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Integration of P2Y receptor-activated signal transduction pathways in G protein-dependent signalling networks

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Abstract The role of nucleotides in intracellular energy provision and nucleic acid synthesis has been known for a long time. In the past decade, evidence has been presented that, in addition to these functions, nucleotides are also autocrine and paracrine messenger molecules that initiate and regulate a large number of biological processes. The actions of extracellular nucleotides are mediated by ionotropic P2X and metabotropic P2Y receptors, while hydrolysis by ecto-enzymes modulates the initial signal. An increasing number of studies have been performed to obtain information on the signal transduction pathways activated by nucleotide receptors. The development of specific and stable purinergic receptor agonists and antagonists with therapeutical potential largely contributed to the identification of receptors responsible for nucleotide-activated pathways. This article reviews the signal transduction pathways activated by P2Y receptors, the involved second messenger systems, GTPases and protein kinases, as well as recent findings concerning P2Y receptor signalling in C6 glioma cells. Besides vertical signal transduction, lateral cross-talks with pathways activated by other G protein-coupled receptors and growth factor receptors are discussed.

Key words C6 glioma · ERK · P2Y receptors · PKB · transactivation · tyrosine kinases

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Abbreviations

AC	adenylate cyclase
Ap_3A	P^1,P^3-di(adenosine-5′)triphosphate
Ap_4A	P^1,P^4-di(adenosine-5′)tetraphosphate
AR	adrenergic receptor
COX	cyclooxygenase
DAG	diacylglycerol
ERK	extracellular signal-regulated kinase
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptor
HT	hydroxytryptamine
IP_3	inositol (1,4,5)-triphosphate
PAP	adenosine-3′,5′-biphosphate
PI	phosphatidylinositol
PL	phospholipase
PI 3-K	phosphatidylinositol 3-kinase
PPADS	pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonate
Pyk2	proline-rich tyrosine kinase 2
RKIP	Raf kinase inhibitory protein
RTK	receptor tyrosine kinase

Introduction

Pharmacological properties of P2Y receptors

Extracellular actions of adenine nucleotides were initially characterised in the cardiovascular system by Drury and Szent-Gyorgyi [1]. It took more than four decades before the concept of purinergic signalling was accepted, but now it is well established that nucleotides initiate and regulate a variety of biological processes, including neurotransmission, inflammation, regulation of blood pressure, platelet aggregation, cell growth and differentiation (Abbracchio et al. [2]; Burnstock and Williams [3]; Burnstock [4]; Ralevic and Burnstock [5]).

Nucleotides are released in the extracellular fluid by cell lysis, exocytosis, secretion of granules, efflux and upon cellular stress such as changes in osmolarity and mechanical perturbations. Once released, they mediate their effect by stimulation of nucleotide receptors.

Based on pharmacological properties, the first suggestion for the existence of ionotropic P2X receptors and metabotropic P2Y receptors was made by Burnstock and Kennedy [6]. After cloning, multiple subtypes of P2X and P2Y receptors were characterised unambiguously (Abbracchio and Burnstock [7] Burnstock and Williams [3]; Fredholm et al. [8]).

Up to now, the P2Y receptor family comprises at least eight subtypes, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and the recently identified $P2Y_{14}$ receptor (Ralevic and Burnstock, [5] Abbracchio et al. [9]; Communi et al. [10]; Hollopeter et al. [11]; Zhang et al. [12]). According to the agonist profile, P2Y receptors can be subdivided into receptors responding to adenine mono- and dinucleotides ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$), and to uridine nucleotides ($P2Y_4$, $P2Y_6$), and receptors for adenine and uridine nucleotides ($P2Y_2$). The pharmacological profile of the recently cloned $P2Y_{14}$ receptor is distinct from the other P2Y receptors since UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine are specific ligands of this receptor (Chambers et al. [13]). Natural P2Y receptor ligands do not exclusively bind to one receptor subtype. ADP is an agonist of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, whereas ATP is a full agonist of $P2Y_2$ and $P2Y_{11}$, but a partial agonist or antagonist of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors. Although the pharmacological properties of P2Y receptors (Table 1) are well conserved between species, some remarkable differences have been observed. While UTP acts as an agonist of both human and rat $P2Y_4$ receptors, ATP is a potent agonist of the rat $P2Y_4$, but an antagonist of the human orthologue. Mutational analysis revealed that the second extracellular loop of the $P2Y_4$ receptor is responsible for the opposing effect of ATP in both species (Herold et al. [14]). A similar phenomenon is observed when human and canine $P2Y_{11}$ receptors were stably expressed in CHO-K1 and 1321N1 astrocytoma cells. Whereas the

Table 1 Pharmacological profile of P2Y receptors and second messenger systems.

	Agonists	Antagonists	Effector	G protein
$P2Y_1$	2MeSADP, ADP, ADPβS, Ap_3A, MRS2365	Suramin, PPADS, PAP, MRS2179, MRS2216, MRS2279, MRS2500, MRS 2603	PLC, $I_{K,\ Ca}$	$G_q/G_{11/12}$
$P2Y_2$	UTPγS, ATPγS, UTP, ATP, INS37217, Ap_4A	Suramin	PLC, $I_{K,\ Ca}$	G_i/G_q
$P2Y_4$	UTPγS, UTP, ATP[a]	ATP[a], PPADS	PLC, I_K	$G_q/G_{11/12}$
$P2Y_6$	UDPβS, UDP, UTP, INS48823	PPADS, suramin, MRS2567	PLC, I_K	$G_q/G_{11/12}$
$P2Y_{11}$	ATPαS, ATPγS, ATP	Suramin	AC, PLC	G_q/G_s
$P2Y_{12}$	2MeSADP, ADP, Ap_3A, ATP, Ap_4A	AR-C69931MX, AR-C67085, AR-C78511KF, clopidogrel, 2MeSAMP, DIDS, suramin, MRS2395	AC, $I_{K,\ Ca}$	$G_{i/o}$
$P2Y_{13}$	2MeSADP, ADP, Ap_3A, ATP	AR-C69931MX, Ap_4A, PPADS, suramin, MRS2211, MRS2603	AC, PLC, I_{Ca}	G_i/G_q
$P2Y_{14}$	UDP-glucose, UDP-galactose, UDP-glucuronic acid, UDP-N-acetylglucosamine		AC, I_{Ca}	G_i

[a] ATP acts as an agonist of the rat $P2Y_4$ but as an antagonist of the human $P2Y_4$ receptor (Herold et al [14]). Reactive blue 2 is not included in the list since it displays lack of specificity towards the different P2Y subtypes. References: Abbracchio et al. [9]; Communi et al. [10, 191]; Chambers et al. [13]; Claes and Slegers [17]; Kim et al. [26]; Xu et al. [27]; Boyer et al. [38, 189, 190]; Grobben et al. [40]; Marteau et al. [47]; Filippov et al. [57–60, 63]; Simon et al. [61]; Wirkner et al. [62]; Korcok et al. [192]; Muller [193]; Skelton et al. [194]; Yerxa et al. [195]; Jacobson et al. [196]; von Kügelgen [197]. Abbreviations: Ap_3A, P^1,P^3-di(adenosine-5′) triphosphate; Ap_4A, P^1,P^4-di(adenosine-5′)tetraphosphate; AR-C69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio) -β,γ-dichloromethylene ATP; AR-C67085, 2-propylthio-D-βγ-dichloromethylene adenosine 5′-triphosphate; AR-C78511KF, (E)-N-[1-[7-(hexylamino)-5-(propylthio)-3H-1,2,3-triazolo-[4,5-d]-pyrimidin-3-yl]-1,5,6-trideoxy-β-D-ribo-hept-5-enofuranuronoyl]-L-aspartic acid monoammonium salt; DIDS, 4,4′-diisothiocyanatostilbene-2, 2′-disulphonic acid; INS37217 [P(1)-(uridine 5′)-P (4)-(2′-deoxycytidine 5′)tetraphosphate tetrasodium salt; INS48823 P^1-((2-benzyl-1, 3-dioxolo-4-yl)uridine 5′)P^3-(uridine 5′) triphosphate; MRS2179, N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate; MRS2211, pyridoxal- 5′-phosphate-6-azo-(2-chloro-5-nitrophenyl)-2,4-disulphonate; MRS2216, 2′-deoxy-2-chloro-N^6-methyladenosine-3′,5′-bisphosphate; MRS2279, 2-chloro-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine 3′,5′-bisphosphate; MRS2365, [(1′S,2′R,3′S,4′R,5′S)-4-[(6-amino-2-methylthio-9H-purin-9-yl)-1-diphosphoryloxymethyl]bicyclo[3.1.0]hexane-2, 3-diol]; MRS2395, 2-dimethyl-propionic acid-3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethylpropionyloxy-methyl)-propylester; MRS2500, 2-iodo-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine 3′,5′-bisphosphate; MRS2567, 1,2-di-(4-isothiocyanatophenyl)ethane; MRS2603, pyridoxal-5′-phosphate-6-azo-(4-chloro-3-nitrophenyl)-2,4-disulphonate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid.

human $P2Y_{11}$ is potently activated by adenosine triphosphate nucleotides, the canine orthologue displayed more selectivity towards the corresponding diphosphates. In this case, the nucleotide selectivity is due to differences in the amino acid sequence at the juxtaposition of TM6 and the third extracellular loop also reported to play an important role in agonist selectivity and signalling of other G protein-coupled receptors (GPCR) (Qi et al. [15]; Lawson and Wheatley [16]).

Despite their chemical stability, extracellular nucleotides are metabolised by several ecto-enzymes (Claes and Slegers [17]; Czajkowski and Baranska [18]; Goding et al. [19]; Zimmerman [20]). Extracellular hydrolysis complicates the evaluation of nucleotide-mediated effects on different cell types and can be overcome by the use of specific non-hydrolysable receptor agonists or ecto-enzyme inhibitors. Some P2Y receptor antagonists, such as pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), suramin, reactive blue 2 and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), are inhibitors of nucleotide hydrolyzing enzymes and are often used in studies of nucleotide-mediated signalling (Grobben et al. [21]). Nevertheless, care must be taken for the interpretation of experimental data since it is also shown that cells can internalise some of these molecules (Claes et al., [22]). Therefore specific P2Y receptor antagonists, developed for therapeutical purposes, have to be used to overcome the lack of specificity (Boeynaems et al. [23]; Lambrecht et al. [24]; Kam and Nethery [25]; Kim et al. [26]; Xu et al. [27]).

P2Y receptor expression on rat C6 glioma cells

Rat C6 glioma is a tumoral cell line of glial origin with oligodendrocytic, astrocytic and neuronal progenitor properties. Due to a point mutation in 'phosphatase and tensin homologue deleted on chromosome ten' (PTEN), the phosphatidylinositol 3-kinase (PI 3-K)/PKB signalling pathway is constitutively active and contributes to the proliferative and invasive properties of these cells (Kubiatowski et al. [28]; Roymans and Slegers [29]; Grobben et al. [30]). In addition, cell proliferation is sustained by secreted growth factors that stimulate growth factor receptors present on these cells. Such autocrine mechanisms are reported for IGFR, bFGFR and PDGFR (Okumura et al. [31]; Resnicoff et al. [32]; Strawn et al. [33]).

In C6 cells, an increase in cAMP by stimulation of the β-adrenergic receptor (β-AR) or by addition of membrane permeable cAMP analogues, e.g., dibutyryl cAMP (dBcAMP) or 8-chloro-cAMP, induces differentiation into an astrocyte type II (Roymans et al. [34]). During this process, cessation of cell growth is accompanied by a shift in intermediate filament synthesis from vimentin to glial fibrillary acidic protein (GFAP) (Backhovens et al. [35]). The latter protein is an astrocytic differentiation marker whose expression is regulated by cAMP at the transcriptional and translational level (Messens and Slegers [36]).

In our laboratory, the signalling pathways activated by extracellular nucleotides, and in particular those affecting cell proliferation and differentiation of C6 cells, were studied in detail. The presence of a P2Y receptor on these cells that negatively affects adenylate cyclase (AC) was postulated for more than a decade (Pianet et al. [37]; Boyer et al. [38]). This receptor is coupled to a G_i protein and has been denominated $P2Y_{AC}$- (Claes et al. [39]; Grobben et al. [40]) before its identification as the $P2Y_{12}$ receptor initially cloned from blood platelets (Czajkowski et al. [41]; Hollopeter et al. [11]; Jin et al. [42]). C6 cells also express the phospholipase (PL)Cβ-coupled $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Czajkowski et al. [41]; Nicholas et al. [43]; Tu et al. [44]; Claes and Slegers [17]). Recently, we also demonstrated the presence of $P2Y_{13}$ mRNA (Van Kolen and Slegers [45]) implicating the expression of three ADP-activated receptors in these cells, i.e., $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$. Although 2MeSADP is reported as a potent $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ agonist, stimulation with this compound inhibits AC, but induces no significant activation of PLC, indicating that the $P2Y_1$ receptor is not activated by ADP in cells grown in chemically defined medium (Grobben et al. [40]). This is confirmed by Czajkowski et al. [46], who showed that, in cells cultivated in the presence of fetal calf serum, ADP signalling is predominantly determined by the $P2Y_1$ receptor. However, upon serum deprivation, expression of the $P2Y_1$ receptor is decreased and the $P2Y_{12}$ receptor becomes the main activated receptor. Characterization of $P2Y_{13}$ receptor function is complicated by the fact that $P2Y_{12}$ and $P2Y_{13}$ receptors have almost the same agonist profile (Table 1). The receptor antagonist N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX), often used as a specific $P2Y_{12}$ antagonist, also blocks the $P2Y_{13}$ receptor (Marteau et al. [47]). While the human and mouse $P2Y_{13}$ receptor, like the $P2Y_{12}$, is more potently activated by 2MeSADP than ADP, the rat $P2Y_{13}$ receptor shows a higher selectivity for ADP (Fumagalli et al. [48]). In C6 cells, further distinction between the signalling of $P2Y_{12}$ and $P2Y_{13}$ receptors can be made by the use of PPADS, a $P2Y_{13}$ antagonist without effect on $P2Y_{12}$, and P^1, P^4-di(adenosine-5′) tetraphosphate (Ap_4A), a $P2Y_{13}$ antagonist that stimulates the $P2Y_{12}$ receptor (Claes et al. [39];

Grobben et al. [40]; Marteau et al. [47]). In addition, recently synthesised PPADS derivatives pyridoxal-5′-phosphate-6-azo-(2-chloro-5-nitrophenyl)-2,4-disulphonate (MRS2211) and pyridoxal-5′-phosphate-6-azo-(4-chloro-3-nitrophenyl)-2,4-disulphonate (MRS2603) have no effect on the $P2Y_{12}$ receptor but antagonise the $P2Y_{13}$ receptor (Kim et al. [26]).

Although P2Y receptor expression in C6 cells depends on the cultivation conditions (Czajkowski et al. [46]), unpublished data of our laboratory revealed that induction of differentiation into astrocytes type II by dbcAMP (1 mM)- or (-)-isoproterenol (5 μM) does not significantly alter the expression of P2Y receptors. These observations are in accordance with previous studies on the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors in glial cells and primary astrocytes (Bianco et al. [49]; Fumagalli et al. [48, 50]; Sasaki et al. [51]). The function of P2Y receptor expression in glial cells is still under investigation, but a number of studies point to an important role in the intercellular communication between astrocytes and neurons (Bezzi and Volterra, [52]). Another well documented effect of extracellular ATP is induction of reactive astrogliosis upon activation of ERK and cyclooxygenase (COX)-2 (Brambilla et al. [53]).

Similar to the observations made in C6 cells, functional responses of P2Y receptor subtypes in microglial cells depend on cultivation conditions. In N9 mouse brain microglia stimulation of expressed P2Y receptors induces Ca^{2+} mobilization but only $P2Y_6$ and $P2Y_{14}$ receptor-mediated responses are increased upon activation of microglia with lipopolysaccharide. The enhanced $P2Y_6$ response is correlated with mRNA increase which was not the case for the $P2Y_{14}$ receptor-mediated Ca^{2+} mobilization (Bianco et al. [49]). Furthermore, stimulation of microglial $P2Y_{12/13}$ receptors induces membrane ruffling and chemotaxis towards injured neurons through $G_{i/o}$ protein-mediated activation of Rac (Honda et al. [54]). The observations made in astrocytes and microglial cells emphasise the importance of P2Y receptors in brain signalling and identify these receptors as putative targets in defective neurotransmission, neuroimmune functioning, cell survival and cell proliferation in response to oxidative stress and brain injury.

P2Y receptor-activated signalling cascades

Second messengers

P2Y receptors are generally linked to PLC activation that catalyses the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate into the intracellular messenger inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Activation of PLC occurs by $G_i\alpha$- and/or $G_q\alpha$-dependent mechanisms (Communi et al. [55]). Besides signalling through Gα subunits, intracellular Ca^{2+} concentration is also affected by Gβγ subunit-dependent interaction with voltage-gated Ca^{2+} channels. Several reports indicated modulation of K^+ currents and PLCβ activation induced by distinct domains of Gβγ (Mirshahi et al. [56]). Co-expression studies performed in rat sympathetic neurons demonstrated that $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors trigger the closing of N-type Ca^{2+} and M-type K^+ channels, whereas $P2Y_4$ receptor stimulation also displayed coupling to M-type K^+ channels producing a less efficient inhibition of Ca^{2+} currents. In rat brain capillary endothelial cells, it was shown that the $P2Y_{12}$ receptor inhibits $I_{Ca(N)}$ and activates a G protein-coupled inward rectifier K^+ (GIRK) channel. Interestingly, stimulation of the $P2Y_1$ receptor also induces a K^+ current that is rapidly followed by inactivation. Inhibition of $I_{Ca(N)}$ by $P2Y_{12}$ receptor stimulation is also reported in PC12 cells while in HEK 293 this inhibition is mediated by the $P2Y_{13}$ receptor (Filippov et al. [57–60]; Simon et al. [61]; Wirkner et al. [62]). From co-expression studies of P2Y receptors with GIRK1 or GIRK2 in rat sympathetic neurons, it was concluded that P2Y receptors activate GIRK channels by the βγ subunits of $G_{i/o}$ and inhibit these channels by the α subunits of G_q (Filippov et al. [63]).

P2Y-induced calcium release is followed by opening of voltage-independent Ca^{2+} channels. Although this response is observed in a variety of cell types, the physiological implications are miscellaneous. In this context, it has been reported that extracellular ATP induces a Ca^{2+} wave that propagates through neighbouring astrocytes by GAP junctions (Suadicani et al. [64]). In situations of increased neuronal activity or cell damage, ATP stimulates a Ca^{2+}-dependent proton-efflux from astrocytes. Acidification of the extracellular environment serves as a negative feedback mechanism for neurotransmitter release, but also increases blood flow by vasodilatation in cerebral arterioles (Dienel and Hertz [65]; Dixon et al. [66]). Although Ca^{2+} signalling is observed in a variety of cell types, the time dependence of the response is cell type specific. This is especially the case for the $P2Y_1$ receptor which triggers persistent or transient Ca^{2+} responses when it is expressed in human 1321N1 astrocytoma or C6 glioma cells, respectively (Czajkowski et al. [41]; Sellers et al. [67]). A recent study also revealed that, in glial cells, prolongation of the $P2Y_1$ receptor-induced Ca^{2+} response is regulated by interaction with the Na^+/H^+

exchanger regulatory factor type-2 which determines the signalling pathways that are ultimately activated in different cell types (Fam et al. [68]). Indeed, while transient $P2Y_1$ receptor signalling increases proliferation in C6 cells, sustained signalling triggers apoptotic cascades in 1321N1 astrocytoma cells (Czajkowski et al. [46]; Sellers et al. [67]).

A well-known response to PLC-generated DAG and IP_3/Ca^{2+} is the activation of classical PKCs that are involved in rapid internalisation and desensitisation of GPCRs through phosphorylation of residues localised in their cytoplasmic tail. In this regard, PKCβI is reported to attenuate phosphatidylinositol (PI)-hydrolysis induced by $P2Y_1$ and $P2Y_2$ receptors in endothelial cells (Chen and Lin [69]). In astrocytes, high frequency stimulation of the $P2Y_1$ receptor by repeated addition of ATP causes rapid suppression of the $P2Y_1$ receptor-induced Ca^{2+} response. This phenomenon, observed as Ca^{2+} oscillations, is mediated by protein kinase C-dependent phosphorylation of Thr339 in the carboxy-terminus of the $P2Y_1$ receptor (Fam et al. [70]). Besides modulation of receptor responsiveness, PKC signalling also affects long term effects. In the human osteoblastic HOBIT cell line, ATP increases expression of the early growth response protein-1 by a mechanism that requires a Ca^{2+}-independent PKC isoform (Pines et al. [71]). In vascular smooth muscle cells, UDP stimulates cell cycle progression by a PLC- and PKCδ-dependent cascade (Hou et al. [72]). The same isoform is involved in ATP-mediated mitogenic signalling in astrocytes, but in these cells PKCδ activation does not involve PLC but requires PLD-dependent choline formation (Neary et al. [73]).

In addition to PLC-coupled receptors, a growing number of P2Y receptors have been shown to affect the activity of AC. Besides the existence of indirect mechanisms linked to an increase in cAMP (discussed in Communi et al. [55]), only the $P2Y_{11}$ receptor is directly coupled to activation of AC and PLC while $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors negatively affect cAMP synthesis (Chambers et al. [13]; Communi et al. [10]; Hollopeter et al. [11]; Zhang et al. [12]). Adenylate cyclase-dependent signalling is often mediated by the cAMP-regulated kinase PKA. Stimulation of the $P2Y_{11}$ receptor with ATP is shown to activate human monocyte-derived dendritic cells by increased cAMP/PKA signalling (Wilkin et al. [74]). In bovine adrenocortical fasciculate cells, ADP and ATP increase cortisol production through PKA activation by an as yet unidentified G_s protein-coupled P2Y receptor (Nishi et al. [75]). Although in unstimulated cells the cytosolic cAMP concentration is already low, its further decrease by G_i protein-coupled receptors is sometimes sufficient to exert a significant inhibitory action towards PKA. Such a response is reported in microglial cells where ATP and ADP binding to $P2Y_{12/13}$ receptors mediate chemotaxis by PKA-dependent translocation of β1 integrins to ruffling regions of the cell (Nasu-Tada et al. [76]).

Despite the fact that cells express a myriad of different GPCRs and downstream acting regulators, receptor stimulation promotes rapid and specific responses. In addition, multiple GPCRs, sharing the same second messenger cascade, can induce different cellular events in one cell type indicating that GPCR signal propagation requires physical interactions in a defined cellular compartment. An example of spatial organised signalling is the β-arrestin-dependent targeting of an activated receptor into clathrin-coated vesicles or enrichment in membrane microdomains (lipid rafts) formed by cholesterol and sphingolipids (Anderson [77]; DeFea et al. [78]). Modulation of receptor function by rafts is confirmed for an increasing number of GPCRs including P2Y receptors (Anderson [77]; Ostrom and Insel [79]). In endothelial cells, it is reported that P2Y receptor-induced vasodilatation is abolished by disruption of caveolae with methyl-β-cyclodextrin (Kaiser et al. [80]). In C6 glioma cells, signalling by $P2Y_2$ and 5-hydroxytryptamine $(HT)_{2A}$ receptors is attenuated after knock-down of caveolin-1 by si-RNA. Moreover, interaction between the $5\text{-}HT_{2A}$ receptor and caveolin-1 facilitates its interaction with $G\alpha_q$. Since $P2Y_2$ receptor mRNA is downregulated by caveolin-1 knock-down, further studies are required to demonstrate localisation of $P2Y_2$ receptors in caveolae of C6 cells (Bhatnagar et al. [81]).

Small GTPases as molecular switches

The processing of extracellular stimuli by GPCRs often involves signalling by second messengers (cAMP, DAG, Ca^{2+}) towards small GTPases and/or cross-talk with tyrosine kinases. G_q protein-coupled receptor signalling via PLCβ induces formation of DAG and IP_3, Ca^{2+} mobilisation and activation of PKC ultimately leading to activation of proline-rich tyrosine kinase 2 (Pyk2). Pyk2 cooperates with Src to recruite Grb2 and SOS, a guanine nucleotide exchange factor (GEF) that activates Ras (Lev et al. [82]). Such a mechanism is reported for Ras-dependent ERK activation induced by the protease-activated receptor-1 in astrocytes (Wang and Reiser [83]). In PC12 cells, stimulation of the $P2Y_2$ receptor also triggers tyrosine phosphorylation of Pyk2, but further signalling to Ras involves EGFR transactivation by Src (Soltoff et al.

[84]). Tyrosine kinase-dependent Ras signalling is also reported for G_i protein-coupled receptors, but this proceeds through Gβγ subunit-mediated activation of PI 3-Kγ and Shc (Ellis et al. [85]; Lopez-Ilasaca et al. [86]).

GEFs can also be regulated in a tyrosine kinase-independent manner that proceeds through direct activation by cAMP, DAG and Ca^{2+} or by interaction with Gα subunits as observed for the $G_{q/11}$ protein-mediated activation of RhoA (Bhattacharya et al. [87]; Bos [88]; Lutz et al. [89]; Walker et al. [90]).

P2Y receptor signalling towards GTPases is involved in short term responses, such as stress fibre formation or modulation of cell adhesion, but also in long term responses like cell proliferation. Mitogenic Ras-dependent P2Y responses are reported for C6 and HEK293 cells where Ras is implicated in $P2Y_2$ receptor-dependent signalling to the ERK pathway (Gao et al. [91]; Tu et al. [44]). On the other hand, increased proliferation of C6 cells by the $P2Y_{12}$ receptor proceeds independently of Ras, but requires RhoA-dependent activation of ERK and Rho-associated coiled-coil-containing protein kinase (ROCK) (Grobben et al. [40]; Van Kolen and Slegers, unpublished data). Interestingly, when the $P2Y_{12}$ receptor is expressed in CHO cells it activates ERK and RhoA/ROCK by independent mechanisms (Soulet et al. [92]).

Another example of cross-talk between P2Y receptors and GTPases is observed in blood platelets. As mentioned above, release of ADP and subsequent $P2Y_1$ and $P2Y_{12}$ receptor binding is essential for collagen-induced platelet aggregation. A crucial step for immediate and sustained aggregation of platelets is the activation of Rap1 that increases the affinity between integrin $\alpha_{IIb}\beta_3$ and fibrinogen. Knock-out studies revealed that ADP-induced GTP loading of Rap1 proceeds through both G_i and G_q signalling by $P2Y_{12}$ and $P2Y_1$ receptors, respectively. The mechanism initiated by the $P2Y_{12}$ receptor is shown to be PI 3-K-dependent while $P2Y_1$-mediated activation of Rap1 requires Ca^{2+} mobilisation (Woulfe et al. [93]; Greco et al. [94]; Larson et al. [95]; Lova et al. [96, 97]). Stimulation of the $P2Y_1$ receptor also contributes to platelet shape changes by a Ca^{2+}-independent pathway. RhoA and its effector ROCK are activated by ADP through $G_{12/13}$ protein-dependent signalling of the $P2Y_1$ receptor and contribute to rapid actin polymerization and shape changes (Paul et al. [98]). Signalling towards Rho GTPases is also important in other systems. In brain, ATP and ADP induce membrane ruffling and chemotaxis of microglial cells through G_i protein-dependent activation of Rac upon stimulation of $P2Y_{12/13}$ receptors (Sasaki et al. [51]; Honda et al. [54]). Stress fibre formation in vascular smooth muscle cells is reported to be mediated by RhoA/ROCK signalling that becomes activated upon stimulation of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Sauzeau et al. [99]). In the latter study, information concerning the signalling towards RhoA is lacking. In a more recent study on endothelial cells, transactivation of VEGFR upon $P2Y_2$ receptor stimulation and recruitment of the RhoGEF Vav is shown to be a possible mechanism to initiate RhoA-mediated cell adhesion (Seye et al. [100]).

Although several P2Y receptors activate RhoA, downstream signalling and physiological consequences are determined by celltype specific mechanisms leading to diverse responses.

ERK signalling

Several GPCRs are coupled to enhanced proliferation by multiple signal transduction pathways that phosphorylate ERK. Activation of this kinase requires Ras or GTPases of the RhoA family and is often modulated by second messenger-activated pathways, although cross-talk with growth factor receptors also triggers ERK signalling.

In neurons G_s protein-mediated activation of AC increases ERK phosphorylation by a PKA/Rap1/B-Raf cascade. In contrast, induction of cAMP synthesis decreases ERK phosphorylation in C6 cells and astrocytes by a negative action of PKA on the Ras/c-Raf1 interaction, or by Rap1-mediated inhibition of c-Raf1. These observations led to the hypothesis that an increase in cAMP stimulates MEK/ERK signalling in B-Raf expressing cells but inhibits this cascade in B-Raf negative cells (Dugan et al. [101]). In both cases, PKA activation has a central role and mediates its effects through Src and Rap1 activation (Stork and Schmitt [102]). Although the majority of cAMP-dependent effects can be explained by this hypothesis, a few exceptions are reported. In some B-Raf positive cells, an increase in cAMP is shown to inhibit B-Raf, suggesting that regulation of this kinase by cAMP also depends on other cell type specific factors. One model suggests the involvement of 14-3-3 proteins acting as scaffolding proteins to shield B-Raf and Raf1 from PKA phosphorylation (Qiu et al. [103]). Other studies indicated that regulation of ERK by cAMP involves multiple cell type specific mechanisms. In COS cells overexpressing β-AR1 or β-AR2, stimulation of these receptors activate AC through a G_s protein-dependent mechanism as expected. However, PKA also phosphorylates these receptors and induces a switch from G_s to $G_{i/o}$ protein binding to β-AR resulting in

activation of ERK upon receptor stimulation (Martin et al. [104]). Modulation of the ERK cascade by cAMP can also occur independently of PKA. In this context, cAMP binds Epac1 or Epac2, "exchange protein directly activated by cAMP," GEFs that activate Rap1 and Rap2 (de Rooij et al. [105, 106]; Kawasaki et al. [107]). Several examples of G_s protein-mediated activation of ERK through Epac are reported (Laroche-Joubert et al. [108]; Lin et al. [109]). Another PKA-independent mechanism of ERK phosphorylation is the $G_s\beta\gamma$/Src-mediated activation of Ras (Schmitt and Stork [110]).

G_q and some $G_{i/o}$ protein-coupled receptors activate PLCβ and trigger formation of IP_3 and DAG, resulting in Ca^{2+} release and PKC activation, respectively. Ca^{2+} increase can activate ERK through Pyk2 that activates Ras as mentioned above. Otherwise, Ca^{2+}-dependent modulation of Ras activity is also mediated by Ras guanine nucleotide-releasing factor (RasGRF), a GEF that contains Ca^{2+}- and DAG-binding domains, (Ebinu et al. [111]) or by Ca^{2+}/calmodulin-dependent kinases CaMK-II and CaMK-IV (reviewed in Agell et al. [112] and Walker et al. [90]). Increase of intracellular calcium and DAG formation also results in activation of cPKCs while DAG formation alone is sufficient to activate nPKCs. Increase in PKC activity modulates the ERK cascade through Ras by inhibition of RasGAPs and/or stimulation of RasGEFs. In addition, PKC can activate Raf independently of Ras. Indeed, it is shown that PKCα phosphorylates Raf at Ser499 (Kolch et al. [113]). However, mutation of this serine residue into alanine does not affect Raf activity in response to phorbol esters (Yip-Schneider et al. [114]). More convincing data were obtained when constitutively active PKC was expressed in rat 6 fibroblasts. These cells display Ras-independent signalling towards ERK by direct phosphorylation of Raf by PKCε. Since activation of Ras is required in several systems this interaction is cell type-dependent (Cacace et al. [115]; Ueffing et al. [116]). Direct phosphorylation of Raf by PKC is also involved in ERK activation by the G_i protein-coupled leukotriene (LT)D_4 receptor in intestinal epithelial cells. Although stimulation of this receptor also triggers a parallel PKC-independent activation of Ras, transfection experiments confirmed that Ras is dispensable for LTD_4 receptor-mediated ERK activation (Paruchuri et al. [117]). When constitutive active point mutants of PKCα, PKCδ and PKCε were introduced in COS cells, only PKCδ activated the ERK cascade (Ueda et al. [118]), indicating that involvement of PKC isoforms in ERK signalling vary among different cell types. This is also confirmed by the observation that, in platelets, cPKCs are involved in thrombin-induced MEK and ERK activation independently of Ras or Raf (Nadal-Wollbold et al. [119]).

$G_{i/o}$ protein-coupled receptors that are not linked to PLC activation can also modulate mitogenic signalling through Gβγ-dependent activation of PI 3-Kγ. Signalling from PI 3-Kγ to ERK proceeds through Shc/Grb2/SOS/Ras (Lopez-Ilasaca et al. [86]). An increasing number of reports point to the involvement of PKCζ in G_i protein-dependent phosphorylation of ERK. The first observation was made in CHO cells where stimulation of the LPA receptor triggers MEK/ERK signalling via a PI 3-Kγ-dependent activation of PKCζ not abrogated by transfection with dominant negative Ras (Takeda et al. [120]). In addition, a recent report indicated that angiotensin II-induced ERK activation in rat vascular smooth muscle cells requires interaction between Ras and PKCζ (Zhao et al. [121]). PKCζ-dependent activation of ERK is mediated by interaction with MEK, a property shared by other PKC isoforms (Schönwasser et al. [122]), or by regulation of Raf1. Studies performed in rat embryonic hippocampal cells indicated that PKCζ can phosphorylate the Raf kinase inhibitory protein (RKIP) resulting in dissociation of the Raf1/RKIP complex (Corbit et al. [123]). In addition, co-immunoprecipitation experiments in COS cells showed that modulation of c-Raf1 by PKCζ is also regulated by 14-3-3 scaffolding proteins (Van Der Hoeven et al. [124]).

Initial studies concerning P2Y receptor-mediated activation of ERK were made in astrocytes where this cascade was shown to be involved in cell proliferation and process elongation (Neary and Zhu [125]; King et al. [126]).

Although ATP triggers pertussis toxin insensitive IP_3 and Ca^{2+} responses in astrocytes, these are not required for the signalling towards ERK which depends on rapid membrane translocation of PKCδ upon phosphatidylcholine hydrolysis by PLD (Neary et al. [73]). In PC12 cells, stimulation of the $P2Y_2$ receptor also induces PKCδ-dependent ERK phosphorylation, although this mechanism requires Ca^{2+} and Pyk2 for the association of Shc and Grb2 to the receptor and for subsequent activation of SOS/Ras/Raf/MEK/ERK (Soltoff et al. [84]). Many reports showed that P2Y receptor-mediated ERK signalling requires PKC activation (Graham et al. [127]; Huwiler and Pfeilschifter [128]; Erlinge [129]), but a PKC-independent mechanism is reported in thyroid FRTL-5 cells (Tornquist et al. [130]). In 1321N1 astrocytoma cells, stimulation of the $P2Y_6$ receptor with UDP activates PKCα, ε and ζ which are correlated with ERK phosphorylation (Kim et al. [131]). Although the use of general PKC inhibitors 3-[1-(dimethylaminopropyl)

indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride (GF109203X) and 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö 6976) diminished ERK signalling, the lack of specificity of these compounds makes it difficult to determine the contribution of each of these PKC isoforms in the mechanism of ERK activation (Way et al. [132]).

Enhanced proliferation by a P2Y receptor-mediated stimulation of the ERK pathway has been reported in a large number of cell types such as human mesangial cells, vascular smooth muscle cells and primary astrocytes (Huwiler and Pfeilschifter [128]; Harper et al. [133]). Transient ERK activation by $P2Y_1$, $P2Y_2$ or $P2Y_{12}$ receptor stimulation also increases cell proliferation in C6 cells indicating that activation of several P2Y receptor subtypes can converge into the same physiological response (Table 2) (Tu et al. [44]; Claes et al. [39]; Czajkowski et al. [46]).

In addition to mitogenesis, P2Y receptor signalling towards ERK elicits other physiological processes including cell survival, inflammation and reactive gliosis. In human lung microvascular endothelial cells, hyperoxia-induced release of ATP results in cell survival through ERK and PI 3-K signalling cascades activated by $P2Y_2$ and/or $P2Y_6$ receptors, while stimulation of the ERK cascade by the $P2Y_6$ receptor protects 1321N1 astrocytoma cells from TNFα-induced apoptosis (Ahmad et al. [134]; Kim et al. [131]). Rapid ERK1/2 and p38 MAPK activation plays an important role in $P2Y_2$ receptor-dependent primary granule release from human neutrophils (Meshki et al. [135]). A similar phenomenon is observed in articular chondrocytes where ATP acts as a pro-inflammatory mediator by increasing arachidonic acid production and release of prostaglandin E_2 through a $P2Y_2$ receptor-dependent activation of p38 and ERK1/2 (Berenbaum et al. [136]). In primary astrocytes, P2Y receptor-mediated ERK activation by ATP is shown to induce reactive astrogliosis, a phenomenon that occurs upon brain injury and is characterised by astroglial proliferation, cellular hypertrophy and up regulation of GFAP. This effect is mediated by an ERK-dependent increase in the expression of COX-2 (Brambilla et al. [53]). In C6 cells, $P2Y_{12}$ and $P2Y_2$ receptor-induced activation of ERK is coupled to an enhanced cell proliferation, while a negative modulation of GFAP synthesis by the $P2Y_{12}$ receptor is reported (Claes et al. [39]; Tu et al. [44]; Van Kolen and Slegers [45]). These differences are probably due to the fact that in C6 cells induction of GFAP expression is not correlated with an enhanced proliferation but requires growth arrest.

In summary, most P2Y receptors are coupled to ERK phosphorylation, but the signalling mechanism and the physiological effect of this pathway are cell type specific and are determined by the cellular context.

PI 3-K/PKB signalling

PKB/Akt is involved in a large variety of cellular processes including glucose metabolism, mitogenesis, differentiation, survival and motility (Brazil et al. [137]). This member of the AGC protein kinase superfamily is recruited to the plasmamembrane upon PI 3-K-mediated PIP3 formation, but is also controlled in a PI 3-K-independent, but calmodulin-dependent, fashion upon intracellular Ca^{2+} mobilisation by stimulation of neuronal NMDA receptors (Cantley [138]; Leevers et al. [139]; Woodgett [140]; Yano et al. [141]).

Modulation of PKB activity is reported for a variety of GPCR ligands including adrenergics, cannabinoids,

Table 2 G protein-dependent modulation of ERK and PKB signalling cascades in C6 cells.

	G protein	ERK	PI 3-K/PKB	Effect
$P2Y_1$	G_q	↑ PLC-PKC-Ca^{2+}-Ras.	↓ Attenuation of PI 3-K activated by growth factors	Proliferation
$P2Y_2$	G_q	↑ PLC-PKC-Ca^{2+}-Ras.	–	Proliferation
$P2Y_{12}$	G_i	↑ RhoA-PKC-Raf-MEK	↑ PI 3-K/PKB	Proliferation/inhibition of astrocytic differentiation
μOR	G_i	↑ FGF transactivation	–	Proliferation
β-AR	G_s	↓ Transient inhibition dependent on cAMP	↓ Transient inhibition by cAMP	Growth arrest/astrocytic differentiation
CB	G_s	↓ Sustained inhibition	↓ Sustained inhibition	Growth arrest/apoptosis

Transient ERK activation by $P2Y_1$ (Czajkowski et al. [46]), $P2Y_2$ (Tu et al. [44]), $P2Y_{12}$ (Grobben et al. [40]) and μ opioid receptors (μOR) (Belcheva et al. [198]) enhances cell proliferation while stimulation of the β-adrenergic receptor (β-AR) transiently inhibits ERK and PKB concomitant with induction of differentiation (Wang et al. [149]; Van Kolen and Slegers [45]). Inhibition of these pathways by cannabinoids (CB) is sustained and induces apoptosis (Ellert- Miklaszewska et al. [184]).

carbachol, glutamate, histamine, nucleotides and thrombin (Dickenson [142]; Franke et al. [143], Iacovelli et al. [144] Murga et al. [145]; Sanchez et al. [146]). Due to the existence of multiple phosphoinositide-dependent cascades, regulation of PKB signalling by GPCRs varies among the studied systems.

In HEK293 cells, stimulation of β-AR with (-)-isoproterenol activates PKB via $G_s\beta\gamma$, Src, Ras and PI 3-K (Schmitt and Stork [110]; Bommakanti et al. [147]) while activation of AC by $G\alpha_s$ exerts differential effects on PKB activity. In cells expressing Epac, cAMP activates PI 3-K/PKB via Rap1 while, in other cells, cAMP activates PKA that exerts a negative action on PI 3-K and PKB (Mei et al. [148]; Wang et al. [149]).

G_i protein-mediated activation of PKB can occur through the coupling of the Gβγ subunit to the catalytic subunit of PI 3-K or via growth factor receptor transactivation. Although only p110γ was initially reported to be activated by Gβγ subunits, this feature is also observed for the p110β isoform (Kurosu et al. [150]; Stoyanov et al. [151]). This mechanism is reported in Vero cells where stimulation with LPA activates Ras upon increase in p110β lipid kinase activity (Yart et al. [152]). G_i protein-mediated transactivation of growth factor receptors is reported in HaCaT, A-431, and HEK293 cells where stimulation of the angiotensin type I receptor by mechanical stress induces transactivation of EGFR leading to activation of the PI 3-K/PKB cascade and protection of these cells from apoptosis (Kippenberger et al. [153]).

In 1321N1 astrocytoma cells, PLCβ activation by the G_q protein-coupled muscarinic M_3 receptor also triggers PI 3-K activation through ErbB3 transactivation, but this mechanism requires Ca^{2+} mobilisation (Tang et al. [154]). In contrast, some reports showed an inhibitory pathway from G_q protein-coupled receptors towards PI 3-K by direct interaction between Gα-subunits released from heterotrimeric G proteins and p110α, as reported for the α_{1A}-AR in rat-1 fibroblasts (Ballou et al. [155, 156]), or by inhibition of insulin receptor substrate-1-associated PI 3-K activity in 1321N1 astrocytoma cells by carbachol, histamine or thrombin. These observations reveal opposing effects of muscarinic receptor stimulation on PI 3-K activity mediated by insulin and ErbB3 receptors in these cells (Batty et al. [157]).

Modulation of PI 3-K/PKB signalling is also reported for a few P2Y receptors. In bovine adventitial fibroblasts, ATP is shown to induce proliferation through parallel but independent ERK and PI 3-K signalling cascades that contribute to mTOR and p70S6K phosphorylation (Gerasimovskaya et al. [158]). In rat mesangial cells, stimulation of the $P2Y_2$ receptor with ATP or UTP activates PKB by a PDK-1-dependent mechanism while, in C6 cells, ADP activates PI 3-K/PKB by the G_i protein-coupled $P2Y_{12}$ receptor but inhibits PI 3-K by stimulation of the $G_q/G_{11/12}$ protein-coupled $P2Y_1$ receptor (Table 2) (Van Kolen and Slegers [45]; Czajkowski et al. [46]; Huwiler et al. [159]). Although most effects of P2Y-mediated activation of PI 3-K signalling are known to be related to cell proliferation, differentiation and survival, this signalling cascade is also involved in other processes. In this regard, it can be mentioned that $P2Y_{12}$ receptor-mediated PI 3-K/PKB activation modulates proliferation and differentiation of C6 cells, but also plays an important role in ADP-induced platelet aggregation (Van Kolen and Slegers [45]; Czajkowski et al. [46]; Chen et al. [160]; Kim et al. [161]).

P2Y receptor-integrated G protein-coupled receptor and receptor tyrosine kinase signalling cascades

G protein-coupled receptor cross-talk

Complementary to vertical downstream signalling upon GPCR stimulation, these receptors also mediate lateral signalling by cross-talk with other receptors (reviewed in Cordeaux and Hill [162]). In human platelets, it was reported that $P2Y_{12}$ receptor activation potentiates $P2Y_1$ receptor-mediated Ca^{2+} signalling, while the $P2Y_1$ receptor negatively regulates this action (Hardy et al. [163]). In renal mesangial cells, P2Y receptors activated by ATP and UTP induce a rapid desensitisation of the sphingosine-1-phosphate (S1P) receptor by PKC-dependent phosphorylation (Xin et al. [164]). A more complex interplay is observed between P2Y receptors and 5-HT receptor subtypes. Studies performed in CHO cells stably expressing $5\text{-}HT_{1A}$ receptors revealed that the responsiveness of this receptor is reduced by a PLD/PKC-dependent phosphorylation upon short (<5 min) pre-treatment with ATP, while the agonist efficacy of the overexpressed $5\text{-}HT_{1B}$ receptor is not altered. Alternatively, longer treatment with ATP alone attenuates $5\text{-}HT_{1B}$ signalling by a mechanism that requires activation of phospholipase A_2 (PLA_2) (Berg et al. [165]). Furthermore, stimulation of P2Y receptors can also modulate the release of transmitter molecules, including dopamine, glutamate and serotonin (Bezzi and Voltera [52]; Krugel et al. [166]; Nedergaard et al. [167]). A recently discovered mechanism of GPCR cross-talk is the assembly of a heteromeric receptor complex displaying the pharmacological profile of one receptor and the signalling properties of the other. Such an interaction is reported in

HEK293 cells overexpressing A_1 and $P2Y_1$ receptors. The heteromeric A_1-$P2Y_1$ receptor complex inhibits AC through $G_{i/o}$ protein, but displays $P2Y_1$ receptor-like pharmacological properties (Yoshioka et al. [168]).

P2Y receptor-mediated transactivation

Many studies reveal that GPCRs and growth factor receptors share a number of signalling modules (e.g., Raf/MEK/ERK, PI 3-K/PDK/PKB) to transduce their effects. In the past decade, it has become clear that the signalling pathways of both receptor systems are interconnected. Stimulation of a GPCR can induce a rapid tyrosine phosphorylation of RTKs. This transactivation mechanism is reported for many GPCRs and proceeds through the Gβγ subunit-dependent activation of Src. Src in turn activates RTKs by phosphorylation of specific tyrosines located in their intracellular domains or induction of matrix metalloproteases-dependent release of growth factor receptor ligands, e.g., release of heparin-bound EGF (Luttrell and Luttrell [169]).

Another target for signal integration of GPCRs and RTKs are docking proteins. Although these proteins contain phospho-tyrosine binding domains that interact with phosphorylated tyrosine residues of RTKs, stimulation of GPCRs can induce growth factor receptor-independent phosphorylation of docking proteins by Src (Bisotto and Fixman [170]).

In addition to GPCR-dependent phosphorylation of RTKs, the opposite activation mechanism is also reported. Binding of PDGF to its cognate receptor induces association of PDGFR with the G_i protein-coupled S1P receptor. Subsequently, Src is recruited to this complex by Gβγ subunits and phosphorylates Grb-2 associated binder-1 resulting in dynamin II-induced "pinching off" of vesicles involved in endocytosis of PDGF-S1P signalling complexes and subsequent activation of ERK1/2 (Waters et al. [171]).

Cross-talk between RTKs and P2Y receptors is reported in Müller glial cells where ATP exerts its mitogenic effect through transactivation of EGF and PDGF receptors resulting in ERK-dependent enhanced proliferation. In these cells, ATP-induced activation of ERK was abolished by treatment with the RTK autophosphorylation inhibitor tyrphostin (AG1478) (Milenkovic et al. [172]). In rat striatal astrocytes, ATP and bFGF activate ERK and induce astrogliosis by a mechanism that is insensitive to RTK inhibition (Abbracchio et al. [173]; Bolego et al. [174]; Neary et al. [175]). More recently, mechanistic studies performed in 1321N1 astrocytoma cells reveal that the human $P2Y_2$ receptor interacts with Src and Pyk2, probably by its proline-rich putative SH3 binding sites (PXXP). This interaction is implicated in $P2Y_2$ receptor-induced transactivation of EGF, PDGF and VEGF receptors (Liu et al. [176]; Seye et al. [100]). Src inhibition abolishes growth factor receptor transactivation and ERK phosphorylation. Although the rat $P2Y_2$ receptor lacks PXXP motives, tyrosine kinase-dependent activation of ERK upon $P2Y_2$ receptor stimulation is reported in a few rat cell lines, including C6 and PC12 cells (Soltoff et al. [84]; Tu et al. [44]). In the latter cases, $P2Y_2$ receptor-dependent activation of Pyk2 is mediated by PKC and Ca^{2+} suggesting that the PXXP sequence is dispensable for $P2Y_2$ receptor-induced tyrosine phosphorylation of Pyk2 and downstream signalling towards ERK. Moreover, $P2Y_2$ mutants lacking PXXP-motives are still able to activate ERK demonstrating the existence of other pathways towards phosphorylation of ERK (Liu et al. [176]). Observations made in human endothelial cells, where UTP-induced signalling to ERK was shown to depend on Ca^{2+}, PKC and integrin-mediated cell anchorage, already pointed to a pathway distinct from the classical Ras/Raf/MEK/ERK cascade (Short et al. [177]). Human and mouse $P2Y_2$ receptors contain a RGD sequence which allows activation of ERK by interaction with $\alpha_V\beta_3/\beta_5$ integrins followed by G_o protein coupling. Since these proteins also mediate cell adhesion and chemotaxis, the observed $P2Y_2/\alpha_V\beta_3/\beta_5$-interaction also points to a possible function of $P2Y_2$ receptors in inflammatory responses (Erb et al. [178]).

It is clear that, in analogy with other GPCRs, cross-talk between P2Y and growth factor receptors may occur at different levels of the signal transduction pathway depending on receptor subtypes and on the studied system. For the $P2Y_2$ receptor, additional transactivation mechanisms are facilitated by the presence of signalling motives (e.g., PXXP or RGD) that allow direct interaction with other signalling components (Src, integrins).

P2Y receptor-activated signal transduction pathways in C6 glioma cells

As mentioned above, the final outcome of nucleotide-mediated signalling is influenced by ecto-enzymes (Claes and Slegers [17]; Czajkowski and Baranska [18]; Grobben et al. [21, 179]). ATP and ADP hydrolysis to adenosine results in growth inhibition by a mechanism that is not yet fully understood. When nucleotide hydrolysis is prevented, ATP, ADP and Ap_nA (in particular Ap_3A and Ap_4A) increase cell

proliferation more than two-fold. Stimulation with 2MeSADP, a P2Y agonist not hydrolysed by the ecto-enzymes present on the plasma membrane of C6 cells, also results in growth enhancement and inhibition of β-AR-induced differentiation into astrocyte type II (Claes et al. [39]; Van Kolen and Slegers [45]). The pathways involved in the P2Y receptor-dependent effects on growth and differentiation of these cells are presented in Figure 1.

Nucleotides stimulate several purinergic receptors that activate the ERK cascade by at least two distinct mechanisms. The $P2Y_2$ receptor, stimulated by UTP and ATP, enhances ERK phosphorylation through a PLCβ/PKC/Ras/Raf/MEK cascade that is attenuated by inhibition of tyrosine kinases and Ca^{2+} chelation by BAPTA-AM (Tu et al. [44]). The Ca^{2+}-dependence of the $P2Y_2$ receptor-mediated activation of ERK suggests the involvement of a cPKC (α, βI, βII or γ). It is also shown that ADP stimulates the $P2Y_1$ receptor and activates ERK through a Ca^{2+}-dependent mechanism (Czajkowski et al. [46]), likely by a similar mechanism as reported for the $P2Y_2$ receptor (Tu et al. [44]). In addition, it has been shown that ADP can activate ERK by stimulation of the $P2Y_{12}$ receptor through a RhoA- and PKC-dependent pathway that does not require Ca^{2+}, Ras or tyrosine kinase activation (Grobben et al. [40]). The fact that Ca^{2+} removal does not affect $P2Y_{12}$ receptor-mediated ERK activation excludes the involvement of cPKCs. Stimulation of the $P2Y_{12}$ receptor does not induce PI-turnover, but nPKCs might be involved since alternative activation mechanisms, based on Ser/Thr and Tyr phosphorylation, have been reported (Steinberg [180]; Parekh et al. [181]). Data from our laboratory suggest an important role for PKCζ in $P2Y_{12}$ receptor-dependent activation of ERK. The fact that no cross-talk between ERK and PI 3-K is observed in C6 cells indicates that PKCζ exerts its actions independently of PI 3-K via a RhoA-

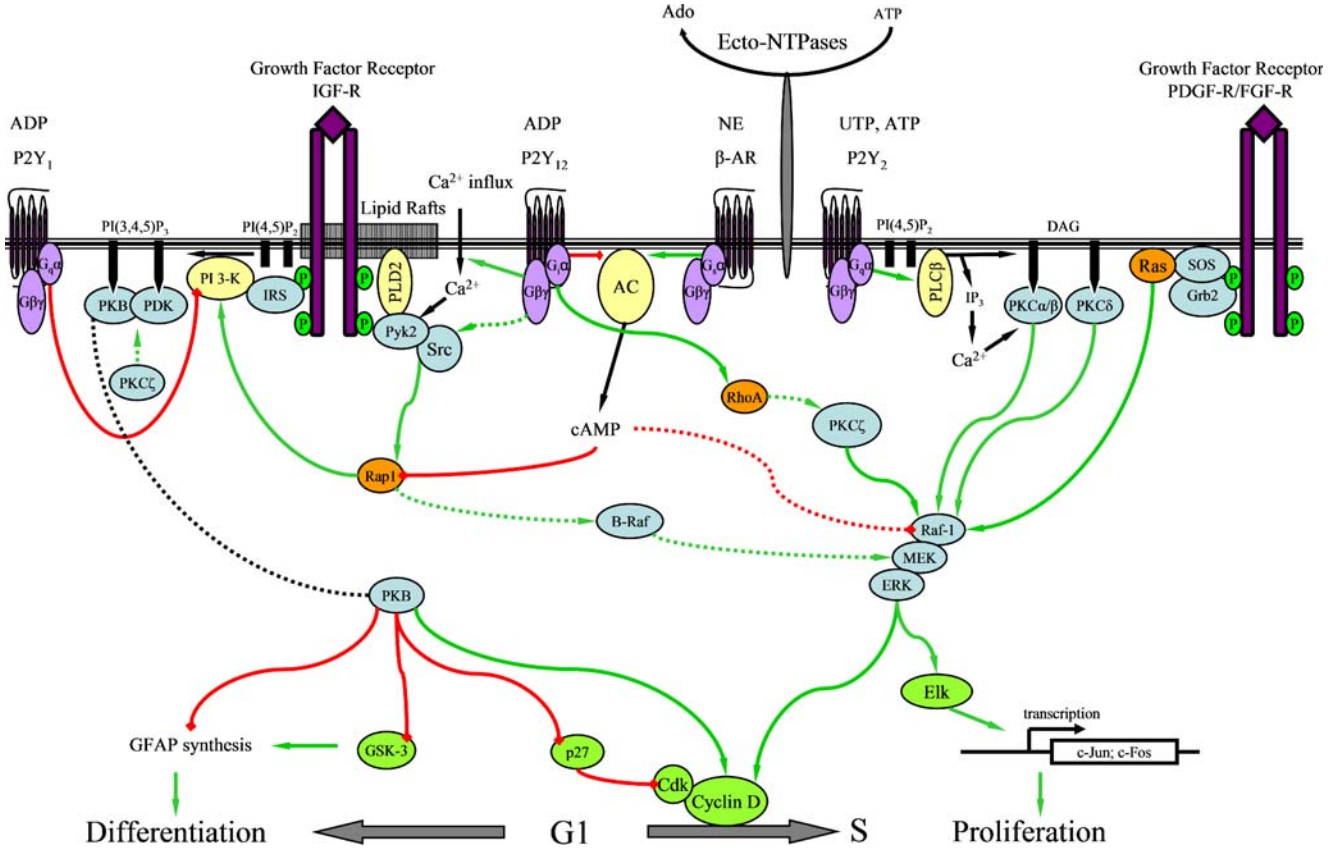

Figure 1 Overview of P2Y receptor-mediated signalling cascades in C6 cells. *Green* and *red lines* represent stimulatory (*green arrows*) and inhibitory (*red squares*) actions respectively. Dashed lines are incomplete characterised pathways. $P2Y_2$ receptor stimulation enhances ERK-dependent proliferation through a PLC-dependent pathway while $P2Y_{12}$ receptor stimulation enhances cell proliferation by RhoA- and PKCζ-dependent activation of ERK (Claes et al. [39]; Grobben et al. [40]; Tu et al. [44]; Van Kolen and Slegers, [199]). $P2Y_{12}$ receptor stimulation also inhibits cAMP-dependent induction of differentiation by reactivation of PKB which requires Src/Pyk2 complex formation and Rap1 activation. Formation of the Src/Pyk2 complex requires Ca^{2+} and PLD2 which is constitutively active (Claes et al. [22]; Van Kolen and Slegers [45]; Van Kolen et al. [185]). Cyclic AMP-dependent inhibition of PKB and ERK is suggested to depend on inhibition of Rap1 (Wang et al. [149]). The negative modulation of PI 3-K by the $P2Y_1$ receptor is only displayed in the presence of serum (Czajkowski et al. [46])

dependent mechanism (Grobben et al. [40]; Van Kolen and Slegers, [199]). Although P2Y receptors use different mechanisms to activate ERK, they all converge to increased cell proliferation by enhanced synthesis of c-Myc, c-Jun and c-Fos (Zhang et al. [182]). Progression through the G1/S phase of the cell cycle is due to a decreased expression of $p27^{Kip}$ and increased expression of cyclinD.

While stimulation of ERK signalling by P2Y receptors has been known for several years, the coupling with PI 3-K activation was discovered more recently. When C6 cells are grown in the presence of serum, $P2Y_1$ receptor signalling predominates and is shown to inhibit PI 3-K (Czajkowski et al. [46]). Upon serum deprivation, $P2Y_1$ receptor expression decreases while $P2Y_{12}$ becomes the main ADP-stimulated receptor that enhances the activity of PI 3-K by a G_i protein-dependent mechanism. These observations demonstrate that, in addition to autocrine growth factor receptor signalling, the constitutive PI 3-K activity in C6 cells is modulated by $P2Y_1$ and $P2Y_{12}$ receptor expression. Another cross-talk at the level of PI 3-K/PKB is observed for $P2Y_{12}$ and β-AR. Increase in cAMP upon stimulation of the latter receptor transiently inhibits PKB phosphorylation. Stimulation of the $P2Y_{12}$ receptor, which negatively affects AC, does not only counteract this inhibition but even enhances PKB activity in comparison to unstimulated cells, suggesting that $P2Y_{12}$ receptor-mediated PI 3-K/PKB activation is not only due to its inhibitory effect on AC (Van Kolen and Slegers [45]; Czajkowski et al. [46]; Baranska et al. [183]). In addition to their opposing effects on PI 3-K/PKB signalling, unpublished data of our laboratory revealed similar modulation of ERK signalling by $P2Y_{12}$ and β-AR. Whether the $P2Y_{12}$ receptor-mediated reversal of ERK inhibition is involved in the inhibition of β-AR-induced GFAP synthesis remains to be determined. The observation that stimulation of the cells with UTP activates ERK, but fails to inhibit the β-AR-induced growth arrest and GFAP synthesis, suggests that ERK activation alone is not sufficient to counteract differentiation (Claes et al. [39]; Tu et al. [44]). Conversely, transfection of C6 cells with constutively active PKB prevented (-)-isoproterenol-induced differentiation indicating that inhibition of PKB signalling is required for cAMP-dependent induction of differentiation. Apparently this observation is in contrast with data showing that cAMP-dependent induction of differentiation requires PI 3-K activity which is not inhibited upon a 48-h treatment with dbcAMP (Roymans et al. [34]). This might be explained by the fact that induction of differentiation by stimulation of β-AR proceeds through transient inhibition of PKB while recovery of this activity is required to prohibit cell death. This hypothesis is confirmed by a recent study where sustained inhibition of PI 3-K/PKB by cannabinoids is shown to induce apoptosis in C6 cells (Table 2) (Ellert-Miklaszewska et al. [184]). Taken together, $P2Y_{12}$ receptor stimulation inhibits cAMP-dependent induction of differentiation by a transient increase in PI 3-K/PKB activity. Ca^{2+} chelation inhibits the basal PKB activity and $P2Y_{12}$ receptor-mediated increase in PKB phosphorylation. Although C6 cells also express the $P2Y_2$ receptor, stimulation with UTP does not enhance the activity of PI 3-K/PKB, which may be explained by a differential coupling to G protein subtypes. $P2Y_2$ receptor-mediated signalling proceeds through G_q proteins while the activation of PDK is G_i protein-dependent (Table 2) (Tu et al. [44]; Huwiler et al. [159]). The lack of G_i protein coupling of the $P2Y_2$ receptor in C6 cells might be a consequence of compartimentalisation into caveolae as reported for some G_q protein-coupled receptors (Bhatnagar et al. [81]).

Although experiments in CHO cells reveal that $P2Y_{12}$ receptor-induced ERK activation requires PI 3-Kγ (Soulet et al. [92]), experiments performed with LY294002 or Wortmannin excluded cross-talk between both cascades in C6 cells (Grobben et al. [40]). These differences in signalling mechanisms can be explained by the fact that the latter PI 3-K-isoform is only moderately expressed in C6 cells (Van Kolen and Slegers [45]). The exact mechanism of $P2Y_{12}$ receptor-induced PI 3-K/PKB activation is not fully understood, but recent data revealed that Src and Pyk2 are involved in $P2Y_{12}$ receptor signalling to PI 3-K (Van Kolen et al., [185]). A similar pathway is observed in PC12 cells where Src, in complex with Pyk2 and PLD2, activates PI 3-K in response to H_2O_2 (Banno et al., [186]). Since PLD2 is constitutively active in C6 cells (Bobeszko et al. [187]), a significant role for this enzyme in PI 3-K/Akt signalling is suggested. Although Soulet et al. [92] reported that transactivation of PDGFR is involved in PI 3-K activation by the $P2Y_{12}$ receptor in CHO cells, the use of receptor kinase inhibitors indicated that PDGFR and EGFR are not transactivated by the $P2Y_{12}$ receptor in C6 cells. Alternatively, a Rap1-mediated activation of PI 3-K by the $P2Y_{12}$ receptor cannot be excluded. Indeed, PI 3-K is postulated as a downstream effector of Rap1 that is inhibited by an increase in cAMP concentration (Wang et al. [149]). Data from our laboratory indicated a rapid $P2Y_{12}$ receptor-induced activation of Rap1 that was abolished by Ca^{2+} chelation and inhibition of Src/Pyk2 complex formation but not by PI 3-K inhibition (Van Kolen et al. [185]). These results positioned Rap1 downstream of Src/Pyk2 but

upstream of PI 3-K. In addition, this mechanism involves Gβγ protein subunits and Ca^{2+}-dependent activation of Pyk2 that requires association to IGF-IR and PLD2 to interact with Src. Although Src and Pyk2 are shown to activate Ras/Raf/MEK/ERK in primary astrocytes (Wang and Reiser [83]), this mechanism did not contribute to $P2Y_{12}$ receptor-mediated ERK activation in C6 cells pointing to a physical separation of both cascades (Grobben et al. [40]; Van Kolen and Slegers, [199]). Indeed, the formation of a Pyk2/Src/PLD2/IGFI-R complex may contribute to compartmentalisation of this signalling pathway that requires intact lipid rafts to be active (Van Kolen et al. [185]). In contrast, in blood platelets Rap1, but also Pyk2 activation by the $P2Y_{12}$ receptor, depends on PI 3-K activity but is insensitive to Ca^{2+} chelation (Lova et al. [96, 97]; Koziak et al. [188]). These findings indicate that different cell specific pathways are involved in $P2Y_{12}$ receptor-mediated activation of PI 3-K/PKB and additional research is required to allow full characterisation of these signalling cascades.

Conclusions

At present, nucleotides are known to regulate a variety of biological processes related to vascular-, immunological- and intestinal functioning. In vitro studies on glial and neuronal cells implicated the P2Y receptor-activated signalling pathways in regulation of cell motility, proliferation, chemotaxis and protection against oxidative stress. Furthermore, investigations on tumoral cells demonstrated that stimulation of P2Y receptors contribute to tumorigenesis by increasing cell proliferation through ERK and PKB signalling pathways activated by independent mechanisms. From these observations, a role of these receptors as potential targets in clinical applications emerges.

P2Y receptors modulate these physiological functions by activation of GTPases and direct or indirect activation of protein kinases. Characterisation of the involved receptor(s) and elucidation of P2Y receptor-induced activation of defined pathways needs to be improved by synthesis of specific P2Y agonists and antagonists.

Studies on P2Y receptor-mediated signalling, discussed in this review, demonstrate that besides vertical signal transduction, lateral cross-talk between growth factor receptors and GPCRs extends the signalling properties of a defined receptor subset. It also becomes clear that signal transduction pathways activated by P2Y receptors largely depend on the cell type and their environment. On the one hand, cellular specificity is determined by differential expression of signalling proteins, but on the other hand also depends on the assembly of signalling modules. Besides specific protein-protein interactions, intracellular compartmentalisation (e.g., lipid rafts, clathrin-coated vesicles) also contributes to the specificity of receptor signalling. Identification of the signalling modules and cellular compartmentalisation will provide more insight into the P2Y receptor-activated signalling cascades.

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