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N-Arachidonoyl glycine, another endogenous agonist of GPR55

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Abstract

Interest in lipoamino acids as endogenous modulators of G-protein coupled receptors has escalated due to their involvement in a variety of physiologic processes. In particular, a role for these amino acid conjugates has emerged in the endocannabinoid system. The study presented herein investigated the effects of N-arachidonoyl glycine (NAGly) on a candidate endocannabinoid receptor, GPR55. Our novel findings reveal that NAGly induces concentration dependent increases in calcium mobilization and mitogen-activated protein kinase activities in HAGPR55/CHO cells. These increases were attenuated by the selective GPR55 antagonist ML193 (N-[4-[(3,4-Dimethyl-5-isoxazolyl)amino]sulfonyl]phenyl]-6,8-dimethyl-2-(2-pyridinyl)-4-quinolinecarboxamide), supporting receptor mediated signaling. To our knowledge this is the first report identifying GPR55 as a target of the endogenous lipoamino acid, NAGly.

Keywords

GPR55; calcium; mitogen activated protein kinase; inositol-3-phosphate receptor; N-arachidonoyl glycine

1. Introduction

Activation of the lipid sensing receptor, GPR55, by lysophosphatidyl inositol (LPI) has been well documented, and implicated in endocannabinoid signaling [1]. Intracellular events resulting from GPR55 activation include; enhanced β -arrestin activity, calcium mobilization and ERK1/2 phosphorylation. Despite the efforts of many investigations, a variety of potent, selective, pharmacologically useful GPR55 ligands are not yet available. LPI remains the

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Chemical compounds studied in this article:

Bafilomycin A1 (PubChem CID 6436223), ML193, (N-[4-[(3,4-Dimethyl-5-isoxazolyl)amino]sulfonyl]phenyl]-6,8-dimethyl-2-(2-pyridinyl)-4-quinolinecarboxamide) (PubChem CID1261822) N-arachidonoyl glycine (PubChem CID 5283389), ryanodine (PubChem CID 11317883), Xestopongin C (PubChem CID 9846431)

principle ligand that synthetic agonists are compared to, and synthetic antagonists are measured against. The role of N-arachidonoyl amino acids (NAAA) as modulators of GPCR activity has been suggested as a consequence of NAAA involvement in a variety of physiologic processes [2–5].

Our group has previously reported activation of GPR18 by NAGly (N-arachidonoyl glycine), supporting studies by McHugh et al [6, 7], yet in conflict with the reports of others [8, 9]. Since both GPR18 and GPR55 have been implicated in endocannabinoid signaling, and are regarded as candidate cannabinoid receptors, we examined the effect of NAGly on GPR55-mediated intracellular events. Employing a triple hemagglutinin-tagged GPR55 stable Chinese Hamster cell line (HAGPR55/CHO), we investigated alterations in levels of intracellular calcium and ERK 1/2 (extracellular receptor kinase 1/2) phosphorylation induced by NAGly in the presence and absence of a previously identified and selective GPR55 antagonist, ML193 [10].

2. Materials and Methods

2.1 Materials

Alexa Fluor 568 goat anti-mouse, and Alexa Fluor555 goat anti-rabbit (Invitrogen, Carlsbad, CA, USA); anti-HA mouse monoclonal antibody (Covance, Princeton, NJ, USA); DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, USA); FBS (Hyclone), G418 and Ham's F12 medium (Corning cellgro™), Fisher Scientific); IRDye 800-conjugated goat anti-mouse IgG, IRDye 680-conjugated goat anti-rabbit IgG, and blocking buffer (Li-Cor Biosciences); monoclonal anti-phosphorylated ERK 1/2 and Poly-D-Lysine (Sigma); NAGly (Focus Biomolecules); rabbit anti-total ERK1/2 (Cell Signaling). All other chemicals were obtained from standard laboratory chemical suppliers.

2.2 Cell Culture

Chinese hamster ovary (CHO) cells were transfected with human GPR55 cDNA, containing a triple sequence of hemagglutinin (HA) at the N-terminus, using Lipofectamine according to manufacturer protocols. Clones of HAGPR55/CHO cells were isolated in the presence of 800ug/ml G418. HAGPR55/CHO cells were maintained in Ham's/F-12 media (Gibco) supplemented with 10% FBS and G418 (350µg/ml) at 37°C, 5% CO₂. Cells were passaged every 2–3 days for a maximum of 15 passages.

2.3 Poly-D-Lysine Coating

All cover slips and 6-well plates were coated with a solution of poly-D-lysine (50ug/ml) for 24 hours (4°C), rinsed three times with sterile water, and allowed to air dry at room temperature.

2.4 Immunocytochemistry

Stable expression of HAGPR55 was confirmed by HA immunocytochemistry and microscopy. Cells were plated on poly-D-lysine coated coverslips and incubated overnight at 37°C in 24-well plates prior to immunocytochemical protocols. Localization of GPR55 receptors in the HAGPR55/CHO cell line was visualized using a mouse antibody to the N-

terminal hemagglutinin and Alexafluor 568. Antibody validation was attained by subjecting untransfected CHO cells to the same immunocytochemical protocols. Coverslips were mounted onto slides using DAPI Fluoromount-G for nuclear identification. Corresponding images from both channels were overlaid in Adobe Photoshop® CS5 (Adobe systems Incorporated).

2.5 Calcium Imaging

HAGPR55/CHO cells were plated on poly-D-lysine coated coverslips (25mm in diameter) in Ham's/F12 media supplemented with 10%FBS and G418 overnight (18–20 hrs). Serum containing media was removed 1 hour prior to calcium studies and replaced with Ham's/F12 in the absence of serum and G418. Intracellular Ca^{2+} measurements were performed as described previously [11]. Briefly, cells were incubated with 5 μM fura-2/AM in Hank's Balanced Salt Solution (HBSS) at room temperature (RT, 19°–22°C) for 45 minutes (min) in the dark, washed with dye-free buffer, and incubated for another 45 min to allow for complete deesterification of the dye (in calcium and calcium-free buffer). Coverslips were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Optical Apparatus Co., Ardmore, PA), and cells were exposed to NAGly, or the GPR55 antagonist ML193 (N-[4-[[[(3,4-Dimethyl-5-isoxazolyl)amino]sulfonyl]phenyl]-6,8-dimethyl-2-(2-pyridinyl)-4-quinolinecarboxamide) for 30 min prior to exposure to NAGly. To elucidate the contribution of various intracellular calcium stores, cells were pretreated with various calcium store receptor inhibitors as previously reported [12–14]. Briefly, cells were treated with either: bafilomycin A1 (1 μM , 1 hour), ryanodine (10 μM , 1h), 2- Aminoethoxydiphenyl borate (2-APB; 100 μM , 15min), or Xestospongine C (XeC; 10 μM , 15 min), prior to NAGly exposure. The microscope was equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Roper Scientific, Optical Apparatus Co.). During the experiments the Perfect Focus System was activated. Fura-2/AM fluorescence (emission = 510nm) following alternate excitation at 340 and 380 nm was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using Nikon NIS-Elements AR 3.1 software (Optical Apparatus Co.). The ratio of the fluorescence signals (340/380nm) was converted to Ca^{2+} concentrations [15].

2.6 Mitogen-activated protein kinase (MAPK) Assay

HAGPR55/CHO cells were plated on poly-D-lysine coated 6-well dishes in Ham's/F12 media supplemented with 10%FBS and G418 (350 $\mu\text{g}/\text{ml}$) until 80–90% confluent. At this time the antibiotic containing media was replaced with Ham's/F12 supplemented with 10% FBS overnight (18–20 hours). Following a one-hour serum starvation (37°C, 5% CO_2), cells were incubated with NAGly for 5 min (37°C, 5% CO_2) or pre-incubated with the GPR55 antagonist ML193 for 15 minutes prior to exposure to NAGly. Cells were lysed with the addition of: HEPES (50mM), NaCl (150mM), EDTA (1mM), EGTA (1mM), glycerol (10%, w/v), Triton X-100 (1%, w/v), MgCl_2 (10 μM), 20 mM *p*-nitrophenyl phosphate, 1 mM Na_3VO_4 , 25 mM NaF, and a EDTA-free protease inhibitor mixture (1:50, pH 7.5). Cell lysates were incubated on ice for 30 min followed by centrifugation at 13,200 rpm (4°C), 30 min, and the concentration of protein was determined by the Bradford method. Proteins (20 μg) were separated on a 10% acrylamide gel by SDS-PAGE followed by standard Western

protocols (phospho ERK1/2, 1:5000; total ERK1/2, 1:1000; conjugated secondary antibodies, IRdye 800CW, 1: 8000; IRDye 680, 1:10,000). Quantification of MAPK activity was accomplished using the Odyssey Infrared Imaging System and software (LI-COR, NE, USA).

Statistical analysis—Results were graphed using GraphPad Prism 5 (GraphPad Software, LaJolla, CA). Data points were graphed as a mean $\pm$ S.E.M. and analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni correction.

3. Results

3.1 GPR55 is localized to the cell membrane and within the cytoplasm

A stable cell line expressing HA incorporated into the N-terminus of the human GPR55 cDNA was generated in CHO cells (HAGPR55/CHO). Verification of expression was determined via immunohistochemistry using a mouse anti-HA antibody (Figure 1). In addition to membrane staining, puncta representing the receptor were also visualized in the cytoplasm.

3.2 Calcium mobilization induced by NAGly in HAGPR55/CHO cells is receptor mediated

Concentration dependent increases in intracellular $[Ca^{2+}]_i$ were observed following exposure to NAGly (Figure 2A). Maximal levels of $[Ca^{2+}]_i$ were apparent within two minutes whereby a concentration of 546 ± 6.2 nM was attained (Figure 2B). These increases in intracellular calcium were transient. Pre-incubation with the selective GPR55 antagonist, ML-193 completely abrogated NAGly-induced increases in $[Ca^{2+}]_i$ as shown in Figure 2B and 2C ($p < 0.05$). Exposure to ML-193 alone did not significantly alter basal levels of calcium (figure not shown). Calcium mobilization was not observed in the presence of NAGly in wild-type CHO cells, devoid of GPR55 expression. This lack of effect supports the specificity of the NAGly/GPR55-mediated calcium response. To determine the contribution of intracellular versus extracellular calcium in the NAGly-GPR55 mediated calcium response, cells were incubated in the absence of calcium. As depicted in Figure 3 (top, left panel), exposure to NAGly (3 μ M) triggered a peak increase in intracellular calcium ($[Ca^{2+}]_i = 347 \pm 3.8$ nM); significantly lower than that observed in the presence of extracellular calcium ($[Ca^{2+}]_i = 546 \pm 6.2$ nM). The time frame of peak response to the return to basal was unchanged in the absence of extracellular calcium. To shed light on the source(s) of intracellular calcium we preincubated cells with various calcium store blockers in the absence of extracellular calcium. Disruption of lysosomal calcium stores with bafilomycin A1 (1 μ M, 1h pre-incubation), an inhibitor of V-type ATPase that prevents lysosomal acidification did not significantly interfere with NAGly/GPR55-mediated increases in intracellular calcium ($[Ca^{2+}]_i = 328 \pm 4.1$ nM (Figure 3, top, right panel). There was no change in amplitude, nor time frame. Similarly, preincubation with the ryanodine receptor blocker, ryanodine (10 μ M, 15 min) did not alter intracellular levels of calcium ($[Ca^{2+}]_i = 335 \pm 3.7$ nM), compared to NAGly in zero calcium alone ($[Ca^{2+}]_i = 347 \pm 3.8$ nM), as depicted in Figure 3 (bottom, left panel). However, pretreatment with IP3 receptor blockers XeC and 2-APB, resulted in a complete loss of NAGly-induced calcium

mobilization ($[Ca^{2+}]_i = 14 \pm 1.6$ nM) as illustrated in the right, bottom panel of Figure 3 ($p < 0.05$).

3.3 Increase in MAPK activity induced by NAGly in GPR55/CHO cells is receptor mediated

In the presence of NAGly, concentration dependent increases in MAPK activity were observed. Significance ($p < 0.0001$) was achieved at both 3 μ M and 10 μ M NAGly, corresponding to a 2–3-fold increase in ERK 1/2 phosphorylation over control levels (Figure 4A). Complete blockade of NAGly-induced MAPK activity was found in the presence of the GPR55 antagonist ML193, verifying that the response is mediated by GPR55 (Figure 4B). Additional support for a specific NAGly/GPR55-mediated event comes from the finding that NAGly does not induce an increase in MAPK activity in wild-type CHO cells, similar to the lack of effect in the calcium studies (Figure 4C).

4. Discussion

Findings from the current study are the first, to our knowledge, to report activation of GPR55 by the N-arachidonoyl amino acid, NAGly. Activation of GPR55 by NAGly was determined using two different signal transduction assays, calcium mobilization and MAPK activity. In the presence of the previously reported GPR55 antagonist, ML193 [10], increases in intracellular calcium and MAPK activity were abrogated. This indicates a specific GPR55 mediated event. Further support for a receptor mediated response is the finding that wild-type CHO cells do not demonstrate an increase in calcium mobilization nor MAPK activity in the presence of NAGly.

Since NAGly/GPR55-mediated increases in calcium were observed in the presence and absence of extracellular calcium, we chose to ascertain the contribution by intracellular calcium stores as well as by TRP channels. The increase in intracellular calcium is not a consequence of calcium release from lysosomal stores as the vacuolar H^+ ATPase inhibitor bafilomycin A1 [16] was without effect on the amplitude and duration of the NAGly-GPR55 mediated calcium mobilization. To parse out the influence of IP3 receptors and TRP channels, the inhibitors 2-APB and XeC were employed. Whereas 2-APB inhibits both IP3 receptors and TRP channels [17, 18], XeC is a specific inhibitor of IP3 receptors [19]. Our findings implicate IP3 receptors rather than TRP channels as NAGly does not activate these plasma membrane channels [20]. Additionally, abolishment of the NAGly/GPR55-mediated response was observed in calcium free buffer. Since CHO cells have been shown to be devoid of ryanodine receptors [21], and ryanodine was without effect on calcium mobilization, IP3 receptors are the likely candidate involved in NAGly/GPR55-mediated calcium mobilization.

The results presented herein indicate that the NAAA, NAGly, is another endogenous ligand of GPR55. NAGly/GPR55-mediated cell signaling events include augmentation of ERK 1/2 phosphorylation and intracellular calcium. Both of these signal transduction pathways have been identified in lysophosphatidyl inositol-induced GPR55 activation [22, 23].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
LPI	lysophosphatidyl inositol
MAPK	mitogen-activated protein kinase
ML193	(N-[4-[[[(3,4-Dimethyl-5-isoxazolyl)amino]sulfonyl]phenyl]-6,8-dimethyl-2-(2-pyridinyl)-4-quinolinecarboxamide)
NAGly	N-arachidonoyl glycine
XeC	Xestopongin C

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Highlights

- N-arachidonyl glycine, an endocannabinoid metabolite, is an agonist at the orphan receptor GPR55
- N-arachidonyl glycine produces increases in intracellular calcium and mitogen activated protein kinase activities via GPR55
- Calcium mobilization by N-arachidonyl glycine is mediated via inositol-3-phosphate receptors
- A GPR55 antagonist blocks the effects of N-arachidonyl glycine

Figure 1. Intra- and extracellular localization of GPR55

Representative micrograph of immunocytochemistry of the stable HAGPR55/CHO cell line using anti-HA antibodies. Staining depicts both intra- and extracellular localization of GPR55 in permeabilized, fixed cells. White line overlaid onto micrograph corresponds to a distance of 30μm.

Figure 2. NAGly induced increases in calcium mobilization in HAGPR55/CHO cells are antagonized by the GPR55 antagonist ML193

A. Concentration dependent increases in intracellular calcium, $[\text{Ca}^{2+}]_i$, following exposure to NAGly in the stable HAGPR55/CHO cell line. **B.** Real time traces of the change in $[\text{Ca}^{2+}]_i$ prior to (basal), and following exposure to $3\mu\text{M}$ NAGly in the absence ($\square$) and presence of $10\mu\text{M}$ ML193 ($\blacksquare$). The transient increases in $[\text{Ca}^{2+}]_i$ peak at 2 minutes. **C.** Graphic representation of NAGly ($\square$) induced increases in $[\text{Ca}^{2+}]_i$ and blockade of the response in the presence of ML193 ($\blacksquare$). Significance was determined by One-way ANOVA, $p < 0.05^*$, $n=30$ cells/concentration with Bonferroni correction).

A

B

Figure 3. NAGly/GPR55-mediated calcium mobilization is dependent upon IP3 receptors, not lysosomal calcium stores nor sarcoplasmic reticulum ryanodine receptor

Real time traces of the change in $[Ca^{2+}]_i$ in the presence and absence of calcium store inhibitors, in the absence of extracellular calcium. The lysosomal disruptor, bafilomycin A1 (**top, right panel**) nor the ryanodine receptor blocker, ryanodine (**bottom, left panel**), affected the amplitude, nor time frame of NAGly-mediated calcium mobilization ($[Ca^{2+}]_i = 328 \pm 4.1$ nM and 335 ± 3.7 nM, respectively). Pretreatment with IP3 receptor blockers XeC and 2-APB, resulted in complete inhibition of NAGly-induced calcium mobilization ($[Ca^{2+}] = 14 \pm 1.6$ nM, **bottom, right panel**). Graphic representation of this data is found to the right of the real time calcium tracings (One-way ANOVA, $p < 0.05^*$ with Bonferroni correction, $n=30$ cells/treatment group).

Figure 4. NAGly induced increases in MAPK activity in HAGPR55/CHO cells is blocked by the GPR55 antagonist ML193

A. Concentration dependent increases in ERK 1/2 phosphorylation was observed in the presence of NAGly (One-way ANOVA, $p < 0.0001$ *** with Bonferroni correction, $n=4-20$).

B. Complete blockade (One-way ANOVA, $p < 0.0001$ *** with Bonferroni correction, $n=5-20$) of 3µM NAGly-induced increases in ERK 1/2 phosphorylation in the presence of the GPR55 antagonist ML193 (10µM). **C.** Effect of 10µM NAGly (solid bar) and DMSO vehicle (hatched bar) on ERK 1/2 phosphorylation in wild-type CHO cells.