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Discovery of endogenous inverse agonists for G protein-coupled receptor 6

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Abstract

The orphan G protein-coupled receptor 6 (GPR6) is highly expressed in the striatum and has been linked to multiple striatal pathologies. The identification of endogenous ligands and their mechanisms of action at GPR6 will help to elucidate the physiological and pathological roles of the receptor. In the current study, we tested the concentration-dependent effects of a variety of endocannabinoid-like N-acylamides on GPR6 signaling. Here, we demonstrate for the first time that N-arachidonoyl dopamine, N-docosahexaenoyl dopamine, N-oleoyl dopamine and N-palmitoyl dopamine exert inverse agonism at GPR6. This effect was concentration-dependent, with potencies in the micromolar range, and functionally selective for β -arrestin2 recruitment. Structure-activity relationship studies demonstrate that both the N-acyl side chain and the dopamine head group are important for these ligands to act on GPR6. Our discovery of these N-acyl dopamines as endogenous inverse agonists for GPR6 moves us one step further in understanding the roles GPR6 play in neurodegenerative and neuropsychiatric disorders related to striatal dysfunction.

Keywords

GPR6; G protein-coupled receptor; N-acyl dopamines; Inverse agonist

1. Introduction

G protein-coupled receptors (GPCRs) comprise one of the largest, most diverse receptor superfamilies and mediate a broad range of biochemical activities. To date, over 800 GPCR transcripts have been discovered, making up close to 2% of the human genome [1]. While a significant portion – approximately 35–50% [2] – of commercially available drugs in the U.S. modulate GPCR function and signaling, only ~10% of the known GPCRs are being targeted by these drugs [3]. There remains a vast number of orphan GPCRs with unknown endogenous ligands whose therapeutic potential have yet to be uncovered. Expanding our understanding of their physiological functions will aid in translational pursuits and drug discovery. Indeed, in the last two decades, deorphanization efforts have identified multiple novel GPCRs and corresponding endogenous ligands involved hunger stimulation, puberty development, reproduction and sleep regulation [4].

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G-protein coupled receptor 6 (GPR6), together with GPR3 and GPR12, constitute a subfamily of Class A orphan receptors that were first molecularly cloned from human genomic DNA in the mid 1990s [5–9]. GPR6 is primarily expressed in the human CNS [9], with the highest protein levels exhibited in striatopallidal neurons where it modulates dopamine signaling [10], motor activity [10], and instrumental learning [11]. Pharmacological targeting of GPR6 may prove therapeutically useful for treatment of striatal-associated neurological and neuropsychiatric diseases, such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, autism spectrum disorders, schizophrenia, and drug addiction [12,13].

Our recent reports have revealed that GPR6 is a novel molecular target for the phytocannabinoid cannabidiol (CBD), a major nonpsychoactive component of *Cannabis sativa*, along with its analog cannabidivarin (CBDV) and the synthetic cannabinoids WIN55,212–2, SR141716A and SR144528 [14,15]. Homology clustering analysis has revealed GPR6's phylogenetic relationship to the cannabinoid receptors CB1 and CB2, sharing 35% amino acid sequence identity in the transmembrane regions [16]. Therefore, we hypothesized that endocannabinoid-like compounds might interact with GPR6.

In the current study, we investigated potential endogenous compounds that may act on GPR6, using both β -arrestin2 recruitment and cAMP accumulation assays. We discovered that several endocannabinoid-like N-acylamides, specifically four N-acyl dopamines, are functionally selective inverse agonists for GPR6.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagles's Medium (DMEM), penicillin/streptomycin, L-glutamine, trypsin, and geneticin were obtained from VWR International (Radnor, PA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA) and dichlorodimethylsilane for silanizing glass tubes was purchased from Sigma-Aldrich (St. Louis, Mo). 384-well, round bottom, low volume white plates were purchased from Greiner Bio One (Monroe, NC). The PathHunter™ Chinese hamster ovary (CHO)–K1 β -arrestin2 human GPR6 eXpress kits were purchased from DiscoverX (Fremont, CA). The homogenous time-resolved fluorescence (HTRF) cAMP Hirange kits were purchased from CisBio International (Bedford, MA). N-acyl dopamines and other N-acylamides were purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Pathhunter™ β -arrestin2 recruitment assay

The GPR6 mediated β -arrestin2 recruitment was measured by using the PathHunter™ Chinese hamster ovary (CHO)–K1 β -arrestin2 human GPR6 eXpress kits. In this cell line, GPR6 receptors are fused with a β -galactosidase N-terminal fragment termed ProLink 1 (GPR6-PK1), and β -arrestin2 are fused to an N-terminal deleted version of β -galactosidase (EA- β -arrestin2). Activation of the receptor induces β -arrestin2 recruitment, causing complementation of the two β galactosidase enzyme fragments. Levels of the active enzyme are the direct result of β -arrestin2 recruitment caused by receptor activation and quantified

using the PathHunter™ detection reagent containing β -galactosidase substrates. The assays were performed following manufacturer's instructions for the PathHunter eXpress kits. Briefly, cells were plated in 384-well plates in DiscoverX cell plating reagent1 for 48 h in a humidified atmosphere at 37 °C and 5% CO₂. Compounds were serially diluted in cell plating reagent! After incubation with ligand at 37 °C following manufacturer's instructions, the cells were incubated with detection reagent for 1 h at room temperature in the dark. Subsequently, the chemiluminescence signal, measured as relative luminescence units, was detected using a TECAN GENios Pro microplate reader.

2.3. Cell-based HTRF cAMP assay

The HTRF cAMP assay was performed as previously published with modifications [17]. Cellular cAMP levels were measured using reagents supplied by Cisbio International (HTRF cAMP HiRange kit). Cultured cells were washed twice with phosphate-buffered saline (8.1 mM NaH₂PO₄, 1.5 mM KH₂PO₄, 138 mM NaCl, and 2.7 mM KCl, pH 7.2), and then dissociated in phosphate-buffered saline containing 1 mM EDTA. Dissociated cells were collected by centrifugation for 5 min at 2000g. The cells were resuspended in cell buffer (DMEM plus 0.2% fatty acid free bovine serum albumin) and centrifuged a second time at 2000g for 5 min at 4 °C. Subsequently, the cells were resuspended in an appropriate final volume of cell buffer plus the phosphodiesterase inhibitor Ro 20-1724 (2 μ M). 5000 cells were added at 5 μ l per well into 384-well, round bottom, low volume white plates (Grenier Bio One, Monroe, NC). Compounds were diluted in drug buffer (DMEM plus 2.5% fatty acid free bovine serum albumin) and added to the assay plate at 5 μ l per well. Cells were treated with drugs or vehicle for 1 h in a humidified incubator at 37 °C and 5% CO₂. d2-conjugated cAMP and Europium cryptate-conjugated anti-cAMP antibody were added to the assay plate at 5 μ l per well. After 1-h incubation at room temperature, the plate was read on a TECAN GENious Pro microplate reader.

2.4. Data analysis

For β -arrestin2 recruitment assays, ligand-induced changes in β -arrestin2 recruitment were calculated by dividing luminescence readings in the presence of different concentrations of ligands by basal luminescence readings, times 100. For both cAMP accumulation and β -arrestin2 recruitment assays, data were subject to non-linear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA) and the graphs were also generated using GraphPad Prism. Data points represent the mean $\pm$ SEM obtained from three independent experiments performed in quadruplicate.

For cAMP accumulation assays, data analyses were performed based on the ratio of fluorescence intensity of each well at 620 nm and 665 nm. Data are expressed as F%, which is defined as [(standard or sample ratio – ratio of the negative control)/ratio of the negative control] x 100. The standard curves were generated by plotting F% versus cAMP concentrations using non-linear least squares fit (Prism software, GraphPad, San Diego, CA). Unknowns are determined from the standard curve as nanomolar concentrations of cAMP. Ligand-induced changes in cAMP accumulation were calculated by dividing cAMP levels in the presence of different concentrations of ligands by basal cAMP levels, times 100.

Statistical analyses were performed using unpaired *t*-test, or one-way ANOVA followed with Bonferroni's post-test. *p*-values of <0.05 were considered significant.

3. Results

While a variety of exogenous compounds, including the phytocannabinoids CBD and CBDV, have been shown to act on orphan receptor GPR6 [14,15], an endogenous ligand has yet to be confirmed for this receptor. The present study was designed to test endocannabinoid-like compounds (N-acylamides) on GPR6-mediated cell signaling with the goal of deorphanizing the receptor.

3.1 Effects of N-acyl dopamines on GPR6-mediated β -arrestin2 recruitment and cAMP accumulation

Due to GPR6's close phylogenetic relationship to the cannabinoid receptors CB1 and CB2, we hypothesized that an endocannabinoid or its analog may be able to interact with the orphan receptor and alter receptor-mediated signaling. In the first set of experiments, we investigated the concentration-dependent effects of the CB1 agonist N-arachidonoyl dopamine (NADA) (Fig. 1) on GPR6-mediated signaling using β -arrestin2 recruitment and cAMP accumulation assays. As shown in Fig. 2A, GPR6-mediated constitutive β -arrestin2 recruitment was concentration-dependently inhibited following treatment with NADA. In contrast, at concentrations ranging from 10 pM–10 μ M, NADA failed to significantly alter GPR6-mediated constitutive cAMP accumulation (Fig. 2B).

Given these results, we next looked at three additional endogenous N-acyl dopamines – N-docosahexaenoyl dopamine (DHDA), N-oleoyl dopamine (OLDA) and N-palmitoyl dopamine (PALDA) (Fig. 1). Similar to NADA, all three compounds concentration-dependently reduced GPR6-mediated constitutive β -arrestin2 recruitment, but not did not alter GPR6-mediated constitutive cAMP accumulation (Fig. 2A and B). Among the four N-acyl dopamines tested, OLDA and PLDA had higher efficacy than DHDA and NADA; the potencies were roughly equivalent for all four compounds, with EC₅₀s in the μ M range.

In order to examine the functional importance of the N-acyl tail, we next tested whether dopamine alone could alter GPR6-mediated signaling. As shown in Fig. 2A and B, dopamine alone did not cause any significant change in either constitutive β -arrestin2 recruitment or cAMP accumulation at concentrations ranging from 10 pM–10 μ M.

Together, these results suggest that the presence of a fatty acid conjugated to the dopamine structure is necessary for these ligands to cause signaling alterations mediated by GPR6. However, the effects of these N-acyl dopamines on GPR6 appear to be exclusive to the β -arrestin2 recruitment pathway, as cAMP accumulation was unchanged by all the N-acyl dopamines tested.

3.2. Effects of head groups on N-oleoyl-conjugated derivatives on GPR6-mediated signaling

We next tested the hypothesis that altering the head group conjugated to the fatty acid moiety might impact the ability of the compounds to alter GPR6 signaling. We chose to

investigate oleic acid derivatives specifically, because OLDA was one of the most efficacious N-acyl dopamines acting on GPR6, and fewer variations of arachidonoyl, docosahexaenoyl and palmitoyl acid-conjugated derivatives were commercially available. Head groups included in this study were alanine, glycine, serine and valine (Fig. 1).

Interestingly, no significant change from constitutive GPR6-mediated β -arrestin2 recruitment was observed with N-oleoyl alanine (OLAla), N-oleoyl glycine (OLGly), N-oleoyl L-serine (OLSer) and N-oleoyl valine (NOVal) at concentrations ranging from 10 pM–10 μ M (Fig. 3A). In addition, none of these oleoyl-conjugated amino acids altered GPR6-mediated constitutive cAMP accumulation (Fig. 3B). These data indicate that the dopamine head group is necessary for reducing GPR6-mediated constitutive β -arrestin2 recruitment.

4. Discussion

4.1. Structure-activity relationship of N-acyl dopamines as inverse agonists for GPR6

The aim of this study was to identify potential endogenous ligands for the class A orphan receptor GPR6 to better understand its physiological role(s). The endogenous ligands for GPR6 has been debated. Both Uhlenbrock et al. (2002) and Ignatov et al. (2003) demonstrated the ability of sphingosine-1-phosphate to activate the receptor [18,19]. However, because these results could not be replicated [20], GPR6 is currently still classified as an orphan GPCR. Here, we report the discovery of endogenous N-acyl dopamines as inverse agonists for GPR6, that are able to interact with the receptor to inhibit GPR6-mediated constitutive β -arrestin2 recruitment.

A number of arachidonic acid derivatives have been reported as endocannabinoids, including anandamide (AEA), 2-arachidonoylglycerol (2-AG), noladin ether, virodhamine and NADA [21,22]. Our previous study tested the first four of these compounds and demonstrated that none of these endocannabinoids (AEA, 2-AG, noladin ether, and virodhamine) were able to alter GPR6-mediated either β -arrestin2 recruitment or cAMP accumulation [15]. However, NADA was not investigated in the previous study, and acted as the starting point for the present study. Our current observation that NADA caused a significant, concentration-dependent reduction of GPR6-mediated β -arrestin2 recruitment, together with the fact that AEA, 2-AG, noladin ether and virodhamine did not affect GPR6-mediated signaling, suggest that the dopamine moiety is crucial for NADA to act on GPR6. Furthermore, the current study showed that replacing the dopamine head group with amino acids (alanine, glycine, serine and valine) lead to a complete loss of inverse agonistic activity. These data reinforce the notion that the dopamine head group is crucial for the inverse agonistic effects of N-acyl dopamines on GPR6.

In this study, we also examined the influence of the length and degree of saturation of the N-acyl tail attached to dopamine on GPR6-mediated signaling. Among the compounds tested – NADA (20 carbon side-chain with 4 double bond), DHDA (22 carbon side chain with six double bonds), OLDA (18 carbon, monounsaturated side chain) and PALDA (16 carbons, saturated side chain) – the rank order of efficacy was PALDA=OLDA > NADA = DHDA in

inhibiting β -arrestin2 recruitment to GPR6. These data suggest that shorter, more saturated side chains favor higher efficacy.

Overall, our current results demonstrate that both the dopamine head group and the fatty acid side chain are important for inhibition of GPR6-mediated β -arrestin2 recruitment. In addition, the lack of activity of N-acyl dopamines in the cAMP accumulation assay further suggests functional selectivity of these endogenous compounds on GPR6 signaling.

4.2. Functional significance of the discovery of N-acyl dopamines as inverse agonists for GPR6

Cloning experiments first conducted in the mid 1990s revealed GPR6 to be expressed predominately in the CNS, with only trace amounts in the periphery [9]. Of the central tissues, the striatum exhibits the highest GPR6 protein levels [9]. Functionally, the striatum, as the principal input structure of the basal ganglia, regulates the motor control system, participates in the reward pathway, and influences learning and memory [12,13]. Pathophysiologically, striatal structures have been implicated in a large number of neurodegenerative diseases and neuropsychiatric disorders, most notably Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, schizophrenia, autism, and drug addiction [12,13]. It makes sense then that GPR6 might be linked to many of these same pathologies. For example, GPR6 knockout has been shown to decrease dyskinesia by altering striatal dopamine levels in a mouse model of Parkinson's disease [10].

Among the four N-acyl dopamines tested in this study, NADA, OLDA and PALDA are found in the mammalian brain regions, including striatum [23,24]. While the biological activity of dopamine in the striatum has been well recognized, the physiological functions of its N-acyl condensation products NADA, DHDA, OLDA and PALDA remain largely unknown, and the molecular targets for these N-acyl dopamines have yet to be fully characterized.

NADA was first identified as a potential endocannabinoid by Bisogno et al., in 2000, after the compound was found to inhibit binding of [3 H]SR141716A, a selective CB1 antagonist, to rat brain membranes [25]. NADA was subsequently suggested to be a putative endovanilloid, exerting potent agonist activity at TRPV1 [23].

DHDA exhibits cannabimimetic properties in vivo, although there is little information available regarding the effects of DHDA on CB1 and TRPV1 [26].

OLDA is a putative endovanilloid because it activates TRPV1 [24]. In addition, OLDA has moderate affinity for the CB1 cannabinoid receptor [24]. Intraperitoneal injection of OLDA to mice/rats has been shown to cause hyperlocomotion. Therefore, it has been suggested as a potential neuromodulator of motor behavior [27]. In terms of signaling in striatum, Sergeeva et al. (2017) found that OLDA interacts with TRPV1 channels and the endocannabinoid system to modulate neural firing of nigrostriatal DA neurons [27].

Palmitic acid accounts for 20–30% of total fatty acids in the human body, making it the most common saturated fatty acid [28]. While PALDA alone is relatively inactive at TRPV1, this palmitic acid derivative is able to produce an entourage effect, potentiating NADA-induced

TRPV1 signaling [29]. It has not been reported whether PALDA interacts with cannabinoid receptors.

To our knowledge, this is the first report that endocannabinoid-like compounds N-acyl dopamines are inverse agonists for GPR6. This discovery has important functional implications. Since GPR6 plays important physiological and pathophysiological roles in striatum, it is highly possible that N-acyl dopamines, by affecting on GPR6, might be involved in the normal function as well as pathological conditions of striatum.

GPR6 is constitutively coupled to both β -arrestin2 and the G_s protein (leading to constitutively enhanced β -arrestin2 recruitment and cAMP accumulation) [15]. The cAMP pathway has been shown to be important for several GPR6 functions, such as enhancing neurite outgrowth, blocking myelin sheath inhibition, and promoting axonal regeneration [30]. Since we found that N-acyl dopamines exhibit functional selectivity for GPR6-mediated β -arrestin2 recruitment over cAMP accumulation, this may shed some light on the mechanism of action of these endogenous compounds on striatal function and pathologies.

In summary, by discovering several N-acyl dopamines as functionally selective inverse agonists for GPR6, this study contributes to our overall knowledge of this orphan receptor and moves us one step closer to understand the functional roles of GPR6 and its endogenous ligands. Further studies are warranted to elucidate the detailed mechanisms by which endogenous N-acyl dopamines exert their effects on striatum function and pathology through GPR6.

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Fig. 2. Effects of side chain length and degree of saturation on the effects of N-acyl dopamines on GPR6-mediated signaling.

(A) Concentration-response curves of N-acyl dopamines on β -arrestin2 recruitment to GPR6. (B) Concentration-response curves of N-acyl dopamines on GPR6-mediated cAMP accumulation. Data shown represent the mean $\pm$ SEM of three experiments performed in quadruplicate. Data were analyzed by one-way analysis of variance followed by Bonferroni's post-test (*OLDA, #PALDA, †NADA, §DHDA, $p < 0.05$ compared to basal recruitment).

Fig. 3. Effects of head groups on N-oleoyl-conjugated derivatives on GPR6-mediated signaling. (A) Concentration-response curves of N-oleoyl derivatives on GPR6-mediated β -arrestin2 recruitment to GPR6. (B) Concentration-response curves of N-oleoyl derivatives on GPR6-mediated cAMP accumulation. Data shown represent the mean $\pm$ SEM of three experiments performed in quadruplicate. Data were analyzed by one-way analysis of variance followed by Bonferroni's post-test (*OLDA $p < 0.05$ compared to basal recruitment).