Purinergic Signalling (2004) 1: 67–74

GPR80/99, proposed to be the $P2Y_{15}$ receptor activated by adenosine and AMP, is not a P2Y receptor

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Received 26 August 2004; accepted in revised form 14 October 2004

Key words: α-ketoglutarate, cyclic AMP accumulation, GPR80, inositol phosphate accumulation, P2Y receptor, $P2Y_{15}$ receptor

Abstract

The orphan receptor GPR80 (also called GPR99) was recently reported to be the $P2Y_{15}$ receptor activated by AMP and adenosine and coupled to increases in cyclic AMP accumulation and intracellular Ca^{2+} mobilization (Inbe et al. J Biol Chem 2004; 279: 19790–9 [12]). However, the cell line (HEK293) used to carry out those studies endogenously expresses A_{2A} and A_{2B} adenosine receptors as well as multiple P2Y receptors, which complicates the analysis of a potential P2Y receptor. To determine unambiguously whether GPR80 is a P2Y receptor subtype, HA-tagged GPR80 was either stably expressed in CHO cells or transiently expressed in COS-7 and HEK293 cells, and cell surface expression was verified by radioimmunoassay (RIA). COS-7 cells overexpressing GPR80 showed a consistent twofold increase in basal inositol phosphate accumulation. However, neither adenosine nor AMP was capable of promoting accumulation of either cyclic AMP or inositol phosphates in any of the three GPR80-expressing cells. A recent paper (He et al. Nature 2004; 429: 188–93 [15]) reported that GPR80 is a Gq-coupled receptor activated by the citric acid cycle intermediate, α-ketoglutarate. Consistent with this report, α-ketoglutarate promoted inositol phosphate accumulation in CHO and HEK293 cells expressing GPR80, and pretreatment of GPR80-expressing COS-7 cells with glutamate dehydrogenase, which converts α-ketoglutarate to glutamate, decreased basal levels of inositol phosphates. Taken together, these data demonstrate that GPR80 is not activated by adenosine, AMP or other nucleotides, but instead is activated by α-ketoglutarate. Therefore, GPR80 is not a new member of the P2Y receptor family.

Abbreviations: ADA – adenosine deaminase; Ado – adenosine; GluDH – glutamate dehydrogenase; HA – hemagglutinin; HBSS – Hank's balanced salt solution; PBS – phosphate-buffered saline; PPADS – pyridoxal-phosphate-6-azophenyl-2′, 4′-disulphonic acid; RIA – radioimmunoassay

Introduction

Extracellular adenine and uridine nucleotides exert their physiological effects through two main receptor families: The ligand-gated ion channel P2X receptors and the G protein-coupled P2Y receptors [1]. Molecular cloning and functional studies by a number of laboratories have identified eight functional mammalian P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) [2]. These receptors can be subdivided pharmacologically into the adenosine nucleotide-preferring P2Y receptors activated primarily by ATP and/or ADP ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$), the uridine nucleotide-preferring receptors ($P2Y_4$, $P2Y_6$, and $P2Y_{14}$) activated by UTP, UDP or UDP sugars, and a receptor ($P2Y_2$) activated equipotently by both ATP and UTP. From a phylogenetic, structural, and signaling point of view, P2Y receptors fall into two subfamilies. The $P2Y_1$ receptor subfamily, which encompasses $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors, shares 35%–52% sequence identity (with exception of 28%–30% identity of the $P2Y_{11}$ receptor to the other four members) and couples to Gq thereby activating phospholipase C. The $P2Y_{12}$ receptor subfamily, which includes $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, is located in a gene cluster on chromosome 3, shares 45%–55% identity, and couples to Gi/o and thereby inhibits adenylyl cyclase.

The identification and naming of new P2Y receptors has been controversial and has led to considerable confusion in P2Y receptor nomenclature. For example, four receptors were originally identified as P2Y receptors ($P2Y_5$, $P2Y_7$, $P2Y_9$, and $P2Y_{10}$) [3, 4] but were shown later either to not respond to nucleotides ($P2Y_5$, $P2Y_7$) [5, 6] and/or to be activated by a non-nucleotide ligand ($P2Y_7$, $P2Y_9$) [7, 8].

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Moreover, two additional receptors, chick $P2Y_3$ [9] and Xenopus $P2Y_8$ [10] receptors, are likely species homologues of human $P2Y_6$ and $P2Y_4$ receptor subtypes, respectively [11]. Problems in identifying new P2Y receptors arise in part because of (1) the low sequence identity between bona fide P2Y receptor subtypes, (2) the lack of a generally applicable radioligand for P2Y receptors, (3) endogenous expression of one or more subtypes of P2Y and adenosine receptors on model cell lines in which potentially new receptors are expressed and characterized, and (4) extracellular metabolism and interconversion of nucleotides, which can lead to unintended or misleading responses to added nucleotides. Thus, identification of a new receptor in the P2Y family requires rigorous demonstration of receptor activity.

Recently, the orphan receptor GPR80 (also called GPR99), which encodes a protein of 337 amino acids with 25%–36% sequence identity (43%–58% similarity) to members of the $P2Y_1$ receptor subfamily, was reported to be the $P2Y_{15}$ receptor and to mediate AMP- and adenosine-promoted increases in cyclic AMP accumulation and mobilization of intracellular Ca^{2+} [12]. However, the cell line (HEK293) used to examine the pharmacological selectivity of recombinant GPR80 endogenously expresses $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{12}$ and $P2Y_{13}$ receptors as well as A_{2A} and A_{2B} adenosine receptors [12–14], which markedly obfuscates the potential study of recombinant receptors activated by either nucleotides or nucleosides. Moreover, a recent study reported activation of GPR80 by the citric acid cycle intermediate α-ketoglutaric acid [15], which further calls into question the assertion that GPR80 is a receptor for adenosine and AMP.

To determine unambiguously whether GPR80 is a new P2Y receptor subtype, HA-tagged GPR80 was stably expressed in CHO cells or transiently expressed in COS-7 and HEK293 cells, and activation by AMP and adenosine was evaluated by measuring both inositol phosphate and cyclic AMP accumulation. Our data demonstrate that GPR80 is not activated by adenosine, AMP, or any other nucleotides, but instead is activated by the citric acid cycle intermediate, α-ketoglutarate, as reported by He et al. [15].

Materials and methods

Materials

All cell culture reagents were supplied by the tissue culture facility in the Lineberger Comprehensive Cancer Center (University of North Carolina at Chapel Hill). AMP, adenosine, ADP, UDP, α-ketoglutarate, adenosine deaminase (ADA), Ro20-1724, glutamate dehydrogenase, and carbachol were from Sigma (St Louis, Missouri, USA). ATP, UTP, and the transfection reagent FuGENE6 were from Roche Biochemicals (Indianapolis, Indiana, USA). Forskolin was purchased from Calbiochem (La Jolla, California, USA). Suramin and PPADS (pyridoxal-phosphate-6-azophenyl-2′, 4′-disulphonic acid) were from RBI (Natick, Massachusetts, USA).

Construction of HA-tagged GPR80 cDNA

The coding sequence of GPR80 was amplified by PCR with *Pfu* polymerase (Stratagene) from human genomic DNA. The 5′ primer (5′-GAGACGCGTCCAATGAGC CACTAGACTATTTA-3′) was complementary to codons 2–7 of GPR80 and contained an *Mlu*I site at the 5′ end, while the 3′ primer (5′-AGACTCGAGTCAAGGGTTGTT TGAGTAACTGATT-3′) was complementary to codons 337–331 and contained a *Xho*I site following the stop codon. The amplified cDNA was digested with *Mlu*I and *Xho*I and ligated in-frame into a similarly digested pLXSN retroviral expression vector containing an upstream *Eco*RI restriction site, Kozak initiation sequence, initiating methionine residue, and the hemagglutinin (HA) epitope tag (YPYDVPDY) to yield pLXSN-GPR80. To construct pcDNA3-GPR80, pLXSN-GPR80 was digested with *Eco*RI and *Xho*I and ligated into the similarly digested expression vector, pcDNA3.

Transient expression of GPR80 in COS-7 and HEK293 cells

All cells were maintained in DMEM-High Glucose (DMEM-H) with 10% serum. Transient transfection of COS-7 and HEK293 cells was performed according to protocols supplied with the FuGENE6 reagent. Briefly, 1.5×10^6 cells per 60-mm dish were incubated overnight in 4 ml medium. The next day, 8 μl FuGENE6 and 2.8 μg of either pcDNA3 or pcDNA3-GPR80 in 400 μl serum-free DMEM-H was added to the cells for 5–6 h. The transfected cells then were trypsinized and seeded in 24-well plates for assays.

Stable expression of GPR80 in CHO cells

The procedure for stable receptor expression was described in detail previously [16]. Briefly, recombinant retrovirus particles were produced by calcium phosphate-mediated transfection of PA317 cells with pLXSN or pLXSN-GPR80 and used to infect CHO cells. Geneticin-resistant cells were selected with DMEM-H containing 1 mg/ml G418.

Assays for inositol phosphate and cyclic AMP accumulation

CHO cells were seeded in 24-well plates at 5×10^4 cells/well and assayed 3 days later. For transiently transfected COS-7 and HEK293 cells, 2×10^5 cells/well were seeded in 24-well plates and assayed 2 days later. Inositol lipids were radiolabelled by overnight incubation of the cells with 200 μl serum-free, inositol-free DMEM containing

0.4 μCi *myo*-[^{3}H]inositol per well. Agonists or antagonists were added at 5× concentration in 50 μl of 50 mM LiCl, 250 mM HEPES, pH 7.25. Following a 10-min incubation (30 min incubation in Figures 1C and 5) at 37 °C, the reaction was terminated by aspirating the medium and adding 0.75 ml boiling EDTA, pH 8.0. [^{3}H]Inositol phosphates were resolved with Dowex AG1-X8 columns.

To monitor cyclic AMP accumulation, cells were labeled with 200 μl serum-free DMEM containing 0.8 μCi of [^{3}H]adenine for 2 h. Twenty minutes prior to the assay, cells were supplemented with 50 mM HEPES, pH 7.4. Ro20-1724 (100 μM), which in contrast to IBMX used by Inbe and coworkers [12] does not inhibit adenosine receptors, was added 10 min prior to the initiation of the assay to inhibit cyclic nucleotide phosphodiesterases. Drugs were added at 6× concentration in 50 μl of Hank's balanced salt solution (HBSS) for 10 min at 37 °C. Reactions were terminated by aspiration of the drug-containing medium and addition of 1 ml of ice-cold 5% trichloroacetic acid. [^{3}H]Cyclic AMP was purified using Dowex and alumina columns and quantified by scintillation counting.

Radioimmunoassay (RIA) to quantify cell surface expression

Cells were seeded in 24-well plates at the densities described above and assayed 2–3 days later as described previously [17]. Cells were fixed in 4% paraformaldehyde, washed, blocked, and then incubated with a 1:1,000 dilution of mouse anti-HA monoclonal antibody (clone HA.11) for 1 h. Cells were washed twice with HBSS containing 1 mM Ca^{2+} and Mg^{2+}, followed by incubation with [^{125}I]-labeled rabbit anti-mouse antibody. After incubating for 2 h, the cells were washed twice with HBSS containing Ca^{2+} and Mg^{2+}, solubilized with 1 M NaOH, and transferred to glass tubes for quantitation of radioactivity by γ-counting.

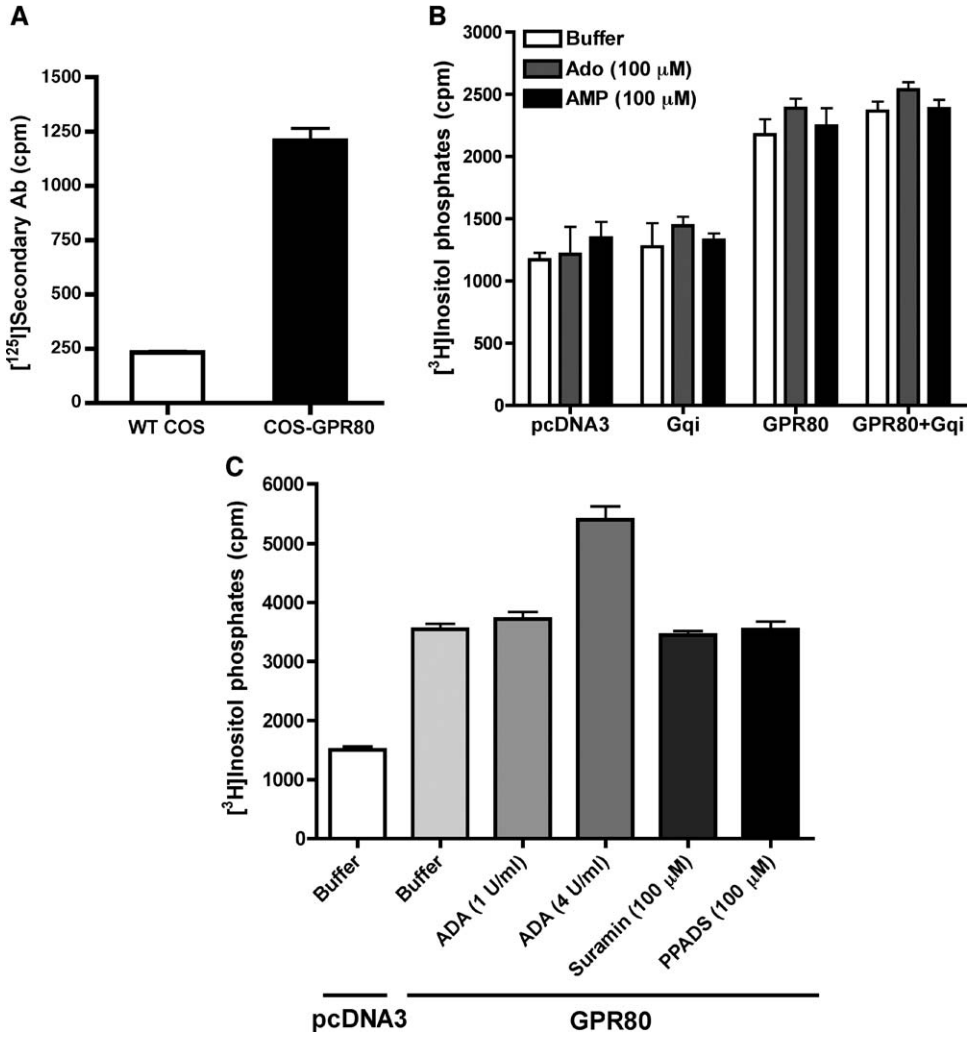

Figure 1. Lack of adenosine- and AMP-promoted inositol phosphate accumulation in COS-7 cells transiently expressing GPR80. COS-7 cells were transfected with pcDNA3 or pcDNA3-HA-GPR80 and analyzed for cell surface expression and receptor activity. A) Quantitation of cell surface expression of HA-tagged GPR80 by RIA. B) Cells were transfected with the indicated plasmids and the capacity of adenosine (Ado) and AMP to increase inositol phosphate accumulation over 10 min was measured. C) GPR80-expressing COS-7 cells were incubated for 30 min with adenosine deaminase (ADA), suramin, or PPADS at the indicated concentrations, followed by addition of LiCl and incubation for a further 30 min. Data shown are the mean from triplicate assays from three separate experiments.

Results

Transient expression of GPR80 in COS-7 cells

To identify the ligand(s) that activate GPR80, we first expressed the HA-tagged receptor transiently in COS-7 cells. GPR80 was well expressed in COS-7 cells as determined by a cell-surface RIA (Figure 1A). To assess the capacity of adenosine or AMP to activate GPR80, COS-7 cells were transfected with pcDNA3 or pcDNA3-GPR80 either with or without a plasmid encoding chimeric Gqiα [18], and then challenged with high concentrations (up to 100 μM) of adenosine or AMP. Gqiα is a chimeric Gα subunit in which the last five amino acids of Gqα have been replaced with the corresponding residues from Giα, allowing Gi-coupled receptors to activate phospholipase C and increase inositol phosphate accumulation.

Neither adenosine nor AMP promoted inositol phosphate accumulation in either vector- or GPR80-transfected cells (Figure 1B). Similarly, ATP, ADP, UTP, UDP and UDP-glucose (all at 100 μM) failed to promote inositol phosphate accumulation (data not shown).[1] However, we consistently observed an approximately twofold increase in basal inositol phosphate accumulation in GPR80-transfected cells compared to vector-transfected cells, and this increase in basal levels was not affected by co-expression of Gqiα. The increase in basal inositol phosphate levels in GPR80-transfected cells and the absence of a further increase when GPR80 was co-expressed with Gqiα suggests that GPR80 is coupled to Gq/phospholipase C.

The GPR80-dependent increase of basal inositol phosphate accumulation could be due either to constitutive activity of the receptor or to the activation of GPR80 by endogenously released compounds. To assess whether adenosine is released and activates the receptor, cells were preincubated with 1 or 4 U/ml of adenosine deaminase for 1 h prior to the addition of Li and incubation for an additional 30 min. No decrease in inositol phosphate accumulation was observed in the presence of the adenosine-metabolizing enzyme (Figure 1C). We also tested the capacity of non-selective P2 receptor antagonists, PPADS and suramin, to inhibit basal accumulation of inositol phosphates, but both of these compounds had no effect.

Stable expression of GPR80 in CHO cells

CHO cells were utilized as an additional cell line to investigate the signaling properties of GPR80. HA-tagged GPR80 was stably expressed in CHO cells following retroviral infection, and immunoassays indicated that the receptor was well expressed in CHO cells (Figure 2A). Neither AMP nor adenosine (at 100 μM) promoted inositol

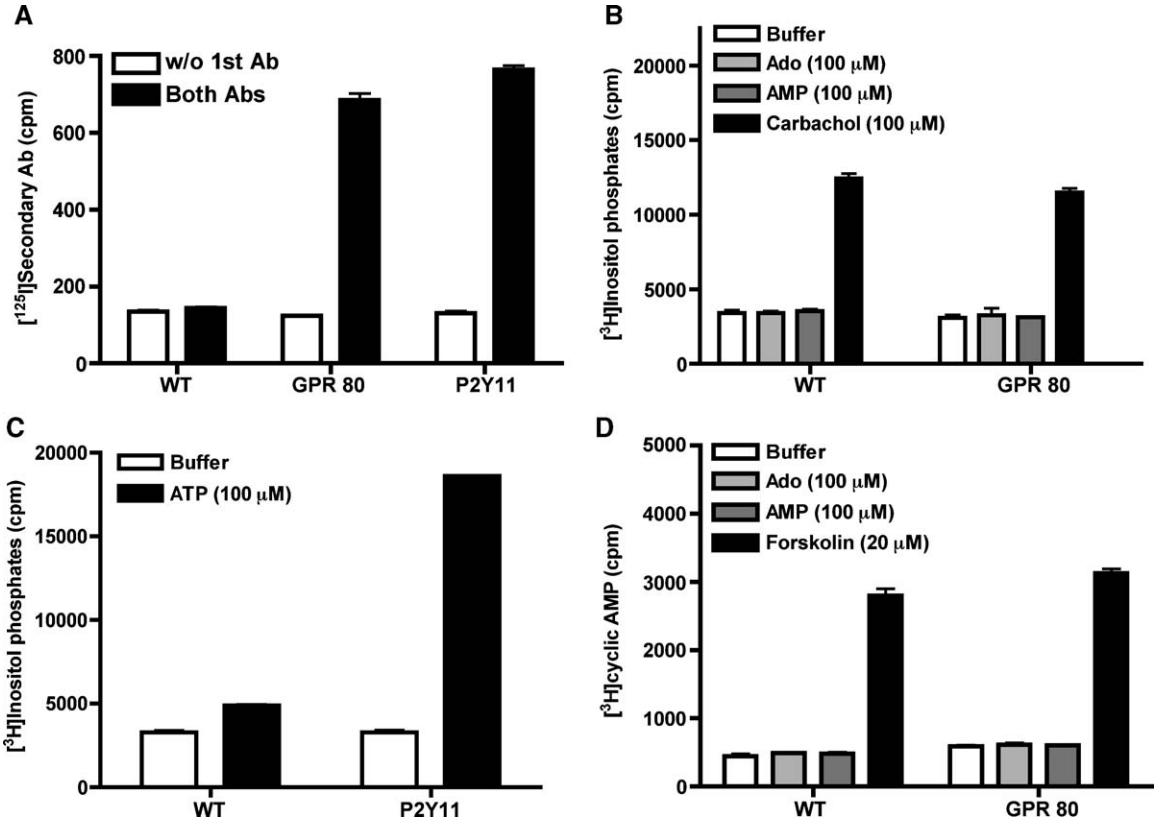

Figure 2. Lack of adenosine- and AMP-promoted second messenger signaling in CHO cells stably expressing GPR80. GPR80 was stably expressed in CHO cells by retroviral infection and selection of G418-resistant cells. A) Quantitation of cell surface expression of HA-tagged receptors by RIA. $P2Y_{11}$ refers to CHO cells stably expressing the HA-$P2Y_{11}$ receptor. B) Adenosine (Ado), AMP and carbachol were added to wild-type and GPR80-expressing CHO cells, and their capacity to promote [^{3}H]inositol phosphate accumulation was assessed. C) ATP (100 μM) was added to wild-type and $P2Y_{11}$ receptor-expressing CHO cells for 10 min, followed by measurement of [^{3}H]inositol phosphates accumulation. D) The capacity of adenosine (Ado), AMP and forskolin to increase [^{3}H]cyclic AMP accumulation in wild-type and GPR-80 expressing CHO cells was measured. Data shown are the mean from triplicate assays from three separate experiments.

phosphate or cyclic AMP accumulation (Figures 2B and D). In contrast, carbachol, which activates an endogenous muscarinic receptor, promoted a robust increase in inositol phosphates in both wild-type and GPR80-expressing cells (Figure 2B), and ATP (100 μM) promoted large increases in inositol phosphate accumulation in CHO cells exogenously expressing the P2Y$_{11}$ receptor (Figure 2C). These data provide additional data in a different cell line demonstrating that neither AMP nor adenosine is an agonist for GPR80.

Transient expression of GPR80 in HEK293 cells

The studies of Inbe et al. [12] reporting AMP- and adenosine-promoted increases in intracellular calcium mobilization and cyclic AMP accumulation were carried out in HEK293 cells transiently transfected with GPR80. Although we did not observe the agonist activity of AMP or adenosine at GPR80 in either COS-7 or CHO cells, the possibility remained that an endogenous factor exists in HEK293 that combines with GPR80 to convey nucleotide/nucleoside sensitivity to the receptor. To test this possibility, we transiently expressed GPR80 in HEK293 cells and tested the capacity of ATP, AMP and adenosine to promote inositol phosphate or cyclic AMP accumulation. Although HA-tagged GPR80 was well expressed in HEK293 cells (Figure 3A), neither AMP nor adenosine (up to 100 μM) promoted increases in inositol phosphate accumulation (Figure 3B) in cells transfected with GPR80 compared to the cells transfected with empty vector. A slight increase in inositol phosphate accumulation was observed with ATP, which presumably occurred due to activation of endogenous P2Y$_1$ and P2Y$_2$ receptors in HEK293 cells [13]. Both AMP and adenosine promoted cyclic AMP accumulation in HEK293 cells, but no difference was observed in cells transfected with empty vector *versus* cells expressing GPR80 (Figure 3C). Taken together, our data in three different cell lines demonstrate that GPR80 is not a receptor for either adenosine or AMP.

A citric acid cycle intermediate, α-ketoglutarate, is an agonist for GPR80

During the course of this work, α-ketoglutarate, a citric acid cycle intermediate, was reported to activate GPR80, promoting intracellular calcium mobilization and inositol phosphate accumulation through a Gq-mediated pathway [15]. To confirm these results, we assessed the capacity of α-ketoglutarate to promote inositol phosphate accumulation in CHO-GPR80 and in GPR80-transfected HEK293 cells. As shown in Figure 4, α-ketoglutarate increased inositol phosphate accumulation in a concentration-dependent manner in both cell lines, with an EC$_{50}$ of 140 ± 10 μM in CHO-GPR80 cells and 160 ± 20 μM in HEK293-GPR80 cells.

As shown above (Figures 1B and C), expression of GPR80 in COS-7 cells resulted in increased basal accumulation of inositol phosphates. To determine if GPR80-dependent increases in inositol phosphate accumulation was

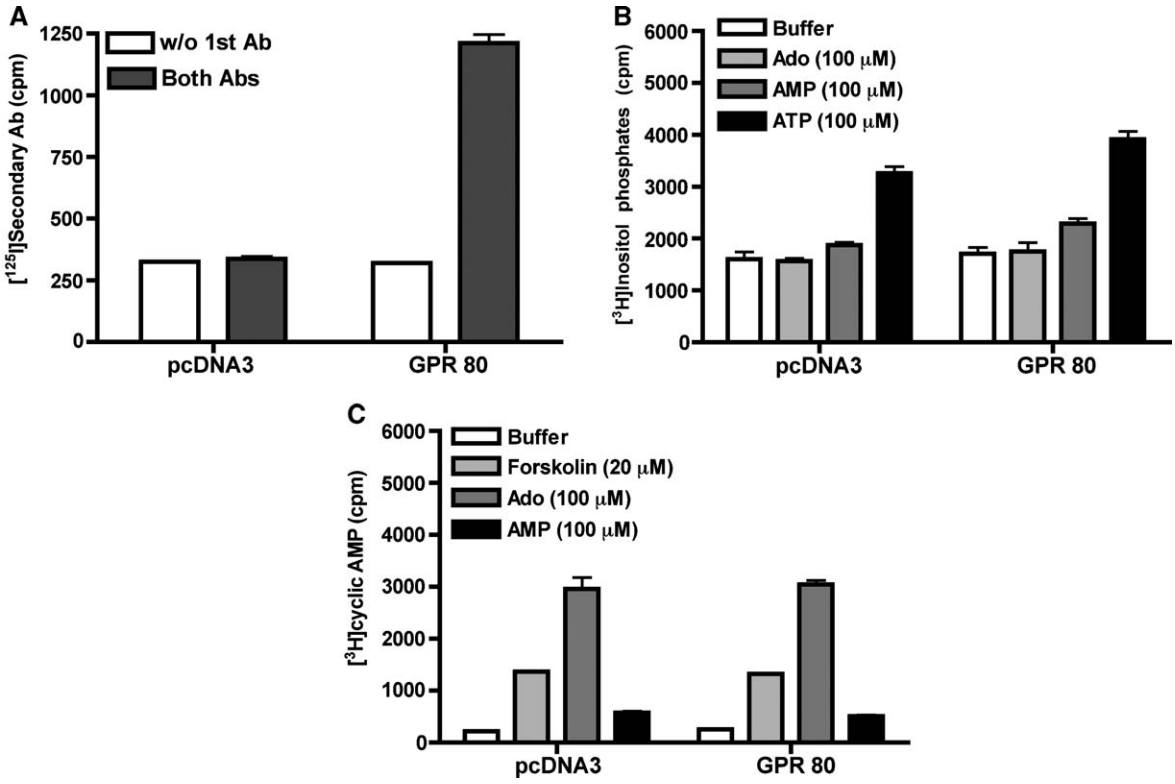

Figure 3. Lack of adenosine- and AMP-promoted second messenger signaling in GPR80-expressing HEK293 cells. HEK293 cells were transiently transfected with empty vector or pcDNA3-HA-GPR80, and analyzed for cell surface expression and receptor activity. A) Quantitation of cell surface expression of HA-tagged GPR80 by RIA. B) Quantitation of [^{3}H]inositol phosphate accumulation in empty vector- and GPR80-transfected HEK293 cells in response to the indicated agents. C) Quantitation of [^{3}H]cyclic AMP accumulation in empty vector- and GPR80-transfected cells in response to the indicated agents. Data shown are the mean from triplicate assays from three separate experiments.

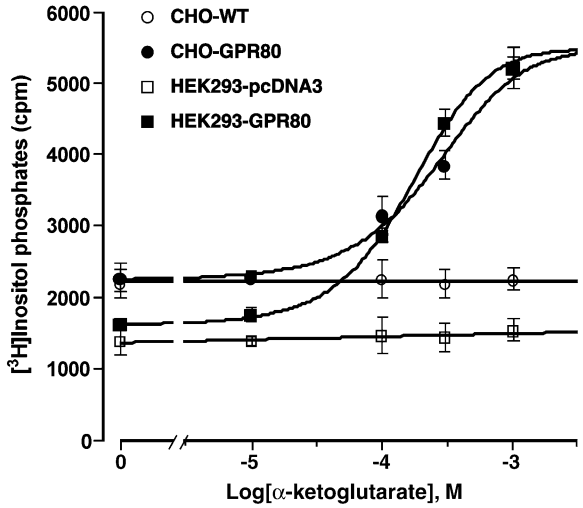

Figure 4. GPR80 is activated by α-ketoglutarate, a citric acid cycle intermediate. CHO and HEK293 cells expressing GPR80 were challenged with α-ketoglutarate and [^{3}H]inositol phosphate accumulation was measured. Data shown are the mean from triplicate assays from three separate experiments.

due to the presence of α-ketoglutarate in the medium, we utilized the enzyme glutamate dehydrogenase (GluDH), which catalyzes the conversion of α-ketoglutarate to glutamate in the presence of NADH, NH_4^+, and ADP. COS-7 cells expressing GPR80 or empty vector were pre-incubated for 60 min with PBS, reaction buffer (135 μM NADH, 200 μM NH_4Cl, 85 μM EDTA, and 100 μM ADP, final concentrations), or reaction buffer plus GluDH, and basal accumulation of inositol phosphate accumulation was assessed. As seen in Figure 5, pre-incubation with GluDH significantly lowered basal inositol phosphate accumulation in GPR80-expressing cells, although the levels were still higher than those in empty vector-transfected COS-7 cells.

Discussion

The nomenclature for P2Y receptors is confusing at best. Six receptors have erroneously been designated as P2Y receptors, which has led to considerable confusion and controversy in the P2 receptor field. Thus, all reports of potential new P2Y receptors should be viewed with caution until the results are independently verified by other labs. We show here that GPR80, which was reported to be the $P2Y_{15}$ receptor activated by adenosine and AMP [12], is not a receptor for these compounds, and, consequentially, should not be included in the P2Y receptor family. By either stably or transiently expressing GPR80 in three different cell lines (COS-7, CHO, and HEK293), we have provided compelling evidence that although GPR80 is a functional Gq-coupled receptor, it is not activated by adenosine, AMP and other common nucleotides and nucleotide sugars, but instead is activated by the citric acid cycle intermediate, α-ketoglutarate, as first reported by He and coworkers [15].

In the study by Inbe et al. [12], AMP and adenosine promoted both intracellular Ca^{2+} mobilization and cyclic AMP accumulation in HEK293 cells expressing GPR80 but not in wild-type cells. However, our data from three different cell lines expressing GPR80, including HEK293 cells, demonstrate that AMP and adenosine have no such activity. What might be the source(s) of this discrepancy? Our data with HEK293 cells demonstrate that adenosine and to a lesser extent AMP promote robust cyclic AMP accumulation, whether or not GPR80 was expressed in these cells. Perhaps the stimulation of cyclic AMP accumulation in HEK293 cells stably expressing GPR80 *versus* wild-type HEK293 cells reflects a difference in the level of endogenous receptors expressed in the two cell lines, leading to the erroneous conclusion that stimulation was due to activation of GPR80. The Ca^{2+} mobilization response to adenosine and AMP observed in GPR80 cells also may have been due to differential expression of endogenous P2Y receptors and their activation by small amounts of contaminating nucleotides or by bioconversion of added nucleotides by ecto-enzymes. This is particularly relevant given the fact that the signal amplification inherent in Ca^{2+} measurements can result in large signals from activation of a receptor even at low receptor occupancy.

Although we were unable to observe a response to adenosine or AMP in GPR80-expressing COS-7 and CHO cells, one possibility to explain the discrepancy between our results and those of Inbe et al. [12] is that the HEK293 cells used to express GPR80 in the previous study might express an endogenous factor that was required for receptor activity. For example, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{12}$ and $P2Y_{13}$ receptors or A_{2a} and A_{2b} receptors, all of which are expressed in HEK293 cells [12–14], might potentially form a heterodimer with overexpressed GPR80 and result in a receptor with novel pharmacological selectivity. For example, this phenomenon has been observed with mu and delta opioid receptors [19, 20] and with $GABA_B$ receptors [21] as well as with other receptors [22]. However, as in

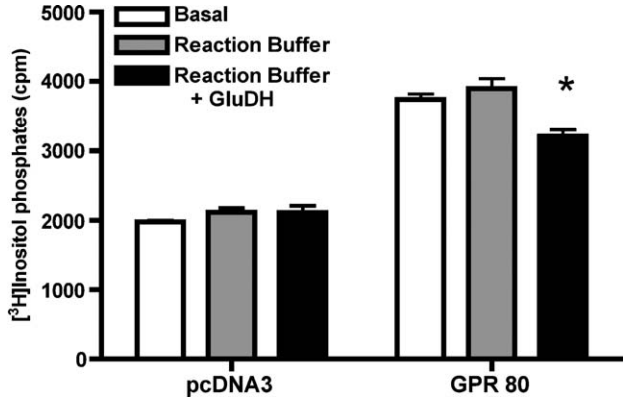

Figure 5. Pre-incubation of COS-7 cells expressing GPR80 with glutamate dehydrogenase decreases basal accumulation of inositol phosphates. Transfected COS-7 cells labeled overnight with *myo*-[^{3}H]inositol were pre-incubated for 60 min with 50 μl PBS, reaction buffer (RB; NADH 135 μM, NH_4Cl 200 μM, EDTA 85 μM, and ADP 100 μM, final concentration), or reaction buffer plus GluDH. Accumulation assays in the absence of added agonist were initiated by adding LiCl (10 mM final) and allowed to proceed for 30 min. Data shown are the mean from triplicate assays from three separate experiments. * $P < 0.05$ (reaction buffer + GluDH *versus* PBS and reaction buffer + GluDH *versus* reaction buffer alone, unpaired *t*-test).

COS-7 and CHO cells, expression of GPR80 in HEK293 cells did not confer capacity of either adenosine or AMP to increase inositol phosphate or cyclic AMP accumulation over levels observed in vector-transfected cells. Thus, heterodimerization or expression of an endogenous factor in HEK293 cells apparently does not explain the results of Inbe et al. [12].

Interestingly, we consistently observed a twofold increase in basal inositol phosphate accumulation when GPR80 was expressed in COS-7 cells. This increase in basal inositol phosphate accumulation was reminiscent of increases observed upon expression of P2Y receptors in 1321N1 astrocytoma and other cell lines [23], which is due to the cellular release of nucleotides and autocrine/paracrine receptor activation [24]. We reasoned that the increased basal accumulation of inositol phosphates might also be due to release of an endogenous compound(s) that activated GPR80. The increased basal accumulation was not affected by increasing adenosine deaminase levels in the medium (Figure 1C), suggesting that adenosine was not the activating endogenous compound. Upon confirmation that GPR80 was activated by α-ketoglutarate, we investigated whether the increased basal inositol phosphates was due to release of α-ketoglutarate and subsequent autocrine/paracrine activation of the receptor. Indeed, addition of GluDH, which converts α-ketoglutarate to glutamate, significantly decreased the basal levels of inositol phosphate accumulation when added 60 min prior to addition of LiCl (to block inositol monophosphatase), although this decrease represented only a fraction of the total increase. Several possibilities might explain the relative impotence of GluDH in reducing basal accumulation of inositol phosphate accumulation in GPR80-expressing cells. These include: (1) addition of a suboptimal amount of enzyme (the maximum amount added was 10 U/ml), (2) inability of the enzyme to reduce α-ketoglutarate levels in the 'unstirred layer' at the extracellular surface of the cells [25], and (3) relatively low levels of inositol monophosphatase in COS-7 cells, which would result in substantial amount of GluDH-insensitive inositol phosphate accumulation prior to the addition of LiCl. Whatever the reason, our results do suggest that α-ketoglutarate release is involved in increased basal activity of GPR80.

Surprisingly, the increase in basal accumulation of inositol phosphates was observed only in COS-7 cells and not in CHO or HEK293 cells. Although this observation suggests that only COS-7 cells release α-ketoglutarate into the medium, an alternative explanation follows from the fact that COS-7 cells contain the SV40 large T antigen, which supports runaway plasmid replication following transfection with plasmids (such as pcDNA3) containing the SV40 origin of replication and eventually results in lytic death [26]. Thus, cell death may occur following transfection of COS-7 cells resulting in release of intracellular compounds, including α-ketoglutarate.

One interesting aspect of this work and that of He et al. [15] is the relatively low potency of α-ketoglutarate for activation of GPR80. The potency of α-ketoglutarate ranged from 32 and 69 μM in the aequorin and FLIPR assays, respectively, described by He and coworkers [15] to ~150 μM in inositol phosphate accumulation assays reported here (Figure 4). These values are considerably higher than the potency of most natural nucleotides for activation of P2Y receptors. However, the citric acid cycle ensures a large reservoir of α-ketoglutarate and the mean level of α-ketoglutarate in rat plasma is about 25 μM [27, 28]. Given that α-ketoglutarate has the potential to be released from cells in a regulated fashion following receptor activation or mechanical stimulation, the levels of extracellular α-ketoglutarate could easily reach levels sufficient for activation of GPR80. Moreover, the recent work by Joseph and coworkers [25] has highlighted the lack of correlation between the concentrations of nucleotides in the bulk medium *versus* at the extracellular surface. Thus, the relatively high EC_{50} may allow relevant extracellular regulation of GPR80 under physiological conditions.

Acknowledgements

This work was supported in part by National Institutes of Health Grants HL71131 (to R.A.N.) and GM38213 (to T.K.H.).

Note

1. Small increases of similar magnitude were observed in both vector- and GPR80-transfected cells upon addition of ATP, ADP, and UTP, presumably from activation of endogenous $P2Y_1$ and $P2Y_2$ receptors.

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