G Protein-Coupled Purinergic P2Y Receptor Oligomerization: Pharmacological Changes and Dynamic Regulation

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Abbreviation

2-MeSADP

2-MeSAMP

A_1R

ADPβS

AMI

Ang II

Ap_3A

AT_1R

ARC69931MX

B2R

BAPTA-AM

BK

BzATP

co-IP

CPA

ADP' 2-methylthio derivatives

2-methylthioadenosine 5′-monophosphate triethylammonium

adenosine A_1 receptor

adenosine-5'-(β-thio)-diphosphate

acute myocardial infarction

angiotensin II

P1-(5'-Adenosyl) P3-(5'-adenosyl) triphosphate

angiotensin II type 1 recepto

N^6-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)-b,c-dichloromethylene-ATP	1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
bradykinin B2 receptor	bradykinin
	benzoylbenzoyl-ATP
	coimmunoprecipitation
	N^6-cyclopentyladenosine
DC	
EL	dendritic cell
ER	extracellular loop
IP3	endoplasmic reticulum
NECA	inositol triphosphate
NO	5′-(N-ethylcarboxamido) adenosine
P2YR	nitric oxide
PAR4	P2Y receptor
PLCβ	Protease-activated receptor 4
PLA_2	phospholipase Cβ
PKC	phospholipase A_2
SNP	protein kinase C
TM	single-nucleotide polymorphism
VCAM-1	transmembrane
ZM241385	vascular cell adhesion molecule 1
	4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino] ethyl) phenol

Abstract

P2Y receptors (P2YRs), a δ group of rhodopsin-like G protein-coupled receptors (GPCRs), have many essential functions in physiology and pathology, such as platelet aggregation, immune responses, neuroprotective effects, inflammation, and cellular proliferation; thus, they are among the most researched therapeutic targets for use in the clinical treatment of diseases (e.g., clopidogrel, an antithrombotic drug, and Prolacria, a treatment for dry eye). Over the past two decades, GPCRs have been revealed to transmit signals as dimers to increase the diversity of signalling pathways or pharmacological activities. Many studies have frequently confirmed dimerization between P2YRs and other GPCRs due to their functions in cardiovascular and cerebrovascular processes in vivo and in vitro. Recently, some P2YR dimers that dynamically balance physiological functions in the body were shown to be involved in effective signal transduction and exert pathological pharmacological effects. In this review, we summarize the types, pharmacological changes, and active regulators of P2YR-related dimerization. In summary, our review delineates that P2YR-related dimers have new functions and pharmacological activities and maybe a novel direction to improve the effectiveness of medications such as thrombotic events associated with COVID-19.

Keywords: GPCR, P2Y, Oligomerization, Dimerization, Pharmacology, Drug discovery, COVID-19

Introduction

G protein-coupled receptors (GPCRs) are one of the largest families of cell surface transmembrane protein receptors in mammalian genomes and mediate a myriad of fundamental physiological processes by converting extracellular information into intracellular signals by coupling to a G protein (trimeric GTP-binding proteins)

(Rosenbaum, Rasmussen, & Kobilka, 2009). Therefore, GPCRs are the targets of more than half of the current therapeutic drugs on the market and play a unique pharmacological role. A large number of new drug candidates are also being designed based on GPCRs. P2Y receptors (P2YRs) are members of the δ subgroup of the A family of GPCRs and respond to adenine and uridine nucleotides in the body(Lagerstrom & Schioth, 2008); they can also bind to some synthetic nucleoside analogues(von Kugelgen, 2006). Similar to all GPCRs, the P2YR family has the same architecture, which consists of an extracellular N-terminal sequence, seven hydrophobic transmembrane domains (TMs; TM1–TM7), and an intracellular C-terminal domain. According to the amino acid sequences and signal transduction pathways, mammalian P2YRs are further divided into two major subfamilies: G_q protein-coupled receptors ($P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, and $P2Y_{11}R$) and G_i protein-coupled receptors ($P2Y_{12}R$, $P2Y_{13}R$, and $P2Y_{14}R$)(Abbracchio et al., 2006; von Kugelgen, 2006). P2YRs contain several potential glycosylation sites in the extracellular N-terminus and several consensus binding/phosphorylation sites for protein kinases in the intracellular C-terminus. Additionally, TM3, TM6, and TM7 contain some positively charged residues crucial for receptor activation and ligand recognition (TM6 in all P2YRs has an H-X-X-R/K sequence)(Abbracchio et al., 2006). Four cysteine residues are located in the extracellular domains, and their positions are different among P2YRs. For $P2Y_1R$, $P2Y_2R$, and $P2Y_{12}R$, cysteines in the N-terminal domain and extracellular loop 3 (EL3) and EL1 and EL2 bind to each other to form two disulfide bridges(Ding, Bynagari, Mada, Jakubowski, & Kunapuli, 2009; Hillmann et al., 2009; Hoffmann, Moro, Nicholas, Harden, & Jacobson, 1999). P2YRs are widely distributed in nearly all mammalian organs and cells and perform different functions such as ion secretion, molecular migration, cellular proliferation, differentiation, and survival(Abbracchio et al., 2006; Ralevic & Burnstock, 1991; von Kugelgen & Wetter, 2000). Moreover, these receptors have been shown to exhibit pathological neurotransmission activity and are involved in Alzheimer's disease and atherosclerosis. Therefore, studies of the P2YR subfamily in the central nervous system and cardiovascular/cerebrovascular diseases are revelant(Burnstock, 1972, 2006; Li et al., 2012).

Until the 1990s, GPCRs were traditionally believed to be activated and transmit signals through allosteric changes, and GPCRs were postulated to complete their actions as monomeric units. However, in recent decades, many GPCRs have been shown to function as dimers or oligomers(Borroto-Escuela et al., 2014; Borroto-Escuela et al., 2017; Ciruela et al., 2012; Fanelli, Hanyaloglu, & Jonas, 2020; Ferre et al., 2014; Sleno & Hebert, 2019; Terrillon & Bouvier, 2004). Investigators first observed cross-communication between GPCRs in 1975 was when site-site interactions among β-adrenergic receptors in purified frog erythrocyte membranes were used to explain the phenomenon of negative or positive cooperativity in agonists and antagonists of complex binding sites. Since then, many studies have focused on receptor dimerization/oligomers. And these oligomers may have entirely new functional properties in signal transduction with clinical effects that differ from their respective monomers (www.gpcr-hetnet.com), including increasing the number of receptor types, changing the biochemical and/or

pharmacological diversity and enriching transduction pathways, which may revolutionize the basic concept of pharmacology and promote the rational drug development to a new level of specificity and efficacy(Borroto-Escuela et al., 2017; Borroto-Escuela & Fuxe, 2019; Borroto-Escuela et al., 2013; Botta, Appelhans, & McCormick, 2020; Ferre et al., 2009; Ferre, Ciruela, Casado, & Pardo, 2020; Navarro, Borroto-Escuela, Fuxe, & Franco, 2016; Sleno & Hebert, 2019).

Oligomerization is also observed between the P2YRs or between P2YRs with other receptors from GPCRs or other families. As early as 1997, one English team found that adenosine A1 receptor (A_1R) activation can increases $P2Y_2$-purinoceptor mediated activation of phospholipase A_2 (PLA_2) activity in CHO cells, which may represent an interaction between these receptors to regulate cell function(Selbie, King, Dickenson, & Hill, 1997). In 2001, one Japanese team found that when A_1R and $P2Y_1R$ were cotransfected in HEK293T cells, A_1R generated a “P2Y-like agonistic pharmacology”(Yoshioka, Saitoh, & Nakata, 2001). Since then, many researchers have focused their attention on the interactions of P2YRs. Many studies have also reported partially or completely different functions of many P2YR oligomers compared with their native functions, including changes in ligand specificity, agonist potency alterations, and signalling pathway changes(Haas, Shaaban, & Reiser, 2014; Nishimura et al., 2016; Schicker et al., 2009; Suzuki, Obara, Moriya, Nakata, & Nakahata, 2011; Yoshioka et al., 2001; Yoshioka, Saitoh, & Nakata, 2002). Similar to GPCRs, the oligomerization of P2YRs can affect the receptor’s ligand-binding pharmacology, internalization, trafficking, and signalling of the receptor to increase the diversity of GPCR phenotypes. Moreover, due to its role in internalization and trafficking, receptor oligomerization may not be an exception but a requirement for some P2YRs to exert their effects(Bouvier, 2001), although most oligomers have been detected in transient over-expression systems. Because of the extensive distributions and functions of P2YRs(Abbracchio et al., 2006; von Kugelgen & Hoffmann, 2016), many recent studies have investigated the possible interaction of two members of the P2YR family to address challenges in optimal drug development and clinical treatment(Botta et al., 2020; Choi, Simon, Tsim, & Barnard, 2008; Ecke et al., 2008; Nishimura et al., 2016; Savi et al., 2006). Heterodimerization may be the general mechanism of cell-specific modulation of GPCR interactions between membrane receptors; therefore, an understanding of GPCR signalling is vital for analysing GPCR dimers. Ligand-ligand binding properties of receptor dimers may be utterly different from that of their receptor monomer. Therefore, studies aiming to determine which receptors interact and how their respective activities are regulated are urgently needed. Here, we summarize the functional properties and regulation of currently known and relatively important P2YR homo- and heterooligomers.

1. P2YR dimers

Currently, P2YR complexes are generally divided into three categories: monomers, dimers, and oligomers. Naturally, P2YRs generally achieve their full function through interactions with other proteins to form complexes instead of completing the function alone. The rapid development of detection technology (Table 1), such as

coimmunoprecipitation (co-IP), Förster resonance energy transfer (FRET), and X-ray crystallography technology, has been valuable for the discovery and research of receptor oligomerization. The classic classification of GPCR oligomerization is divided into homo-oligomerization and heterodimerization. Homooligomers are composed of two of the same receptors; hetero-oligomers includes distantly related receptor complexes and isoreceptor complexes. Notably, the concept of isoreceptor complexes was introduced in the classification of GPCR dimers based on ligand and structure(Agnati, Guidolin, Cervetto, Borroto-Escuela, & Fuxe, 2016; Gurevich & Gurevich, 2008). Furthermore, their respective oligomers have varying degrees of changes in their functions, signal transduction pathways, and pharmacological effects. The timeline of P2YRs discovery is shown in Figure 1. This review will describe oligomers identified in some relatively straightforward studies, and additional possible receptor complexes are shown in Table 1.

1.1. $P2Y_1R$-related dimers

The $P2Y_1$ receptor ($P2Y_1R$)(Abbracchio et al., 2006) is widely distributed in many tissues in humans, and is activated by ADP, ATP, or their respective 2-methylthio derivatives(Ayyanathan et al., 1996). $P2Y_1R$ is coupled to G_q/G_{11} to promote the intracellular activation of phospholipase Cβ (PLCβ) generation of inositol triphosphate (IP3), the release of intracellular Ca^{2+} stores, and activation of monomeric G proteins (RhoA and Rac)(Soulet et al., 2005; Waldo et al., 2002). The significant effects of $P2Y_1R$ coupled to G_q/G_{11} include platelet aggregation or changes in the platelet shape changes (by activating p160 Rho kinase to respond to ADP better)(Barragan-Iglesias et al., 2015; Soulet et al., 2005), as well as vasodilation, pain sensation, inflammation, and astroglial signalling. These effects are subsequently involved in pathological signalling responses, such as atherosclerosis, cardiovascular and vascular diseases, inflammatory nociceptive hyperalgesia, and neuromuscular transmission(Zerr et al., 2011). The primary domain for agonist-induced receptor internalization and clathrin-mediated endocytosis is the last 19 amino acids of the C-terminus of $P2Y_1R$, and this function is essential for receptor desensitization and resensitization required for normal physiological functions. Several $P2Y_1R$-related oligomers have been identified, including $P2Y_1R$ with P2YR subfamilies, which include $P2Y_1R$ homodimers(Choi et al., 2008), P2Y heteroreceptor complexes that include $P2Y_1R$-$P2Y_{11}R$(Ecke et al., 2008), $P2Y_1R$-$P2Y_2R$(Moccia et al., 2001; Ribeiro-Filho et al., 2016), $P2Y_1R$-$P2Y_4Rs$(Franke & Illes, 2006; Ribeiro-Filho et al., 2016), $P2Y_1R$-$P2Y_6R$(D'Ambrosi, Iafrate, Saba, Rosa, & Volonte, 2007), $P2Y_1R$-$P2Y_{12}R$ (Hardy, Jones, Mundell, & Poole, 2004) and $P2Y_1R$-$P2Y_{13}R$(Schicker et al., 2009), as well as $P2Y_1R$ oligomers that interact with non-P2YR subfamilies, which include $P2Y_1$-A_1R heterodimers(Yoshioka, Saitoh, et al., 2002), $P2Y_1R$-$A_{2A}R$ heterodimers(Nakata, Suzuki, Namba, & Oyanagi, 2010; Suzuki et al., 2011), $P2Y_1R$-M71R(Bush et al., 2007) and $P2Y_1R$-$A_{2A}R$-$P2Y_{12}R$ oligomeric heterodimers(Suzuki et al., 2011) (Table 1).

1.1.1. Homo-dimerization of $P2Y_1R$

In 2008(Choi et al., 2008), Choi et al. found that constitutive dimeric $P2Y_1R$ accounts for approximately 44% ± 3% of the $P2Y_1R$ in the resting-state in HEK293 cell membranes that display full activity similar to the activity of monomeric $P2Y_1R$. Moreover, agonist-induced dimerization follows desensitization and is completely reversible upon withdrawal of the agonist(Barragan-Iglesias et al., 2015). Therefore, agonist-induced dimerization

has not yet been shown to result in active receptor. Additionally, increases in intracellular Ca^{2+} concentrations promote the dimerization of $P2Y_1R$; however, further research is needed to determine whether $P2Y_1R$ homodimers are active. In 2002, Wang et al. reported the potential function of $P2Y_1R$ homodimer in smooth muscle cells, although they did not provide associated evidence(Wang et al., 2002).

The essential structural parts of the receptor for constitutive dimers and agonist-induced homodimerization are not the same (Figure 2). The last four amino acids (DTSL) at the C-terminus are essential for constitutive $P2Y_1R$ dimerization, although the determinant sequence for agonist-induced dimerization consists of the last 19 amino acids of the C-terminus (EDMTLNILPEFKQNGDTSL)(Ayyanathan et al., 1996). Both of these C-terminal sequences are important for the internalization process.

1.1.2. Hetero-dimerization of $P2Y_1R$

1.1.2.1. $P2Y_1R$-$P2Y_{11}R$

$P2Y_1R$ is the isoreceptor of $P2Y_{11}R$ and shares approximately 33% amino acid identity with $P2Y_{11}R$(Communi, Govaerts, Parmentier, & Boeynaems, 1997); both receptors are activated by adenine nucleotides or their derivatives and are coupled to G_q proteins to mediate an increase in the intracellular calcium concentration. Scholars have revealed that ligand specificity is not equivalent to endogenously and heterologously expressed receptors(Adams et al., 1995), suggesting an association between these two isoreceptors(Schnurr et al., 2003; White, Webb, & Boarder, 2003). In 2008, Ecke et al. (Ecke et al., 2008) identified a physical association between $P2Y_1R$ and $P2Y_{11}R$ in HEK293 cells. 2-MeSADP causes the internalization of the $P2Y_1R$-$P2Y_{11}R$ heterodimer, and the dimerization of $P2Y_1R$-$P2Y_{11}R$ can promotes agonist-induced internalization. $P2Y_{11}R$ alone does not undergo internalization and endocytosis, suggesting a significant functional consequence of P2YR heterodimerization, because heterodimerization is known to control desensitization and resensitization of GPCRs and may also specifically maintaining a constant number of cell membrane receptors(Prinster, Hague, & Hall, 2005). One of the crucial residues for agonist-induced $P2Y_{11}R$ internalization is R268 at its extracellular N-terminus, although this amino acid does not alter the formation of $P2Y_1R$-$P2Y_{11}R$ heterodimers. The heterodimer of $P2Y_1R$ and $P2Y_{11}R$ (with a single-nucleotide polymorphism (SNP) that causes the A87T mutation) may be a new target to treat vascular inflammation-related diseases because the pathological effect appears only when these receptors are coexpressed(Amisten, Melander, Wihlborg, Berglund, & Erlinge, 2007; Haas et al., 2014).

In addition, changes in ligand specificity have been observed for heterodimers of these two receptors compared with their monomers. For example, 2-MeSADP and the specific $P2Y_1R$ antagonist MRS2179 both have functional activity with $P2Y_{11}R$, and 2-MeSADP results in $P2Y_{11}R$ internalization by promoting the internalization of $P2Y_1R$-$P2Y_{11}R$. MRS2179 inhibits Ca^{2+} release and agonist-induced internalization mediated by the potent $P2Y_{11}R$ agonist benzoylbenzoyl-ATP (BzATP) in HEK293 cells that coexpress $P2Y_1R$ and $P2Y_{11}R$. The changes in ligand specificity and internalization may be due to conformational changes in the ligand-binding domain during the formation of the $P2Y_1R$-$P2Y_{11}R$ heterodimer; however, structural evidence has not been reported. For the $P2Y_1R$-$P2Y_{11}R$ heterodimer, the ligands BzATP and MRS2179 may bind to the dimer-shared

domain, but this area is not the only $P2Y_1R$ binding site because BzATP and MRS2179 do not present activity with $P2Y_1R$ and $P2Y_{11}R$, respectively.

1.1.2.2. $P2Y_1R$-$P2Y_{12}R$

$P2Y_1R$ and $P2Y_{12}R$ are colocalized in platelets, endothelial cells, smooth muscle cells, neurons (such as Schwann cells and oligodendrocytes), and mesangial cells. Moreover, $P2Y_1R$ and $P2Y_{12}R$ are both involved in the regulation of platelet aggregation and cell apoptosis(Jin & Kunapuli, 1998; Mamedova, Gao, & Jacobson, 2006). $P2Y_1R$ initiates platelet activation and aggregation, although $P2Y_1R$ alone cannot complete platelet aggregation to make platelets form stable plaques(Leon et al., 1999; Ohlmann et al., 2000). Furthermore, studies have suggested a relationship between the signalling pathways of $P2Y_1R$ and $P2Y_{12}R$(Daniel et al., 1998). In 2004, Hardy et al. found that $P2Y_{12}R$ promotes $P2Y_1R$-induced platelet aggregation through phosphoinositide 3-kinase (PI3K) activation and AC inhibition, similar to previous findings; conversely, $P2Y_{12}R$ antagonists can partially inhibit $P2Y_1R$-dependent Ca^{2+} release. $P2Y_1R$, however, decreases $P2Y_{12}R$-induced Ca^{2+} mobilization through the activation of Src kinase(Hardy et al., 2004). Therefore, $P2Y_{12}R$ enhances the function of $P2Y_1R$, but $P2Y_1R$ inhibits the function of $P2Y_{12}R$ in Ca^{2+} release, thereby maintaining the dynamic balance of thrombosis in the body. Some thrombotic pathological states, such as cardiovascular and cerebrovascular inflammatory diseases and atherosclerosis, as well as diseases related to coagulopathy may be potentially treated by adjusting the balance of the $P2Y_1R$-$P2Y_{12}R$ heterodimer, which represents a new research area for chemical ligands. However, further research is needed to determine the clear interaction mechanism and conformation of the $P2Y_1R$-$P2Y_{12}R$ heterodimer.

1.1.2.3. $P2Y_1R$-A_1R

A_1R belongs the GPCR family (belonging to P1 receptor subgroup) activated by the selective agonist N^6-cyclopentyladenosine (CPA); the distribution of A_1R is similar to that of $P2Y_1R$. Additionally, both $P2Y_1$ and A_1R have been implicated in brain damage and neurodegenerative processes(Franke & Illes, 2006; Neary, Kang, Willoughby, & Ellis, 2003; Ralevic & Burnstock, 1998). Co-IP and bioluminescence resonance energy transfer (BRET) experiments confirmed the presence of the $P2Y_1R$-A_1R heterodimer was found in humans, at least in cotransfected cells, and this heterodimer has a new functional activity that differs from the functional activities of their respective monomers(Yoshioka et al., 2001; Yoshioka, Saitoh, et al., 2002). In 2002(Yoshioka, Saitoh, et al., 2002), Yoshioka et al. identified a heterodimer between $P2Y_1R$ and A_1R in cotransfected HEK293T cells. Moreover, their respective agonists CPA and ADPβS promote this heteromeric process, although this effect is not stable. $P2Y_1R$-A_1R heterodimerization occurs in rat brain tissues in the natural state, including the cortex and hippocampus cerebellum, in primary cultures of cortical neurons, and in human astroglial cells(Tonazzini, Trincavelli, Montali, & Martini, 2008; Yoshioka, Hosoda, Kuroda, & Nakata, 2002). Therefore, the $P2Y_1R$-A_1R heterodimer indeed exists in vivo. The pharmacological properties and functional changes in the $P2Y_1R$-A_1R heterodimer have been compared with their respective monomers. In cotransfected cells, $P2Y_1R$ agonists impair A_1R coupling to G proteins and change the A_1R agonist potency; conversely, A_1R stimulation promotes the functional response of $P2Y_1R$ to $P2Y_1R$ agonists. In addition,

the A_1R antagonist inhibits $P2Y_1R$ function(Nakata, Yoshioka, Kamiya, Tsuga, & Oyanagi, 2005), consistent with results from a previous study showing that $P2Y_1R$ is sensitive to an A_1R antagonist(Mendoza-Fernandez, Andrew, & Barajas-Lopez, 2000; Tonazzini et al., 2008). According to a previous study, this heterodimer inhibits neurotransmitter release involving in adenine nucleotides (ADPβS displaces the A_1R ligand from the [^{3}H]NECA binding site to inhibit excitatory synaptic transmission in the receptor complex, particularly glutamatergic synapses in the rat hippocampus)(Nakata, Yoshioka, & Kamiya, 2004); therefore, this heterodimer may be a new target to treat some neurosynaptic diseases.
In this complex(Yoshioka et al., 2001), the primary domain involved in heterodimerization is not clear, but the C-terminus is excluded because a C-terminal deletion does not affect heterodimer generation in coexpressed HEK293T cells.

1.1.2.4. $P2Y_1R$-$A_{2A}R$-$P2Y_{12}R$ and $P2Y_1R$/$P2Y_{12}R$-$A_{2A}R$

$A_{2A}R$ is an adenosine receptor that is activated by adenosine, couples to G_s proteins to activate AC, and subsequently promotes the activation of the second messenger cAMP and finally inhibits platelet aggregation. Its function is inhibited by ZM241385 and SCH442416, which are both specific and selective antagonists of $A_{2A}R$(Fredholm, AP, Jacobson, Linden, & Muller, 2011; Lasley, Kristo, Keith, & Mentzer, 2007). This type of receptor is distributed in many cells and tissues in humans, such as astrocytes, microglia, oligodendrocytes, immune cells, leukocytes, platelets, the striatum, blood vessels, heart, and lung, and regulates the inhibition of platelet aggregation, vasodilation, and neuronal excitatory activity(Borea, Gessi, Merighi, & Varani, 2016; Kull, Svenningsson, & Fredholm, 2000). $P2Y_1R$ or $P2Y_{12}R$ has been reported to contribute to platelet activation and aggregation through different pathways(Ledent et al., 1997; Leon et al., 1999).
These three receptors are all distributed in the platelet membrane and regulate platelet aggregation. In 2005, Nakata et al. detected $P2Y_1R$-$A_{2A}R$, $P2Y_{12}R$-$A_{2A}R$, and $P2Y_1R$-$A_{2A}R$-$P2Y_{12}R$ heterooligomers in human platelets and cotransfected HEK293T cells using co-IP, although the functional activities and associated structures were not described(Kunapuli, Dorsam, Kim, & Quinton, 2003; Nakata et al., 2005; Schicker et al., 2009). In 2011(Suzuki et al., 2011), Suzuki et al. found that the $A_{2A}R$- and $P2Y_{12}R$-specific antagonists ZM241385 (4-(2- [7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino] ethyl) phenol) and ARC69931MX (N^6-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)-b,c-dichloromethylene-ATP), respectively, inhibit or decrease $P2Y_1R$-induced Ca^{2+} release when coexpressed in HEK293T cells. In addition, when used alone or together, these two antagonists, ZM241385 and ARC69931MX, exerted similar dose-dependent effects. However, their potency in regulating Ca^{2+} release differs; thus, $P2Y_{12}R$ and $A_{2A}R$ directly modulate the function of $P2Y_1R$ in different ways. Importantly, $P2Y_{12}R$ antagonists inhibit platelet aggregation through both $P2Y_{12}R$ and $P2Y_1R$ but do not directly inhibit the functional activity of $P2Y_{12}R$; $A_{2A}R$ antagonists inhibit $A_{2A}R$-induced aggregation and $P2Y_1R$-induced platelet shape changes. Therefore, these three receptors coregulate platelets to ensure a delicate balance by changing the platelet state between the active and resting conditions.
The specific domain interactions and functional activities of these three receptors' interactions have not been clarified; thus, further research is needed. However, their

interactions are undoubtedly essential to maintaining the normal physiological function of platelets.

1.2. $P2Y_2R$-related dimers

$P2Y_2R$(Franke & Illes, 2006; Neary et al., 1999; von Kugelgen, 2006) is completely activated by ATP and UTP, although this complete activation is not observed for ADP(Lazarowski, Watt, Stutts, Boucher, & Harden, 1995). The coupling of $P2Y_2R$ with $G_{q/11}$ activates PLCβ and promotes the generation of second messengers, such as IP3 and diacylglycerol (DAG). The latter regulates Ca^{2+} release from intracellular stores, promotes protein kinase C (PKC) activation of nitric oxide (NO) emission and vascular cell adhesion molecule 1 (VCAM-1) expression, and affects physiological functions, such as vasodilation, mitogenic actions, ion/cytokine secretion, wound healing, visual/auditory transmission, and blood pressure/intraocular pressure regulation, which also result in pathologies such as dry eye disease, airway inflammation, and atherosclerosis(Burnstock, 2008; Yu, Erb, Shivaji, Weisman, & Seye, 2008). In addition, $P2Y_2R$ couples to G_{12} to activate RhoA/Rac and regulate cytoskeletal rearrangements, cell migration, and phagocytosis, such as microglial phagocytosis. Currently, several $P2Y_2R$ oligomers have been identified, including $P2Y_2R$ homodimers(Abe, Watanabe, Kuroda, Nakagawa, & Higashi, 2018; Kotevic, Kirschner, Porzig, & Baltensperger, 2005), P2YR-related complexes that include $P2Y_1R$-$P2Y_2R$(Ribeiro-Filho et al., 2016), $P2Y_2R$-$P2Y_4R$, $P2Y_2R$-$P2Y_6R$(D'Ambrosi et al., 2007), $P2Y_2R$-$P2Y_{12}R$, and $P2Y_2R$-$P2Y_{13}R$ dimers(Schicker et al., 2009); in addition, $P2Y_2R$ interacts with non-P2YR subfamily members, such as A_1R(Bush et al., 2007; Namba, Suzuki, & Nakata, 2010; Safhill, 1975), $A_{2A}R$, bradykinin B2 receptor (B2R)(Nakagawa, Takahashi, Matsuzaki, Kuroda, & Higashi, 2018; Yashima et al., 2015), and M71R(Bush et al., 2007). However, some of the receptor complexes are only observed in physical proximity, and their functional activity requires additional investigation. Some of these dimers were described above.

1.2.1. Homo-dimerization of $P2Y_2R$

In 2005(Kotevic et al., 2005), Kotevic et al. applied FRET and detected that there are $P2Y_2R$ homodimers in HEK293 and K562 cells that cannot be influenced by agonists; the activity of these homodimers was not detected. In 2018(Abe et al., 2018), Abe et al. found that in HEK293 cells, the disulfide bonds that form the $P2Y_2R$ homodimer are also not agonist-induced. Both Cys25Ser at the N-terminus and Cys278Ser in the EL3 will promote homodimerization; in addition, Cys106 and Cys183 are essential for forming disulfide bonds and are involved in homodimerization. The specific functions of $P2Y_2R$ homodimers are not clear, although they may exhibit a functional activity similar to $P2Y_2R$ monomers.

1.2.2. Hetero-dimerization of $P2Y_2R$

1.2.2.1. $P2Y_2R$-A_1R

Both $P2Y_2R$ and A_1R are described above, and these two receptors are expressed in the airway and neutrophils, as well as in cells of central nervous system (e.g., astrocytes); besides, they also regulate platelet function, although they exert opposite effects(Chen, Shukla, Namiki, Insel, & Junger, 2004; Rugolo et al., 1993). In the 1990s(McCoy, Schwiebert, Karlson, Spielman, & Stanton, 1995), the interaction between these two receptors was reported. As shown by McCoy et al., when an agonist activates A_1R to

regulate Cl^- transport, both the potency and concentration of the agonist regulate A_1R, indicating another receptor may be involved in this regulation. Then, in 1998, Ralevic V et al. reported the coexpression of $P2Y_2R$ and A_1R in some tissues and CHO cells, and that this coexpression synergistically promotes the activation of MAP kinase signalling(Ralevic & Burnstock, 1998). This same phenomenon was documented in a study by Burnstock et al. Both Yoshioka (2001) and Yu Chen et al. (2004) observed the $P2Y_2R$-A_1R heterodimeric complex in cotransfected cells(Burnstock & Williams, 2000; Chen et al., 2004). However, they did not identify a specific functional or pharmacology activity related to this complex. In 2006(Suzuki, Namba, Tsuga, & Nakata, 2006), Suzuki et al. discovered a $P2Y_2R$ and A_1R heterodimer and determined its functional activity. When both the $P2Y_2R$ agonist (at high concentrations) and the A_1R agonist were added simultaneously, the function of the A_1R agonist decreased in coexpressed HEK293 cells due to decreases in the number of ligand-binding sites and affinity. In addition, the $P2Y_2R$ agonist UTP and the A_1R agonist 5′-(N-ethylcarboxamido) adenosine (NECA) synergistically induce Ca^{2+} release and MAPK activation in coexpressing cells. It is unclear whether the synergistic functions result from the heterodimerization of these two receptors or if A_1R functions alone(Namba et al., 2010). Moreover, $P2Y_2R$ agonists also decrease forskolin-stimulated cAMP production(Fredholm et al., 2011). The $P2Y_2R$-A_1R heterodimer, the function of which changes to increase the $P2Y_2R$-coupled G_q signal but decreases the A_1R-coupled G_i signal, also alters the ligand affinity or pharmacological features. The $P2Y_2R$-A_1R heterodimer also exists in neurons in the cortex, cerebellum, and cerebellar Purkinje cells in vivo, and very high concentrations of the $P2Y_2R$-A_1R heterodimer are present in pyramidal cells(Namba et al., 2010).

However, clear data showing the interface or conformational changes in the heterodimer are not available, and further investigations are required. For this pharmacological change in the $P2Y_2R$-A_1R heterodimer, a better approach may be to use the receptor interactions to treat thrombosis-related diseases.

1.2.2.2. $P2Y_2R$-B2R

B2R is a member of the GPCR family that belongs to the bradykinin (BK) receptor of the rhodopsin family(Figueroa et al., 2001). It is primarily activated by BK and couples to G_q through the PLC pathway to promote the formation of IP3 and subsequently regulate Ca^{2+} release. additionally, B2R regulates arachidonic acid release, thereby playing a major role in regulating pain, blood pressure, healing of vascular injury, and inflammation(Gainer, Morrow, Loveland, King, & Brown, 1998; Higashida, Streaty, Klee, & Nirenberg, 1986; Krankel et al., 2013). $P2Y_2R$ and B2R are both expressed in endothelial cells, smooth muscle cells, and nerve cells, and they couple to G_q and promote the generation of PLC, thereby regulating vascular tone and inflammation. Most studies on the interaction of $P2Y_2R$ and B2R have focused on their concurrence.

In 2015(Yashima et al., 2015), Yashima et al. identified an association between $P2Y_2R$ and B2R, thus confirming that this heterodimer/heterooligomer exists in H1321N1 cells and HEK293 cells. Before this study, many studies reported functional interactions between $P2Y_2R$ and B2R, although no clear outcomes were confirmed a physical interaction(Lopez-Valdes, Beltran-Parrazal, Brennan, & Charles, 2010; Reetz & Reiser, 1994; Tamesue, Sato, & Katsuragi, 1998). BK, a ligand of B2R, activates $P2Y_2R$,

promotes $P2Y_2R$ internalization, and increases this potency by increasing B2R expression. However, $P2Y_2R$ agonists can promote β-arrestin recruitment to B2R and internalization. Therefore, $P2Y_2R$ and B2R interact and regulate the activation and internalization of the other receptor. This interaction is most likely due to the formation of a heterodimer or heterooligomer(Nakagawa et al., 2018). In COS-7 cells, $P2Y_2R$ can promote the recruitment of B2R to the endoplasmic reticulum (ER)(Nakagawa et al., 2017). Moreover, UTP induces the complete endocytosis of both $P2Y_2R$ and B2R, although BK induces the incomplete endocytosis of $P2Y_2R$ and B2R, and BK and B2R are not colocalized when endocytosed into cells. However, no clear outcome of this phenomenon has been identified, perhaps colocalization affects endocytosis in different ways, due to differences in the speed of endocytosis of dimers or other effects on the endocytosis of heterodimers/heterooligomers.

Regarding the structure, the $P2Y_2R$ N9Q/N13Q mutant or N-linked sugar chain do not alter the interaction of $P2Y_2R$ with B2R, and the $P2Y_2R$ N9Q/N13Q mutant also generates a heterodimer but remains in the cytoplasm instead of being transported to the PM, suggesting that the heterodimerization of $P2Y_2R$ and B2R does not rely on their extracellular ligands(Michineau, Alhenc-Gelas, & Rajerison, 2006). In addition, the degree of glycosylation of B2R (the N-glycosylation site has been suggested to be an important site for B2R homodimerization in rats) may be essential for heterodimerization, and glycosylation may inhibit the dimerization process. In humans, 3 N-glycosylation sites have been identified: N30, N39, and N207.

1.3. $P2Y_4R$-related dimers

$P2Y_4R$ is a purine receptor with no potential N-glycosylation sites (sites involved in posttranslational N-glycosylation)(Bogdanov, Wildman, Clements, King, & Burnstock, 1998; Burnstock, 2004). In humans and other mammals, $P2Y_4R$ is distributed in many cells, such as astrocytes, microglia, eosinophils, lymphocytes, monocytes, and endothelial cells, and in tissues of the brain, kidney tubule and glomerulus, intestine, and vascular smooth muscles(Burnstock, 2004; Neary et al., 2003; Neary, Rathbone, Cattabeni, Abbracchio, & Burnstock, 1996). In humans, $P2Y_4R$ is activated by UTP, ITP, and GTP, which is then coupled to $G_{q/o}$ and subsequently activates PLC and regulates Ca^{2+} release(Communi, Motte, Boeynaems, & Pirotton, 1996; Nguyen et al., 1995). Among the ligands, UTP is the most potent $P2Y_4R$ agonist, and ATP is a competitive antagonist or a low-potency partial agonist of $P2Y_4R$(Herold, Qi, Harden, & Nicholas, 2004). However, in rats or mice, ATP is the agonist of $P2Y_4R$. $P2Y_4R$ plays a vital role in regulating immune cell recruitment and phagocytosis, intraocular pressure, vasodilatation, and epithelial K^+/Cl^- secretion(Burnstock, 2002). Currently, several $P2Y_4R$ oligomers are known and include $P2Y_4R$ homodimers(D'Ambrosi et al., 2007; D'Ambrosi et al., 2006), and $P2Y_1R$-$P2Y_4R$(Ribeiro-Filho et al., 2016), $P2Y_2R$-$P2Y_4R$, $P2Y_4R$-$P2Y_6R$, and $P2Y_4R$-$P2Y_{11}R$ heterodimers(D'Ambrosi et al., 2007)(shown in Table 1). Some of these oligomers are described above.

1.3.1. Homo-oligomerization of $P2Y_4R$

In 2006(D'Ambrosi et al., 2006), D'Ambrosi et al. detected two protein bands for $P2Y_4R$ at 41 kDa and 80 kDa in the rat pheochromocytoma PC12 cell variant nnr5 and human neuroblastoma SH-SY5Y cell line. However, based on the amino acid sequence, the

molecular mass of $P2Y_4R$ is 41 kDa(Bogdanov et al., 1998; Rodrigues, Almeida, Richardson, Oliveira, & Cunha, 2005). Therefore, based on these results suggest, $P2Y_4R$ homodimers exist or are generated and are stable in human cells. Some chemical agents (dithiothreitol (DTT) and 5% β-mercaptoethanol) damage $P2Y_4R$ homodimers, although 5% β-mercaptoethanol also promotes resistance to certain agents; therefore, a structural connection, such as covalent disulfide bonds may exist between two receptors or among receptors. In addition, in native neuronal systems (such as rat pheochromocytoma PC12 cells), a high-order $P2Y_4R$ complex disaggregates into homodimers and monomers at a ratio of approximately 4:1; thus, a high homodimer content is regarded as the minimum functional unit. Additionally, the homodimer distribution also differs; e.g., in rat cerebellar tissue, the dimer distributes primarily in lipid rafts. In mature rat cerebellar granule neurons, the molecular mass of $P2Y_4R$ homodimers is approximately 90 kDa, as observed in Western blots or in cerebellar granule neurons; moreover, in 2005, 88 kDa $P2Y_4R$ molecules were also detected in the rat hippocampus(Rodrigues et al., 2005). Therefore, the homodimer was initially described as a functional unit for $P2Y_4R$. More experiments are needed to understand the functional activity of $P2Y_4R$. No clear experiments have determined the associated structure and interface of the $P2Y_4R$ homodimer/homooligomer, although $P2Y_4R$ must form covalent disulfide bonds among its subunits because of its stable structure.

1.3.2. Hetero-dimerization of $P2Y_4R$-$P2Y_6R$

$P2Y_4R$ and $P2Y_6R$ are also isoreceptors(Hebebrand, Friedl, & Propping, 1988), both of which are present in several immune cell membranes and vascular smooth muscle cells, and they couple to G_q to activate the PLC pathway and are involved in inflammation. In 2002(Sak & Webb, 2002), Sak et al. confirmed approximately 40% amino acid sequence homology between $P2Y_4R$ and $P2Y_6R$. In 2007(D'Ambrosi et al., 2007), D'Ambrosi et al. used SDS-PAGE and Western blotting to detect $P2Y_4R$ and $P2Y_6R$ in the native rat pheochromocytoma PC12 cells. Although they transfected pheochromocytoma PC12 cells with other P2YRs, such as $P2Y_1R$, these cells only formed $P2Y_4R$-$P2Y_6R$ heterooligomers; therefore, a specific domain may regulate the formation of certain heterooligomers. Moreover, this phenomenon is specific among cells; thus, $P2Y_4R$-$P2Y_6R$ heterooligomers are not generated in human neuroblastoma SH-SY5Y cells or form heterooligomers with other P2YRs.

Regardless of the presence of the homo- or heterodimers of $P2Y_4R$ and $P2Y_6R$ in the cell membrane or a lipid association, clear data are not available to confirm the physical association sites of $P2Y_4R$ or $P2Y_6R$. Both $P2Y_4R$ and $P2Y_6R$ are generated from the disaggregation of high-order $P2Y_4R$-$P2Y_6R$ heterooligomers, and some are native aggregates. More experiments are needed to determine their conformations and activities by elucidating their structures and specific functions.

1.4. $P2Y_6R$-related dimer

$P2Y_6R$ is distributed in many cells, such as epithelial cells, vascular smooth muscle cells, placenta cells, thymus cells, and immune cells. In particular, $P2Y_6R$ is involved in inflammation in immune cells(Nguyen et al., 1995). In humans, $P2Y_6R$ couples to G_q upon activation by UDP, and partial agonists UTP, ADP, and 2-methylthio-ATP, subsequently activating PLC to regulate Ca^{2+} release in the cells(Koizumi et al., 2007).

Alternatively, $P2Y_6R$ couples to $G_{12/13}$ activate Rho and regulate vascular tone and inflammation, and this process is involved in cardiac fibrosis in mice. Currently, several $P2Y_6R$-related oligomers have been identified, including $P2Y_6R$ homooligomers(Brinson & Harden, 2001; D'Ambrosi et al., 2007) and $P2Y_2R$-$P2Y_6R$, $P2Y_4R$-$P2Y_6R$, $P2Y_6R$-$P2Y_{11}R$ (D'Ambrosi et al., 2007), and $P2Y_6R$-angiotensin II (Ang II) type 1 receptor (AT_1R) heterodimers(Nishimura et al., 2016). regarding the evidence for the study of the receptor complexes, in this review, we describe only $P2Y_6R$-AT_1R heterodimers, and the information about the other complexes is presented in Table 1.

1.4.1. Homo-dimerization of $P2Y_6R$

$P2Y_6R$ is expressed in human neuroblastoma SH-SY5Y cells and PC12 cells. It exists as a native high-order oligomer with molecular masses of approximately 500 and 230 kDa, respectively, which is disaggregated by 1% SDS to homodimers (approximately 80 kDa) and monomers (approximately 40 kDa) at a 1:1 ratio(D'Ambrosi et al., 2007). The agonist of $P2Y_6R$ stabilizes the 500 kDa high-order $P2Y_6R$ complexes but decreases the level of the 230 kDa high-order $P2Y_6R$ complexes in SH-SY5Y cells and PC12 cells; however, over time, t the constitutive distribution is restored, and a similar tendency is observed for $P2Y_6R$ internalization(Brinson & Harden, 2001).

1.4.2. Hetero-dimerization of $P2Y_6R$-AT_1R

In humans and other mammals, AT_1R is a GPCR that is distributed in nearly all tissues and many types of cells, particularly in vascular smooth muscle cells(Heart Outcomes Prevention Evaluation Study et al., 2000). AT_1R signalling maintains vascular homeostasis in the human body and has pathological consequences, such as hypertension, atherosclerosis, thrombosis, vascular remodelling, endothelial dysfunction, and cardiovascular disease(Garg & Yusuf, 1995; Heart Outcomes Prevention Evaluation Study et al., 2000; Ohtsu et al., 2006). However, AT_1R signalling is blocked by AT_1R blocking (ARB) agents, such as losartan. As described above, $P2Y_6R$ is also coupled to G_q, which is primarily involved in cardiovascular function and results in vascular remodeling(Stachon et al., 2014). Both receptors can couple to G_q and are expressed in vascular smooth muscle cells(Heart Outcomes Prevention Evaluation Study et al., 2000). In 2016(Nishimura et al., 2016), Nishimura et al. observed the natural formation of a stable heterodimer of two receptors in transfected HEK293 cells and rat aortae. $P2Y_6R$-AT_1R coexpression inhibits β-arrestin recruitment and the internalization of AT_1R induced by Ang II. Therefore, this heterodimer is a pathological complex that increases AT_1R function to result in a series of pathologies, as stated above. The noncompetitive $P2Y_6R$-selective antagonist MRS2578 inhibits Ca^{2+} release and reactive oxygen species (ROS) generation and subsequently inhibits $P2Y_6R$-AT_1R heterodimer generation, which blocks Ang II-induced vascular hypertrophy, but not basal pressure(Mamedova, Joshi, Gao, von Kugelgen, & Jacobson, 2004). $P2Y_6R$ abundance increases with age, and when incorporated in heterodimers, this property of $P2Y_6R$ alter Ang II responses in vascular smooth muscle cells. β-Arrestin-dependent proliferation switches to G protein-dependent hypertrophy in vascular smooth muscle cells induced by Ang II with increasing age(Deguchi, Makuuchi, Nakaoka, Collins, & Takuwa, 1999). Thus, $P2Y_6R$ may promote or increase pathological function and weaken AT_1R signal transmission, but dose not alter the expression of AT_1R. Therefore, we may use $P2Y_6R$ antagonists to inhibit Ang II

in clinicopathological conditions, which will provide a complete approach to treat and even prevent AT_1R-induced vascular events. Reports have not clearly determined the interface or sites involved in heterodimerization, although many scholars proposed that TM4-TM6 are very likely to be involved in heterodimerization, similar to other GPCR heterodimers or heterooligomers(Filizola & Weinstein, 2005).

1.5. $P2Y_{12}R$-related dimers

$P2Y_{12}R$(Franke & Illes, 2006; Neary et al., 1999; von Kugelgen, 2006) is one of the GPCRs in the δ group class A subfamily(Fredriksson, Lagerstrom, Lundin, & Schioth, 2003). $P2Y_{12}R$ has a distinct straight conformation of helix V, which distinguishes it from other P2YR family members. ADP and its 2-methylthio derivatives (2-MeSADP) are potent agonists (the former is a native agonist)(F. L. Zhang et al., 2001). Partial agonist activity of ATP and its analogues is observed for $P2Y_{12}R$, and they function as agonists or antagonists(Chang et al., 2012; Kauffenstein, Hechler, Cazenave, & Gachet, 2004) at different dosages and under specific circumstances. The active metabolite of clopidogrel binds to Cys97 or Cys175 to inhibit $P2Y_{12}R$. Agonists targeting $P2Y_{12}R$ coupled to Gi/o to activate the downstream signalling of $P2Y_{12}R$, such as PI3K activation, Rap 1B activation, cAMP inhibition, and vasodilator-stimulated phosphoprotein dephosphorylation, to inhibit AC, modulate the activity of physiological functions, such as platelet aggregation/stabilization, dendritic cell (DC) activation, microglial activation/migration/polarization and vasoconstriction, and induce pathological effects, such as thrombosis, myocardial infarction, and atherosclerosis(Conley & Delaney, 2003; Foster et al., 2001; Kim, Jin, & Kunapuli, 2004). Compared with other P2YRs, except $P2Y_6R$, $P2Y_{12}R$ does not undergo agonist-induced receptor internalization, which is important for some physiological or pathological functions. Currently, several known $P2Y_{12}R$ oligomers have been identified, including $P2Y_{12}R$ homooligomers(Savi et al., 2006), $P2Y_1R$-$P2Y_{12}R$ heterodimers(Hardy et al., 2004), $P2Y_{12}R$-$P2Y_{13}R$ heterodimers(Schicker et al., 2009), $P2Y_{12}R$-PAR4 heterodimers(Khan, Li, Ibrahim, Smyth, & Woulfe, 2014; Smith, Li, Dores, & Trejo, 2017), $P2Y_{12}R$-$A_{2A}R$ heterodimers, and $P2Y_1R$-$A_{2A}R$-$P2Y_{12}R$ oligomers(Nakata et al., 2005). Some of the receptor complexes have been described above.

1.5.1. Homo-dimerization of $P2Y_{12}R$

In 2006(Savi et al., 2006), Savi et al. detected that the homooligomeric complex of $P2Y_{12}R$ at the surface of mammalian cells and in freshly isolated platelets. In the resting state, $P2Y_{12}R$ in platelets is located in lipid rafts mainly as a homooligomer, with only a small amount of $P2Y_{12}R$ existing as a homodimer or monomer. Moreover, upon the addition of clopidogrel, $P2Y_{12}R$ homooligomers preferentially disassociate to nonfunctional $P2Y_{12}R$ homodimers in vitro in HEK293 cells (as the active metabolite of clopidogrel inhibits 2-MeSADP by binding to $P2Y_{12}R$)(Savi et al., 2001), and $P2Y_{12}R$ homodimers are subsequently redistributed outside of lipid rafts shown in Figure 3. A similar outcome has been observed in vivo (in platelet membranes) when rats are treated with clopidogrel; therefore, homooligomers may be the functional form of $P2Y_{12}R$. Also, DTT (a thiol-reducing reagent) causes $P2Y_{12}R$ homooligomers to almost completely disassociate into nonfunctional monomers and homodimers that are redistributed to outside of lipid rafts. Therefore, $P2Y_{12}R$ antagonists, particularly those with a thiol group,

may promote or stabilize $P2Y_{12}R$ homodimers, and their distribution and the location of lipid rafts may be important for $P2Y_{12}R$ activity. Because $P2Y_{12}R$ oligomers preferentially localize to certain lipid-rich domains in the PM, such as lipid rafts, this microdomain may be very important for oligomer-targeted therapy(Bickel et al., 1997). Compared with the oligomerization or dimerization of other GPCRs, the $P2Y_{12}R$ agonist binding site does not appear to function in receptor homo-oligomerization/homodimerization. Therefore, the domains of $P2Y_{12}R$ related to homo-oligomerization may be part of constitutive complexes that undergo oligomerization during the receptor assembly process.

Based on this information, thiol groups of Cys residues are essential for $P2Y_{12}R$ oligomer activity. Some of these cysteine residues are resistant to clopidogrel. Moreover, the Cys97 mutation (in EL1) in the $P2Y_{12}R$ homooligomer prevents clopidogrel binding, thereby impeding the dissociation of the $P2Y_{12}R$ homooligomer into monomers; therefore, this site is involved in the binding of antagonists. The $P2Y_{12}R$ homooligomer is pharmacologically active and displays membrane localization. In the X-ray crystallography structure, which was resolved in 2014, one Chinese team observed the interaction of $P2Y_{12}R$ monomers via helix V and two cholesterol molecules in the extracellular area: one is located in between the interface of helices III and V, and is involved in oligomerization(K. Zhang et al., 2014); and the other is located in the interface of helices I and VII, although it is not involved in crystal contacts. The effect of cholesterol requires further study. Therefore, for this homooligomer, the chemical structure of cholesterol may change the activity and structure of $P2Y_{12}R$ for treatment purposes.

1.5.2. Hetero-dimerization of $P2Y_{12}R$-PAR4

Protease-activated receptor (PAR) 4 is a thrombin receptor with low affinity for thrombin, and a member of the GPCR family that is activated by thrombin or collagen and then couples to $G_{q/12}$ to promote a steady and sustained increased in Ca^{2+} release(Holinstat et al., 2006; Smith et al., 2016). Upon thrombin stimulation, PAR4 recruits β-arrestin-2 in human platelets to promote arrestin-dependent platelet aggregation.

Both $P2Y_{12}R$ and PAR4 are expressed in platelets, smooth muscle cells, and endothelial cells and promote platelet aggregation by coupling to G proteins(Kahn et al., 1998; Kataoka et al., 2003) and both of them are associated with vascular inflammation in COVID-19(Sriram & Insel, 2020). $P2Y_{12}R$ has a low-affinity binding site for thrombin and slowly regulates intracellular calcium mobilization, subsequently maintaining intracellular calcium levels for a long time(Covic, Singh, Smith, & Kuliopulos, 2002; Dorsam, Tuluc, & Kunapuli, 2004), and the interactions between $P2Y_{12}R$ and PAR4 cause complete Akt activation. For β-arrestin-2 recruitment to PAR4, which is induced by PAR4 agonists, PAR4 must coexist with $P2Y_{12}R$. In addition, PAR4 activation results in the colocalization of PAR4 and $P2Y_{12}R$ in human platelets, indicating a synergistic function of these receptors(Baqi, Atzler, Kose, Glanzel, & Muller, 2009). This outcome is consistent with results from a previous study showing that $P2Y_{12}R$ promotes PAR4-induced β-arrestin-2 recruitment by inhibiting DAG kinase and blocking PKC phosphorylation level. In 2014(Khan et al., 2014), the Khan group identified a physically associated PAR4 and $P2Y_{12}R$ heterodimer in transfected HEK293T cells stimulated with thrombin. This heterodimerization requires the activation of both PAR4 and $P2Y_{12}R$. The calcium chelator 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid

tetrakis(acetoxymethyl ester) (BAPTA-AM) blocks PAR4-induced calcium mobilization and internalization of the $P2Y_{12}R$-PAR4 complex. Heterodimerization is promoted by a PAR4 agonist and inhibited by a $P2Y_{12}R$ antagonist. Smith and his team(Smith et al., 2017) further documented a role for PAR4 in Ca^{2+} mobilization, and it binds $P2Y_{12}R$ to regulate platelet aggregation; the findings of their study differed from the previous hypothesis that PAR1 (not PAR4) is directly involved with $P2Y_{12}R$ in this process. A BRET analysis showed that $P2Y_{12}R$ and PAR4 also formed PAR4-induced heterodimers or high-order complexes in cotransfected COS-7 cell membranes. In COS-7 cells, the activation of PAR4 in the $P2Y_{12}R$-PAR4 heterodimer promotes cointernalization(Nisar et al., 2012). However, the recruitment of β-arrestin to endosomes is required for PAR4-mediated internalization, and PAR4 must be coexpressed with $P2Y_{12}R$(Khan et al., 2014; Smith et al., 2017); this is another functional change in the $P2Y_{12}R$-PAR4 heterodimer that is caused by PAR4 and is a β-arrestin-independent $P2Y_{12}R$ internalization pathway that may be important for the stabilization of platelet thrombi.

Three amino acids (L194, G195, and L196) in TM4 of PAR4 are involved in heterodimerization, although the regions of these residues are not the only structures that regulate this process. The mechanism by which L194-L196 regulates heterodimerization is unclear, although these residues do not inhibit receptor trafficking to the membrane. More research is needed to understand how other domains are involved in heterodimerization.

1.6. $P2Y_{13}R$-related dimerization

$P2Y_{13}R$(Perez-Sen et al., 2017) is expressed in the brain, spleen, lymph nodes, and bone marrow and activated by the native agonists P1-(5'-Adenosyl) P3-(5'-adenosyl) triphosphate (Ap_3A) and ADP; it couples to G_i to inhibit AC, and activates PLC and RhoA to regulate bone formation, neuroprotection, trophic activity and cholesterol, and glucose metabolism. Fewer studies have focused on $P2Y_{13}R$-related dimers, and only FRET or other detection methods have identified its colocation or oligomers in the membrane; thus, its interactions and functional activity are unclear. The $P2Y_{13}R$ homooligomer was detected in co-expressing HEK293 cells and accidentally detected when studying other receptors(Savi et al., 2006). Both monomers and homooligomers are located in lipid rafts in the membrane. Similarly, $P2Y_{13}R$ interacts with other GPCRs, such as $P2Y_1R$, $P2Y_2R$, $P2Y_{12}R$, as well as A1R and $A_{2A}R$(Schicker et al., 2009), as shown in Table 1.

2. The function of P2YR oligomers

Currently, many studies are focused on GPCR dimers. However, only a few studies have focused on the function of dimers in vivo in the natural state(Borroto-Escuela et al., 2018; Ferre et al., 2020; Nai et al., 2010; Pei et al., 2010; Shah, Toneatti, Gaitonde, Shin, & Gonzalez-Maeso, 2020), particularly P2YRs, as they generally use in vitro or transient overexpression analyses. In addition, few studies have focused on the physiological and pathological functions of P2YR complexes. In this review, we have summarized several changes in the functions of P2YR dimers.

2.1. Changes in signal transduction and new functional activities of dimers may be used for the development of new drugs

Due to receptor oligomerization, some pharmacological properties of P2YRs change. For example, the specificity and potency of a ligand will change and are independent of the

ligand, but dependent on the conformation. Ligand bias has been observed for the $P2Y_1R$-A_1R heterodimer(Yoshioka, Hosoda, et al., 2002), as the affinity of A_1R for A_1R ligands is decreased while the affinity for $P2Y_1R$ agonists is significantly increased, potentially because of a conformational change in the ligand-binding sites of A_1R caused by heterodimerization; therefore, the A_1R binding pocket better fits the $P2Y_1R$ ligand when A_1R and $P2Y_1R$ form a heterodimer. Importantly, researchers have not determined whether A_1R displays tissue specificity, although both $P2Y_1R$ and A_1R are expressed at high levels in the hippocampus and astrocytes. These brain tissues also exhibit colocalization of the $P2Y_1R$-A_1R heterodimer in vivo in rats, and increased specificity is commonly for heterodimers (which is not limited to GPCRs). Therefore, the next step is to investigate whether this heterodimer displays tissue-specific expression and determine whether it can be targeted to improve the treatment effect and reduce side effects on other organs.

A pathological effect is observed for some receptors when certain mutants form a dimer with other receptors; however, a mutant alone is not sufficient to cause pathological effects. The SNP in $P2Y_{11}R$ that results in the A87T mutation is associated with acute myocardial infarction (AMI) and inflammation by damaging the functions of immune cells and increasing levels of C-reactive protein(Haas et al., 2014). This pathological feature appears only when $P2Y_{11}R$ forms a heterodimer with $P2Y_1R$, and $P2Y_{11}R$ A87T signaling transduction impaired when co-operating with $P2Y_1R$.

Moreover, some other P2YR dimers alter the GPCR pathway or β-arrestin pathway to promote receptor internalization and cycling. For example, the $P2Y_1R$-A_1R heterodimer mediates a $P2Y_1R$ agonist-induced A_1R signal and $P2Y_{11}R$ internalizes with $P2Y_1R$; additionally, $P2Y_{12}R$ internalization is independent of the β-arrestin pathway when $P2Y_{12}R$ and PAR4 form a heterodimer, which is important for maintaining receptor numbers in the membrane and signalling diversity.

However, in terms of heterodimers' conformations, more technology is required to precisely detect changes or downstream signals that influence and contribute to these changes. In conclusion, P2YR oligomerization significantly enhances signal transduction; however, common rules do not appear to apply to P2YR oligomerization, and the pharmacological properties of receptor oligomers are more closely related to the properties of receptor monomers.

2.2. Regulation of molecular membrane trafficking

For some P2YRs, dimerization or oligomerization is the premise for correct membrane trafficking or facilitates membrane transport. Formation of the $P2Y_1R$-M71R heterodimer(Bush et al., 2007; Gayle & Burnstock, 2005; Hegg, Greenwood, Huang, Han, & Lucero, 2003), increases M71R membrane expression, allowing researchers to heterogeneously express M71R and study its pharmacological effects. Studies of M71R will provide insights into other receptors with dysregulated membrane transport and provide new ideas in receptor research. Due to the tissue-specific distribution of M71R, the interaction of $P2Y_1R$-M71R heterodimers in vivo is also tissue-specific. Therefore, the targeting of this complex may be more directed. In addition, for some constitutive oligomers, the oligomerization state and conformation may be more suitable for membrane transport; for example, the majority of $P2Y_{12}Rs$ exist as homooligomers in the

lipid rafts on the surface of mammalian cells and in platelets(Savi et al., 2006).

2.3. Promotion of receptor internalization

In the monomer state, some receptors are internalized slowly or are not internalized at all. According to previous study, the role of internalization or endocytosis is not limited to desensitization(Irannejad et al., 2013); thus, complete activation of signalling pathways may be achieved by recycling or activation of other signal pathways may be activated through receptor internalization. Some P2YR dimers are internalized by internalization after forming dimers because they may lack conventional internalization mechanisms, such as agonist-induced internalization. In some cases, P2YRs must form a dimer to be internalized, which may be due to the conformational changes that occur during dimer formation and exposure or occupation of the binding domain of the internalization-related protein, resulting in a series of subsequent reactions. For instance, formation of the $P2Y_1R$ homodimer promotes the receptor's internalization, which differs from receptor desensitization; because $P2Y_1R$ homodimer desensitization occurs before internalization(Choi et al., 2008), internalization may be attributed to the intracellular activation of other signals or resensitization. However, based on in vitro experiment's, the time of internalization is obviously different from of the results obtained from platelets and smooth muscle cells in vivo; thus, its authenticity has yet to be verified. However, this discovery allows us to re-examine the functions of receptor desensitization and internalization of the $P2Y_1R$ homodimer. Other P2YR dimers have shown to cointernalize. For instance, when a receptor is activated and internalized, another monomer in the dimer will also internalize, which has been described for both $P2Y_1R$-$P2Y_{11}R$ and $P2Y_{12}R$-PAR4 heterodimers(Ecke et al., 2008; Smith et al., 2016). For $P2Y_{12}R$-PAR4 heterodimers, only PAR4 needs to be activated to promote $P2Y_{12}R$-PAR4 heterodimer internalization, whereas PAR4 and $P2Y_{12}R$ must be coexpressed in the membrane for this process to occur. Therefore, the activation of only one receptor results in the internalization of another receptor. In particular, this process has been reported for the $P2Y_1R$ homodimers, $P2Y_1R$-$P2Y_{11}R$ heterodimers, and $P2Y_{12}R$-PAR4 heterodimers and, interestingly, represent a different pathway to promote internalization and desensitization. For example, a PAR4 agonist promotes the internalization of the $P2Y_{12}R$-PAR4 heterodimer, and PAR4 activation in this complex promotes $P2Y_{12}R$ internalization through a mechanism independent of the β-arrestin pathway; while, the $P2Y_1R$ is not internalized without homo-oligomerization. Either internalization or desensitization decrease signalling, and internalization promotes receptor recycling. Therefore, P2YR dimers can also regulate signal transduction.

2.4. Active form of receptors

Some P2YRs must for dimers and bind to ligands to exert their physiological functions; for example, $P2Y_{12}R$ forms an oligomer that binds endogenous ligands(Savi et al., 2006), and the $P2Y_4R$ homodimer is believed to be the active form of $P2Y_4R$(D'Ambrosi et al., 2007). Therefore, for these receptors, we are able to promote or inhibit their ability to oligomerize to treat diseases.

3. Dynamic regulation of P2YR oligomerization

As described above, P2YR oligomerization is important for the activities of living organisms; for instance, oligomerization increases the diversity of signalling pathways,

molecular membrane trafficking, and internalization and changes the affinity of ligands or agonists/antagonists for one receptor in a complex. Therefore, researchers must determine methods to dynamically regulate P2YR oligomerization for clinical applications. However, clear evidence has not been reported for the oligomerization certain of P2YRs, and the activity has not been documented for some P2YR oligomers, such as the $P2Y_1R$, $P2Y_4R$, and $P2Y_6R$ homodimers(Choi et al., 2008; D'Ambrosi et al., 2007) have no activity. In addition, different research groups utilize different methodologies and obtain different results. For P2YRs, common rules for oligomerization do not exist. However, most studies focus on the effects of agonists and antagonists.

Agonists and antagonists exert different effects on the dynamic regulation of P2YR complexes. In some complexes ($P2Y_1R$-A_1R, $P2Y_{12}R$-PAR4, $P2Y_1R$-$P2Y_{12}R$-A_{2A}, $P2Y_2R$-B2R, and $P2Y_6R$-AT_1)(Nishimura et al., 2016; Ribeiro-Filho et al., 2016; Smith et al., 2016; Yoshioka, Saitoh, et al., 2002), agonists and antagonists promote or inhibit the formation of oligomerization and subsequently increase or decrease signal transduction; in other P2YR complexes, agonists and antagonists may have no effects. For example, the interaction of the $P2Y_{12}R$-PAR4 heterodimer(Smith et al., 2017) is promoted by the PAR4 agonist AYPGKF and inhibited by the $P2Y_{12}R$ antagonist 2-methylthioadenosine 5′-monophosphate triethylammonium (2-MeSAMP), as well as the calcium chelator BAPTA-AM; thus, calcium mobilization can also modulate the dynamics of receptor oligomerization. Upon the oligomerization of $P2Y_1R$-$P2Y_{12}R$-A_{2A}(Suzuki et al., 2011), 2-MeSADP/ADP promotes Ca^{2+} release; conversely, ARC69931MX and ZM241385 inhibit this effect. However, dynamic regulation of the complex can also occur through the costimulation of two receptors, such as $P2Y_2R$-A_1R(Suzuki et al., 2006). When both A_1R and $P2Y_2R$ agonists are added to this heterodimer, two opposite outcomes occur; A_1R signalling decreases while $P2Y_2R$ signalling increases.

In addition, the oligomerization of several GPCRs is constitutive and generates a receptor complex in the ER or other organelles in the early stages of their biosynthesis. Thus, these types of receptors, they can theoretically use receptor oligomerization as a common prerequisite for receptor maturation, thereby transiting to the PM and acquiring functional activity, which is not altered by extracellular ligands or receptor agonists, such as purines for P2YR (i.e., does not require receptor activation). This type of receptor complex is not a transient response to extracellular agonists but a required process in their functional maturation and transport to the PM. Therefore, researchers have hypothesized that receptor oligomerization may play a role in the quality control or ER export of P2YRs. Examples of these types of oligomers are the human melatonin receptors MT1 and MT2 and the constitutive oligomerization that occurs early after the biosynthesis of CCR5 in the absence of ligands; moreover, extracellular ligands of these receptors may promote conformational changes but not alter receptor oligomerization. In 2002(Terrillon et al., 2003), Terrillon stated that ‘GPCR dimerization is a constitutive process’ in their study of interaction between vasopressin V1a and V2 receptors using BRET. In fact, this hypothesis obtained some experimental support from studies on the insulin and insulin-like growth factor I receptors and some other membrane receptors. However, not all receptors need to dimerize or oligomerize to transit and mature (an example is B2R). In the P2YR oligomerization system(Choi et al., 2008), $P2Y_1R$ exists as

a homodimer in HEK293 cells, although its monomer accounts for 44% of the total $P2Y_1R$. Although studies have utilized the scavenger apyrase to remove receptor agonists, 28% of $P2Y_1Rs$ are still homodimers and are not altered by extracellular ligands in the studies of the intracellular factors influencing $P2Y_1R$ oligomerization. Therefore, a method or technology to detect the effect of the ligand site or receptor state on subcellular receptor oligomerization is needed.

4. Conclusions

P2YRs are expressed in many cell types in mammals and exert extensive physiological and pathological effects, including neuroprotective effects and immune responses, and they are also involved in platelet aggregation, inflammation, and cellular proliferation(Abbracchio et al., 2006; Ralevic & Burnstock, 1991; von Kugelgen & Wetter, 2000). Currently, many drugs target GPCR to cure diseases, but only a few of them target to P2YRs; moreover, the side effects and tissue-specific activities of chemical ligands are limitations of GPCR drugs that have been neglected(Bishnoi et al., 2019; Cowan, Kehner, & Inan, 2015; Maybrook et al., 2015; Zhou et al., 2020). In addition, the effects of these ligands differ from the expected and theoretical effects among P2YRs, and the majority of ligands do not actually target specific P2YRs. Research on GPCR oligomerization, including P2YR oligomerization, has focused on their functions. Interactions between receptors may change their signalling pathways, which differ from those of their respective monomers; however, other explanations for these changes are possible, such as changes in downstream signals or direct effects on the affinity of the ligand for the receptor in the dimer. Moreover, ligands of P2YRs and their coexpressed receptors may function as agonists or antagonists to regulate receptor oligomerization. Alternatively, in some types of P2Y oligomers that form complexes in the ER during biosynthesis, oligomerization is not directly influenced by extracellular ligands; futhermore, the formation of dimers may be a prerequisite for the correct regulation of receptor maturation, transportation to the PM and regulation of signalling pathway functions.

P2YR oligomers have been shown to have new functions that differ from the function of their native monomers, such as receptor internalization and the regulation of ligand affinity and specificity, signalling pathways, and molecular membrane trafficking(Choi et al., 2008; Ecke et al., 2008; Hardy et al., 2004; Nishimura et al., 2016; Savi et al., 2006; Yoshioka, Saitoh, et al., 2002). All of these changes indicate potentially new pharmacological targets for drug development. In addition, the tissue specificity of P2YR dimers or oligomers may enhance drug or ligand specificity; for example, $P2Y_1R$-M71R(Gayle & Burnstock, 2005; Hegg et al., 2003) localization in the airway and $P2Y_1R$-$P2Y_{11}R$(A87T) coexpression in cells result in pathological effects. Thus, investigations of P2YR oligomers may help determine the binding sites of GPCR ligands and resolve the side effects of current drugs. These new potential targets will also provide a new direction for the development of new chemical ligands for receptor dimers.

Although the approximate mechanism underlying the formation of several GPCRs, such as gamma-aminobutyric acid receptors ($GABA_BRs$), is known, many problems remain to be resolved in P2YR oligomerization, including the following i) specific and accurate interacting structures and molecular mechanisms, ii) conformational changes occurring

during oligomerization, iii) additional experimental methods to detect and confirm the physiological or pathological effects in vivo or in natural states. Additionally, detection methods may require further improvement, because most current technologies are unable to precisely recognize dimers or high-order oligomers or determine their interacting domains. Most current studies probing the interaction between receptors have shown that the receptors are in a state of transient overexpression, specifically through coexpression in the membrane; more investigations are required in the natural state. The interaction between receptors may be transient rather than stable, and thus many interactions will be ignored. Therefore, more precise research methods must be invented to study oligomers.

In conclusion, P2YR oligomers increase the diversity of P2YR signalling pathways by changing the native pharmacological properties of their respective monomers. Novel P2YR oligomer binding sites require further exploration to develop new drugs.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY(Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

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Author Contributions

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Conflict of Interest

The authors declare no conflicts of interest.

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