

Identification of 9-Hydroxyoctadecadienoic Acid and Other Oxidized Free Fatty Acids as Ligands of the G Protein-coupled Receptor G2A*

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G2A is a G protein-coupled receptor that is predominantly expressed in lymphoid tissues and macrophages. G2A can be induced by diverse stimuli to cause cell cycle arrest in the G₂/M phase in pro-B and T cells. G2A is also expressed in macrophages within atherosclerotic lesions, suggesting G2A involvement in atherosclerosis. Recently, G2A was discovered to possess proton-sensing ability. In this paper, we report another function of G2A, that is, as a receptor for 9-hydroxyoctadecadienoic acid (9-HODE) and other oxidized free fatty acids. G2A, expressed in CHO-K1 or HEK293 cells, showed 9-HODE-induced intracellular calcium mobilization, inositol phosphate accumulation, inhibition of cAMP accumulation, [³⁵S]guanosine 5'-3-O-(thio)triphosphate binding, and MAP kinase activation. Furthermore, G2A was activated by various oxidized derivatives of linoleic and arachidonic acids, but it was weakly activated by cholesteryl-9-HODE. Oxidized phosphatidylcholine (1-palmitoyl-2-linoleoyl) when hydrolyzed with phospholipase A₂ also evoked intracellular calcium mobilization in G2A-expressing cells. These results indicate that G2A is activated by oxidized free fatty acids produced by oxidation and subsequent hydrolysis of phosphatidylcholine or cholesteryl linoleate. Thus, G2A might have a biological role in diverse pathological conditions including atherosclerosis.

G2A (derived from G₂ accumulation) was first identified as a stress inducible G protein-coupled receptor (GPCR)² (1). G2A causes cell cycle arrest in the G₂/M-phase in pro-B and T cells when these cells are treated with various DNA-damaging stimuli (1). Mice lacking G2A were shown to develop a late-onset autoimmune syndrome similar to systemic lupus erythematosus (2), suggesting that G2A plays a critical role in controlling peripheral lymphocyte homeostasis. Involvement of G2A in the pathogenesis of atherosclerosis was also suggested, as G2A is

expressed in macrophages in murine, rabbit, and human atherosclerotic plaques (3).

Although G2A was initially identified as an orphan receptor, Kabarowski *et al.* (4) once reported that lysophosphatidylcholine (LPC) and sphingosylphosphorylcholine (SPC) were potent ligands for G2A. By using G2A-expressing cells, they demonstrated that radiolabeled LPC and SPC specifically bound to cell membranes, and that LPC and SPC induced intracellular signaling such as a transient [Ca²⁺]_i increase and mitogen-activated protein kinase (MAP kinase) activation. Furthermore, in their subsequent papers, they showed T cell (5) and macrophage (6) chemotaxis to LPC via G2A. However, they recently retracted the first paper (4) because they were unable to reproduce the specific binding of LPC to G2A-expressing cells (7). Although the possibility of a direct interaction between LPC and G2A cannot be ruled out, the indirect action of LPC on G2A via an as yet identified mechanism is more likely.

G2A forms a GPCR subfamily defined by amino acid sequence homology along with three other GPCRs, namely, G protein-coupled receptor 4 (GPR4) (8, 9), T cell death-associated gene 8 (TDAG8, also known as GPR65) (10), and ovarian cancer G protein-coupled receptor 1 (OGR1, also known as GPR68) (11). A series of recent studies have shown that these four receptors function as proton-sensing receptors (12–15). Murakami *et al.* (13) demonstrated that G2A mediates acidic pH-sensitive accumulation of inositol phosphates (IP) and activation of *zif268* promoter, and that LPC inhibits these pH-dependent activations of G2A in a dose-dependent manner. However, Radu *et al.* (16) reported more recently that G2A is less sensitive to pH fluctuations than the other three receptors in terms of IP and cAMP accumulation. By using receptor-deficient mice, they showed that TDAG8, but not G2A, is required for acidic pH-induced cAMP accumulation in splenocytes, although both the receptors are coexpressed in the cells (16). Thus, G2A might have another function in immune cells that has not yet been identified.

During the course of a de-orphaning project of some orphan GPCRs, we found that G2A expressed in mammalian cells responded to some oxidized free fatty acids, such as 9-hydroxyoctadecadienoic acid (HODE). 9-HODE is an oxidized derivative of linoleic acid and one of the major lipid components of oxidized low-density lipoproteins (LDLs). In this report, we demonstrate that 9-HODE causes G2A activation resulting in intracellular calcium mobilization, [³⁵S]GTPγS binding, inhibition of cAMP formation, and MAP kinase activation. 9-HODE in its free fatty acid form, but not in the form incorporated in phosphatidylcholine (PC) or cholesteryl ester, is effective in activating G2A. Based on the expression of G2A in lymphocytes, macrophages, and atherosclerotic plaques, our results suggest the newly identified aspects of G2A that might be involved in atherosclerosis and other oxidative pathological conditions.

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² The abbreviations used are: GPCR, G protein-coupled receptor; LPC, lysophosphatidylcholine; SPC, sphingosylphosphorylcholine; MAP kinase, mitogen-activated protein kinase; GPR4, G protein-coupled receptor 4; TDAG8, T cell death-associated gene 8; OGR1, ovarian cancer G protein-coupled receptor 1; IP, inositol phosphate; HODE, hydroxyoctadecadienoic acid; LDL, low density lipoprotein; PC, phosphatidylcholine; HODE, hydroperoxyoctadecadienoic acid; HETE, hydroxyeicosatetraenoic acid; PTX, pertussis toxin; CHO-K1, Chinese hamster ovary K1; HEK293, human embryonic kidney 293; BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase; AMVN, 2,2'-azobis(2,4-dimethylvaleronitrile); PLA₂, phospholipase A₂; sPLA₂, secretory type of phospholipase A₂; sPLA₂-X, group X secretory type of phospholipase A₂; GTPγS, guanosine 5'-3-O-(thio)triphosphate; EPPS, N'-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid; OxPC, oxidized phosphatidylcholine; MES, 2-(N-morpholino)ethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials—Various HODE, hydroperoxyoctadecadienoic acid (HPODE), and hydroxyeicosatetraenoic acid (HETE) species, and cholesteryl-9-HODE were purchased from Cayman Chemical. Linoleic acid, arachidonic acid, LPC (1-palmitoyl), and PC (1-palmitoyl-2-linoleoyl) were from Sigma. Pertussis toxin (PTX) was from Calbiochem. [³⁵S]GTPγS was from PerkinElmer Life Sciences.

Synthesis of 9(S)-HODE—Potato 5-lipoxygenase that functions as 9-lipoxygenase of linoleic acid was purified from potato tubers according to the method described by Shimizu *et al.* (17). Linoleic acid was incubated with potato 5-lipoxygenase in a reaction buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM diethylenetriaminepentaacetic acid), followed by reduction with NaBH₄. The reaction products were separated from nonreacted substrates by silica column chromatography and analyzed by liquid chromatography-mass spectrometry. The purified reaction products mainly consisted of 9-HODE (purity >95%) and trace amounts of 13-HODE (data not shown).

Plasmid Constructions—An additional sequence of the FLAG epitope tag was inserted between the amino-terminal initiator methionine and the second amino acid of human G2A (NCBI accession number AF083955) by PCR using Pyrobrest (Takara) and oligonucleotides (sense primer containing BamHI and FLAG tag sequences, 5'-CGGG-ATCCACCATGGATTACAAGGACGACGATGACAAGTGCCCA-ATGCTACTGAAAAAC-3'; antisense primer containing EcoRI sequence, 5'-GGAATTCTCAGCAGGACTCCTCAATC-3'), and then subcloned into mammalian expression vector pCXN2.1, which is a slightly modified version of pCXN2 (18) with multiple cloning sites. This construct was designated as pCXN2.1-G2A. A DNA sequence containing the entire open reading frame of Gα_{qi} was subcloned into a pcDNA3.1/Zeo vector (Invitrogen). Gα_{qi} protein is a chimeric protein in which 9 carboxyl-terminal peptides of murine Gα_q protein (NCBI accession number M55412) were replaced with corresponding residues of murine Gα_i protein (NCBI accession number M13963). The sequence of each construct was confirmed by DNA sequencing using a LIC-4200L DNA sequencing system (Aloka).

Cell Culture, Transfection, and Flow Cytometry—Chinese hamster ovary K1 (CHO-K1) and human embryonic kidney 293 (HEK293) cells were maintained in Ham's F-12 medium (Sigma) and Dulbecco's modified Eagle's medium (Sigma), respectively, containing 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO₂. Cells were transfected with plasmid DNAs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. To observe the expression of FLAG-tagged G2A proteins on cell surfaces, cells were incubated with 10 μg/ml M5 anti-FLAG antibodies (Sigma) for 1 h at room temperature in phosphate-buffered saline containing 1% bovine serum albumin (BSA) without cell permeabilization, followed by staining with fluorescein isothiocyanate-conjugated anti-mouse IgG for 30 min at room temperature and analysis using an EPICS XL flow cytometer system (Beckman Coulter).

Stable Expression of G2A and/or Gα_{qi} Protein in CHO-K1 Cells—CHO-K1 cells were transfected with pCXN2.1-G2A using Lipofectamine 2000 reagent. Stably transfected clones resistant to 1 mg/ml Geneticin (Invitrogen) were selected, and the expression levels of G2A were confirmed by reverse transcriptase-PCR and flow cytometric analysis. Some clones that highly expressed G2A (designated as CHO-G2A cells) were further transfected with pcDNA3.1/Zeo-Gqi, and the stably transfected clones were selected in the presence of 1 mg/ml Zeocin (Invitrogen) and reverse transcriptase-PCR analysis (CHO-G2A-Gqi cells). CHO-K1 cells stably expressing Gα_{qi} protein were also cloned (CHO-Gqi cells).

Measurement of Intracellular Calcium Concentration—CHO cells were loaded with 5 μM Fura-2 AM (Dojindo) in HEPES/Tyrode's/BSA buffer (25 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1.0 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM D-glucose, 0.37 mM NaH₂PO₄, 0.49 mM MgCl₂, and 0.01% fatty acid-free BSA) containing 1.25 mM probenecid and 0.02% Pluronic F127 for 1 h at 37 °C. The cells were washed with HEPES/Tyrode's/BSA buffer, and changes in intracellular calcium concentrations upon ligand stimulation were monitored with a FLEXstation scanning fluorometer system (Molecular Devices) in 96-well microtiter plates or with a RF5300PC spectrofluorometer (Shimadzu) in glass tubes.

Measurement of Intracellular cAMP Concentration—CHO cells seeded onto the 96-well plates were washed with HEPES/Tyrode's/BSA buffer and pretreated with 1 mM 3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor) for 10 min at 37 °C. The cells were costimulated with various concentrations of 9(S)-HODE and 3 μM forskolin for 30 min at 37 °C, and the accumulated cAMP concentrations were measured using a cAMP-Screen System (Applied Biosystems) according to the manufacturer's instructions. In some cases, the cells were pretreated with 50 ng/ml PTX for 16 h before stimulation.

GTPγS Binding Assay—CHO-G2A or HEK293 cells transfected with pCXN2.1-G2A were disrupted by sonication in a homogenizing buffer (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 10 mM MgCl₂, 1 mM EDTA, and Complete protease inhibitor mixture (Roche)). The homogenates were centrifuged for 10 min at 12,000 × g, and the resulting supernatants were further centrifuged for 60 min at 100,000 × g. The precipitates (membrane fractions) were resuspended in the homogenizing buffer, and the protein concentrations were determined by BCA protein assay reagent (Pierce) using BSA as a standard. The membrane fractions (20 μg of proteins) were incubated with 0.5 nM [³⁵S]GTPγS and various concentrations of 9(S)-HODE in 200 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 μM GDP, and 0.1% fatty acid-free BSA) for 30 min at 30 °C in the presence or absence of unlabeled 20 μM GTPγS. The reactions were terminated by rapid filtration through GF/C glass fiber filters (Whatman). The filters were intensively washed with phosphate-buffered saline, dried at 50 °C, and then immersed in the Aquasol II scintillation mixture (Packard). The radioactivity of the filters was measured with a LS6500 scintillation system (Beckman).

Analysis of MAP Kinase Activation—CHO cells were disrupted by sonication after various stimulations, and the cell debris was removed by centrifugation at 10,000 × g for 5 min. An aliquot of protein (15 μg) was separated on 10% SDS-polyacrylamide gel electrophoresis, transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences), blocked in Tris-buffered saline containing 20% Applie Duo blocking solution (Seikagaku Corp.) at 4 °C overnight, and then incubated with primary antibodies in Tris-buffered saline containing 2% Applie Duo blocking solution for 1 h at room temperature. The primary antibodies to detect total and phosphorylated extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun NH₂-terminal kinase (JNK) were supplied as a part of the MAP Kinase Activation Sampler Kit (BD Bioscience), and used according to the manufacturer's instructions. After incubation with horseradish peroxidase-conjugated secondary antibodies, the signals were visualized using an ECL plus Western blotting Detection System (Amersham Biosciences).

In Vitro Peroxidation and PLA₂ Treatment of PC—*In vitro* peroxidation of 1-palmitoyl-2-linoleoyl PC by a radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), was performed according to the method described by Yoshida *et al.* (19). In brief, PC (2 mg) was dissolved in acetonitrile/*tert*-butyl alcohol (4:1 in volume) at a final con-

G2A Is a 9-HODE Receptor

centration of 5 mM, and peroxidation was induced by 2 mM AMVN for 3 h at 37 °C with mild agitation. The reaction was terminated by adding excess ethanol, and the solvent was evaporated under nitrogen gas. For the hydrolysis by phospholipase A₂ (PLA₂), the lipids were redissolved in diethyl ether, and mixed with the same volume of 100 mM Tris-HCl (pH 7.4), and CaCl₂ was added at a final concentration of 2.5 mM. Hydrolysis was induced by adding 20 units of PLA₂ from *Naja mossambica mossambica* (Sigma) followed by incubation for 2 h at room temperature. The reaction was terminated by adding EDTA (2.5 mM), and the lipids were extracted by the Bligh and Dyer method (20). After evaporation of the solvent under nitrogen gas, the lipids were dissolved in HEPES/Tyrosine's/BSA buffer for the FLEXstation assay.

Measurement of Proton-sensing Activity—As described by Ludwig *et al.* (12), Ham's F-12 medium containing 0.1% fatty acid-free BSA and HEPES/EPPS/MES (7.5 mM each) was prepared to achieve a wide range of pH (referred as F12-HEM). EPPS was from Wako. The pH of the buffer was adjusted under the experimental conditions using a carefully calibrated pH meter (Beckman). IP accumulation assay was performed as described by Murakami *et al.* (13). In brief, CHO cells seeded onto 12-well plates were labeled with 1 μCi/ml *myo*-[³H]inositol (Amersham Biosciences) for 18 h in Ham's F-12 medium containing 10% fetal bovine serum. Cells were washed twice and exposed to F12-HEM containing 20 mM LiCl with or without 10 μM 9(S)-HODE for 45 min at 37 °C. Accumulated IPs were isolated by anion-exchange chromatography (AG 1-X8 resin, Bio-Rad), and the radioactivity was measured with a LS6500 scintillation system.

RESULTS

Intracellular Calcium Mobilization Evoked by 9(S)-HODE in CHO Cells Expressing G2A—While determining ligands for orphan GPCRs by a calcium mobilization assay using a FLEXstation system, we found that G2A, which was stably expressed in CHO-K1 cells (CHO-G2A), responded to 9(S)-HODE. As shown in Fig. 1A, 9(S)-HODE evoked intracellular calcium mobilization in CHO-G2A cells in a dose-dependent manner. The concentration of 9(S)-HODE required to induce half-maximal activation was ~2 μM. On the other hand, parental CHO-K1 cells did not respond to 9(S)-HODE at all. To facilitate the detection of calcium signals, we utilized Gα_{qi} chimeric protein in which 9 COOH-terminal peptides of Gα_{qi} protein were replaced with the corresponding residues of the Gα_i protein. The COOH-terminal region of the Gα protein plays an important role in specifying receptor interactions, and this chimeric protein can mediate stimulation of phospholipase C by receptors otherwise coupled exclusively to Gα_i (21). As illustrated in Fig. 1A, cells that stably expressed both G2A and Gα_{qi} (CHO-G2A-Gqi) showed largely enhanced responses to 9(S)-HODE. In these cells, the concentration of 9(S)-HODE required to induce half-maximal activation was reduced to submicromolar levels. On the contrary, CHO cells that stably expressed only Gα_{qi} (CHO-Gqi) did not respond to 9(S)-HODE.

To eliminate the possibility of specific stable clones responding to 9(S)-HODE because of some unexpected changes during the selection processes, we performed the calcium mobilization assay in multiple stable clones and obtained essentially the same results (data not shown). We also analyzed the calcium response of G2A to 9(S)-HODE in a transient expression system. CHO-Gqi cells were transfected with pCXN2.1-G2A or the empty vector (pCXN2.1), and the calcium response to 9(S)-HODE was examined after 24 h. Only after the pCXN2.1-G2A was transfected, did the CHO-Gqi cells respond to 9(S)-HODE (Fig. 1B). These results show that G2A responds to 9(S)-HODE

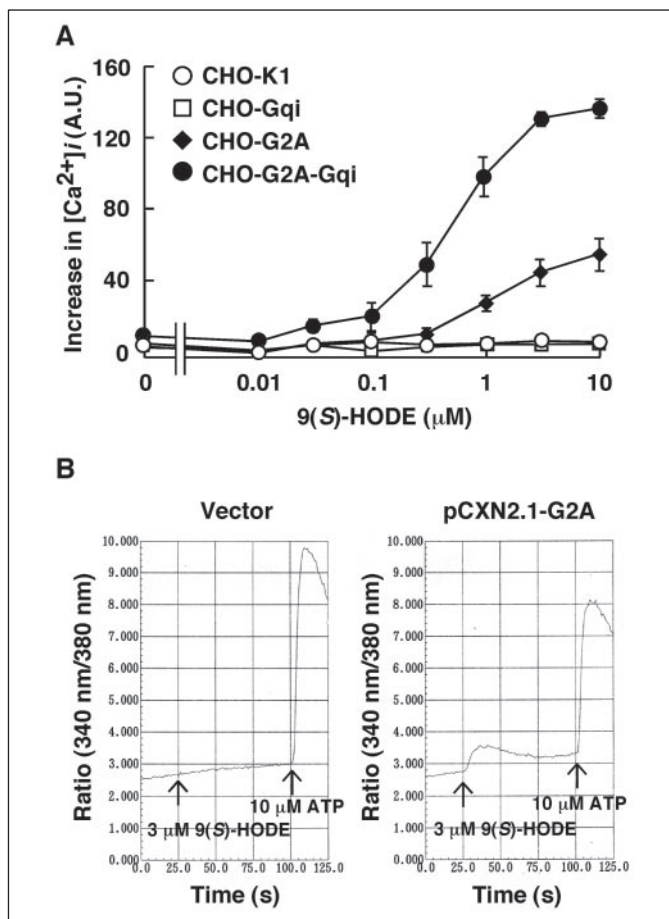


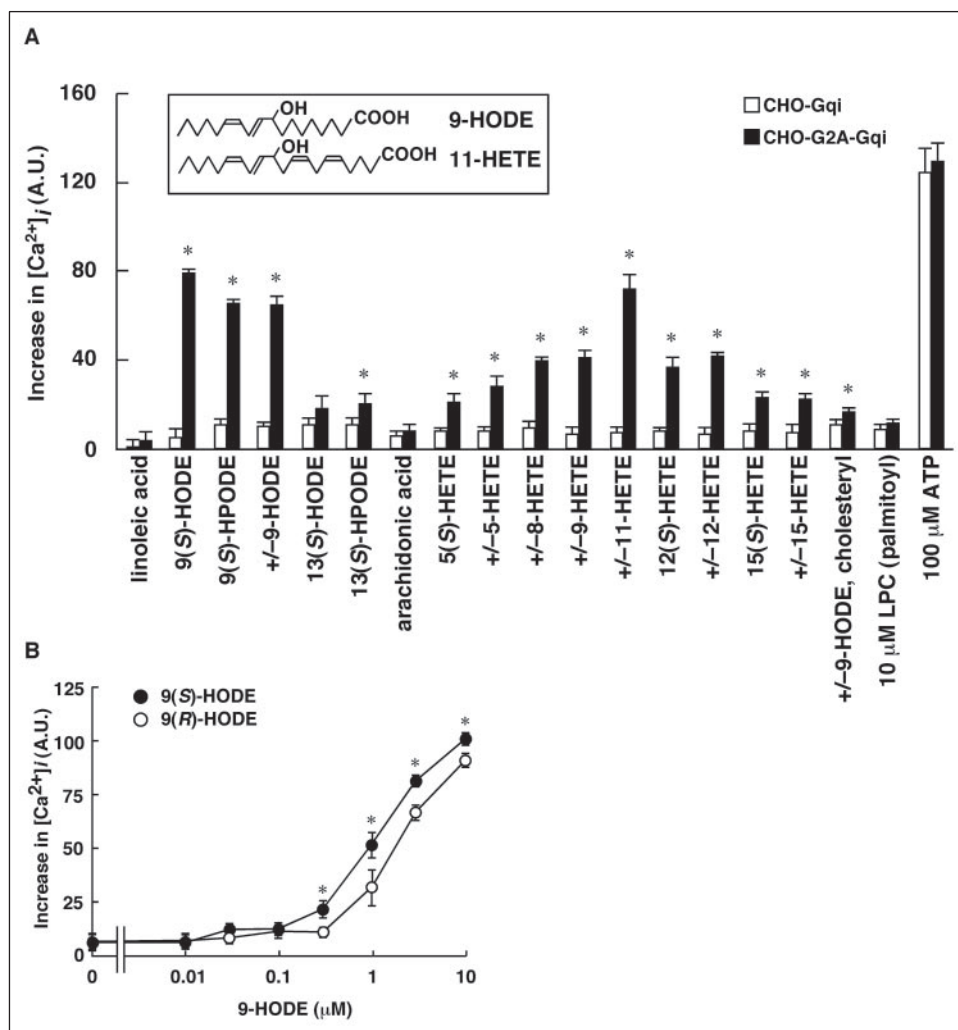
FIGURE 1. Intracellular calcium mobilization via G2A evoked by 9(S)-HODE. A, CHO cells stably expressing G2A (CHO-G2A; filled diamond) with or without Gα_{qi} chimeric protein (CHO-G2A-Gqi; filled circle or CHO-Gqi; open square) and parental CHO-K1 cells (open circle) were loaded with Fura-2 AM. Intracellular calcium mobilization evoked by increasing concentrations of 9(S)-HODE was measured by a FLEXstation system (mean ± S.D., n = 4). Data are typical of three independent experiments. B, CHO-Gqi cells were transfected with pCXN2.1 empty vector or pCXN2.1-G2A. After 24 h, the cells were loaded with Fura-2 AM and changes in the intracellular calcium concentration after stimulation with 3 μM 9(S)-HODE were monitored by a RF5300C spectrofluorometer. Furthermore, the cells were stimulated with 10 μM ATP as a positive control. The intracellular calcium concentration is shown by the ratio of fluorescence at 510 nm by excitations at 340 and 380 nm. Data are typical of four independent experiments.

and mediates intracellular calcium mobilization, which is greatly enhanced by coexpression of Gα_{qi} protein.

Ligand Specificity of G2A—To elucidate the ligand specificity of G2A, various oxidized derivatives of linoleic and arachidonic acid, 1 μM each, were assessed in the calcium mobilization assay. We chose a series of oxidized free fatty acids, as they could be produced *in vivo* by the enzymatic reactions of lipoxygenases and cytochrome P450 enzymes (22–24), or by nonenzymatic radical reactions (19). Among them, 9(S)-HODE and 11-HETE showed the strongest ability to mobilize intracellular calcium in CHO-G2A-Gqi cells (Fig. 2A; the molecular structures of these two lipids are shown in the inset). In this respect, the ability of 9(S)-HODE was comparable with that of 9(S)-HODE, but that of 13(S)-HODE and 13(S)-HODE were much weaker. 9-HODE in its cholesteryl ester form showed little activity. We also examined the effects of LPC (1-palmitoyl) and SPC in the range from 10 nM to 10 μM on calcium mobilization, but neither showed any significant response under these assay conditions. The results with 10 μM LPC are shown in Fig. 2A.

Next, we investigated the chiral specificity of the ligands. The concentrations of 9(S)- and 9(R)-HODE (Cayman Chemical) were adjusted

FIGURE 2. Ligand specificity of G2A. *A*, CHO-Gqi (open square) and CHO-G2A-Gqi (filled square) cells were stimulated with various oxidized derivatives of linoleic and arachidonic acids, 1 μM each, and increases in intracellular calcium concentration were measured by a FLEXstation system (mean \pm S.D., $n = 4$). Structures of 9-HODE and 11-HETE are shown in the inset. *B*, CHO-G2A-Gqi cells were stimulated with increasing concentrations of 9(*S*)- (filled circle) and 9(*R*)- (open circle) HODE, and increases in the intracellular calcium concentration were measured by a FLEXstation system (mean \pm S.D., $n = 4$). Data are typical of three independent experiments. Asterisk, $p < 0.01$ (Student's *t* test) compared with the corresponding values of the CHO-Gqi (*A*) or 9(*R*)-HODE (*B*).



by quantification using a spectrophotometer just prior to use. As shown in Fig. 2B, 9(*S*)-HODE showed little but significantly higher ability to mobilize intracellular calcium in CHO-G2A-Gqi cells than 9(*R*)-HODE in concentrations above 0.3 μM . However, the chiral selectivity of G2A was not highly specific. These results indicate that the common structure from the hydroxy or hydroperoxy group to the ω end (2*Z*,4*E*-deca-dien-1-hydro(pero)xide) of 9-H(P)ODE and 11-HETE is important for ligand recognition of G2A.

9(*S*)-HODE-induced GTP γ S Binding—Next, we examined whether 9(*S*)-HODE stimulated the GDP/GTP exchange on $G\alpha$ protein using membrane preparations from G2A-expressing cells. As shown in Fig. 3A, the membrane of CHO-G2A cells exhibited 9(*S*)-HODE-evoked [^{35}S]GTP γ S binding in a concentration-dependent manner, whereas the membrane of CHO-K1 cells did not evoke such a binding. To confirm the involvement of G2A in 9(*S*)-HODE-evoked [^{35}S]GTP γ S binding, we performed another analysis in the transient expression system using HEK293 cells. Membranes were prepared from HEK293 cells 24 h after transfection of plasmid DNAs. Although 9(*S*)-HODE-evoked [^{35}S]GTP γ S binding was not observed in the membranes transfected with G2A alone (data not shown), the specific binding became apparent in the membranes after cotransfection of $G\alpha_i$ with G2A (Fig. 3B). On the other hand, transfection of $G\alpha_i$ with the empty vector did not show the 9(*S*)-HODE-evoked [^{35}S]GTP γ S binding. These results indicate that 9(*S*)-HODE can stimulate the activation of $G\alpha$ protein via G2A.

Involvement of $G\alpha_i$ Protein in G2A-mediated Signal—As the intracellular calcium mobilization evoked by 9(*S*)-HODE was largely potentiated by $G\alpha_{qi}$ (Fig. 1A), and the GTP γ S binding was observed by coexpression of G2A with $G\alpha_i$ in HEK293 cells (Fig. 3B), it was inferred that $G\alpha_i$ protein is involved in G2A-mediated signaling. To examine this possibility, CHO-G2A cells were pretreated with 50 ng/ml PTX for 16 h, and analyzed in a calcium mobilization assay. PTX pretreatment partially suppressed intracellular calcium mobilization evoked by 9(*S*)-HODE, although PTX did not affect the calcium mobilization induced by 100 μM ATP (Fig. 4A). This result indicates that G2A induces intracellular calcium mobilization through both $G\alpha_i$ and $G\alpha_{qi}$ pathways.

Next, we examined the effects of 9(*S*)-HODE on adenylyl cyclase activity by measuring cAMP accumulation. When CHO-K1 or CHO-G2A cells were stimulated with 9(*S*)-HODE, no increase in cAMP concentration was observed (data not shown). On the other hand, in the presence of 3 μM forskolin (adenylyl cyclase activator), 9(*S*)-HODE inhibited cAMP accumulation in a dose-dependent manner, and this inhibition was abolished almost completely by PTX pretreatment (Fig. 4B). These results indicate that G2A couples with $G\alpha_i$ and inhibits adenylyl cyclase activity.

JNK Activation by 9(*S*)-HODE—The family of serine/threonine kinases referred to as MAP kinases is activated after cell stimulation by various stimuli and plays pivotal roles in multiple signal transduction pathways. Three important kinases, namely, ERK1/2, p38, and JNK,

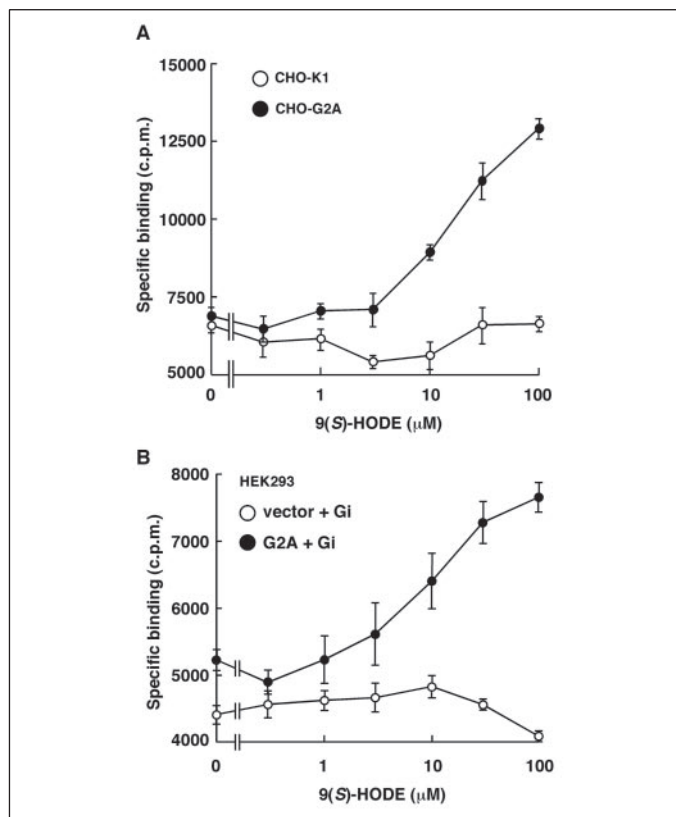


FIGURE 3. 9(S)-HODE-induced [35 S]GTP γ S binding to the membrane fractions prepared from G2A-expressing cells. Membrane fractions were prepared from CHO-K1 (open circle) and CHO-G2A (filled circle) cells (A), or from HEK293 cells transiently expressing G α_i with (filled circle) or without (open circle) G2A (B). The membrane proteins (20 μ g) were incubated with 0.5 nM [35 S]GTP γ S and the indicated concentrations of 9(S)-HODE for 30 min at 30 °C in the presence (nonspecific binding) or absence (total binding) of unlabeled 20 μ M GTP γ S. Specific binding values were obtained by subtracting nonspecific binding from total binding. Data are the mean \pm S.D. ($n = 4$) and are typical of three independent experiments.

belong to this family. We examined whether 9(S)-HODE activated these MAP kinases. CHO-K1 and CHO-G2A cells were treated with 9(S)-HODE for 0–30 min, and whole cell lysates were prepared by sonication. Activation of MAP kinases was assessed by Western blotting using phospho-specific antibodies. As shown in Fig. 5A, JNK was activated by 9(S)-HODE in CHO-G2A cells, but not in CHO-K1 cells. The activation of JNK was maximal at \sim 10 min with 1 μ M 9(S)-HODE stimulation, and it was diminished after 30 min. As shown in Fig. 5B, 9(S)-HODE activated JNK in a dose-dependent manner in CHO-G2A cells. On the other hand, activation of neither ERK1/2 nor p38 was observed with 9(S)-HODE both in CHO-K1 and CHO-G2A cells (Fig. 5A).

Oxidation and Hydrolysis of Phosphatidylcholine—Linoleic acid esters are the most abundant polyunsaturated fatty acid esters both in human plasma and membrane lipids, and are continuously exposed to oxidative stresses to yield hydroxy and hydroperoxy esters. To mimic the production of oxidized free fatty acids *in vitro*, 1-palmitoyl-2-linoleoyl PC was oxidized by AMVN and then followed by hydrolysis of the ester bond of phospholipids at the *sn*-2 position by PLA $_2$. AMVN, used as a radical initiator, was reported to hydroperoxidize 1-palmitoyl-2-linoleoyl PC to generate 9- and 13-HPODE esters (19). PLA $_2$ from *N. mossambica mossambica* hydrolyzed almost all PC to produce free fatty acids and LPC in our assay conditions, as judged from thin layer chromatography analysis (data not shown). As shown in Fig. 6, neither PC nor oxidized PC (OxPC) had the ability to mobilize intracellular calcium in CHO-G2A cells. However, PLA $_2$ -treated oxidized PC (OxPC-PLA $_2$)

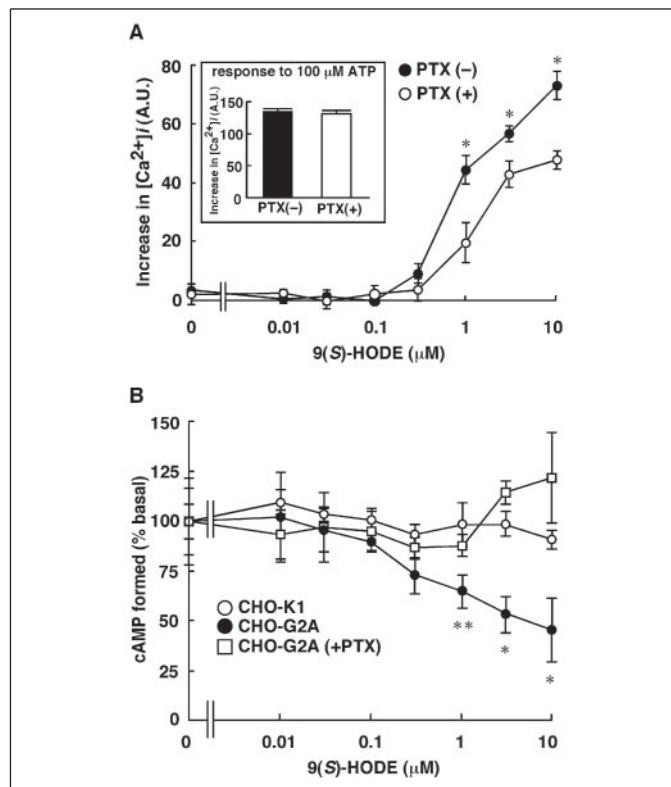


FIGURE 4. Involvement of G α_i protein in G2A-mediated signal transduction. A, CHO-G2A cells were pretreated with 50 ng/ml PTX (open circle) or vehicle (filled circle) for 16 h, and stimulated with 9(S)-HODE. The increases in the intracellular calcium concentration were measured by a FLEXstation system (mean \pm S.D., $n = 4$). The inset shows the increases in the intracellular calcium concentrations evoked by 100 μ M ATP that were not affected by PTX pretreatment. Data are typical of three independent experiments. B, CHO-K1 (open circle) and CHO-G2A (filled circle) cells were stimulated with 9(S)-HODE in the presence of 3 μ M forskolin for 20 min at 37 °C, and the accumulated cAMP concentrations were measured. Furthermore, CHO-G2A cells pretreated with 50 ng/ml PTX for 16 h (open square) were measured. Data are the mean \pm S.D. ($n = 4$) and are typical of four independent experiments. Asterisk, $p < 0.01$; double asterisk, $p < 0.05$ (Student's t test) compared with the corresponding values of the PTX-treated cells.

showed a response comparable with 1 μ M 9(S)-HODE. Hydrolyzed PC without oxidation (PC-PLA $_2$) also showed little ability, which is possibly because of oxidization of PC during the experimental procedures. On the other hand, LPC (1-palmitoyl), with or without AMVN treatment, possessed little ability to mobilize intracellular calcium. The generation of free 9- and 13-HPODE in our assay conditions was confirmed by liquid chromatography-mass spectrometry analysis. The concentration of HPODE in the OxPC-PLA $_2$ sample was estimated at 1–5 μ M from liquid chromatography-mass spectrometry analysis (data not shown). These results indicate that oxidized free fatty acids, which are not in the ester forms, can activate G2A to induce intracellular calcium mobilization.

Proton-sensing Activity of G2A and the Effects of 9(S)-HODE—Finally, we examined the effects of 9(S)-HODE on the proton-sensing activity of G2A by measuring IP accumulation (Fig. 7). In CHO-G2A cells, the IP accumulation was increased at pH 7.5 compared with CHO-K1 cells, possibly because of a constitutive activity of G2A. The IP accumulation was slightly enhanced at acidic pH, indicating a proton-sensing activity of G2A. 9(S)-HODE caused an exaggerated accumulation of IPs in CHO-G2A but not in CHO-K1 cells at pH 7.5. At acidic pH, 9(S)-HODE-dependent IP accumulation was slightly enhanced compared with pH 7.5 in CHO-G2A cells. However, 9(S)-HODE had little effect on the proton-sensing activity of G2A. Thus, we concluded

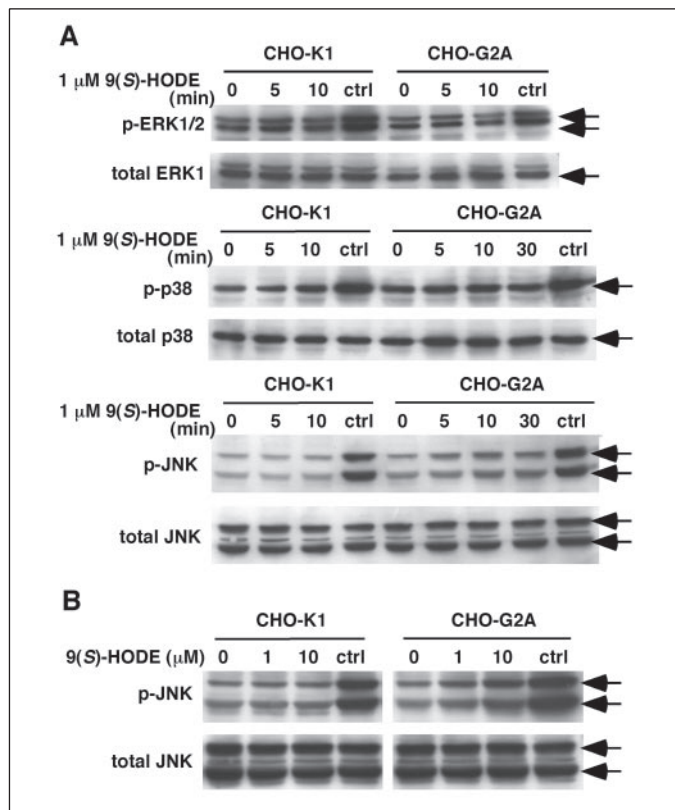


FIGURE 5. 9(S)-HODE activates JNK MAP kinase via G2A. CHO-K1 and CHO-G2A cells were stimulated with $1 \mu\text{M}$ 9(S)-HODE for 0–30 min at 37°C (A), or with increasing concentrations of 9(S)-HODE for 10 min at 37°C (B), and whole cell lysates were prepared by sonication. Activations of ERK1/2, p38, and JNK MAP kinases were examined by Western blotting analysis using antibodies specific to phosphorylated forms. Total amounts of each MAP kinase was also shown. The cells were stimulated with 500 nM 12-O-tetradecanoylphorbol 13-acetate for 10 min as a positive control for ERK1/2, and with $10 \mu\text{g/ml}$ anisomycin for p38 and JNK. Data are typical of four independent experiments.

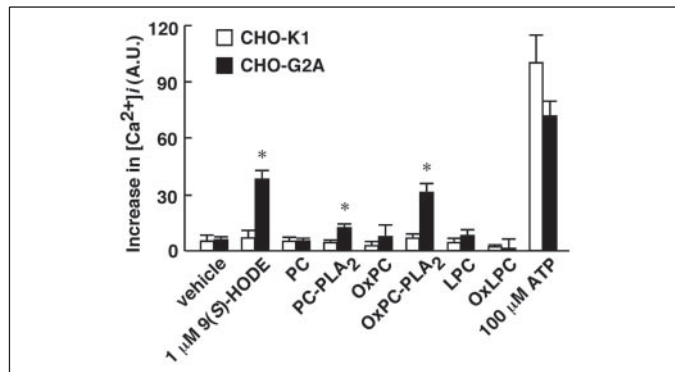


FIGURE 6. Hydrolysis of oxidized PC induces G2A activation. PC (1-palmitoyl-2-linoleoyl) and LPC (1-palmitoyl), 2 mg each, were peroxidized by 2 mM AMVN for 3 h at 37°C (OxPC and OxLPC). OxPC was further hydrolyzed with 20 units of PLA₂ for 2 h at room temperature (OxPC-PLA₂). Extracted lipid fractions were dissolved in HEPES/Tyrosine/BSA buffer. Activity to mobilize intracellular calcium was examined in CHO-K1 (open square) and CHO-G2A (filled square) cells by a FLEXstation system. Data are the mean \pm S.D. ($n = 4$) and are typical of four independent experiments. Asterisk, $p < 0.01$ (Student's *t* test) compared with the corresponding values of the CHO-K1 cells.

that 9(S)-HODE has an additive effect but neither synergistic nor antagonistic effect on the proton-sensing activity of G2A.

DISCUSSION

In the present study, we showed that G2A, expressed in CHO-K1 or HEK293 cells, is activated by 9(S)-HODE and other oxidized free fatty acids derived from linoleic and arachidonic acids, and then mediates

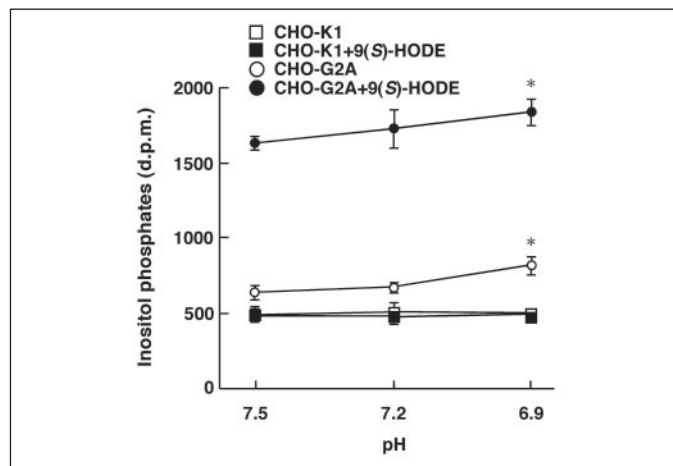


FIGURE 7. Proton-sensing activity of G2A and the effects of 9(S)-HODE. CHO-K1 (square symbols) and CHO-G2A (circle symbols) cells were labeled with $1 \mu\text{Ci/ml}$ myo-[^3H]inositol for 18 h and subsequently exposed to F12-HEM (pH 7.5, 7.2, and 6.9) containing 20 mM LiCl in the presence (filled symbols) or absence (open symbols) of $10 \mu\text{M}$ 9(S)-HODE for 45 min at 37°C . Accumulated IPs were isolated, and radioactivity was measured. Data are the mean \pm S.D. ($n = 3$) and are typical of three independent experiments. Asterisk, $p < 0.05$ (Student's *t* test) compared with the corresponding values at pH 7.5.

intracellular calcium mobilization, GTP γ S binding to the membrane fractions, inhibition of cAMP accumulation, and JNK activation. We also discovered that the ω end structure of 9-HODE and 11-HETE, and their free fatty acids forms, but not their esterified forms, are important for the ligand recognition of G2A. Furthermore, there exists a chiral specificity of the hydroxy group for ligand recognition, that is, 9(S)-HODE is better at ligand recognition as compared with 9(R)-HODE. These results strongly indicate that G2A is a receptor for oxidized free fatty acids.

Kabarowski *et al.* (4) once reported that G2A was a high affinity receptor for LPC and SPC (4). However, the authors have withdrawn the paper because they were unable to reproduce the results of the direct binding of radioactive LPC to G2A (7). In our study, G2A responded to neither LPC nor SPC up to the concentration of $10 \mu\text{M}$ in the calcium mobilization assay in CHO cells that expressed G2A. We prepared [^3H]9(S)-HODE that was derived from [^3H]linoleic acid by the enzymatic reaction of 5-lipoxygenase from potato tubers, and attempted a direct binding study with the membrane fractions from G2A-expressing cells. However, high background binding of 9(S)-HODE to the membranes prevented us from observing the specific binding; the presence of excess amounts of unlabeled 9(S)-HODE ($30 \mu\text{M}$) also increased the binding of 100 nM [^3H]9(S)-HODE (data not shown). Although the exact reason has not yet been elucidated, it is possible that high concentrations of 9(S)-HODE promoted the formation of micelle-like structures with [^3H]9(S)-HODE and its fusion with the membrane lipids. Hence, we performed an agonist-induced [^{35}S]GTP γ S binding assay. 9(S)-HODE induced [^{35}S]GTP γ S binding in a dose-dependent manner with the membrane fractions from both CHO-G2A cells (Fig. 3A) and HEK293 cells that were transiently transfected with G2A (Fig. 3B). Agonist-induced GTP γ S binding to the membranes is a well established method to identify and evaluate ligands for GPCRs, especially when the ligands are lipids and the direct binding assay of radiolabeled ligands is difficult.

There is an apparent discrepancy between the dose-response curves obtained from the calcium assay (Figs. 1A and 2B) and the GTP γ S binding assay (Fig. 3). The comparison of the dose-response curve in the calcium assay (Fig. 1A, filled diamond) with that in the GTP γ S binding assay (Fig. 3A, filled circle), both obtained from CHO-G2A cells, showed

G2A Is a 9-HODE Receptor

a certain discrepancy. We could not obtain saturable curves or EC_{50} values in both assays. This might be partially because of some detergent effects at higher concentrations of 9-HODE as discussed previously. In the calcium assay, 30 μM 9-HODE starts to cause cell lysis, and increases intracellular calcium concentrations in a nonspecific manner. The discrepancy of the dose-response curves might be explained by the difference of signal amplification processes. Namely, the calcium signal can be amplified by many steps between the activation of G protein and the release and/or the influx of calcium ion, whereas GTP γ S binding to G α protein is quite a direct process after the ligand binding. The species of G α proteins required and their coupling efficiency with GPCR after ligand binding may vary with the signal and the cell type.

G2A was found to couple with the G α_{13} family of G α proteins leading to RhoA-dependent actin rearrangement (25). Activation of a specific combination of G proteins including G α_s , G α_q , and G α_{13} by G2A was also reported (26). In our experiments, coupling of G2A with G α_i was indicated by [^{35}S]GTP γ S binding to the membranes from HEK293 cells transfected with G2A and G α_i (Fig. 3B). 9(S)-HODE-induced [^{35}S]GTP γ S binding was also observed by cotransfection of G2A with G α_{13} , but not with G α_{16} in HEK293 cells (data not shown). 9(S)-HODE did not induce accumulation of cAMP in CHO-G2A cells. Instead, 9(S)-HODE induced the inhibition of adenylyl cyclase in forskolin-stimulated CHO-G2A cells, which was sensitive to PTX pretreatment (Fig. 4B). Furthermore, the intracellular calcium mobilization response was partially resistant to PTX (Fig. 4A), suggesting that G2A may activate G α_q in addition to G α_i . In summary, our results indicate that G2A couples with the G α_i , G α_{13} , and G α_q families of G α protein.

G2A, along with the three other structurally related GPCRs, namely, GPR4, OGR1, and TDAG8, has been shown to possess proton-sensing activity (12–15). Murakami *et al.* (13) demonstrated the acidic pH-sensitive production of the IP species and activation of the *zif268* promoter in G2A-overexpressing COS-7 and PC12h cells, respectively. We examined the possibility of whether the application of oxidized free fatty acids at a micromolar concentration caused the acidification of the cell environment and induced the responses. The pH of the buffer (adjusted by 25 mM HEPES-NaOH to pH 7.4 prior to the assay) was not affected during and after the intracellular calcium mobilization assay with 10 μM 9(S)-HODE (data not shown). We concluded that the intracellular calcium increase evoked by 9(S)-HODE was not mediated by a change in the environmental pH. Murakami *et al.* (13) reported that LPC has antagonistic effects on the pH-dependent activation of G2A. So, we investigated the effects of 9(S)-HODE on the proton-sensing activity of G2A by measuring IP accumulation. As reported by Murakami *et al.* (13), G2A showed IP accumulation at pH 7.5 that was further enhanced at acidic pH. 9(S)-HODE caused an exaggerated accumulation of IP via G2A, as expected from the results of the intracellular calcium mobilization with 9(S)-HODE (Fig. 1A). The proton-sensing activity of G2A was not changed in the presence or absence of 9(S)-HODE. We concluded that 9(S)-HODE has an additive effect but neither synergistic nor antagonistic effect on the proton-sensing activity of G2A. It might be possible that the local environment becomes acidic under the conditions where oxidative stresses promote the production of oxidized fatty acids such as 9-HODE, and that low pH and oxidized fatty acids participate together in the pathogenesis of certain diseases.

Some oxidized derivatives of linoleic and arachidonic acids induced G2A-mediated intracellular calcium mobilization (Fig. 2A). Among the lipids examined, 9-HODE and 11-HETE were the most potent in mediating intracellular calcium mobilization. In this regard, 9-HODE was also a potent ligand, but 13-HODE and 13-HPODE showed weaker ability. Linoleic acid is the most abundant polyunsaturated fatty acid in

human plasma. Most of the linoleic acid exists in esterified forms as PC and cholesteryl linoleate, both are major components of LDL, and are continuously exposed to many kinds of oxidative stresses to generate hydroxy and hydroperoxy species. Biochemical analyses of copper-oxidized (27) or human monocyte-oxidized (28) LDL revealed that major oxidized fatty acid products were esterified 9- and 13-hydroxy/hydroperoxy derivatives of linoleic acid. Furthermore, increased levels of lipid oxidation products such as 9-HODE and 13-HODE were reported in LDLs of patients suffering from rheumatoid arthritis (29) and atherosclerosis (30). In our present study, esterified 9-HODE was not effective to induce intracellular calcium mobilization via G2A. To assess the possibility of the production of free 9-HODE, we hydrolyzed oxidized PC (1-palmitoyl-2-linoleoyl) with PLA $_2$, and showed that oxidation and subsequent hydrolysis of PC were required for inducing intracellular calcium mobilization via G2A (Fig. 6).

Some secretory types of PLA $_2$ (sPLA $_2$) are known to be stored in the secretory granules of immune cells such as macrophages and mast cells and secreted in response to stimulation (31). Among the nine different groups of sPLA $_2$ identified so far in humans, group X sPLA $_2$ (sPLA $_2$ -X) was found to induce potent hydrolysis of PC in LDL leading to the production of large amounts of free fatty acids and LPC (32). It is possible that sPLA $_2$ -X, secreted from macrophages in response to oxidative stress, hydrolyzes oxidized PC in LDL to generate free hydroxy or hydroperoxy fatty acids in micromolar concentrations in the local environment. Similarly, cholesteryl-9-HODE could conceivably be de-esterified by macrophage lysosomal esterases to 9-HODE.

In addition to plasma LDL, phospholipids in plasma and intracellular membranes would also be the targets of oxidation by reactive oxygen species derived from mitochondrial oxidative phosphorylation processes, NADPH oxidases, and various other processes within cells (33). Under normal conditions, reactive oxygen species are quickly eliminated by various antioxidants such as superoxide dismutases, glutathione peroxidase, and vitamin E (33, 34). However, the concentration of reactive oxygen species is increased by stresses such as UV irradiation and smoking (35), or during pathological conditions like cancer and ischemia (34). In such cases, oxidized membrane phospholipids might be hydrolyzed by some types of cytoplasmic PLA $_2$ or Ca $^{2+}$ -independent PLA $_2$ to generate oxidized free fatty acids that act on G2A in an autocrine or paracrine manner. Interestingly, G2A was first identified as a stress inducible GPCR that causes cell cycle arrest (1). Thus, oxidative stresses might induce both receptor expression and ligand production to stimulate appropriate cellular responses.

Rikitake *et al.* (3) showed that G2A was expressed predominantly in macrophages within atherosclerotic lesions at the aortic root of apolipoprotein E-deficient mice and the thoracic aortas of Watanabe heritable hyperlipidemic rabbits. Furthermore, they showed that G2A was expressed in macrophages within the lipid-rich regions in atherosclerotic plaques of human coronary arterial specimens, whereas no immunoreactivity of G2A was observed in fibrous plaques where macrophages did not exist. Moreover, the amounts of esterified 9- and 13-HODE in human atherosclerotic lesions were reported to correlate directly with the severity of the lesion (36), and marked expression of sPLA $_2$ -X was immunohistochemically detected in foam cell lesions in the arterial intima of high fat-fed apolipoprotein E-deficient mice (32). G2A may play a role in the formation and progression of atherosclerotic lesions by functioning as a receptor for oxidized free fatty acids that may be produced by free radical oxidation of LDL and subsequent hydrolysis by sPLA $_2$ -X.

Besides the oxidative stresses, H(P)ODEs and H(P)ETEs could be produced by enzymatic reactions of lipoxygenases, prostaglandin

endoperoxide H synthases, and cytochrome P450 enzymes. Mouse 8-lipoxygenase has been identified and found to convert linoleic acid to 9(S)-HODE (37), but there is no report on the function of human 8-lipoxygenase so far. Although the potato 5-lipoxygenase is known to convert linoleic acid to produce 9-HODE (17), mammalian 5-lipoxygenase is not known to react with linoleic acid. Thus, the contribution of lipoxygenases to the production of 9-HODE in mammalian tissues remains to be elucidated. In addition to cyclooxygenase activity converting arachidonic acid to prostaglandin G₂, prostaglandin endoperoxide H synthases were found to exhibit some lipoxygenase activities producing certain amounts of 11- and 15-HETE from arachidonic acid (38, 39). Many of the cytochrome P450 enzymes are also involved in the metabolism of linoleic and arachidonic acids to generate various hydroxy and epoxy fatty acids including HODE and HETE (23, 24). However, most of these results were obtained from *in vitro* analyses using purified or recombinant enzymes. Further studies are required to understand the biological effects of these enzymes on the production of oxidized free fatty acids as the G2A ligands.

We showed JNK MAP kinase activation by 9(S)-HODE in G2A-expressing CHO cells (Fig. 5). Activation of JNK leads to multiple transcriptional events that induce the expression of various genes including proinflammatory cytokines. Expression of interleukin-1 β was induced by 9-HODE in monocyte-derived macrophages obtained from human peripheral blood (40). It would be interesting to examine whether 9-HODE-induced interleukin-1 β and other cytokine expressions are mediated by G2A through a JNK pathway.

Hydrolysis of oxidized PC by PLA₂ yields both 9-HODE and LPC. Recently, Wang *et al.* (41) reported that LPC-induced surface redistribution is important to regulate the signaling of murine G2A. Moreover, LPC was found to induce chemotaxis of T lymphocytes (5) and macrophages (6) in a G2A-dependent manner. LPC and 9-HODE might function in tandem while attracting T cells and macrophages, changing the surface distribution of G2A, and activating the receptor.

In summary, we showed that G2A is activated by 9-HODE and other oxidized free fatty acids, and it mediates intracellular calcium mobilization, GTP γ S binding to the membrane fractions, inhibition of cAMP accumulation, and JNK activation. G2A may play an important role functioning as a receptor for oxidized free fatty acids produced in various conditions such as inflammation, atherosclerosis, cancer, UV irradiation, and other oxidative stresses.

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Identification of 9-Hydroxyoctadecadienoic Acid and Other Oxidized Free Fatty Acids as Ligands of the G Protein-coupled Receptor G2A

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