0026-895X/06/6901-338–345$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 69:338–345, 2006

Vol. 69, No. 1
14654/3073295
Printed in U.S.A.

Identification of a Potent Inverse Agonist at a Constitutively Active Mutant of Human $P2Y_{12}$ Receptor

Zhongren Ding, Soochong Kim, and Satya P. Kunapuli

Departments of Physiology (Z.D., S.K., S.P.K.) and Pharmacology (S.P.K.) and the Sol Sherry Thrombosis Research Center (Z.D., S.P.K.), Temple University School of Medicine, Philadelphia, Pennsylvania

Received May 11, 2005; accepted October 18, 2005

ABSTRACT

Human platelets express two P2Y receptors: G_q-coupled $P2Y_1$, and G_i-coupled $P2Y_{12}$. Both $P2Y_1$ and $P2Y_{12}$ are ADP receptors on human platelets and are essential for ADP-induced platelet aggregation that plays pivotal roles in thrombosis and hemostasis. Numerous constitutively active G protein-coupled receptors have been described in natural or recombinant systems, but in the P2Y receptors, to date, no constitutive activity has been reported. In our effort to identify G protein coupling domains of the human platelet ADP receptor, we constructed a chimeric hemagglutinin-tagged human $P2Y_{12}$ receptor with its C terminus replaced by the corresponding part of human $P2Y_1$ receptor and stably expressed it in Chinese hamster ovary-K1 cells. It is interesting that the chimeric $P2Y_{12}$ mutant exhibited a high level of constitutive activity, as evidenced by decreased cAMP levels in the absence of agonists. The constitutive activation of the chimeric $P2Y_{12}$ mutant was dramatically inhibited by pertussis toxin, a G_i inhibitor. The constitutively active $P2Y_{12}$ mutant retained normal responses to 2-methylthio-ADP, with an EC_{50} of 0.15 ± 0.04 nM. The constitutively active $P2Y_{12}$ mutant caused Akt phosphorylation that was abolished by the addition of pertussis toxin. Pharmacological evaluation of several $P2Y_{12}$ antagonists revealed (*E*)-*N*-[1-[7-(hexylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]-pyrimidin-3-yl]-1,5,6-trideoxy-β-D-ribo-hept-5-enofuranuronoyl]-L-aspartic acid (AR-C78511) as a potent $P2Y_{12}$ inverse agonist and 5′-adenylic acid, *N*-[2-(methylthio)ethyl]-2-[(3,3,3-trifluoropropyl)thio]-, monoanhydride with (dichloromethylene)bis[phosphonic acid] (AR-C69931MX) as a neutral antagonist. In conclusion, this is the first report of a cell line stably expressing a constitutively active mutant of human platelet $P2Y_{12}$ receptor and the identification of potent inverse agonist.

Extracellular nucleotides influence many biological functions, including vascular tone, cell division, cardiac and skeletal muscle contraction, platelet aggregation, and peripheral and central neurotransmission (Burnstock, 2004). ATP and ADP are released from several sources in the body, including purinergic nerve endings, platelets, chromaffin cells, and endothelial cells (Gordon, 1986). Extracellular nucleotides can trigger intracellular effects by specifically binding to and activating cell-surface membrane proteins known as P2 receptors (Dubyak and Cowen, 1990; Burnstock, 2004). Two main families of receptors for extracellular nucleotides have been described: P2X receptors, which are ligand-gated ion channels, and P2Y receptors, which belong to the superfamily of G protein-coupled receptors (GPCRs) (Burnstock, 2004). Eight distinct P2Y receptors are expressed in human tissues: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (Abbracchio et al., 2003; Burnstock, 2004). Pharmacologically, P2Y receptors can be subdivided into five G_q-coupled subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) and three G_i-coupled subtypes ($P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$). In addition, $P2Y_{11}$ receptor can also couple to G_s to activate adenylyl cyclase, and $P2Y_2$ can activate the G_i-dependent pathway (Meshki et al., 2004).

Of the P2Y receptors, G_q-coupled $P2Y_1$ and G_i-coupled $P2Y_{12}$ are found in human platelets and are the receptors of ADP, which plays an important role in platelet activation and therefore in hemostasis and thrombosis (Leon et al.,

This work was supported by Research grants HL60683 and HL80444 from the National Institutes of Health (to S.P.K.).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.014654.

ABBREVIATIONS: GPCR, G protein-coupled receptor; 2-MeSADP, 2-methylthio-ADP; PTX, pertussis toxin; IBMX, 3-isobutyl-1-methylxanthine: HA, hemagglutinin; CHO, Chinese hamster ovary; Ab, antibody; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20; BSA, bovine serum albumin; PI, propidium iodide; CS-747, prasugrel; AR-C69931MX, 5′-adenylic acid, *N*-[2-(methylthio)ethyl]-2-[(3,3,3-trifluoropropyl)thio]-, monoanhydride with (dichloromethylene)bis[phosphonic acid]; AR-C66096, 2-propylthio-D-β,γ-difluoromethylene ATP, trisodium salt; AR-C67085, 2-propylthio-β,γ-dichloromethylene-d-ATP; AR-C78511, (*E*)-*N*-[1-[7-hexylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]-pyrimidin-3-yl]-1,5,6-trideoxy-β-D-ribo-hept-5-enofuranuronoyl]-L-aspartic acid; AR-C69581, 5′-*O*-[{[[dichloro(phosphono)methyl](hydroxy)phosphoryl]oxy}(hydroxy)phosphoryl]-*N*-phenyl-2-(propylthio)adenosine.

1999, 2004; Hollopeter et al., 2001; Zhang et al., 2001). Costimulation of both P2Y$_1$ and P2Y$_{12}$ is essential for ADP-induced platelet aggregation and thromboxane generation (Jin and Kunapuli, 1998; Jin et al., 2002). The P2Y$_{12}$ receptor generally potentiates other agonist-induced platelet functional responses, including dense granule release (Storey et al., 2000; Dangelmaier et al., 2001). In addition, downstream signaling events from the P2Y$_{12}$ receptor are essential for Akt activation by other agonists in platelets (Kim et al., 2004). Of the two P2Y receptors found in human platelets, P2Y$_1$ receptor is ubiquitously expressed in tissues, whereas P2Y$_{12}$ receptor is almost exclusively found in human platelets and brain glioma cells and is most extensively studied in platelets (Zhang et al., 2001). The P2Y$_{12}$ receptor plays a central role in platelet activation (Dorsam and Kunapuli, 2004) and therefore attracts tremendous interest from pharmaceutical companies to develop the P2Y$_{12}$ antagonist as potential antithrombotic agents (Kunapuli et al., 2003). Clopidogrel and ticlopidine (Yoneda et al., 2004) are the two thienopyridine compounds widely used as antithrombotic drugs that target platelet P2Y$_{12}$ receptor, with clopidogrel exhibiting greater overall benefits than aspirin in the prevention and treatment of thrombotic events. CS-747 is another more potent thienopyridine antithrombotic agent targeting the platelet P2Y$_{12}$ receptor that exerts its role via hepatic metabolism which is currently under clinical trial (Sugidachi et al., 2000, 2001; Niitsu et al., 2005). The AR-C compounds are another series of P2Y$_{12}$ receptor antagonists that directly block the platelet P2Y$_{12}$ receptor (Jin et al., 2001; Vasiljev et al., 2003).

As members of the GPCR superfamily, both P2Y$_1$ and P2Y$_{12}$ share the common overall structure feature of GPCRs. Both of these receptors are encoded on chromosome 3, suggesting gene duplication. Furthermore, the P2Y$_1$ and P2Y$_{12}$ receptors have identical agonist profiles: both ADP and 2-MeSADP are agonists. Considerable efforts have been made to locate the ligand binding domain in the extracellular region and the G protein-coupling domain in the intracellular region in attempts to identify targeting sites on the platelet ADP receptors for novel antithrombotic drug development (Jiang et al., 1997; Hoffmann et al., 1999; Ding et al., 2003, 2005). Clopidogrel, for example, targets the extracellular cysteines of the P2Y$_{12}$ receptor (Savi et al., 2001). In addition, several small-molecule antagonists at the P2Y$_{12}$ receptor such as AR-C69931MX have been developed as potential antithrombotic drugs (Huang et al., 2000; Jacobsson et al., 2002).

In the past decade, the discovery of constitutive activity of GPCRs has provided a significant contribution to our understanding of receptor activation and drug action at molecular levels. Numerous constitutively active GPCRs have been described in natural or recombinant systems, and some GPCRs with constitutive activity have been reported to be disease-causing. According to the two-state model, GPCRs exist in a balance between two functionally and conformationally different states: an inactive state (R), and an active state (R*) capable of activating G proteins in the absence of ligands. The basal level of receptor activity is determined by the proportion of the R* state. The classic agonists have a high affinity for R* and shift the balance to the R* state, resulting in an increase of G protein activity, whereas the inverse agonists have a high affinity for R and shift the balance to R, leading to the decrease of G protein activity. Neutral competitive antagonists bind both R and R* equally and do not displace the balance but can competitively antagonize the effects of both agonists and inverse agonists. Some mutations of the GPCR can also shift the balance to the R* state, increasing G protein activity in the absence of agonists and leading to the constitutive activation of GPCRs.

In our attempt to identify the G_q-coupling domain of the human P2Y$_1$ receptor, we found that the C terminus of the P2Y$_1$ receptor is essential for G_q coupling and further identified two arginine residues essential for G_q activation (Ding et al., 2005). To further study the role of the P2Y$_1$ receptor C terminus, we introduced human P2Y$_1$ receptor C terminus into human P2Y$_{12}$ receptor to explore whether the P2Y$_1$ receptor C terminus is sufficient for G_q coupling and therefore confer P2Y$_{12}$ receptor with G_q-coupling ability. In this study, we report the constitutive activity of the chimeric P2Y$_{12}$ receptor with the P2Y$_1$ carboxyl terminus and the characterization of inverse and neutral antagonists at this receptor. To our knowledge, this is the first report of P2Y receptors with constitutive activity. The establishment of a cell line stably expressing a constitutively active mutant of P2Y$_{12}$ receptor may provide a useful tool for exploring the inverse agonist activity of other P2Y$_{12}$ antagonists.

Materials and Methods

Materials. All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). FITC-labeled monoclonal antibody (HA.11) against the hemagglutinin epitope (HA-tag) was purchased from Covance Research Products (Berkeley, CA). 2-MeSADP, 2′,3′-*O*-(4-benzoylbenzoyl)-ATP, forskolin, pertussis toxin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). AR-C69931MX, AR-C66096, AR-C67085, AR-C69581, and AR-C78511 were gifts from AstraZeneca Pharmaceuticals LP (Loughborough, UK). [^{3}H]Adenine was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Anti-Akt and anti-phospho-Akt (Ser473) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Alkaline phosphatase-labeled secondary antibody was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). CDP-Star chemiluminescent substrates were purchased from Applied Biosystems (Foster City, CA). All other reagents were reagent-grade, and deionized water was used throughout.

Construction of Human P2Y$_{12}$ Wild-Type and P2Y$_{12}$/P2Y$_1$ Chimera Plasmids. Human platelet P2Y$_{12}$ receptor (GenBank accession number AF313449) (Hollopeter et al., 2001) was cloned into pcDNA3.1/Hygro(+) with an HA tag (YPYDVPDYA) inserted at the beginning of the translation initiation by polymerase chain reaction (PCR). Forward primer containing KpnI restriction site and HA-tag sequence is 5′-GCGCGGTACCACCATGTACCCATACGATGTTCC-AGATTACGCTCAAGCCGTCGACAATCTC-3′. Human P2Y$_{12}$/P2Y$_1$ chimera was constructed by overlap-extension PCR with the C terminus of the human platelet P2Y$_{12}$ receptor replaced by that from the human platelet P2Y$_1$ receptor (GenBank accession number U42029) as described previously (Ding et al., 2005).

Cell Culture. Chinese hamster ovary (CHO-K1) cells were grown in Ham's F-12 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin, and amphotericin B at 37°C with 5% CO_2. CHO-K1 cells stably expressing P2Y$_{12}$ wild-type or P2Y$_{12}$/P2Y$_1$ chimeric receptors were grown in the same medium supplemented with 400 μg/ml hygromycin or 500 μg/ml G418, respectively.

Stable Expression of Human P2Y$_{12}$ Wild-Type and P2Y$_{12}$/P2Y$_1$ Chimera Receptor in CHO-K1 Cells. The expression construct for the wild-type P2Y$_{12}$ receptor or P2Y$_{12}$/P2Y$_{12}$ chimera (1

μg) was used to transfect CHO-K1 cells using Lipofectamine as described previously (Akbar et al., 1996). The growth medium was replaced after 6 h with fresh medium. Stable transfectants were selected on medium containing 400 μg/ml hygromycin or 500 μg/ml G418 and screened for the expression of wild-type or chimeric $P2Y_{12}$ receptor by HA-tag detection via flow cytometry.

HA-Tag Detection by Flow Cytometry. CHO-K1 cells (naive, vector-transfected, or stably transfected with wild-type or chimeric $P2Y_{12}$ receptors) were cultured in 100-mm dishes, washed twice with PBS (137 mM NaCl, 2.68 mM KCl, 4.29 mM Na_2HPO_4, and 1.47 mM KH_2PO_4), and detached with Versene (0.5 mM Na_4EDTA, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 15 mM KH_2PO_4, and 1 mM glucose). After spinning at 700 rpm for 3 min, the pellets were resuspended in Tyrode's solution (137 mM NaCl, 2.67 mM KCl, 2 mM $MgCl_2$, 2.03 mM NaH_2PO_4, 5.6 mM glucose, 10 mM HEPES, and 0.2% bovine serum albumin, pH 7.4), and cell concentrations were adjusted to 10^7 cells/ml. Aliquots of 100-μl cell suspension were mixed with 4 μl of 1:10 diluted FITC-labeled monoclonal antibody against HA (Covance) in the presence of 2 mM Ca^{2+}. After incubation at 4°C for 1 h in the dark, cell suspensions were briefly spun, and the supernatant was discarded. Cells were resuspended in 400 μl of Tyrode's solution and analyzed by flow cytometry using FACScan (BD Biosciences, San Jose, CA). Untransfected CHO-K1 cells or vector-transfected cells were used as negative controls.

cAMP Assay. Intracellular cAMP assays were conducted by a modification of a protocol described previously (Ding et al., 2003). In brief, cells were cultured in six-well plates and labeled with 2 μl/ml [^{3}H]adenine (74 kBq/ml) overnight at 37°C. The radiolabeling medium was replaced by fresh growth medium containing 0.5 mM IBMX and incubated for 10 min at 37°C. In the presence of 20 μM forskolin, various concentrations of agonist and antagonist were added and incubated at 37°C for 10 min unless otherwise indicated. The reactions were terminated by the addition of 1 ml of stop solution containing 5% trichloroacetic acid, 1 mM ATP, and 1 mM cAMP. cAMP levels were determined, and cAMP conversion from ATP was calculated as described by Berlot (1999) using the following formula: cAMP conversion from ATP = [^{3}H]cAMP/([^{3}H]ATP + [^{3}H]cAMP) $\times$ 10^3.

Measurement of Phosphorylation of Akt. Phosphorylation of Akt in lysates from CHO-K1 cells stably expressing $hP2Y_{12}$ was estimated by immunoblotting using phospho-Akt (Ser473) antibody (1:1000 dilution) (Cell Signaling) as described previously, with some modification (Kim et al., 2004). Cells grown in six-well plates were stimulated with 2-MeSADP (1 μM) for 5 min at 37°C, and the reaction was stopped by washing with ice-cold PBS and the addition of 250 μl of cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. In some experiments, cells were incubated overnight with 200 ng/ml pertussis toxin, a G_i inhibitor. Samples were boiled for 5 min, and proteins were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Nonspecific binding sites were blocked by incubation in Tris-buffered saline/Tween 20 [TBST; 20 mM Tris, 140 mM NaCl, and 0.1% (v/v) Tween 20] containing 0.5% (w/v) milk protein and 3% (w/v) bovine serum albumin (BSA) for 30 min at room temperature, and membranes were incubated overnight at 4°C with primary antibody (1:1000 in TBST and 2% BSA) with gentle agitation. After three washes for 5 min each with TBST, the membranes were probed with alkaline phosphatase-labeled goat anti-rabbit IgG (1:5000 in TBST and 2% BSA) for 1 h at room temperature. After additional washing steps, membranes were then incubated with a CDP-Star chemiluminescent substrates for 10 min at room temperature, and immunoreactivity was detected using Fujifilm Luminescent Image Analyzer (model LAS-1000 CH; Fujifilm, Tokyo, Japan).

Results

Construction and Stable Expression of Wild-Type and Chimeric Human $P2Y_{12}$ Receptor in CHO-K1 Cells. A chimeric human $P2Y_{12}$ mutant with its C terminus replaced by the corresponding human $P2Y_1$ receptor C-tail (Ding et al., 2005) was constructed by overlapping PCR and subsequently cloned into pcDNA3 (outlined in Fig. 1). The nucleotide sequence encoding the $P2Y_{12}/P2Y_1$ chimera in the expression plasmid was confirmed by DNA sequence analysis, and the construct was transfected into CHO-K1 cells. After culturing in the presence of 500 μg/ml G418 for 2 weeks, stable clones were screened by flow cytometry analysis for expression of the HA-tagged receptor. Of 50 clones screened, one of the high-expression clones in terms of fluorescence of HA-tagged receptor (5-A6) was chosen for further study and designated $P2Y_{12}/P2Y_1$. Likewise, the wild-type $P2Y_{12}$ receptor in pcDNA3.1/Hygro(+) was stably transfected into CHO-K1 cells, and one high-expression clone (6-E11) was chosen after screening more than 60 stable clones resistant to 400 μg/ml hygromycin and designated $P2Y_{12}$WT. As shown in Fig. 2, both wild-type and chimeric $P2Y_{12}$ receptors were successfully expressed in CHO-K1 cells with comparable levels. We also evaluated the HA-tag expression and compared it with cells without receptor expression using counterstaining with propidium iodide. The data are shown in Fig. 2 (C–E). The number in each quadrant represents the percentage of cells stained with PI(+)/FITC-Ab(+), PI(+)/FITC-Ab(−), PI(−)/FITC-Ab(−), and PI(−)/FITC-Ab(+). This result indicates that both wild-type and chimeric receptor transfected cell lines have increased HA-tag expressions (81 versus 3% and 74 versus 3%, respectively) compared with CHO-K1 cells alone. We admit that there is a high proportion of dead cells detected by PI staining, but our data clearly indicate that the antibody only binds HA-tag-expressing cells, and cell status (live or dead) does not affect antibody binding.

Functional Characteristics of the Chimeric $P2Y_{12}/P2Y_1$ Receptor. We have shown previously that the C ter-

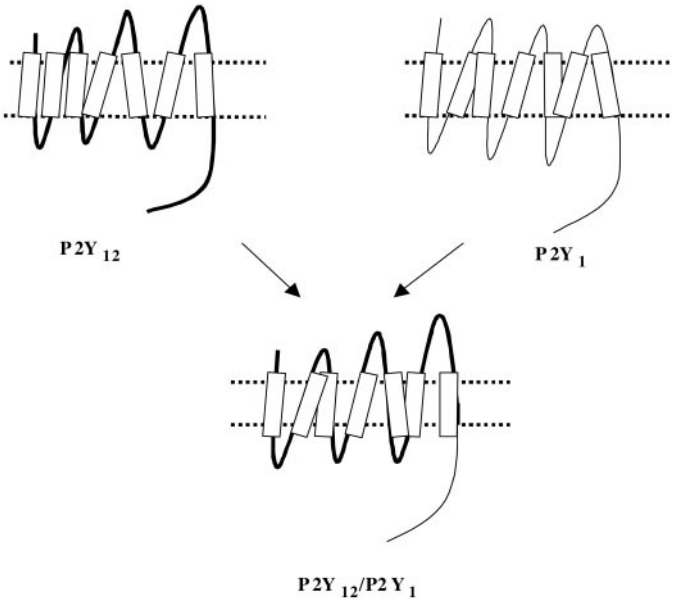

Fig. 1. Schematic representation of the construction of the $P2Y_{12}/P2Y_1$ chimeric receptor.

minus of the human $P2Y_1$ receptor is essential for G_q coupling (Ding et al., 2005). We also demonstrated that this $P2Y_1$ receptor C terminus, when introduced into $P2Y_{12}$ receptor, failed to activate G_q upon agonist stimulation although the chimeric $P2Y_{12}$ receptor expressed very well on the surface of CHO-K1 cells (Ding et al., 2005). To address whether the chimeric $P2Y_{12}$ receptor still functions and activates G_i upon agonist stimulation, we evaluated 2-MeSADP-induced inhibition of adenylyl cyclase in chimeric $P2Y_{12}$-expressing cells. We found that 2-MeSADP dose-dependently decreased cAMP levels in chimeric $P2Y_{12}$-expressing cells stimulated with forskolin with an EC_{50} of 0.15 ± 0.04 nM (Fig. 3A), which is nearly equal to that of wild-type $P2Y_{12}$-expressing cells (EC_{50} = 0.14 ± 0.03 nM) (Ding et al., 2003).

When forskolin-stimulated cAMP levels of the chimeric $P2Y_{12}$ receptor-expressing cells in the absence of $P2Y_{12}$ agonists were compared with those of the wild-type $P2Y_{12}$-expressing cells, we observed that the cAMP levels were markedly decreased in the chimeric $P2Y_{12}$ receptor-expressing cells compared with cells expressing the wild-type $P2Y_{12}$ receptor (Fig. 3B), suggesting that the chimeric $P2Y_{12}$ receptor is constitutively activated. The cAMP levels in chimeric $P2Y_{12}$ receptor-expressing cells in the absence of $P2Y_{12}$ agonists were approximately 28% of that in the wild-type $P2Y_{12}$-expressing cells, which is close to the maximal response induced by ADP or 2-MeSADP in wild-type $P2Y_{12}$-expressing cells (Ding et al., 2003). To confirm this constitutive activation of the G_i pathways, we used pertussis toxin (PTX), a G_i inhibitor. cAMP levels in the chimeric $P2Y_{12}$ receptor-expressing cells were dramatically increased upon inhibition of G_i with PTX (Fig. 3B). However, PTX did not affect the cAMP levels in the cells expressing the wild-type $P2Y_{12}$ receptor (Fig. 3B). These results further confirmed that the chimeric $P2Y_{12}$ receptor constitutively activated the G_i pathways.

Constitutive Activation of Akt in CHO-K1 Cells Stably Expressing Chimeric Human $P2Y_{12}$ Receptor. Serine-threonine kinase Akt has been established as an important downstream signal molecule of G_i pathway in platelets (Kim et al., 2004). Therefore, we evaluated whether this signaling molecule downstream of G_i pathways is activated in the cells expressing the constitutively active $P2Y_{12}$ receptor. We found that Akt is constitutively phosphorylated in the chimeric $P2Y_{12}$ mutant expressing cells in the absence of an agonist and that this phosphorylation is further enhanced by 2-MeSADP stimulation (Fig. 4). Similar to the effects on cAMP levels, this constitutive phosphorylation is PTX-sensitive and can be inhibited by PTX pretreatment (Fig. 4). This further confirmed that the chimeric $P2Y_{12}$ receptor is consti-

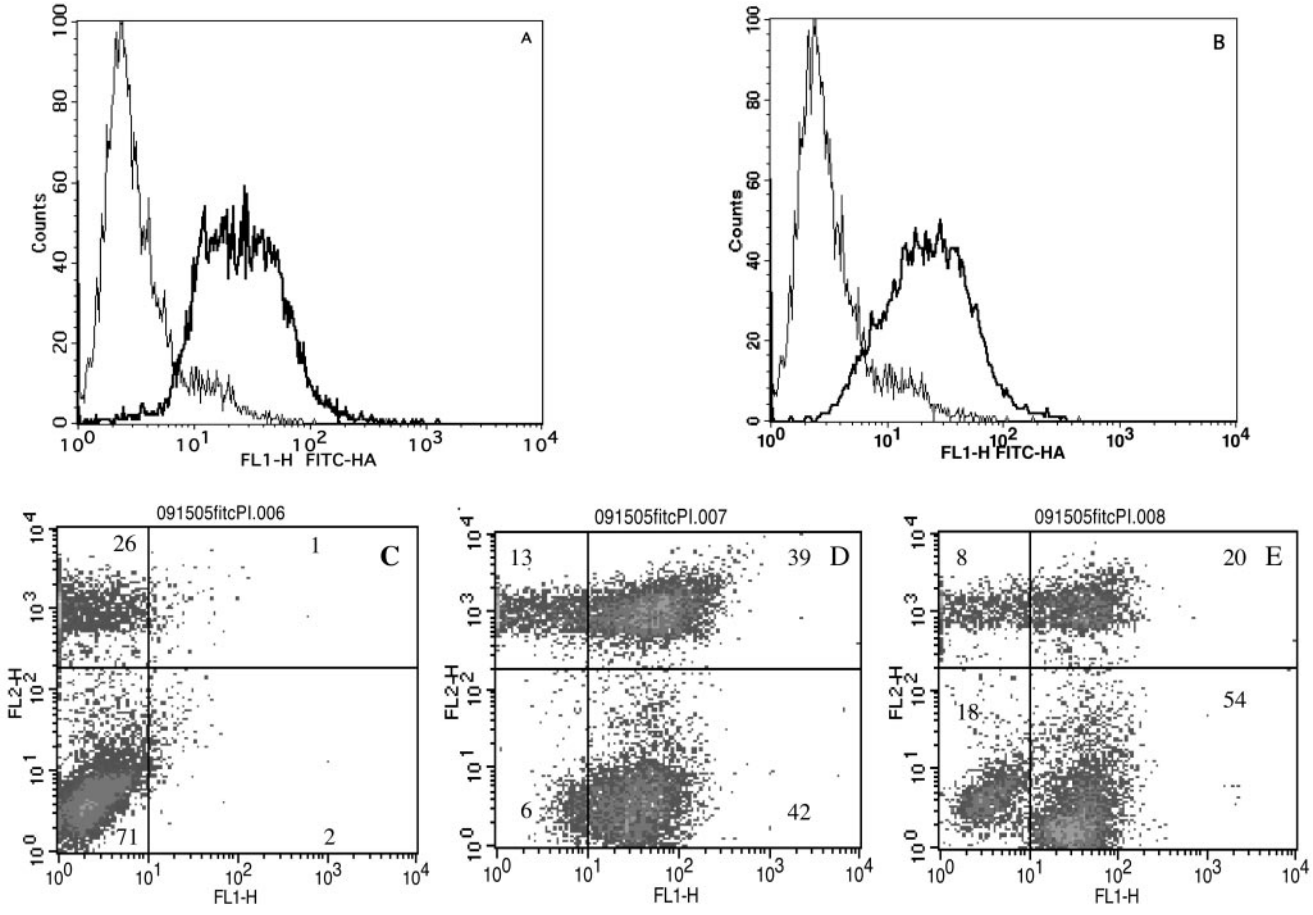

Fig. 2. Flow cytometry analysis of HA-tagged $P2Y_{12}$ receptor on the surface of CHO-K1 cells. A, thin line, CHO-K1 cells; thick line, $P2Y_{12}$WT. B, thin line, CHO-K1 cells; thick line, $P2Y_{12}$/$P2Y_1$. The diagram shows representative results for at least two experiments as described under *Materials and Methods*. Bivariate plots (propidium iodide versus antibody-associated binding) of CHO-K1, P2Y12WT, and P2Y12/P2Y1 cells are shown in C through E. Propidium iodide 0.625 μg/ml was added 5 min before scanning. The number in each quadrant represents the percentage of cells stained with PI(−)/FITC-Ab(−) (bottom left quadrant), PI(−)/FITC-Ab(+) (bottom right quadrant), PI (+)/FITC-Ab(+) (top right quadrant), and PI (+)/FITC-Ab(−) (top left quadrant).

tutively activated and stimulates G_i pathways in the absence of an agonist.

Pharmacological Characterization of the Constitutively Active $P2Y_{12}$ Receptor. AR-C78511 is a selective $P2Y_{12}$ receptor antagonist (Jin et al., 2001; Vasiljev et al., 2003) and the most potent of the 2-alkylthio-substituted ATP analogs (AR-C compounds) developed by AstraZeneca targeting $P2Y_{12}$ receptor (Vasiljev et al., 2003). In this study, we found that AR-C78511 dose-dependently increased the basal cAMP levels of the $P2Y_{12}/P2Y_1$-expressing cells with an IC_{50} of 17.4 ± 4.9 nM (Fig. 5A). This is consistent with the reported pIC_{50} value of AR-C78511 to reverse 2-MeSADP- or ADP-induced response in rat and human platelets and in C6-2B cells (Jin et al., 2001; Vasiljev et al., 2003). The maximal increase of intracellular cAMP over control (Fig. 5, A and C) and the IC_{50} of 17.4 nM indicate that AR-C78511 is a potent inverse agonist on the $P2Y_{12}$ receptor.

AR-C69931MX is a $P2Y_{12}$ antagonist, which has entered into phase II clinical trial and was recently halted in development as an antiplatelet drug by AstraZeneca because of poor oral availability and the lack of commercial potential as an injectable antiplatelet drug (Ingall et al., 1999; Huang et al., 2000; Jacobsson et al., 2002; Collins and Hollidge, 2003). We found that AR-C69931MX has no effects on the constitutive activity of the chimeric $P2Y_{12}$ up to 300 nM (Fig. 5A). The efficacy of AR-C69931MX as a $P2Y_{12}$ receptor antagonist was confirmed by its effect antagonizing ADP-induced adenylyl cyclase inhibition in $P2Y_{12}$ wild-type receptor-expressing cells (Fig. 5B). Thus, we conclude that AR-C69931MX is a neutral $P2Y_{12}$ antagonist.

As shown in Fig. 5A, AR-C69931MX at 300 nM did not demonstrate inverse agonistic activity on the constitutively active $P2Y_{12}$ mutant. However, at this concentration AR-C69931MX nearly completely reversed the increased cAMP level induced by 100 nM AR-C78511 (Fig. 5C). This result further confirmed that AR-C69931MX is a pure antagonist, whereas AR-C78511 is an inverse agonist on human $P2Y_{12}$ receptor.

AR-C66096, AR-C-67085, and AR-C69581 are other AR-C compounds developed by AstraZeneca as potential antithrombotic drugs targeting platelet $P2Y_{12}$ receptor (Humphries et al., 1994; Daniel et al., 1998; Ingall et al., 1999; Vasiljev et al., 2003). AR-C66096 has a pK_B value of 7.6 on B10 cells (cAMP) (Simon et al., 2001) and a pK_B value of 8.66 on ADP-induced human platelet aggregation (Humphries et al., 1994). In this study, we found that at 3 μM, which is more than 300-fold greater than that needed to completely inhibit ADP-induced human platelet aggregation (Daniel et al., 1998), AR-C66096 only partially increased the

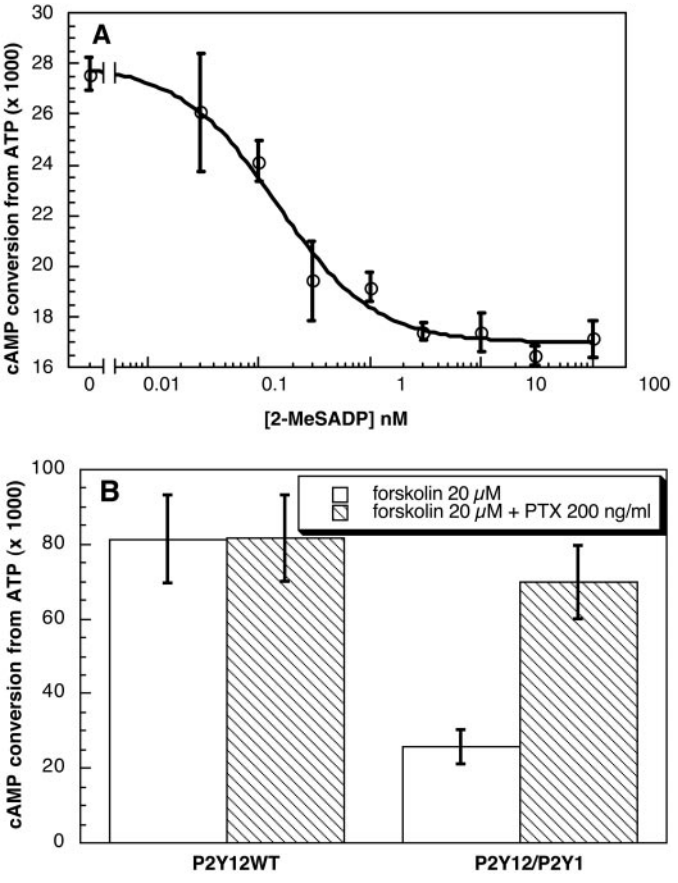

Fig. 3. Functional characteristics of the chimeric $P2Y_{12}/P2Y_1$ receptor. A, 2-MeSADP dose-dependently activates human $P2Y_{12}/P2Y_1$ stably expressed in CHO-K1 cells. 2-MeSADP-induced inhibition of adenylyl cyclase stimulated with 20 μM forskolin in CHO-K1 cells stably expressing human $P2Y_{12}/P2Y_1$. Data were expressed as mean ± S.E.M. representing at least three separate experiments. B, chimeric human $P2Y_{12}/P2Y_1$ is activated in the absence of agonists, whereas pertussis toxin inhibits its constitutive activity. CHO-K1 cells stably expressing $P2Y_{12}$WT or $P2Y_{12}/P2Y_1$ were pretreated with or without pertussis toxin overnight before forskolin stimulation for 10 min. Data were expressed as mean ± S.E.M. representing at least three separate experiments.

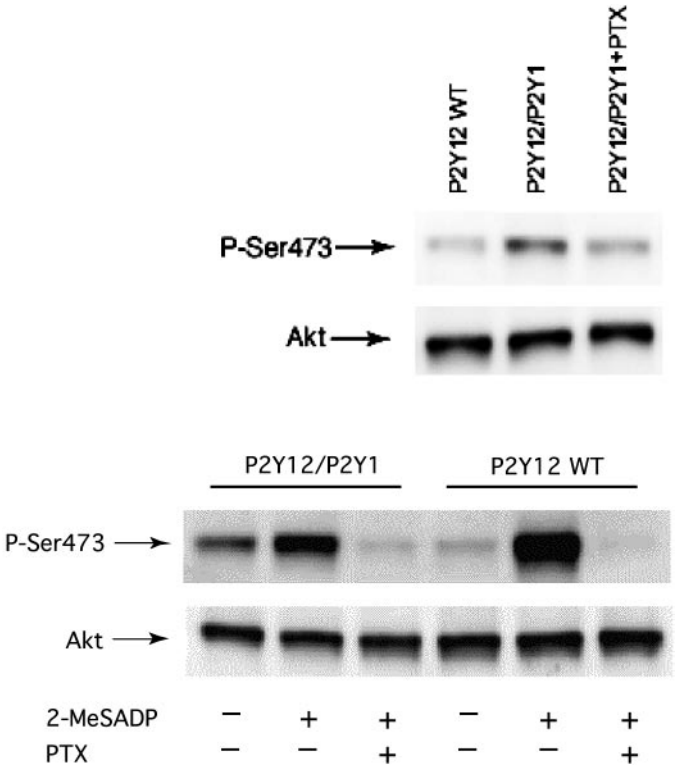

Fig. 4. Constitutively activated phosphorylation of Akt in $P2Y_{12}/P2Y_1$-expressing cells. The constitutively activated phosphorylation is PTX-sensitive (top) and is further enhanced by 2-MeSADP stimulation (bottom). Cells grown in six-well plates were incubated overnight in the absence or presence of PTX 200 ng/ml and then were stimulated with 2-MeSADP (1 μM) for 5 min at 37°C. The reaction was stopped by washing with ice-cold PBS and the addition of 250 μl of ice-cold lysis buffer. Akt phosphorylation was estimated by immunoblotting using phospho-Akt (Ser473) antibody (1:1000 dilution) (Cell Signaling).

decreased cAMP levels by the constitutively active mutant of human P2Y$_{12}$ receptor and thus only weakly inhibited the constitutive activity of P2Y$_{12}$ mutant (Table 1). Therefore, in contrast to AR-C78511 and AR-C69931MX, AR-C66096 is a partial inverse agonist on P2Y$_{12}$ receptor. Likewise, we found that AR-C67085 and AR-C69581 are also partial inverse agonists at the constitutively active human P2Y$_{12}$ receptor. AR-C67085 was reported to reverse 2-MeSADP-induced P2Y$_{12}$ receptor activation on human platelet and rat brain

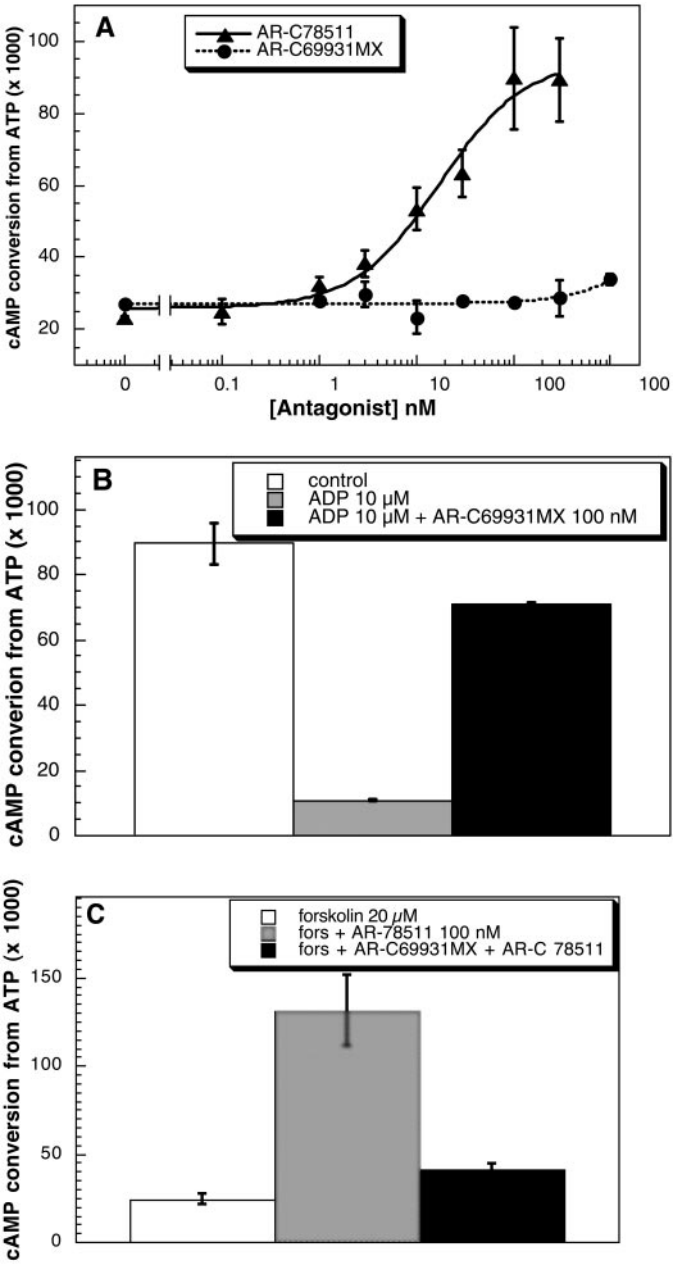

Fig. 5. Effects of AR-C compounds on forskolin-stimulated cAMP levels in cells expressing the constitutive active mutant of human P2Y$_{12}$ receptor. A, cells were stimulated with varying concentrations of AR-C78511 or AR-C69931MX for 10 min at 37°C. B, cells were stimulated with 10 μM ADP in the absence (▨) or presence (■) of 100 nM AR-C69931MX for 10 min at 37°C. C, cells were treated with AR-C78511 in the absence (▨) or presence (■) of 300 nM AR-C69931MX for 10 min at 37°C. cAMP levels were assayed as described under *Materials and Methods*. Data were normalized to the response obtained in the presence of 20 μM forskolin alone taken as 100% and expressed as mean ± S.E.M. representing at least three separate experiments.

with a pIC$_{50}$ value of 6.7 to 8.6 (Vasiljev et al., 2003). In agreement with this, we found that AR-C67085 dose-dependently reversed ADP-induced adenylyl cyclase inhibition in wild-type P2Y$_{12}$-expressing CHO-K1 cells with an IC$_{50}$ value of 66 ± 8 nM. When the inverse agonistic activity of AR-C67085 was evaluated, we found that in the range of 0.1 to 3000 nM, AR-C67085 dose-dependently increased cAMP levels in CHO-K1 cells stably expressing the constitutively activated human P2Y$_{12}$ chimeric receptor with a IC$_{50}$ value of 20.3 ± 8.1 nM. Compared with its efficacy antagonizing ADP-induced adenylyl cyclase inhibition on wild-type P2Y$_{12}$ (data not shown) and the potent efficacy of AR-C78511 as an inverse agonist, AR-C67085 demonstrated partial inverse agonist activity on constitutively activated human P2Y$_{12}$ mutant (Table 1).

We have compared the relative abilities of the AR-C compounds in a separate assay evaluating the phosphorylation of Akt downstream of the P2Y$_{12}$ receptor. As shown in Fig. 6, AR-C78511 dramatically inhibited the Akt phosphorylation caused by the constitutively active P2Y$_{12}$ receptor. Other AR-C compounds have exhibited a smaller extent of inhibition. These data compare well with cAMP levels as readout (Table 1).

Vasiljev et al. (2003) found that AR-C69581 has a pIC$_{50}$ value of 5.7 ± 0.1 in reversing 2-MeSADP-stimulated 5′-*O*-(3-[^{35}S]thio)triphosphate binding to human platelet membrane. Consistent with this pIC$_{50}$ value, in this study, we found that in the range of 100 nM through 3 μM, AR-C69581 concentration-dependently reversed ADP-induced adenylyl cyclase inhibition in CHO-K1 cells stably expressing P2Y$_{12}$ wild-type receptor. Compared with its dramatic effects in antagonizing ADP-induced P2Y$_{12}$ activation at 1 and 3 μM, AR-C69581 exhibited only weak inverse agonistic activity toward the constitutively active mutant of P2Y$_{12}$ receptor at 3 μM (Table 1).

2′,3′-*O*-(4-Benzoylbenzoyl)-ATP, an ADP receptor antagonist that antagonizes P2Y$_{12}$ receptor with a IC$_{50}$ value of 116 ± 23 μM (Ding et al., 2003), concentration-dependently increased the cAMP level of the constitutively activated P2Y$_{12}$ mutant in the range of 100 to 3000 μM, thus exhibiting its inverse agonistic activity at high concentration (data not shown).

Discussion

From a traditional point of view, GPCRs are activated upon agonist binding to its receptors; however, this concept

TABLE 1
Comparison of the activities of AR-C compounds on constitutively activated P2Y$_{12}$ chimera stably expressed in CHO-K1 cells
cAMP assays were performed in the presence of 20 μM forskolin as described under *Materials and Methods*. Inhibition of phosphorylated Akt (pAkt) was compared with control through densitometric analysis of the Western blots. Data are expressed as mean ± S.E.M. representing at least three separate experiments.

	cAMP Conversion from ATP (×10^3)	Inhibition of pAkt
		%
Control	26.7 ± 2.9	0
AR-C78511 100 nM	88 ± 12	76 ± 9
AR-C69931MX 300 nM	27.8 ± 4	9.7 ± 2.4
AR-C66096 3 μM	40 ± 6	44 ± 5
AR-C67085 1 μM	48 ± 6	24 ± 4
AR-C69581 3 μM	44 ± 4	17 ± 4

has changed in the past decade. Numerous investigators have reported that GPCRs can be activated in the absence of agonists (i.e., constitutively activated). Constitutive activation can be induced by receptor overexpression or receptor mutation, both of which have been reported to be the causes of a variety of human diseases (Parma et al., 1993; Parfitt et al., 1996; Pearce et al., 1996; Smits et al., 2003; Montanelli et al., 2004), such as familial syndrome of hypocalcemia with hypercalciuria hyperfunctioning (Pearce et al., 1996), thyroid adenomas (Parma et al., 1993), etc. To treat these diseases, the classic GPCRs antagonists that antagonize agonist binding to the receptors are ineffective, whereas the inverse agonists are believed to have advantages (Lefkowitz, 2004). Although numerous constitutively active GPCRs have been described in the past decade, no constitutive activation was reported in the P2Y subfamily, including human platelet ADP receptors $P2Y_1$ and $P2Y_{12}$.

We observed that replacement of the C terminus of human $P2Y_{12}$ receptor with the corresponding part of human $P2Y_1$ receptor confers the mutated $P2Y_{12}$ high constitutive activity when stably expressed in CHO-K1 cells. At an expression level similar to that of the wild-type $P2Y_{12}$ receptor, the basal level of the chimeric receptor activity is 3.6-fold higher than that of the wild-type receptor, as evaluated by adenylyl cyclase inhibition. Compared with other recombinant constitutively active receptor systems, for example, the constitutive activity of cholecystokinin type 2 receptor mutants, which are 3 to 17% of the agonist-induced maximal response in the wild-type receptor (Beinborn et al., 2004), the constitutive activity of the chimeric $P2Y_{12}$ receptor is 88% of the agonist-induced maximal activity in the corresponding wild-type receptor. Moreover, eliminating the activation of G_i protein through the addition of PTX abolished the constitutive activity of $P2Y_{12}/P2Y_1$, demonstrated by the fact that the basal cAMP level in the absence of $P2Y_{12}$ receptor agonists was restored nearly to the level of wild type. All of these results clearly indicate that the chimeric $P2Y_{12}$ receptor is constitutively activated. Furthermore, Akt, a downstream signal molecule of G_i pathway, is also constitutively phosphorylated in the chimeric $P2Y_{12}$ receptor-expressing cells, which was abolished in the presence of PTX, providing further evidence that the chimeric $P2Y_{12}$ receptor is constitutively activated.

Inverse agonists are believed to bear advantages over pure antagonists to treat diseases caused by constitutive activation of GPCRs. Although there are no clinical data indicating that an inverse agonist demonstrates superior clinical efficacy over the pure antagonists, data from numerous in vitro (Dupre et al., 2004; Mahe et al., 2004; Tryoen-Toth et al., 2004; Vermeulen et al., 2004; Vertongen et al., 2004) and some in vivo studies (Bond et al., 1995; Adan and Kas, 2003; Schwartz et al., 2003) have demonstrated the potential therapeutic advantage of inverse agonists. Many clinically important medicines have been demonstrated to behave as inverse agonists when tested against either wild-type or mutated GPCRs (Milligan, 2003); we believe, to some extent, that this evidence highlights the potential advantage of inverse agonists over neutral antagonists.

Inverse agonism is very common among GPCR antagonists (Kenakin, 2004). At the α_{1A} adrenergic receptor, 5-hydroxytryptamine-2A receptor, and histamine H1 receptor, the majority of the known antagonists are actually inverse agonists (Rossier et al., 1999; Bakker et al., 2001; Weiner et al., 2001). Considering the therapeutic implication, it is suggested that all new antagonists should be routinely tested for their potential inverse agonistic activity in future drug development programs (Behan and Chalmers, 2001; Chalmers and Behan, 2002; Seifert and Wenzel-Seifert, 2002). Using the cell line expressing high constitutive activity of the human $P2Y_{12}$ mutant, we further explored the inverse agonist activities of a series of $P2Y_{12}$ receptor antagonists, including the AR-C compounds that were developed as potential antithrombotic drugs by AstraZeneca. Of the five AR-C compounds screened, AR-C78511 exhibits a potent full inverse agonist activity, whereas AR-C69931MX is a pure $P2Y_{12}$ antagonist in the range of 0.1 to 300 nM. AR-C69931MX completely antagonized the inverse agonist activity of AR-C78511, further confirming that AR-C78511 is an inverse agonist, whereas AR-C69931MX is a pure antagonist.

The present results offer new perspectives on the functionality of the human $P2Y_{12}$ receptor and on the pharmacological properties of a selective $P2Y_{12}$ antagonist. Despite numerous disease-causing constitutively active mutations described in GPCRs, no constitutively active mutation leading to the induction of thrombotic diseases has been reported to date. The identification of a constitutively active $P2Y_{12}$ mutation in this study raised the possibility that there may be unidentified constitutively active mutation of $P2Y_{12}$ receptor underlying some thrombotic disorders with unknown causes.

In conclusion, this is the first report of the constitutive activity of the human $P2Y_{12}$ receptor. The establishment of a cell line stably expressing the constitutively active human $P2Y_{12}$ receptor provides a very useful tool for studying the inverse agonist activity of $P2Y_{12}$ receptor antagonists. Using the cell line, we successfully identified a $P2Y_{12}$ receptor antagonist with potent inverse agonist activity that is believed to have advantages over neutral $P2Y_{12}$ receptor antagonists against thrombotic diseases.

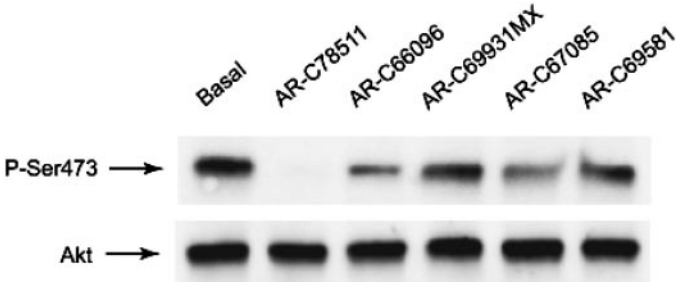

Fig. 6. Effects of AR-C compounds on the phosphorylation of Akt in constitutively activated $P2Y_{12}/P2Y_1$-expressing cells. Cells grown in six-well plates were stimulated with AR-C compounds for 10 min at 37°C. The reaction was stopped by washing with ice-cold PBS and the addition of 250 μl of ice-cold lysis buffer. Akt phosphorylation was estimated by immunoblotting using phospho-Akt (Ser473) antibody (1:1000 dilution) (Cell Signaling).

Acknowledgments

We thank Drs. James L. Daniel, Barrie Ashby, and Todd M. Quinton, Temple University School of Medicine, for critically reading the manuscript.

References

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, et al. (2003) Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* **24:**52–55.

Adan RA and Kas MJ (2003) Inverse agonism gains weight. *Trends Pharmacol Sci* **24:**315–321.
Akbar GK, Dasari VR, Webb TE, Ayyanathan K, Pillarisetti K, Sandhu AK, Athwal RS, Daniel JL, Ashby B, Barnard EA, et al. (1996) Molecular cloning of a novel P2 purinoceptor from human erythroleukemia cells. *J Biol Chem* **271:**18363–18367.
Bakker RA, Schoonus SB, Smit MJ, Timmerman H, and Leurs R (2001) Histamine H_1-receptor activation of nuclear factor-κB: roles for Gβγ- and $G\alpha_{q/11}$-subunits in constitutive and agonist-mediated signaling. *Mol Pharmacol* **60:**1133–1142.
Behan DP and Chalmers DT (2001) The use of constitutively active receptors for drug discovery at the G protein-coupled receptor gene pool. *Curr Opin Drug Discov Devel* **4:**548–560.
Beinborn M, Ren Y, Blaker M, Chen C, and Kopin AS (2004) Ligand function at constitutively active receptor mutants is affected by two distinct yet interacting mechanisms. *Mol Pharmacol* **65:**753–760.
Berlot C, ed (1999) *Expression and Functional Analysis of G Protein Alpha Subunits in Mammalian Cells.* CRC Press, Boca Raton.
Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, McMinn TR, Apparsundaram S, Hyek MF, Kenakin TP, Allen LF, et al. (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. *Nature (Lond)* **374:**272–276.
Burnstock G (2004) Introduction: P2 receptors. *Curr Top Med Chem* **4:**793–803.
Chalmers DT and Behan DP (2002) The use of constitutively active GPCRs in drug discovery and functional genomics. *Nat Rev Drug Discov* **1:**599–608.
Collins B and Hollidge C (2003) Antithrombotic drug market. *Nat Rev Drug Discov* **2:**11–12.
Dangelmaier C, Jin J, Smith JB, and Kunapuli SP (2001) Potentiation of thromboxane A2-induced platelet secretion by Gi signaling through the phosphoinositide-3 kinase pathway. *Thromb Haemost* **85:**341–348.
Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, and Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* **273:**2024–2029.
Ding Z, Kim S, Dorsam RT, Jin J, and Kunapuli SP (2003) Inactivation of the human P2Y12 receptor by thiol reagents requires interaction with both extracellular cysteine residues, Cys17 and Cys270. *Blood* **101:**3908–3914.
Ding Z, Tuluc F, Bandivadekar KR, Zhang L, Jin J, and Kunapuli SP (2005) Arg333 and Arg334 in the COOH terminus of the human P2Y1 receptor are crucial for Gq coupling. *Am J Physiol* **288:**C559–C567.
Dorsam RT and Kunapuli SP (2004) Central role of the P2Y12 receptor in platelet activation. *J Clin Investig* **113:**340–345.
Dubyak GR and Cowen DS (1990) Activation of inositol phospholipid-specific phospholipase C by P2-purinergic receptors in human phagocytic leukocytes. Role of pertussis toxin-sensitive G proteins. *Ann NY Acad Sci* **603:**227–245.
Dupre DJ, Le Gouill C, Gingras D, Rola-Pleszczynski M, and Stankova J (2004) Inverse agonist activity of selected ligands of the cysteinyl-leukotriene receptor 1. *J Pharmacol Exp Ther* **309:**102–108.
Gordon JL (1986) Extracellular ATP: effects, sources and fate. *Biochem J* **233:**309–319.
Hoffmann C, Moro S, Nicholas RA, Harden TK, and Jacobson KA (1999) The role of amino acids in extracellular loops of the human P2Y1 receptor in surface expression and activation processes. *J Biol Chem* **274:**14639–14647.
Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, et al. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature (Lond)* **409:**202–207.
Huang J, Driscoll EM, Gonzales ML, Park AM, and Lucchesi BR (2000) Prevention of arterial thrombosis by intravenously administered platelet P2T receptor antagonist AR-C69931MX in a canine model. *J Pharmacol Exp Ther* **295:**492–499.
Humphries RG, Tomlinson W, Ingall AH, Cage PA, and Leff P (1994) FPL 66096: a novel, highly potent and selective antagonist at human platelet P2T-purinoceptors. *Br J Pharmacol* **113:**1057–1063.
Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, et al. (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. *J Med Chem* **42:**213–220.
Jacobsson F, Swahn E, Wallentin L, and Ellborg M (2002) Safety profile and tolerability of intravenous AR-C69931MX, a new antiplatelet drug, in unstable angina pectoris and non-Q-wave myocardial infarction. *Clin Ther* **24:**752–765.
Jiang Q, Guo D, Lee BX, Van Rhee AM, Kim YC, Nicholas RA, Schachter JB, Harden TK, and Jacobson KA (1997) A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. *Mol Pharmacol* **52:**499–507.
Jin J and Kunapuli SP (1998) Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* **95:**8070–8074.
Jin J, Quinton TM, Zhang J, Rittenhouse SE, and Kunapuli SP (2002) Adenosine diphosphate (ADP)-induced thromboxane A_2 generation in human platelets requires coordinated signaling through integrin $alpha_{IIb}beta_3$ and ADP receptors. *Blood* **99:**193–198.
Jin J, Tomlinson W, Kirk IP, Kim YB, Humphries RG, and Kunapuli SP (2001) The C6–2B glioma cell $P2Y_{AC}$ receptor is pharmacologically and molecularly identical to the platelet $P2Y_{12}$ receptor. *Br J Pharmacol* **133:**521–528.
Kenakin T (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol* **65:**2–11.
Kim S, Jin J, and Kunapuli SP (2004) Akt activation in platelets depends on Gi signaling pathways. *J Biol Chem* **279:**4186–4195.
Kunapuli SP, Ding Z, Dorsam RT, Kim S, Murugappan S, and Quinton TM (2003) ADP receptors–targets for developing antithrombotic agents. *Curr Pharm Des* **9:**2303–2316.
Lefkowitz RJ (2004) Historical review: a brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharmacol Sci* **25:**413–422.
Leon C, Alex M, Klocke A, Morgenstern E, Moosbauer C, Eckly A, Spannagl M, Gachet C, and Engelmann B (2004) Platelet ADP receptors contribute to the initiation of intravascular coagulation. *Blood* **103:**594–600.
Leon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, Dierich A, LeMeur M, Cazenave JP, and Gachet C (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. *J Clin Investig* **104:**1731–1737.
Mahe C, Loetscher E, Feuerbach D, Muller W, Seiler MP, and Schoeffter P (2004) Differential inverse agonist efficacies of SB-258719, SB-258741 and SB-269970 at human recombinant serotonin 5-HT7 receptors. *Eur J Pharmacol* **495:**97–102.
Meshki J, Tuluc F, Bredetean O, Ding Z, and Kunapuli SP (2004) Molecular mechanism of nucleotide-induced primary granule release in human neutrophils: role for the P2Y2 receptor. *Am J Physiol* **286:**C264–C271.
Milligan G (2003) Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol* **64:**1271–1276.
Montanelli L, Delbaere A, Di Carlo C, Nappi C, Smits G, Vassart G, and Costagliola S (2004) A mutation in the follicle-stimulating hormone receptor as a cause of familial spontaneous ovarian hyperstimulation syndrome. *J Clin Endocrinol Metab* **89:**1255–1258.
Niitsu Y, Jakubowski JA, Sugidachi A, and Asai F (2005) Pharmacology of CS-747 (prasugrel, LY640315), a novel, potent antiplatelet agent with in vivo P2Y12 receptor antagonist activity. *Semin Thromb Hemostasis* **31:**184–194.
Parfitt AM, Schipani E, Rao DS, Kupin W, Han ZH, and Juppner H (1996) Hypercalcemia due to constitutive activity of the parathyroid hormone (PTH)/PTH-related peptide receptor: comparison with primary hyperparathyroidism. *J Clin Endocrinol Metab* **81:**3584–3588.
Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, and Vassart G (1993) Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature (Lond)* **365:**649–651.
Pearce SH, Williamson C, Kifor O, Bai M, Coulthard MG, Davies M, Lewis-Barned N, McCredie D, Powell H, Kendall-Taylor P, et al. (1996) A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. *N Engl J Med* **335:**1115–1122.
Rossier O, Abuin L, Fanelli F, Leonardi A, and Cotecchia S (1999) Inverse agonism and neutral antagonism at α_{1a}- and α_{1b}-adrenergic receptor subtypes. *Mol Pharmacol* **56:**858–866.
Savi P, Labouret C, Delesque N, Guette F, Lupker J, and Herbert JM (2001) $P2y_{12}$, a new platelet ADP receptor, target of clopidogrel. *Biochem Biophys Res Commun* **283:**379–383.
Schwartz JC, Morisset S, Rouleau A, Ligneau X, Gbahou F, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, and Arrang JM (2003) Therapeutic implications of constitutive activity of receptors: the example of the histamine H3 receptor. *J Neural Transm Suppl* 1–16.
Seifert R and Wenzel-Seifert K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **366:**381–416.
Simon J, Vigne P, Eklund KM, Michel AD, Carruthers AM, Humphrey PP, Frelin C, and Barnard EA (2001) Activity of adenosine diphosphates and triphosphates on a $P2Y_T$-type receptor in brain capillary endothelial cells. *Br J Pharmacol* **132:**173–182.
Smits G, Olatunbosun O, Delbaere A, Pierson R, Vassart G, and Costagliola S (2003) Ovarian hyperstimulation syndrome due to a mutation in the follicle-stimulating hormone receptor. *N Engl J Med* **349:**760–766.
Storey RF, Sanderson HM, White AE, May JA, Cameron KE, and Heptinstall S (2000) The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. *Br J Haematol* **110:**925–934.
Sugidachi A, Asai F, Ogawa T, Inoue T, and Koike H (2000) The in vivo pharmacological profile of CS-747, a novel antiplatelet agent with platelet ADP receptor antagonist properties. *Br J Pharmacol* **129:**1439–1446.
Sugidachi A, Asai F, Yoneda K, Iwamura R, Ogawa T, Otsuguro K, and Koike H (2001) Antiplatelet action of R-99224, an active metabolite of a novel thienopyridine-type G_i-linked P2T antagonist, CS-747. *Br J Pharmacol* **132:**47–54.
Tryoen-Toth P, Decaillot FM, Filliol D, Befort K, Lazarus LH, Schiller PW, Schmidhammer H, and Kieffer BL (2004) Inverse agonism and neutral antagonism at wild-type and constitutively active mutant delta opioid receptors. *J Pharmacol Exp Ther* **313:**410–421.
Vasiljev KS, Uri A, and Laitinen JT (2003) 2-Alkylthio-substituted platelet P2Y12 receptor antagonists reveal pharmacological identity between the rat brain Gi-linked ADP receptors and P2Y12. *Neuropharmacology* **45:**145–154.
Vermeulen ES, van Smeden M, Schmidt AW, Sprouse JS, Wikstrom HV, and Grol CJ (2004) Novel 5-HT7 receptor inverse agonists. Synthesis and molecular modeling of arylpiperazine- and 1,2,3,4-tetrahydroisoquinoline-based arylsulfonamides. *J Med Chem* **47:**5451–5466.
Vertongen P, Langlet C, Langer I, Gaspard N, and Robberecht P (2004) Ac His1 [D-Phe2, K15, R16, L27] VIP (3–7)/GRF (8–27)—a VPAC1 receptor antagonist—is an inverse agonist on two constitutively active truncated VPAC1 receptors. *Peptides* **25:**1943–1949.
Weiner DM, Burstein ES, Nash N, Croston GE, Currier EA, Vanover KE, Harvey SC, Donohue E, Hansen HC, Andersson CM, et al. (2001) 5-Hydroxytryptamine2A receptor inverse agonists as antipsychotics. *J Pharmacol Exp Ther* **299:**268–276.
Yoneda K, Iwamura R, Kishi H, Mizukami Y, Mogami K, and Kobayashi S (2004) Identification of the active metabolite of ticlopidine from rat in vitro metabolites. *Br J Pharmacol* **142:**551–557.
Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao X, Liu YH, Chen G, Pramanik B, Laz TM, et al. (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. *J Biol Chem* **276:**8608–8615.

Address correspondence to: Dr. Satya P. Kunapuli, Department of Physiology, Temple University School of Medicine, 3420 N. Broad Street, Philadelphia, PA 19140. E-mail: spk@temple.edu