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Review

Delineation of ligand binding and receptor signaling activities of purified P2Y receptors reconstituted with heterotrimeric G proteins

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Abstract

P2Y receptors are G protein coupled receptors that respond to extracellular nucleotides to promote a multitude of signaling events. Our laboratory has purified several P2Y receptors with the goal of providing molecular insight into their: (1) ligand binding properties, (2) G protein signaling selectivities, and (3) regulation by RGS proteins and other signaling cohorts. The human $P2Y_1$ receptor and the human $P2Y_{12}$ receptor, both of which are intimately involved in ADP-mediated platelet aggregation, were purified to near homogeneity and studied in detail. After high-level expression from recombinant baculovirus infection of Sf9 insect cells, approximately 50% of the receptors were successfully extracted with digitonin. Purification of nearly homogeneous epitope-tagged P2Y receptor was achieved using metal-affinity chromatography followed by other traditional chromatographic steps. Yields of purified P2Y receptors range from 10 to 100 μg/l of infected cells. Once purified, the receptors were reconstituted in model lipid vesicles along with their cognate G proteins to assess receptor function. Agonist-promoted increases in steady-state GTPase assays demonstrated the functional activity of the reconstituted purified receptor. We have utilized this reconstitution system to assess the action of various nucleotide agonists and antagonists, the relative G protein selectivity, and the influence of other proteins, such as phospholipase C, on P2Y receptor-promoted signaling. Furthermore, we have identified the RGS expression profile of platelets and have begun to assess the action of these RGS proteins in a reconstituted P2Y receptor/G protein platelet model.

Abbreviations: 2MeSADP – 2-methylthio-ADP; CHAPS – 3-(3-cholamidopropyl)dimethyl-ammonio-1-propanesulfonate; DDM – n-dodecyl-β-D-maltose; GAP – GTPase activating protein; GDI – guanine nucleotide dissociation inhibitor; GEF – guanine nucleotide exchange factor; GPCR – G protein coupled receptor; Ni-NTA – nickel-nitrilotriacetic acid; octylglucoside – n-octyl-β-D-glucopyranoside; PLC – phospholipase C; RGS – regulator of G protein signaling

Introduction

G protein-coupled receptors (GPCRs) represent one of the largest targets for therapeutic development. The P2Y receptor family of GPCRs are heptahelical transmembrane proteins that allow extracellular signals, in the form of nucleotides, to initiate intracellular signaling cascades that result in a wide range of physiological responses [1–3]. P2Y receptors have been subdivided into two groups based on sequence homology and G protein selectivity. Subtypes of the $P2Y_1$-like family, comprised of the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors, all couple to $G\alpha_q$ to activate phospholipase C (PLC). These five receptors were the first P2Y receptors cloned and were illustrated to be activated by ADP ($P2Y_1$), ATP and UTP ($P2Y_2$), UTP ($P2Y_4$), UDP ($P2Y_6$), and ATP ($P2Y_{11}$). Existence of a Gi/adenylyl cyclase-coupled receptor for ADP was initially reported by Cooper and Rodbell [4] in 1979, but molecular identification of this Gi-coupled P2Y receptor remained elusive for two decades. However, the $P2Y_{12}$ receptor was ultimately cloned in 2001 and a $P2Y_{12}$-like subfamily of P2Y receptors exists that includes the ADP-activated $P2Y_{12}$ receptor, the ADP-activated $P2Y_{13}$ receptor, and the nucleotide-sugar-activated $P2Y_{14}$ receptor [5–10].

Unambiguous delineation of the ligand selectivity of P2Y receptors is difficult due to the nature of nucleotide-promoted signaling. All cell systems used to study P2Y receptor-mediated signaling release nucleotides basally and/or after mechanical stimulation. Moreover, cell surface expression of enzymes that metabolize or interconvert nucleotides confounds the interpretation of results obtained with exogenously presented nucleotides. Although radio-ligand binding assays provide useful means to study P2Y receptor binding properties in ways that circumvent some of these problems, lack of high-affinity, stable, and

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selective antagonists for these receptors has largely prevented the development of such direct assays.

A recent goal of our laboratory has been purification of the P2Y receptor subtypes to near homogeneity. These purified receptors allow unequivocal determination of ligand binding selectivities and also provide a well controlled system to study both G protein selectivity and influences of other proteins on the signaling activities of P2Y receptors. Thus, recombinant P2Y receptors are solubilized in detergent, purified, and reconstituted into model phospholipid vesicles with heterotrimeric G proteins. Our data to date indicate maintenance of the native structure and signaling properties of the two ADP-activated receptors of platelets, the $P2Y_1$ and $P2Y_{12}$ receptors, when studied in purified form. This approach was initially applied by Elliot Ross and coworkers to address questions of the molecular mechanism(s) of β-adrenergic receptor/G protein interaction [11]. Detergent-solubilized β-adrenergic receptors maintained ligand affinities equivalent to those of the natively expressed receptor, and hormone-promoted activation of G proteins was observed when these signaling partners were combined by reconstitution in model phospholipid vesicles. This approach has been greatly simplified with the advent of molecular techniques designed to epitope-tag proteins and through the use of high-level expression systems. Application of this technology to purinergic receptors provides a reliable and precise means to study the ligand and G protein selectivity of P2Y receptors in an environment independent of nucleotide interconversion, release, or metabolism.

Our lab has attempted to purify several P2Y receptors, including the human $P2Y_1$, human $P2Y_2$, human $P2Y_2$ fused to its cognate G protein $G\alpha_q$ ($P2Y_2$-Gq), human $P2Y_{12}$, and an avian P2Y receptor [12]. The avian P2Y receptor is unique among all species members of the P2Y family in that it couples equally well to both $G\alpha_q$ and $G\alpha_i$, essentially encompassing the properties of the two mammalian subfamilies, the $P2Y_1$-like Gq-coupled and the $P2Y_{12}$-like Gi-coupled families [12, 13]. Among other physiological actions, the $P2Y_2$ receptor is critical in lung airway physiology and is a current target for the development of cystic fibrosis therapeutics [14, 15]. The $P2Y_1$ receptor and the $P2Y_{12}$ receptor are mediators of the physiological action of ADP in platelets, and activation of both receptors is required to initiate and sustain platelet aggregation [16–18]. A clinically important drug clopidogrel targets the $P2Y_{12}$ receptor, and many more therapeutic agents currently are in development targeting both the $P2Y_1$ and $P2Y_{12}$ receptors [19].

Receptor expression and solubilization

To allow purification of sufficient quantities of P2Y receptors, a baculovirus/Sf9 (*Spodoptera frugaperda*) insect cell system was used, which provides high-level expression of post-translationally modified proteins. Receptor genes were subcloned into either PVL1393 (for the $P2Y_1$, $P2Y_2$, $P2Y_2$-Gq and the avian P2Y clones), which contains a C-terminal hexahistidine epitope-tag and an N-terminal FLAG epitope-tag, or pFastbac HTb (for the $P2Y_{12}$ clone),

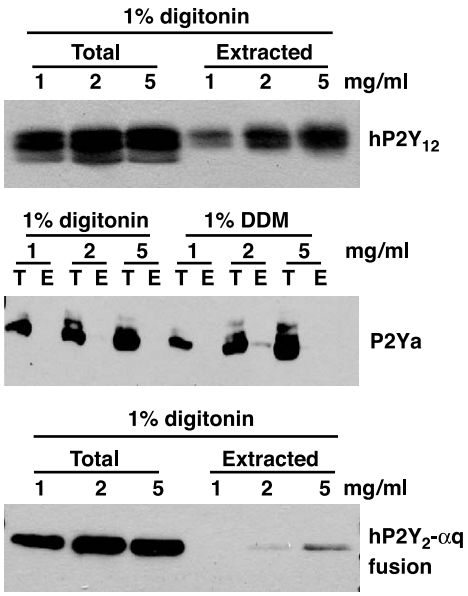

Figure 1. Immunoblot analysis of extraction of recombinant P2Y receptors with detergent. Recombinant P2Y receptors tagged with His-6 ($P2Y_{12}$ and avian P2Y) or FLAG ($P2Y_2$-α_q) epitopes were extracted from Sf9 insect cell plasma membranes with the indicated detergent and at protein concentrations of 1, 2, or 5 mg/ml. Extractions were carried out at 4 °C for 1 h followed by high-speed centrifugation. Avian P2Y receptor (P2Ya), Total (T), high speed supernatant extract (E).

which contains only an N-terminal hexahistidine epitope tag. High-titer receptor-expressing viral stocks were produced and used for infections of Sf9 insect cells. Following centrifugation and subsequent lysis of cells, receptor-containing membranes were collected. The pelleted membranes were resuspended in digitonin-containing extraction buffer to a concentration of 3–5 mg of protein/ml, and the extraction was carried out by gentle agitation for 1 h at 4 °C. Solubilized membrane proteins were recovered by collection of the supernatant following centrifugation at 100,000 *g* for 1 h. The use of 1% digitonin resulted in extraction of nearly 50% of receptors from the membrane for the $P2Y_{12}$ receptor (Figure 1) as well as the $P2Y_1$ and the $P2Y_2$ receptors (data not shown). Under identical extraction conditions, neither the avian P2Y receptor nor the $P2Y_2$-Gq fusion protein was solubilized from the membrane (Figure 1). Other detergents, such as 1% DDM (Figure 1 for avian P2Y), CHAPS, or octylglucoside (data not shown) were tested for their capacity to extract the receptors. However, both the avian P2Y receptor and $P2Y_2$-Gq fusion protein remained insoluble in these conditions. The cause of this insolubility remains unclear. One possibility is the proteins are misfolded. The co-expression of other protein(s) may be required to stabilize these otherwise insoluble receptors for proper processing and expression at the plasma membrane.

Receptor purification

Solubilized $P2Y_1$, $P2Y_2$, and $P2Y_{12}$ receptors were purified utilizing the hexahistidine tag, which binds with high

affinity to bivalent cations such as Ni^{2+}. Thus, one-step purification of the solubilized receptor is achieved following incubation with Ni-NTA resin (for greater detail of receptor purification, see Bodor et al. [20] and Waldo et al. [21]). This single step provides active receptor that is functional in subsequent reconstitution assays. However, the level of purity is low (Figure 2a, lane 3). Further chromatographic steps involving ion exchange and/or gel filtration resulted in near homogeneous receptor preparations as seen for the $P2Y_1$, $P2Y_2$, and $P2Y_{12}$ receptors in Figures 2 and 3. Furthermore, all three receptors were expressed as glycosylated proteins as determined by a decrease in apparent molecular mass following treatment with *N*-glycosidase F as shown in Figure 3 for the $P2Y_2$ receptor and as shown previously for the $P2Y_1$ and $P2Y_{12}$ receptors [20, 21]. Many GPCRs contain at least one consensus site for *N*-linked glycosylation that may confer some functional properties. For example, glycosylation of thrombin receptors appears to be necessary for thrombin binding whereas glycosylation of the β2-adrenergic receptor has no effect on ligand binding but is necessary for normal G protein coupling [22, 23]. The availability of an excellent radioligand binding assay for $P2Y_1$ receptors permits quantification of functional $P2Y_1$ receptor binding sites at each step of purification using the radiolabeled antagonist, [^{3}H]MRS2279 [24]. As reported earlier, the $P2Y_1$ receptor was purified approximately 1000-fold, resulting in a specific activity of approximately 3.0 nmol/mg [21]. Qualitative assessment of the $P2Y_2$ and $P2Y_{12}$ receptor purifications suggest similar fold increases in purity. Yields of purified P2Y receptors typically range from 10 to 100 μg/l of infected cells. Our laboratory also routinely purifies Gα-subunits [25], Gβγ-subunits [25], and PLC-β isozymes [26] after expression of recombinant proteins from baculoviruses in Sf9 insect cells.

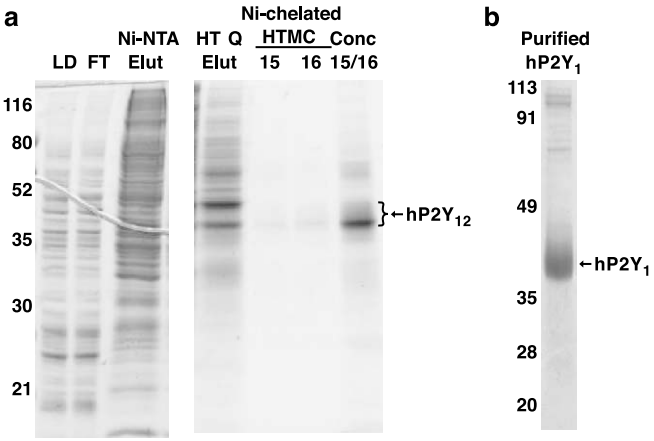

Figure 2. Purified $P2Y_{12}$ and $P2Y_1$ receptors. (a) Following solubilization of $P2Y_{12}$ expressing Sf9 membranes with 1% digitonin, the solubilized fraction was batch incubated with Ni-NTA and then eluted with buffer containing 150 mM imidazole. This eluate was then loaded onto a 1-ml HT Q ion exchange column and protein eluted by a gradient of NaCl. The receptor was further purified by loading the HT Q eluate onto a 1-ml Ni^{2+}-charged HT metal chelate (HTMC) column. Receptor was eluted by an imidazole gradient and concentrated using a Centricon YM-30 centrifugal filter device. Load (LD), flow through (FT). (b) Protein-stained SDS-PAGE gel of the purified $P2Y_1$ receptor following purification as detailed by Waldo et al. [21].

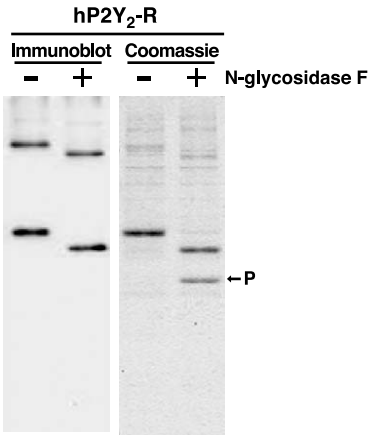

Figure 3. SDS-PAGE and immunoblot analysis of purified recombinant human $P2Y_2$ receptor. Recombinant human $P2Y_2$ receptor tagged with His-6 and FLAG epitopes was purified as described previously for the $P2Y_1$ and $P2Y_{12}$ receptors [20, 21]. Purified $P2Y_2$ receptor was treated with *N*-glycosidase F (+) or untreated (−) and subjected to SDS-PAGE analysis. The resulting gel was stained with Coomassie blue or was transferred to nitrocellulose and immunoblotted with anti-FLAG antibody. The *N*-glycosidase F is identified (P) in the Coomassie-stained gel.

Functional reconstitution of purified receptors

Retention of native signaling activities has been confirmed with several of the purified P2Y receptors after reconstitution with heterotrimeric G proteins in model phospholipid vesicles. The initial step in forming protein-containing phospholipid vesicles is the preparation of detergent/phospholipid mixed micelles. The type of lipids used and their relative amounts is critical for proper membrane fusion, protein incorporation, and orientation of integral-membrane proteins (for a detailed review, see Racker [27]). Phosphatidylethanolamine, phosphatidylserine, and cholesteryl hemisuccinate, which all have been used previously in successful reconstitution experiments with GPCRs, are dried and then resuspended in deoxycholate-containing buffer. This preparation is then combined with 15 pmol of purified receptor, 50 pmol of Gα, and 150 pmol of $G\beta_1\gamma_2$ and immediately loaded onto a G-50 Sephadex column. The void volume, which contains the vesicles, is eluted and collected. Although G-50 Sephadex resin does not separate the formed vesicles from free proteins of the molecular sizes used in these studies, contamination of vesicles with free protein is inconsequential. Alternatively, we have prepared vesicles with either an ACA-34 column or Sephacryl S-300 resin, both of which readily separate vesicles from free proteins.

Activities of purified P2Y receptors

To assess the activity of P2Y receptor-containing phospholipid vesicles, agonist-promoted receptor-mediated

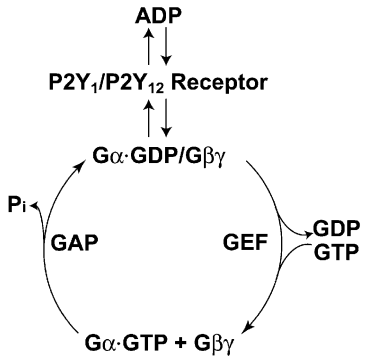

Figure 4. Schematic representation of the $P2Y_1$- or $P2Y_{12}$-activated G protein cycle. Agonist activation of either the $P2Y_1$ or the $P2Y_{12}$ receptor causes an increase in the rate of guanine nucleotide exchange on $G\alpha_q$ or $G\alpha_i$, respectively, whereby GTP replaces GDP, creating the active species Gα-GTP. The signaling pathway is turned off by hydrolysis of bound GTP to GDP by the G protein. GAPs, such as RGS proteins, or PLC-β in the case of $G\alpha_q$, augment the rate of GTP hydrolysis. Hence, two important rate limiting steps exist in the signaling pathway: (1) the rate of guanine nucleotide exchange (regulated by the agonist-activated receptor acting as a GEF) and (2) the rate of GTP hydrolysis (regulated by GAPs).

activation of Gα proteins is monitored. The steady-state GTPase activity of Gα subunits depends on both the rate of GTP hydrolysis and the rate of guanine nucleotide exchange (Figure 4). Typically, guanine nucleotide exchange by heterotrimeric G proteins is rate-limiting, and increases in GTP catalysis by the presence of GTPase activating proteins (GAPs), such as RGS proteins, results in only small increases in measured GTPase activity under basal conditions. This is particularly true for steady-state GTPase measurements with members of the $G\alpha_q$ family that bind GDP tightly in the basal state. However, in the presence of robust agonist-promoted guanine nucleotide exchange, GTP hydrolysis becomes rate-limiting and remarkably large increases in GTPase activity are observed due to the catalytic activity of RGS proteins or other GAPs such as PLC-β isozymes. Steady-state GTPase activity of a Gα subunit under the concerted regulation of a GPCR and an RGS protein is the summation of multiple steps in a complex regulatory cycle [28]. In its simplest application, this system provides an optimal means to assess agonist or antagonist activity of nucleotides and other molecules at P2Y receptors. As an extension, this is a powerful system to establish G protein selectivities of a given P2Y receptor, as well as to elucidate the regulatory activities of other molecules central to G protein signaling.

To assess steady-state GTP hydrolysis, GTP labeled at the γ-phosphate with ^{32}P is incubated with vesicles reconstituted with P2Y receptors and heterotrimeric G proteins. Liberated [^{32}P]Pi is then quantified as described previously to determine basal activity and activities promoted by P2Y receptor activation and/or stimulation of GTPase activity by RGS proteins or effectors such as PLC-β, which are GAPs [20, 21]. As shown in previous publications from our laboratory, both the purified $P2Y_1$ and the $P2Y_{12}$ receptors maintain activities expected based on previous studies of the native receptors [20, 21]. Briefly, 2MeSADP was the most potent agonist tested in reconstitutions with purified $P2Y_1$ receptor and $G\alpha_q\beta_1\gamma_2$, while ATP was a partial agonist. Both RGS4 and PLC-β were effective GAPs in the $P2Y_1$ receptor/Gq reconstitution experiments [21]. $P2Y_{12}$ reconstitution experiments also revealed 2MeSADP as the most potent agonist tested while the cognate $P2Y_{12}$ agonist, ADP, was three orders of magnitude less potent. ATP exhibited no agonist activity but inhibited the action of 2MeSADP at the $P2Y_{12}$ receptor. Following reconstitution with $G\beta_1\gamma_2$ and Gα-subunits of the Gi and Gq families, we demonstrated selective coupling of the $P2Y_{12}$ receptor to $G\alpha_{i2}$ [20].

In contrast to the robust activities observed with purified $P2Y_1$ and $P2Y_{12}$ receptors, no UTP or ATP promoted activity was observed with the purified $P2Y_2$ receptor reconstituted in proteoliposomes with $G\alpha_q\beta_1\gamma_2$ (Figure 5a). Various detergents were examined to determine if the nature of the detergent influenced the retention of activity

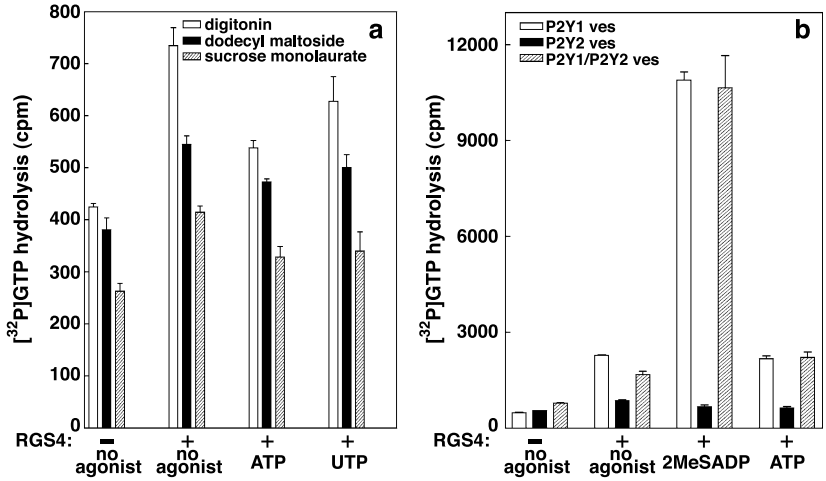

Figure 5. $P2Y_2$ receptor agonists do not promote steady-state GTP hydrolysis in proteoliposomes containing $P2Y_2$ receptor/$G\alpha_q\beta_1\gamma_2$: Purified $P2Y_2$ receptor, $G\alpha_q$, and $G\beta_1\gamma_2$ were reconstituted in proteoliposomes. (a) Steady-state GTP hydrolysis was measured in proteoliposomes containing $P2Y_2$ receptor extracted with the indicated detergent in the absence (−) or presence (+) of 100 nM RGS4 and 10 μM of the indicated agonist. (b) Steady-state GTP hydrolysis was measured in proteoliposomes containing either purified $P2Y_1$ receptor, $P2Y_2$ receptor, or both incubated in the absence (−) or presence (+) of 100 nM RGS4 and 10 μM of the indicated nucleotide agonist.

of this receptor. Thus, in addition to digitonin, which was effective for extraction of the $P2Y_1$ receptor and the $P2Y_{12}$ receptor in active forms, both dodecyl maltoside and sucrose monolaurate were used to solubilize active $P2Y_2$ receptor but with little success (Figure 5a). To be certain that inhibitory factors did not co-purify with the $P2Y_2$ receptor, the $P2Y_1$ and $P2Y_2$ receptors were reconstituted either independently or were co-reconstituted into vesicles along with purified $G\alpha_q$ and $G\beta_1\gamma_2$. While the $P2Y_1$ receptor maintained its activity in the presence of RGS4 and agonist when reconstituted alone or with the $P2Y_2$ receptor, the $P2Y_2$ receptor exhibited no activity under either condition (Figure 5b). These results suggest that no inhibitory components are co-purified in the $P2Y_2$ receptor preparation. The inactivity of the purified $P2Y_2$ receptor is puzzling. While purified under similar conditions to the active purified $P2Y_1$ and $P2Y_{12}$ receptors, the $P2Y_2$ receptor is unable to stimulate GTPase activity. Unfortunately, a $P2Y_2$ receptor-specific radioligand is not available to determine if the receptor is active when initially expressed in the Sf9 insect cells or if binding activity is lost subsequent to solubilization. Alternatively, the purified receptor may be modified in such a way as to interfere with G protein coupling or may be lacking a necessary binding partner(s) to maintain activity.

Utility of purified receptor preparations

The successful purification and functional reconstitution of P2Y receptors allows studies of P2Y signaling and its regulation in a system free of nucleotide release, interconversion, or metabolism, which is critical in studies of nucleotide-based agonists and antagonists. Indeed, we have functionally reconstituted an entire P2Y receptor–G protein–effector system by illustrating that an effector of the $P2Y_1$ receptor, PLC-β1, acts as a GAP on $G\alpha_q$ in phospholipid vesicles composed of the $P2Y_1$ receptor and $G\alpha_q\beta_1\gamma_2$ [21].

$P2Y_1$ and $P2Y_{12}$ receptor-containing vesicles also have been used to assess the action of RGS proteins in these systems. For example, RGS4, a GAP for Gα-subunits of the Gi and Gq families, and RGS2, a GAP primarily for Gq, are nearly equivalent in action in the proteoliposomes reconstituted with the $P2Y_1$ receptor and $G\alpha_q$ (Figure 6a). Furthermore, at a single concentration, both RGS4 and RGS5 are nearly equivalent in stimulating GTP hydrolysis in $P2Y_{12}/G\alpha_{i2}$ reconstituted vesicles (Figure 6b). The relative action of various other purified RGS proteins is currently under investigation. Other components of G protein signaling, including GDIs (guanine dissociation inhibitors) and various Gβγ dimer compositions, can be studied in this reconstituted system. For example, Kimple et al. [29] identified a GoLoco motif(s) in RGS12 and RGS14 that inhibits the dissociation of GDP from members of the Gi family of G proteins. Garrison and coworkers [30–32] have demonstrated the impact of Gβγ dimer composition on receptor/Gα coupling. The application of these and other proteins to this P2Y receptor-reconstitution system will illuminate complexities of signaling related to individual P2Y receptor subtypes.

RGS proteins in a reconstituted GPCR/G protein platelet model

RGS proteins are classically defined as GTPase activating proteins. However, RGS proteins likely play more complex roles in GPCR signaling. In addition to their conserved RGS box domain, members of a number of RGS subfamilies contain multiple domains that allow protein–protein interactions, potentially conferring to these proteins activities in addition to their capacity to promote GTP hydrolysis by Gα-subunits. For example, the aforementioned GoLoco domain, as well as PDZ and PTB domains exist in RGS12 and RGS14, a GGL domain exists in RGS6, 7, 9, and 11 that confers to these RGS proteins

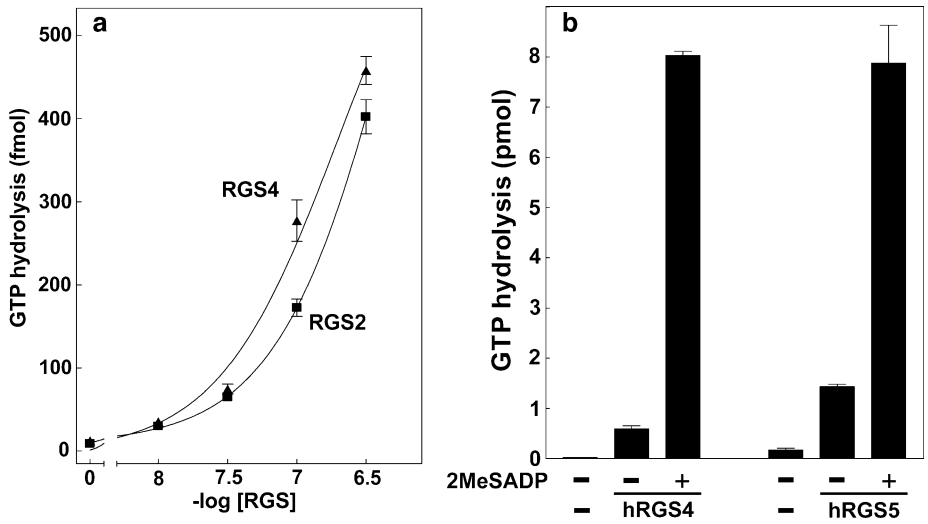

Figure 6. Capacity of RGS proteins for stimulation of steady-state GTPase activity. (a) Purified $P2Y_1$ receptor, $G\alpha_q$, and $G\beta_1\gamma_2$ were reconstituted in proteoliposomes. Steady-state GTP hydrolysis was measured in proteoliposomes incubated with 10 μM 2MeSADP and increasing concentrations of either RGS2 (■) or RGS4 (▲). (b) Purified $P2Y_{12}$ receptor, $G\alpha_{i2}$, and $G\beta_1\gamma_2$ were reconstituted in proteoliposomes. Steady-state GTP hydrolysis was measured in the absence or presence of 10 μM 2MeSADP, 100 nM hRGS4, or 100 nM hRGS5.

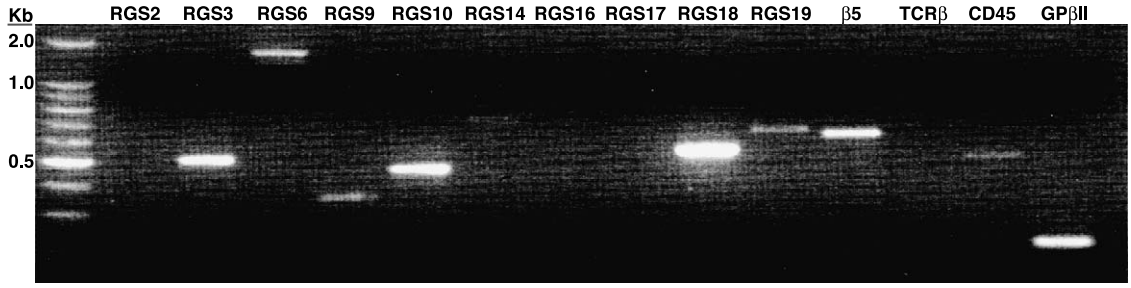

Figure 7. Expression of RGS proteins in platelet. RGS-specific primers were used to screen cDNA generated from human platelet mRNA. Amplification was optimized to minimize background signal, as determined by the negative controls TCRβ and CD45. GPβII is a positive control for a platelet-specific expressing protein.

capacity to exist as functional heterodimers with $G\beta_5$, and a DH/PH domain exists in the RGS protein p115RhoGEF that confers activity as a guanine nucleotide exchange factor for Rho (for an RGS review, see Neubig and Siderovski [33]).

Because of the critical role both the $P2Y_1$ and $P2Y_{12}$ receptors play in platelets, we have identified which RGS proteins are expressed in human platelets with the goal of focusing on a subset of RGS proteins that may regulate P2Y receptor/G protein coupling in the physiological response to ADP. The apparent expression profile of RGS proteins in platelets was determined using specific primers designed to 26 different RGS proteins (for a comprehensive list of RGS box-containing proteins, see Neubig and Siderovski [33]). These primers then were used to screen cDNA generated from human platelet mRNA. To control for message potentially present from sources other then platelets, we also utilized specific primers for TCRβ and CD45, both of which are expressed at high levels in other blood cells such as T cells, B cells, and natural killer cells, but are not expressed in platelets. Messages for RGS3, RGS10, and RGS18 (Figure 7, note that not all RGS proteins screened are shown) were identified in this screen of human platelet mRNA. While the level of RGS6 appears to only moderately exceed the negative controls, the existence of RGS6 in platelets is bolstered by the high level of $G\beta_5$ message, which is the necessary binding cohort for RGS6 and other members of the R7 family of RGS proteins (Figure 7). Previous reports also have identified RGS1, RGS2, and RGS16 in platelets [34–36]. The message for these RGS proteins may be too low in our platelet RNA sample, thus rendering them undetectable. Intriguingly, most of the RGS proteins expressed in platelets are members of the R4 family of RGS proteins, and thus principally consist of an RGS domain with very short amino- and carboxy-termini that lack any other discernable motifs or domains. Several recent reports suggest that these small RGS proteins confer receptor-selectivity in G protein signaling. Indirect evidence was initially reported by Wilkie and coworkers who observed that RGS1, RGS4, and RGS16 differentially inhibited the capacity of three different Gq-coupled receptors to promote Ca^{2+} signaling [37]. Recently, Bernstein et al. [38] showed that RGS2 directly interacts with the 3rd intracellular loop of the human M1-muscarinic receptor. Our reconstitution model will enable us to examine the functional interaction of this defined set of RGS proteins on $P2Y_1$ and $P2Y_{12}$ receptor-dependent signaling.

Conclusion

Despite high level expression of various P2Y receptors in Sf9 insect cells, only a subset of these receptors proved to be solubilized by detergents. Of the three receptors that were successfully solubilized, only two were active upon reconstitution with their cognate heterotrimeric G proteins. The reason for the insolubility/inactivity of some receptors is unknown. Analyses of the two active receptors, the $P2Y_1$ and the $P2Y_{12}$ receptors, have provided novel and unequivocal insight into the signaling properties of these receptors. Future studies of RGS proteins, GDIs and Gβγ dimers in our reconstitution systems should lead to further delineation of the intricacies of P2Y signaling.

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