPC activates Ca\(^{2+}\) influx pathway independently of these pathways, where TRPM7 may be partly involved. Thus, TRP protein appears to be a target molecule of Ca\(^{2+}\) signaling in hCASMCs elicited by SAA and LPC, an inflammatory substance.

**Methods**

Cell culture of human coronary artery smooth muscle cells (hCASMCs): Human coronary artery smooth muscle cells (hCASMCs) were obtained from Clonetics Corporation (Palo Alto, CA, USA). Cells were maintained at 37°C under 5% CO\(_2\) in Smooth Muscle Growth Medium-2 (ShmGM-2, Clonetics) containing 5% fetal bovine serum (FBS), recombinant human epidermal growth factor (rhEGF, 0.5 μg/mL), recombinant human fibroblast growth factor (rhFGF, 1 μg/mL), recombinant human insulin (5 μg/mL), gentamicin (50 μg/mL), and amphotericin-B (0.05 μg/mL). At confluence, the cells were detached using 0.05% trypsin in 0.02% EDTA, and cultured into the medium. Medium was replaced more than twice weekly. Cells before confluence at passage 5-9 were detached from the culture dishes with 0.05% trypsin in 0.02% EDTA, and cultured into the medium. Medium was replaced more than twice weekly. Cells before confluence at passage 5-9 were detached from the culture dishes with 0.05% trypsin in 0.02% EDTA, and used for later experiments.

Solutions and drugs: Serum amyloid A (SAA) was obtained from Pepro Tech, Inc. (Rocky Hill, NJ) and dissolved in PBS at a concentration of 1 mg/mL. Lysophosphatidylcholine (LPC, palmitoyl, C16:0) was purchased from Sigma (Poole, UK) and dissolved in PBS at a concentration of 5 mM. Pertussis toxin (PTX, Funakoshi, Japan) and cholera toxin (LIST Biological Laboratories, Inc., Campbell, California, USA) were prepared as a stock solution of 200 μg/mL in H.2O. Fura-2 AM and mag-fura-2 AM were purchased from Sigma.

Measurement of intracellular Ca\(^{2+}\) concentration: Intracellular Ca\(^{2+}\) concentration was measured by a fura-2 fluorescence dual-wavelength excitation method as described previously. Cells were incubated with 2 μM fura-2 AM for 30 minutes at 37°C under 5% CO\(_2\). After fura-2 loading, cells were isolated with 0.25% trypsin and diluted into 10\(^5\) cells/mL. Tyrode without Ca\(^{2+}\) and with 0.1 mM EGTA. The fluorescence ratio between excitation and emission was continuously measured with constant stirring with an intracellular ion analyzer CAF-110 (JASCO Corporation, Japan). Fura-2 fluorescence was measured by excitation at 340 nm and 380 nm and emission at 500 nm.

The selective fluorescent probe, mag-fura-2 AM, was used to measure [Mg\(^{2+}\)]. Cells were loaded with mag-fura-2 AM (4 μM), which was dissolved in dimethyl sulfoxide with 0.02% pluronic acid. After mag-fura-2 loading, cells were isolated with 0.25% trypsin and diluted into 10\(^5\) cells/mL. Tyrode without Mg\(^{2+}\) and with 0.1 mM EDTA. The fluorescence ratio between excitation and emission was continuously measured with constant stirring with an intracellular ion analyzer CAF-110 (JASCO Corporation) in a similar manner to fura-2 AM.
RNA extraction and reverse transcription/polymerase chain reaction (RT-PCR): To determine the expression of TRP channels in hCASMCs by RT-PCR, total cellular RNA was extracted from the cultured cells using an RNeasy mini kit (Qiagen, Cambridge, MA). For RT-PCR, complementary DNA (cDNA) was synthesized from 1 µg of total RNA with reverse transcriptase with random primers (Toyobo, Osaka). The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 35 cycles consisting of heat denaturation, annealing, and extension. The cycling conditions were: denaturation at 98°C for 20 seconds, annealing at 52-56°C for 10 seconds, extension at 74°C for 1 minute. PCR products were size-fractionated on 2% agarose gels, and stained with ethidium bromide and visualized under UV light. Primers were chosen based on the sequence of human TRP family genes (TRPC1, 3, 4, 5, 6, 7, TRPV1-6, and TRPM1-8) as shown in the Table.

Data analysis: All values are expressed as the mean ± SEM. Differences between groups were compared by ANOVA. Two-group analysis was performed with the Student t-test. Differences were considered significant if P < 0.05

Results

Effects of SAA and LPC on [Ca\(^{2+}\)] mobilization in hCASMCs: The effects of SAA and LPC on [Ca\(^{2+}\)] were investigated using the Ca\(^{2+}\)-sensitive dye fura 2-AM. As shown in Figure 1A, both SAA (10 µg/mL, Figure 1Aa) and LPC (10 µM, Figure 1Ab) increased [Ca\(^{2+}\)], in the presence of extracellular Ca\(^{2+}\). The SAA-induced [Ca\(^{2+}\)], rise quickly returned to a near control level within several minutes (Figure 1Aa), while LPC induced a sustained rise in [Ca\(^{2+}\)], in the presence of extracellular Ca\(^{2+}\) (Figure 1Ab). After the SAA-induced [Ca\(^{2+}\)], rise returned to a control level, additional application of LPC induced a sustained rise in [Ca\(^{2+}\)] (Figure 1Ac). On the other hand, in cells immersed in Ca\(^{2+}\)-free standard solution, SAA (10 µg/mL, Figure 1B) failed to increase [Ca\(^{2+}\)]. The subsequent application of histamine (100 µM) transiently increased [Ca\(^{2+}\)], due to Ca\(^{2+}\)-release from intracellular storage sites, suggesting that the SAA-increased [Ca\(^{2+}\)], rise was mainly due to the entry of extracellular Ca\(^{2+}\). The effects of extracellular Ca\(^{2+}\) on LPC-induced [Ca\(^{2+}\)], rise are presented in Figure 1C. In the absence of

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Figure 1. Effects of SAA and LPC on [Ca\(^{2+}\)], in hCASMCs. The Ca\(^{2+}\)-free bathing solution contained 0.1 mM EGTA in the absence of extracellular Ca\(^{2+}\). 0.9 mM Ca\(^{2+}\) was added into the solution to obtain Ca\(^{2+}\)-containing solution. A: Effects of SAA (Aa and Ac) and LPC (Ab and Ac) in the presence of extracellular Ca\(^{2+}\). Note that SAA transiently increased [Ca\(^{2+}\)], and LPC induced a sustained rise in [Ca\(^{2+}\)]. B: Effects of SAA and histamine in the absence of extracellular Ca\(^{2+}\). Note that SAA failed to increase [Ca\(^{2+}\)], but the additional application of histamine transiently increased [Ca\(^{2+}\)], due to Ca\(^{2+}\)-release from Ca\(^{2+}\) store sites. C: Effects of LPC on [Ca\(^{2+}\)]. The [Ca\(^{2+}\)], rise elicited by Ca\(^{2+}\)-entry due to the addition of Ca\(^{2+}\) was compared in a cell bathed with LPC (10 µM, Ca) or without (Cb). Note that LPC only slightly increased [Ca\(^{2+}\)], but the additional application of Ca\(^{2+}\) markedly increased [Ca\(^{2+}\)], due to Ca\(^{2+}\)-entry. The data are representative of three different experiments.
extracellular Ca\(^{2+}\), LPC (10 \(\mu\)M) only slightly increased [Ca\(^{2+}\)], while the addition of Ca\(^{2+}\) into the bath solution markedly induced a sustained rise in [Ca\(^{2+}\)], due to Ca\(^{2+}\) entry (Figure 1C). In contrast, in the absence of LPC, Ca\(^{2+}\) entry was minimal (Figure 1Cb). These results suggest that LPC induced Ca\(^{2+}\) entry in hCASMCs as reported previously in rabbit coronary artery smooth muscle cells.\(^{30}\)

Figure 2 shows the concentration-dependent effects of SAA on [Ca\(^{2+}\)]. SAA (Figure 2A, 0.1-10 \(\mu\)g/mL) transiently increased [Ca\(^{2+}\)], in a concentration-dependent manner. The concentration-dependency of SAA is shown in Figure 2B (n = 3). SAA at concentrations above 0.3 \(\mu\)g/mL significantly increased [Ca\(^{2+}\)], in a concentration-dependent manner.

Figure 3 illustrates the concentration-dependent effects of LPC on [Ca\(^{2+}\)]. Since LPC at high concentrations exceeding 50 \(\mu\)M has been shown to induce a toxic and detergent effect, we tested concentrations lower than 25 \(\mu\)M. As shown in Figure 3A, LPC (3-25 \(\mu\)M) significantly increased [Ca\(^{2+}\)], in a concentration-dependent manner. The concentration-dependency of LPC is presented in Figure 3B. LPC at concentrations above 3 \(\mu\)M increased [Ca\(^{2+}\)], in a concentration-dependent manner.

Effects of various drugs on SAA- and LPC-induced [Ca\(^{2+}\)], rise: The above results suggest that SAA and LPC increase [Ca\(^{2+}\)], via enhancement of Ca\(^{2+}\) entry. Therefore, we investigated whether several blockers inhibit calcium mobilization induced by SAA and LPC. First, we added 10 \(\mu\)M nifedipine, an L-type Ca\(^{2+}\) channel blocker, into the cuvette before the addition of extracellular calcium (0.9 mM) and SAA or LPC. As shown in Figures 4Aa and 4Ab, inclusion of 10 \(\mu\)M nifedipine in the bath solution did not significantly affect SAA- and LPC-induced [Ca\(^{2+}\)], rise (Figure 4Ab), compared with the control cells (Figure 4Aa), suggesting that the L-type Ca\(^{2+}\) channel does not significantly contribute to the Ca\(^{2+}\) rise. Similarly, nifedipin (10 \(\mu\)M), a T-type Ca\(^{2+}\) channel blocker, did not affect the SAA- and LPC-induced [Ca\(^{2+}\)], rise (Figures 4B and C). These results suggest that L-type and T-type calcium channels do not participate in SAA- and LPC-induced [Ca\(^{2+}\)], rise.

Therefore, to further determine the type of channels involved in calcium influx, we next examined the effects of the relatively nonspecific TRP channel blocker Gd\(^{3+}\) (100 \(\mu\)M, Figure 4Ac). Both SAA (10 \(\mu\)g/mL) and LPC (10 \(\mu\)M) failed to increase [Ca\(^{2+}\)], in cells pretreated with Gd\(^{3+}\) (100 \(\mu\)M, Figure 4Ac). These results suggest that LPC as well as SAA increase [Ca\(^{2+}\)], via a non-voltage-dependent Ca\(^{2+}\) channel such as TRP, and the toxic or detergent effect of LPC is unlikely. Furthermore, the effects of various drugs on SAA- and LPC-induced [Ca\(^{2+}\)], rise were also investigated. We used 2-aminoethoxydiphenyl borate (2-APB) and SKF96365, which are blockers of TRP channels. Pretreatment of 2-APB (100 \(\mu\)M, Figure 4Ad) or SKF96365 (50 \(\mu\)M, Figure 4 Ae) significantly inhibited SAA-induced [Ca\(^{2+}\)], rise, while it failed to inhibit LPC-induced [Ca\(^{2+}\)], rise. The inhibitory effects of various blockers are summarized in Figures 4B and 4C. SKF96365 almost completely abolished SAA-induced Ca\(^{2+}\) mobilization. Similarly, 300 \(\mu\)M 2-APB reduced it by 81.3 \(\pm\) 5.4 % compared to the control (n = 3). In contrast, pretreatment of SKF96365 (50 \(\mu\)M, Figure 4d) or 2-APB (300 \(\mu\)M, Figure 4e) did not significantly inhibit the LPC-induced [Ca\(^{2+}\)] rise.

Effects of preincubation of pertussis toxin on [Ca\(^{2+}\)], mobilization in hCASMCs: SAA is known to be coupled to multiple trimeric G proteins.\(^{27,45-47}\) Therefore, we investigated the effects of pertussis toxin (PTX), which selectively inactivates Gi protein, on SAA- and LPC-induced Ca\(^{2+}\) mobilization in hCASMCs. The cells were pretreated with PTX (Figure 5A, 2 \(\mu\)g/mL) for 2 hours, and the Ca\(^{2+}\) response to SAA (10 \(\mu\)g/mL), histamine (100 \(\mu\)M) and LPC (10 \(\mu\)M) in the presence of extracellular Ca\(^{2+}\) was compared with control cells not treated with these agents. As shown in Figure 5A, PTX markedly sup-
pressed SAA-induced Ca\(^{2+}\) mobilization (Figure 5Ab), compared with control cells (Figure 5Aa). On the other hand, PTX did not significantly inhibit histamine- and LPC-induced Ca\(^{2+}\) mobilization. The data are summarized in Figure 5B. Thus, SAA, but not LPC, appears to activate PTX-sensitive G proteins (Gi), and then Ca\(^{2+}\) mobilization in hCASMCs. In addition, the cells were pretreated with choler toxin (an activator of Gs protein), 2 \(\mu\)g/mL for 2 hours, and the Ca\(^{2+}\) response to SAA (10 \(\mu\)g/mL) and histamine (100 \(\mu\)M) in the presence of extracellular Ca\(^{2+}\) was compared with control cells. Cholera toxin did not affect SAA- and LPC-induced Ca\(^{2+}\) mobilization (data not shown).

**Effects of U73122 on SAA and LPC-induced [Ca\(^{2+}\)]\(_i\) rise in hCASMCs:** In addition, we investigated the effects of U-73122, a specific inhibitor of phospholipase C (PLC), which is known to be activated upon Go/\gamma liberation from trimeric G proteins, on SAA- and LPC-induced Ca\(^{2+}\) mobilization in hCASMCs (Figure 5C). U-73122 abolished the SAA-induced Ca\(^{2+}\) mobilization (Figures 5Cb and D), compared with control cells (Figure 5Ca). On the other hand, it failed to inhibit LPC-induced Ca\(^{2+}\) mobilization (Figures 5Cb and D). These results suggest that G protein coupled PLC is required for SAA-induced Ca\(^{2+}\) mobilization, but not for LPC-induced Ca\(^{2+}\) mobilization in hCASMCs.

**Expression of TRP family mRNA in hCASMCs:** We investigated the expression of the TRP channel gene members TRPC, TRPV, and TRPM in hCASMCs (Figure 6, left part). Mouse adult brain was used as a positive control (right part). Figures 6A, 6B, and 6C show the RT-PCR analysis of the transcripts of TRPC1-C7, TRPV1-V6 and TRPM1-8, respectively. Among TRPCs, TRPC2 is a pseudo-gene in Homo sapiens, and therefore, we investigated the expression of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7. TRPC1 and TRPC4 mRNA were detected in hCASMCs, as compared with mouse adult brain. The amplitude of cDNA fragments was of the predicted molecular size, identical to cDNA fragments amplified from reversely transcribed mRNA. Among TRPV1-6, TRPV1, TRPV2 and TRPV4 were detected in hCASMCs, but TRPV3, TRPV5, TRPV6 were not detected. The only signifi-
The major findings of the present study are as follows. 1) Both SAA and LPC increased intracellular calcium concentration ([Ca$^{2+}$]) due to calcium influx from extracellular medium in hCASMCs. 2) The SAA-induced Ca$^{2+}$ entry was inhibited by Gd$^{3+}$, SKF96365 and 2-aminoethoxydiphenyl borate (2-APB), while the LPC-induced Ca$^{2+}$ entry was blocked by Gd$^{3+}$, but not by SKF96365 and 2-APB. 3) SAA-induced [Ca$^{2+}$], rise required Gi protein and PLC activity, while on the contrary, LPC-induced [Ca$^{2+}$], rise required neither G protein nor PLC activity. 4) LPC, but not SAA, increased Mg$^{2+}$ influx as well as Ca$^{2+}$. 5) The RT-PCR analysis revealed the expression of TRPC1/4, TRPV1/2/4, and TRPM7/8 mRNA. These results suggested that SAA and LPC activate Ca$^{2+}$ influx in hCASMCs; SAA activates it via PTX-sensitive G-protein, PLC and TRPC pathways, while LPC activates it independently of these pathways, where TRPM7 may be partly involved. Thus, TRP protein appears to be a target molecule of Ca$^{2+}$ signaling in hCASMCs elicited by SAA/LPC, which may play roles in coronary muscle dysfunction in pathophysiological and inflammatory conditions such as atherosclerosis.

Transient receptor potential (TRP) channels were initially identified in Drosophila. Later, TRP channels were found in vertebrates. They are ubiquitously expressed in various cell types and play vital roles in the regulation of cellular functions such as vasoconstriction and cell proliferation.42) The existence of TRP channels has also been described in smooth muscle cells.43) However, the molecular identities and function of TRP channels remain largely unexplored in hCASMCs, and only a few studies have examined the expression of TRP channels. To date, 7 TRPC subtypes have been identified: TRPC1 to TRPC7, and classified into 4 subgroups based on their amino acid sequences: TRPC1, TRPC3/6/7, and TRPC4/5. TRPC2 is a pseudo-gene in humans. Takahashi, et al (2007)44) reported using standard RT-PCR analysis that hCASMCs constitutively expressed TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 genes in cultured hCASMCs. In the present study, we confirmed the expression of TRPC1 and TRPC4 among TRPC homologues but failed to detect TRPC3, TRPC5, TRPC6 and TRPC7, which is inconsistent with Takahashi, et al (2007).45)
The reason for this discrepancy is unclear, but it may be due to the different cell culture conditions. In addition, we provided the first evidence showing that TRPV1, TRPV2, TRPV4, TRPM7 and TRPM8 were also detected among TRPV and TRPM homologues in hCASMCs.

A few studies have reported that SAA increases Ca\(^{2+}\) influx in human monocytes\(^{35}\) and neutrophils.\(^{40}\) SAA has also been reported to use a receptor that functionally couples to a Gi protein in several cells such as neutrophils,\(^{27,40,46}\) Badolato, et al. (1995)\(^{37}\) described that SAA induces Ca\(^{2+}\) mobilization and chemotaxis by enhancing Ca\(^{2+}\) entry in human monocytes, both of which were blocked by pertussis toxin (PTX). PTX selectively mediates adenosine diphosphate (ADP) ribosylation of the Gi class of G proteins, suggesting that the effects of SAA are mediated by a receptor that functionally couples to a Gi protein. On the other hand, it has been reported that SAA induces CCL2 production in human umbilical vein endothelial cells via a PTX-insensitive pathway.\(^{47}\) Thus, multiple signaling pathways may exist in SAA actions. The present study showed that PTX and U-73122, an inhibitor of PLC, inhibited SAA-induced Ca\(^{2+}\) influx pathways via PTX-sensitive G protein (Gi)-PLC pathways in hCASMCs. Kumon, et al. (2002)\(^{38}\) showed that SAA induced and PTX inhibited cell migration in cultured rat aortic smooth muscle cells. Since intracellular Ca\(^{2+}\) plays an essential role in cell migration, SAA may induce cell migration via enhancing the Ca\(^{2+}\) mobilization pathway shown in our study. In fact, we observed that SAA enhanced the cell migration of hCASMCs (data not shown). Furthermore, we investigated the possibility of involvement of TRP channels in SAA-induced [Ca\(^{2+}\)]\(_i\) rise. The voltage-dependent Ca\(^{2+}\) channel (L-type and T-type) serves diverse biological functions in vascular smooth muscle cells, and has been reported to be expressed in vascular smooth muscle cells in primary culture.\(^{40}\) However, neither nifedipine nor mibebradil inhibited SAA-induced Ca\(^{2+}\) mobilization, indicating that SAA induces Ca\(^{2+}\) mobilization through a non-voltage-dependent Ca\(^{2+}\) channel. Alternatively, the compounds Gd\(^{3+}\), SKF96365 and 2-aminoethoxydiphenyl borate (2-APB), a nonselective transient receptor potential (TRP) blocker, inhibited SAA-induced Ca\(^{2+}\) mobilization, suggesting that the involvement of TRP channels is likely. As mentioned above, we confirmed gene expression of TRPC1/C4, TRPV1/V2/V4 and TRPM7/M8. 2-APB, which has been reported to inhibit TRPC1, TRPC4 and TRPM8, blocked the SAA-induced [Ca\(^{2+}\)]\(_i\) rise. In addition, 2-APB, an activator of TRPV1-3,\(^{49}\) and menthol, an activator of TRPM8, and 4α-phorbol 12,13-didecanoate (4 α-PDD), an activator of TRPV4,\(^{50}\) failed to induce Ca\(^{2+}\) mobilization significantly. From these observations, it is likely that TRPC1 and/or TRPC4 can be activated by SAA via PTX-sensitive G protein-PLC pathways. Histamine, but not SAA, transiently increased [Ca\(^{2+}\)]\(_i\) due to Ca\(^{2+}\) release from intracellular storage sites in the absence of extracellular Ca\(^{2+}\), suggesting that SAA increased [Ca\(^{2+}\)]\(_i\), rise was independent of the filling state of internal Ca\(^{2+}\) stores, but dependent on the activation of PLC. Since many studies have suggested that TRPC1 is assembled in a Ca\(^{2+}\) signaling complex composed of a PTX-insensitive Gq subunit,\(^{51}\) TRPC4 appears to be a target molecule of SAA via PTX-sensitive G protein and PLC pathways. In fact, it has been reported that TRPC4 can be activated by G protein-coupled receptors.\(^{52,53}\) Shafer, et al. (2000)\(^{54}\) showed that TRPC4 forms nonselective cation channels that integrate signaling pathways from G-protein-coupled receptors, and the activity of TRPC4 is dependent on the activation of PLC. However, further studies using a specific blocker of TRPC4 such as siRNA are required to clarify the possibility. The TRPC3/6/7 subfamily of TRPCs has been reported to be activated subsequent to PLC stimulation, either as a result of In sP3-mediated signaling cascades\(^{55,56}\) or the lipid second messenger diacylglycerol.\(^{57,58}\) In contrast to TRPC3/6/7, diacylglycerol does not activate TRPC4. In the present study, OAG, a diacylglycerol analogue, did not increase [Ca\(^{2+}\)], (data not shown). Thus, the steps leading to channel activation following PLC activation remain unclear, and further studies are also needed to clarify the signaling pathways.

A previous report suggested that LPC induces Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels.\(^{59}\) However, in our study, neither nifedipine nor mibebradil blocked Ca\(^{2+}\) influx induced by LPC, suggesting that LPC induced Ca\(^{2+}\) mobilization through a non-voltage-dependent Ca\(^{2+}\) channel. On the other hand, several papers have suggested that LPC activates some types of TRP channels. So, et al. (2005)\(^{47}\) reported that LPC causes an increase of [Ca\(^{2+}\)]\(_i\) via TRPC6 in cultured human corporal smooth muscle cells. Furthermore, LPC induces migration of the monocyte cell line THP-1 requiring TRPC6/TRPV1 activity.\(^{48}\) LPC can also activate TRPC5 on vascular smooth muscle cells and HEK293 cells over-expressing this channel,\(^{49}\) and promote TRPM8 channel activity.\(^{50}\) On the other hand, Monet, et al. (2009)\(^{51}\) reported that LPC and lysophosphatidylglycerol (LPI) stimulate prostate cancer cell migration via TRPV2 channel activation. Thus, the target molecule of LPC on Ca\(^{2+}\) entry pathways may be different, depending on the cell type or the concentration of LPC used in the study. In our study, to clarify the molecular target of LPC in hCASMCs, we examined the expression of TRP channel family using conventional RT-PCR analysis. In our study, among these TRPC5, TRPC6, TRPV1, TRPV2 and TRPM8, the expression of only TRPV1 and TRPM8 was found in hCASMCs. In addition, OAG (TRPC3/6/7 agonist),\(^{52}\) 2-APB (TRPV1-3 agonist), 4α-PDD (TRPV4 agonist),\(^{53}\) and menthol, an activator of TRPM8,\(^{54}\) did not mimick the effects of LPC on Ca\(^{2+}\) mobilization (data not shown), suggesting that the involvement of TRPV1-4, TRPC3/6/7 and TRPM8 in LPC-induced Ca\(^{2+}\) entry is unlikely. In addition, involvement of TRPC1 and TRPC4 was also excluded, because 2-APB blocked SAA-induced Ca\(^{2+}\) mobilization, but not LPC-induced Ca\(^{2+}\) mobilization. Furthermore, LPC caused an increase in Mg\(^{2+}\) influx as well as Ca\(^{2+}\). TRPM7 is permeable to both of the divalent cations, Ca\(^{2+}\) and Mg\(^{2+}\).\(^{55}\) Thus, it is very likely that LPC activates Mg\(^{2+}\)- and Ca\(^{2+}\)-permeable channel pathway in hCASMCs, where TRPM7 may be partly involved. The activation of TRPM7 induced by other substances such as dimethylphytosphingosine and Bradykinin has been reported in U937 monocytes and vascular smooth muscle cells.\(^{56,57}\)

The plasma level of SAA in healthy subjects without acute inflammation is approximately 2-4 μg/mL or less. On the other hand, it may be increased by as much as 1000-fold above its baseline levels, reaching a concentration of 80 μM in acute inflammation recognized as a hallmark of atherosclerotic lesion.\(^{60,61}\) Johnson, et al (2004)\(^{62}\) reported that women with definite coronary artery diseases had mean plasma SAA levels of 17.9 μg/mL (ranging from 0.2 to 731 μg/mL), and higher lev-
els of plasma SAA were positively associated with the severity of cardiovascular disease and the risk of cardiovascular events. Thus, the concentrations of SAA used in our study (0.1-10 µg/mL) are clinically relevant. In the present study, SAA induced Ca\(^{2+}\) mobilization at concentrations lower than 1 µg/mL, which are near to the plasma level of SAA in healthy subjects without any inflammation. However, SAA in the blood is usually bound to high-density lipoprotein (HDL). Also, the effects of SAA, including chemotactic activity, have been reported to be inhibited by HDL, suggesting that the effects of SAA on hCASMCs shown in the present study may be inactive in circulation.\(^4\) On the other hand, in pathophysiological and inflammatory conditions where the production of SAA is enhanced, or SAA dissociates from HDL in tissue, the effects of SAA on hCASMCs may appear.

On the other hand, LPC increases during ischemia, and the concentration may reach 990 µM.\(^5\) In addition, atherosclerotic arteries have been reported to be chronically exposed to high concentrations of LPC as compared with normal arteries.\(^5\) \(^6\) Wells, et al (1986)\(^6\) reported that the changes in LPC concentrations from 87 to 292 µM tend to parallel the plasma activity levels of LCAT, which are increased when the severity of coronary atherosclerosis increases. Thus, LPC may accumulate in pathophysiological conditions such as ischemia and atherosclerosis. The present study showed that LPC at concentrations of 3-25 µM induced [Ca\(^{2+}\)]\(_i\) rise. Thus, the effects of LPC observed in this study may play significant roles in pathophysiological conditions such as ischemia and atherosclerosis. LPC is known to be mainly generated from PLA2-dependent hydrolysis of PC. Recently, Sullivan, et al (2010)\(^6\) reported that SAA increased secretory PLA2 in cultured smooth muscle cells. These observations suggest that SAA may potentiate LPC production in pathophysiological conditions such as ischemia and atherosclerosis.

In conclusion, SAA and LPC activate Ca\(^{2+}\) influx in hCASMCs; SAA activates it via PTX-sensitive G-protein, PLC and TRPC pathways, while LPC activates it independently of these pathways, where TRPM7 may be partly involved. Thus, TRP protein appears to be a target molecule of Ca\(^{2+}\) signaling in hCASMCs elicited by SAA and LPC, an inflammatory substance, which may play a role in coronary muscle dysfunction in pathophysiological and inflammatory conditions such as atherosclerosis.

**References**

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