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Identification of a potent inverse agonist at a constitutively active mutant of human $P2Y_{12}$ receptor

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Running title: Constitutively active $P2Y_{12}$ mutant and inverse agonist

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Abbreviations: GPCRs: G protein-coupled receptors; ATP: adenosine triphosphates; ADP: adenosine diphosphate; 2-MeSADP: 2-methylthio-ADP; PTX: pertussis toxin; IBMX: 3-isobutyl-1-methylxanthine: BzATP: 2',3'-O-(4- benzoylbenzoyl) adenosine 5'-triphosphate; HA-tag: hemagglutinin epitope

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Abstract

Human platelets express two P2Y receptors: Gq-coupled $P2Y_1$ and Gi-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 human platelet ADP receptor we constructed a chimeric HA-tagged human $P2Y_{12}$ receptor with its C-terminus replaced by the corresponding part of human $P2Y_1$ receptor and stably expressed it in CHO-K1 cells. Interestingly, 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 Gi inhibitor. The constitutively active $P2Y_{12}$ mutant retained normal responses to 2-MeSADP, 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 AR-C78511 as a potent $P2Y_{12}$ inverse agonist whereas 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.

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Introduction

Extracellular nucleotides influence many biological functions, including vascular tone, cell division, cardiac and skeletal muscle contraction, platelet aggregation, as well as peripheral and central neurotransmission (Burnstock, 2004). Adenosine triphosphate (ATP) and adenosine diphosphate (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 (Burnstock, 2004; Dubyak and Cowen, 1990). 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 super family 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 Gq-coupled subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) and three Gi-coupled subtypes ($P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$). In addition, $P2Y_{11}$ receptor can also couple to Gs to activate adenylyl cyclase, and $P2Y_2$ can activate Gi-dependent pathway (Meshki et al., 2004).

Of the P2Y receptors, Gq-coupled $P2Y_1$ and Gi-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 (Hollopeter et al., 2001; Leon et al., 2004; Leon et al., 1999; Zhang et al., 2001). Co-stimulation of both $P2Y_1$ and $P2Y_{12}$ is

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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 (Dangelmaier et al., 2001; Storey et al., 2000). 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 most extensively studied in platelets (Zhang et al., 2001). $P2Y_{12}$ receptor plays a central role in platelet activation (Dorsam and Kunapuli, 2004) and therefore attracts tremendous interest from pharmaceutical companies to develop $P2Y_{12}$ 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 clopidogel exhibiting 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 that is currently under clinical trial (Niitsu et al., 2005; Sugidachi et al., 2000; Sugidachi et al., 2001). 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 these receptors are encoded on chromosome 3, suggesting gene duplication. Furthermore, the $P2Y_1$ and $P2Y_{12}$ receptors have identical

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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 (Ding et al., 2005) (Ding et al., 2003; Hoffmann et al., 1999; Jiang et al., 1997). 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 anti-thrombotic 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 classical 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

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GPCR can also shift the balance to the R* state, increasing G protein activity in the absence of agonists and leading to constitutive activation of GPCRs.

In our attempt to identify the Gq-coupling domain of the human $P2Y_1$ receptor, we found that the C-terminus of the $P2Y_1$ receptor is essential for Gq coupling and further identified two Arg residues essential for Gq 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 Gq coupling and therefore confer $P2Y_{12}$ receptor with Gq-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 to explore 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 HA-tag (hemagglutinin epitope) was purchased from Covance Research Products (Berkeley, CA). 2-MeSADP, 2',3'-O-(4- benzoylbenzoyl) adenosine 5'-triphosphate (BzATP), forskolin, pertussis toxin and IBMX (3-isobutyl-1-methylxanthine) were purchased from Sigma Chemical (St Louis, MO). AR-C69931MX, AR-C66096, AR-C67085, AR-C69581 and

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AR-C78511 were gifts from Astra-Zeneca (Loughborough, UK). [^{3}H] Adenine was purchased from NEN Life Science Products (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 & 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 No: AF313449] (Hollopeter et al., 2001) was cloned into pcDNA3.1/Hygro (+) with a HA tag (YPYDVPDYA) inserted at the beginning of the translation initiation by polymerase chain reaction (PCR). Forward primer containing Kpn I restriction site and HA tag sequence is 5'-GCGCGGTACCACCATGTACCCATACGATGTTCCAGATTACGCTCAAGCCGTC GACAATCTC-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 No: U42029] as described previously (Ding et al., 2005).

Cell culture

Chinese hamster ovary (CHO-K1) cells were grown in Ham's F12 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

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$P2Y_{12}$ 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 hours 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, 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, 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_2PO4, 5.6 mM glucose, 10 mM Hepes, 0.2% bovine serum albumin, pH 7.4) and cell concentrations were adjusted to 10^7/ml. Aliquots of 100 µl cell suspension were mixed with 4 µl of 1:10 diluted FITC-labeled monoclonal antibody against HA (Covance, Berkeley, CA) in the presence of 2 mM Ca^{2+}. After incubation at 4°C for 1 hour in the

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dark, cells 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). Untransfected CHO-K1 cells or vector-transfected cells were used as negative controls.

Cyclic AMP assay

Intracellular Cyclic AMP assays were conducted by a modification of a previously described protocol (Ding et al., 2003). Briefly, cells were cultured in 6-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 (3-isobutyl-1-methylxanthine) and incubated for 10 minutes 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 minutes unless indicated. The reactions were terminated by addition of 1 ml stop solution containing 5% trichloroacetic acid, 1 mM ATP and 1 mM cAMP. Cyclic AMP levels were determined and cAMP conversion from ATP was calculated as described by Berlot (Berlot, 1999) using the following formula: cAMP conversion from ATP = [^{3}H] cAMP/([^{3}H] ATP + [^{3}H] cAMP) x 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 (Ser^{473}) antibody (1:1000 dilution) (Cell Signaling) as described previously with some modification (Kim et al., 2004). Cells grown in 6-well plates were stimulated with 2-MeSADP (1 µM) for 5 min at 37°C, and

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the reaction was stopped by washing with 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, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1µg/ml leupeptin, and 1 mM PMSF. In some experiments, cells were incubated overnight with 200 ng/ml pertussis toxin, a Gi inhibitor. Samples were boiled for 5 min, and proteins were separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Nonspecific binding sites were blocked by incubation in Tris-buffered saline/Tween (TBST; 20 mM Tris, 140 mM NaCl, 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, 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, 2% BSA) for 1 h at RT. After additional washing steps, membranes were then incubated with a CDP-Star® chemiluminescent substrates for 10 min at RT, and immunoreactivity was detected using Fujifilm Luminescent Image Analyzer (model LAS-1000 CH, 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

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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 two weeks, stable clones were screened by flow cytometry analysis for expression of the HA-tagged receptor. Out 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 $P2Y_{12}/P2Y_1$. Similarly, 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 to cells without receptor expression using counter staining with propidium iodide. The data are shown in Fig. 2 (C - E). 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 WT and chimeric receptor transfected cell lines have increased HA tag expressions (81% vs 3% and 74% vs 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 previously shown that the C-terminus of the human $P2Y_1$ receptor is essential for Gq coupling (Ding et al., 2005). We also demonstrated that this $P2Y_1$ receptor C-terminus, when introduced

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into $P2Y_{12}$ receptor, failed to activate Gq upon agonist stimulation although the chimeric $P2Y_{12}$ receptor expressed very well on the surface of CHO-K1 cells (Ding et al., 2005). In order to address whether the chimeric $P2Y_{12}$ receptor still functions and activates Gi 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} is 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 that 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 about 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). In order to confirm this constitutive activation of the Gi pathways, we used pertussis toxin (PTX), a Gi inhibitor. cAMP levels in the chimeric $P2Y_{12}$ receptor expressing cells were dramatically increased upon inhibition of Gi 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 Gi pathways.

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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 Gi pathway in platelets (Kim et al., 2004). Therefore, we evaluated whether this signaling molecule downstream of Gi 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 constitutively activated and stimulates Gi 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 analogues (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 IC_{50} of 17.4 ± 4.9 nM (Fig. 5A). This is consistent with the reported pIC_{50} of AR-C78511 to reverse 2-MeSADP- or ADP-induced response in rat and human platelets, as well as in C6-2B cells (Jin et al., 2001; Vasiljev et al., 2003). The maximal increase of intracellular cAMP over control (Fig. 5 A & C) and the IC_{50} of 17.4 nM indicate that AR-C78511 is a potent inverse agonist on $P2Y_{12}$ receptor.

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AR-C69931MX is a $P2Y_{12}$ antagonist, which has entered into phase II clinical trial and was recently halted to develop as an antiplatelet drug by AstraZeneca because of poor oral availability and the lack of commercial potential as an injectable antiplatelet drug (Collins and Hollidge, 2003; Huang et al., 2000; Ingall et al., 1999; Jacobsson et al., 2002). 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 while 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 (Daniel et al., 1998; Humphries et al., 1994; Ingall et al., 1999; Vasiljev et al., 2003). AR-C66096 has pK_B 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

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inhibit ADP-induced human platelet aggregation (Daniel et al., 1998), AR-C66096 only partially increased the 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. Similarly, we found 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 with pIC_{50} value of 6.7 – 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 nM – 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} of 20.3 ± 8.1 nM. Compared to its efficacy antagonizing ADP-induced adenylyl cyclase inhibition on wild type $P2Y_{12}$ (data not shown) and the potent efficacy of AR-C78511 as a 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

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constitutively active $P2Y_{12}$ receptor. Other AR-C compounds have exhibited smaller extent of inhibition. These data compare well with cAMP levels as readout (Table 1).

Vasiljev et al found that AR-C69581 has a pIC_{50} of 5.7 ± 0.1 in reversing 2-MeSADP-stimulated [^{35}S]GTPγS binding to human platelet membrane (Vasiljev et al., 2003). Consistent with this pIC_{50}, 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 to its dramatic effects in antagonizing ADP-induced $P2Y_{12}$ activation at 1 μM 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).

BzATP, a ADP receptor antagonist which antagonizes $P2Y_{12}$ receptor with a IC_{50} 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 μM – 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 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, which have been reported to be the causes of a variety of human diseases

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(Montanelli et al., 2004; Parfitt et al., 1996; Parma et al., 1993; Pearce et al., 1996; Smits et al., 2003), 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 classical GPCRs antagonists that antagonize agonist binding to the receptors are ineffective while the inverse agonists are believed to have advantages (Lefkowitz, 2004). Though 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 a similar expression level as 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 to other recombinant constitutively active receptor systems, for example, the constitutive activity of cholecystokinin type 2 receptor (CCK-2R) 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 Gi protein through the addition of PTX abolished the constitutive activity of $P2Y_{12}/P2Y_1$, demonstrated by that the basal cAMP level in the absence of $P2Y_{12}$ receptor agonists was restored nearly to the level of wild type. All these results clearly indicate that the chimeric $P2Y_{12}$ receptor is constitutively activated. Furthermore, Akt, a

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downstream signal molecule of Gi 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. Though there is 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 (Adan and Kas, 2003; Bond et al., 1995; 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 think, to some extent, 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 (Bakker et al., 2001; Rossier et al., 1999; 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 program (Behan and Chalmers, 2001; Chalmers and Behan, 2002; Seifert and Wenzel-Seifert, 2002). Using the cell line expressing high constitutive activity of human $P2Y_{12}$ mutant, we further explored the

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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 while AR-C69931MX is a pure $P2Y_{12}$ antagonist in the range of 0.1 nM – 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 induction of thrombotic diseases have 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 to study 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.

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Footnote

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Figure legends

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

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 vs. antibody associated binding) of CHO-K1, P2Y12WT and P2Y12/P2Y1 cells are shown in Panels C - E. Propidium iodide 0.625 μg/ml was added 5 min before scanning. 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 (+) [upper right quadrant], and PI (+)/FITC-Ab(-) [upper left quadrant].

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 ± SEM representing at least three separate experiments. B. Chimeric human $P2Y_{12}/P2Y_1$ is activated in the absence of agonists while 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 prior to forskolin stimulation for 10 min. Data were expressed as mean ± SEM representing at least three separate experiments.

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Fig. 4. Constitutively activated phosphorylation of Akt in $P2Y_{12}$/$P2Y_1$-expressing cells. The constitutively activated phosphorylation is PTX-sensitive (top panel) and is further enhanced by 2-MeSADP stimulation (bottom panel). Cells grown in 6-well plates were incubated overnight in the absence of presence of PTX 200 ng/ml, then were stimulated with 2-MeSADP (1 µM) for 5 min at 37°C. The reaction was stopped by washing with cold PBS and the addition of 250 µl cold lysis buffer. Akt phosphorylation was estimated by immunoblotting using phospho-Akt (Ser^{473}) antibody (1:1000 dilution) (Cell Signaling).

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 (hatched) or presence (filled) of 100 nM AR-C69931MX for 10 min at 37°C. C: Cells were treated with AR-C78511 in the absence (hatched) or presence (filled) 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 ± SEM representing at least three separate experiments.

Fig. 6. Effects of AR-C compounds on the phosphorylation of Akt in constitutively activated $P2Y_{12}$/$P2Y_1$-expressing cells. Cells grown in 6-well plates were stimulated with AR-C compounds for 10 min at 37°C. The reaction was stopped by washing with cold PBS and the addition of 250 µl cold lysis buffer. Akt phosphorylation was estimated by immunoblotting using phospho-Akt (Ser^{473}) antibody (1:1000 dilution) (Cell Signaling).

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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 Material and Methods. Inhibition of phosphorylated Akt was compared to control through densitometric analysis of the western blots. Data are expressed as mean ± SEM representing at least three separate experiments.

	cAMP conversion from ATP (x 1000)	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 3μM	44 ± 4	17 ± 4

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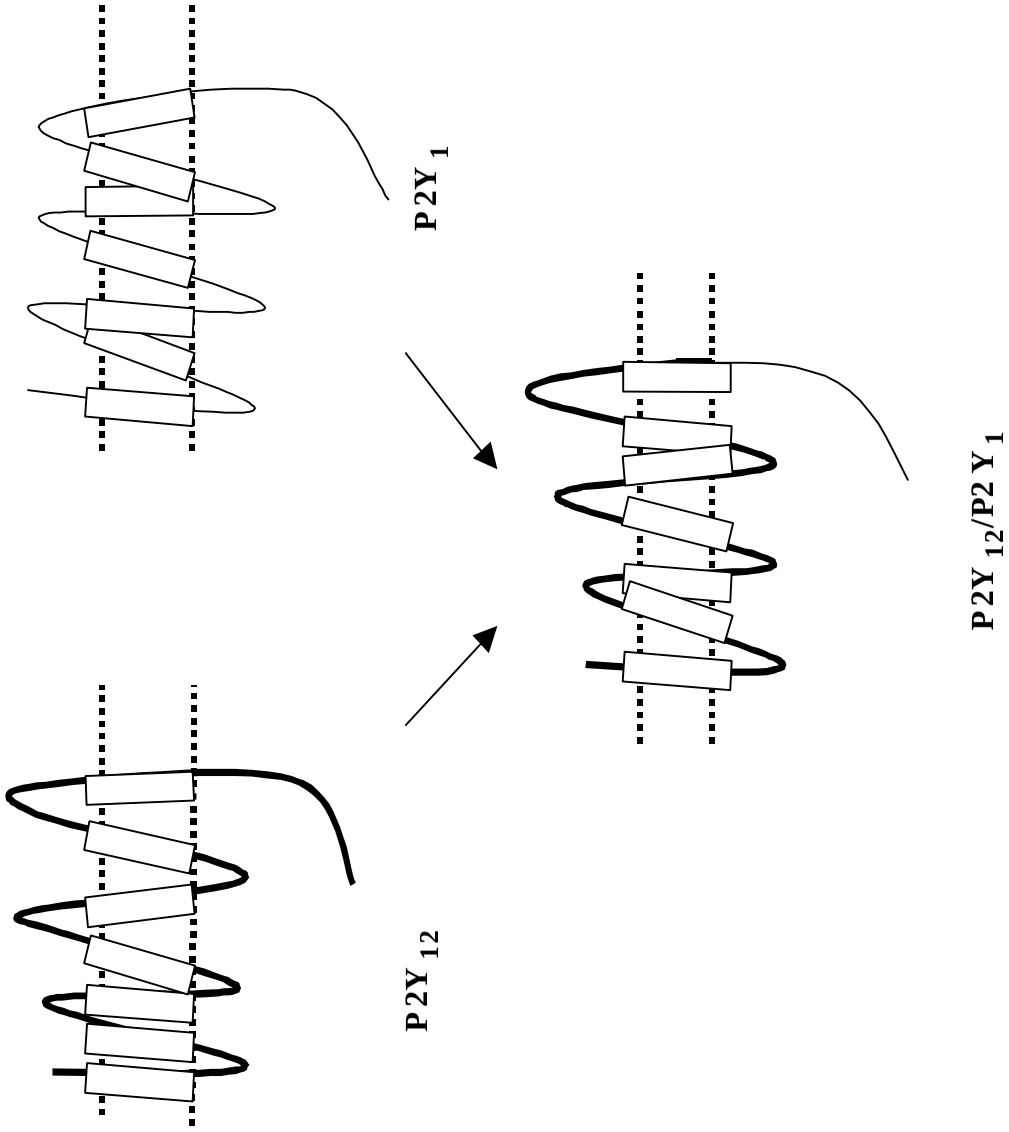

Fig. 1

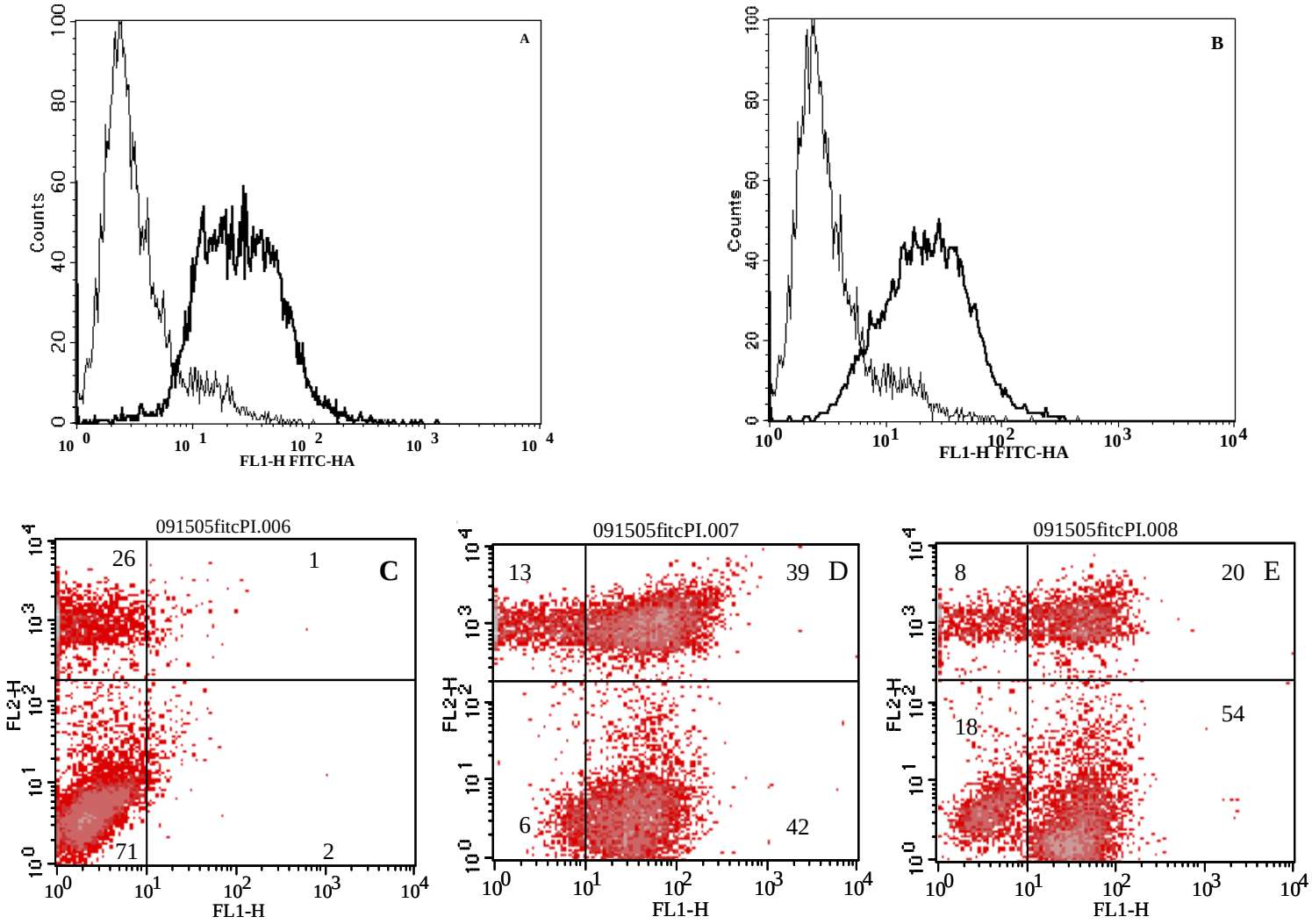

Fig. 2

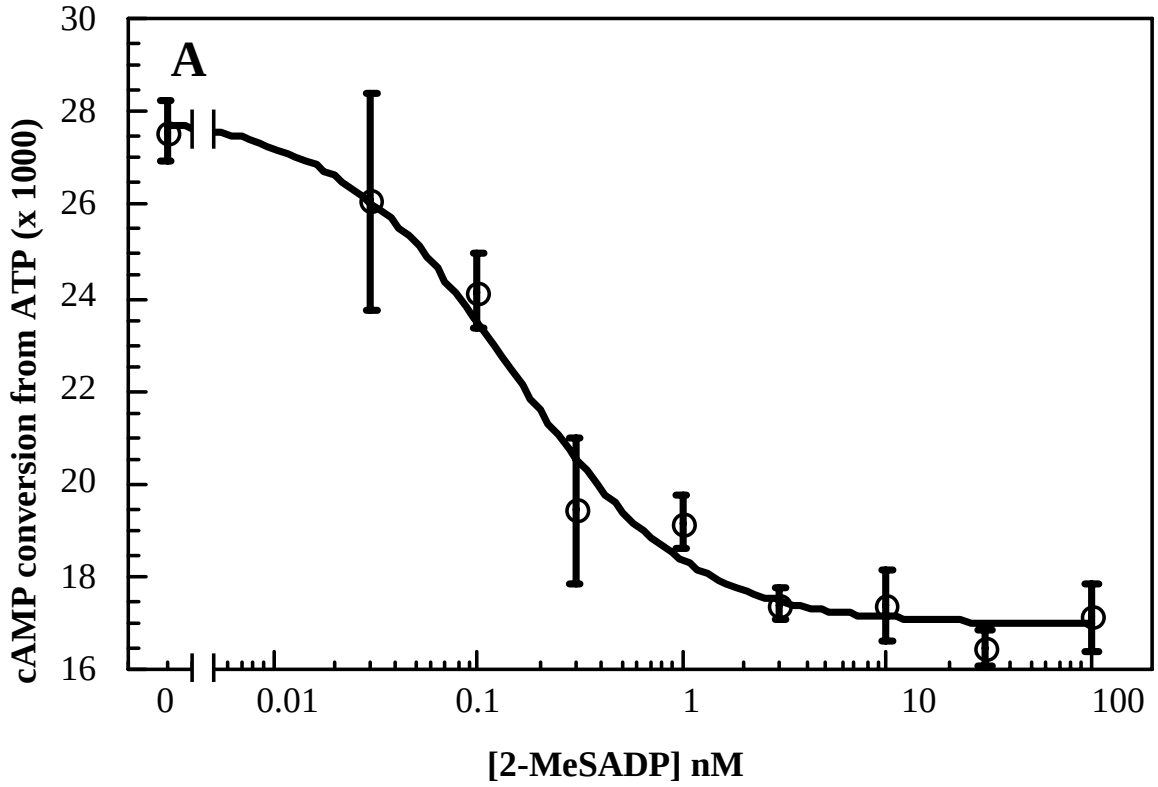

Fig. 3A

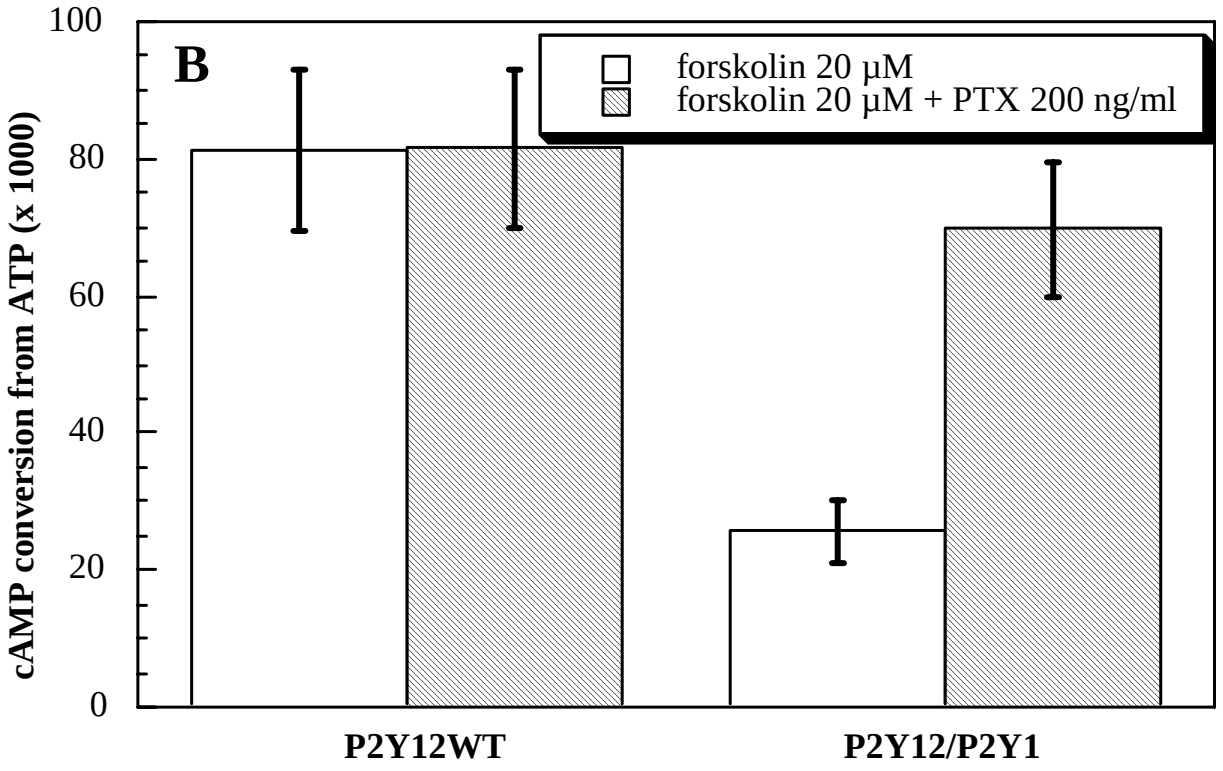

Fig. 3B

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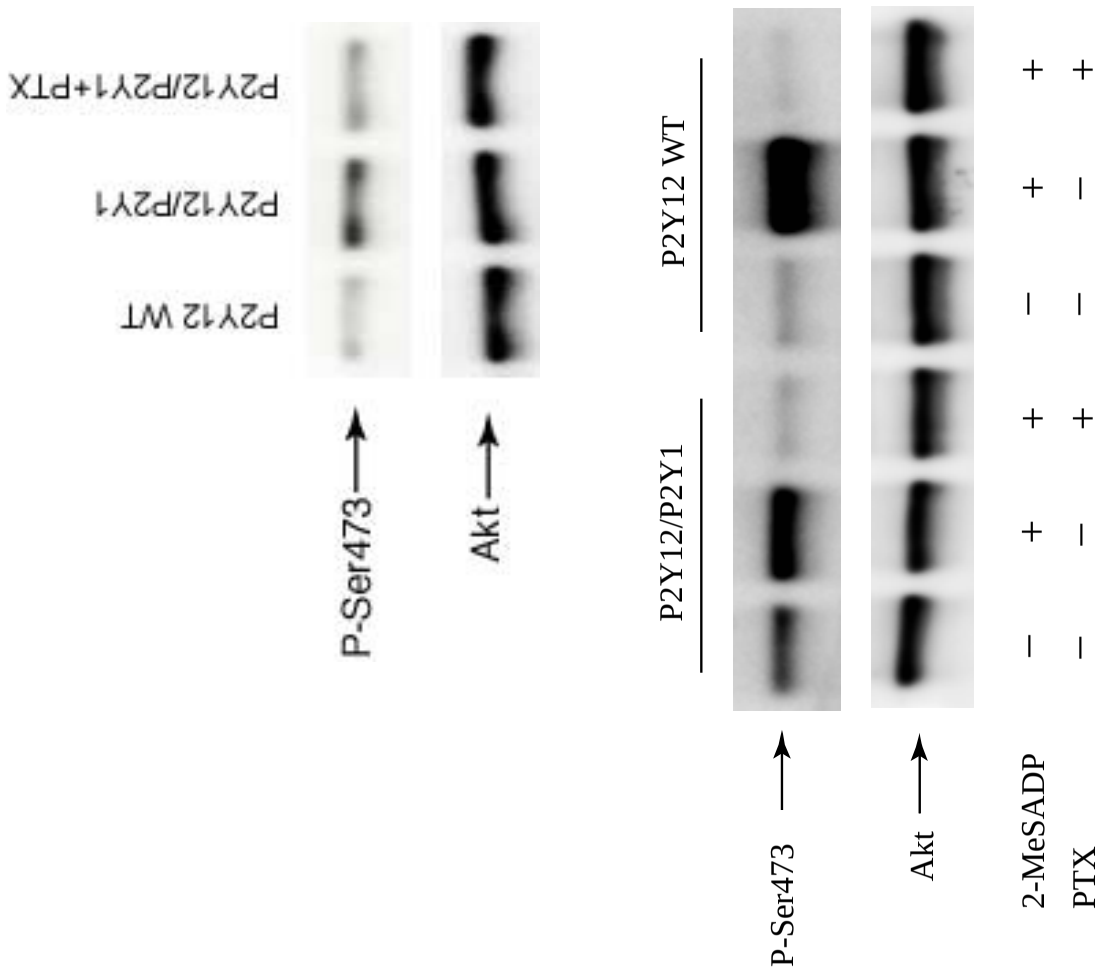

Fig. 4

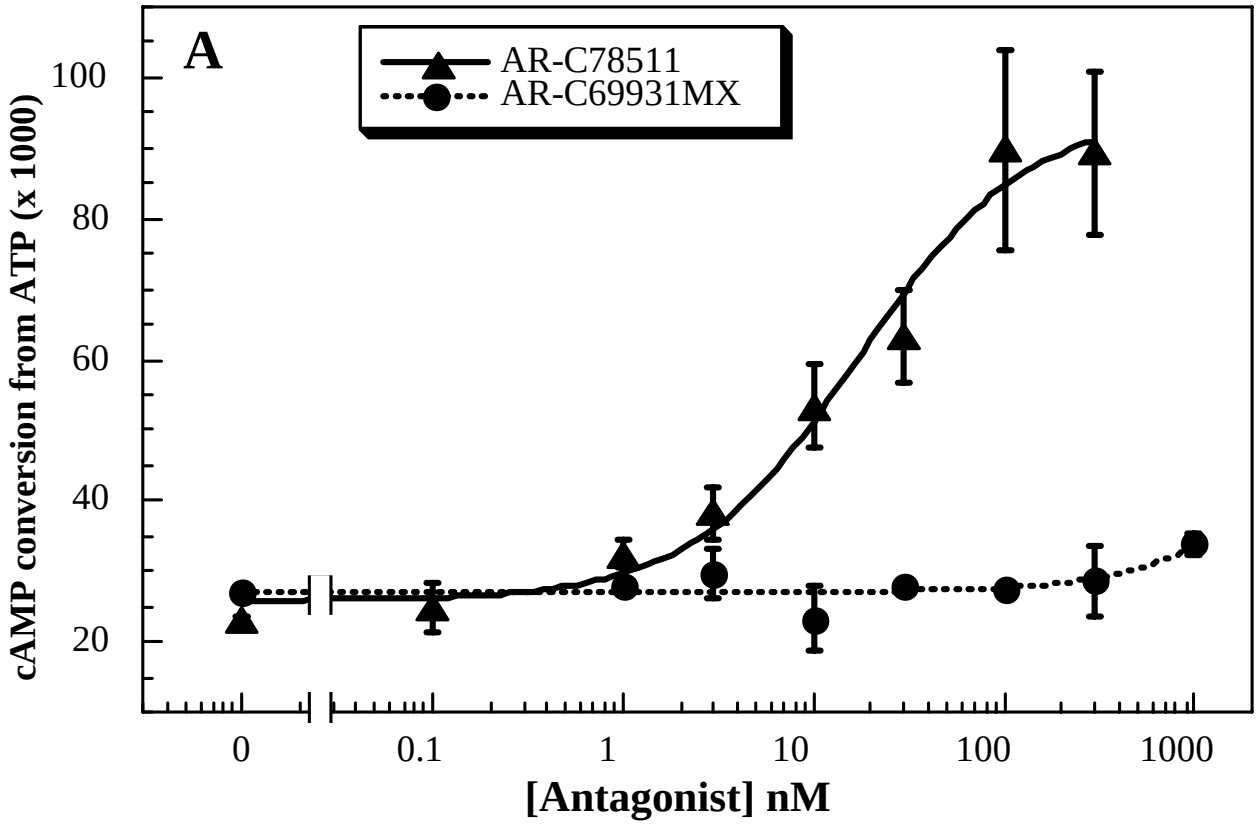

Fig. 5A

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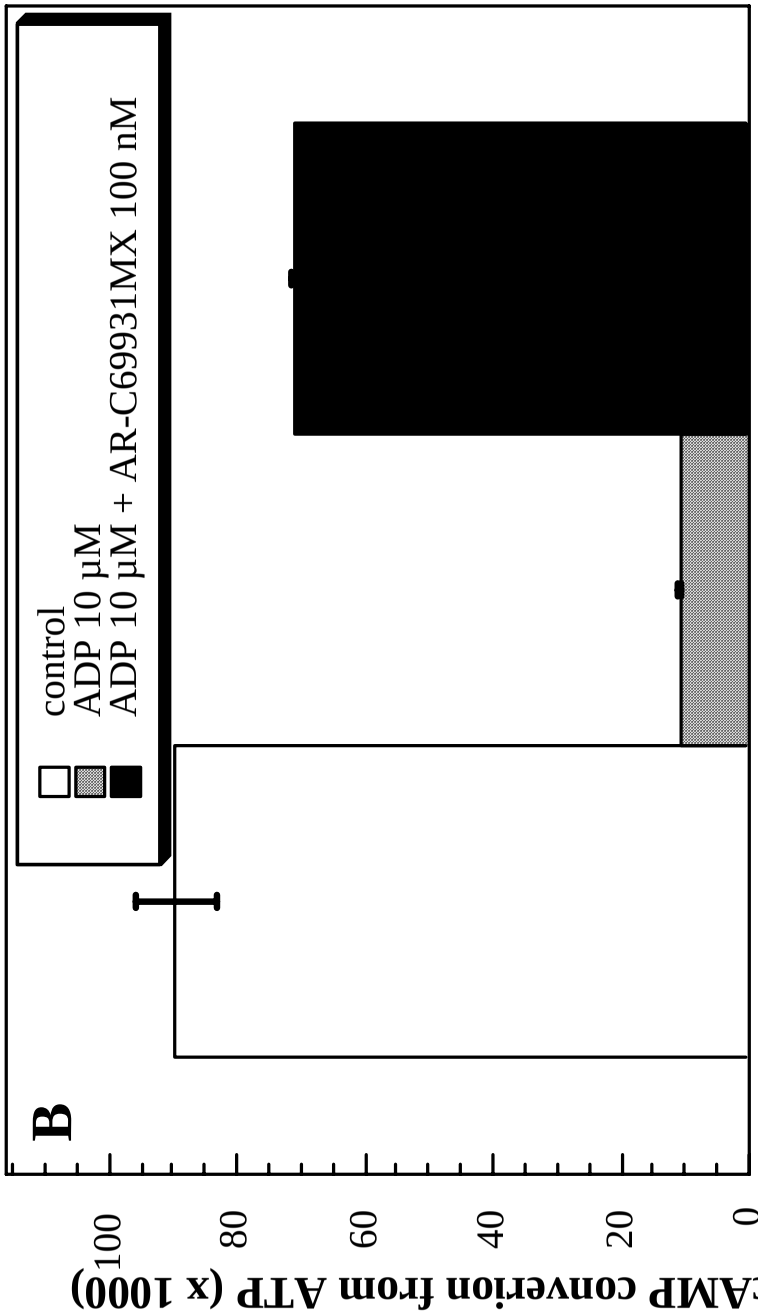

Fig. 5B

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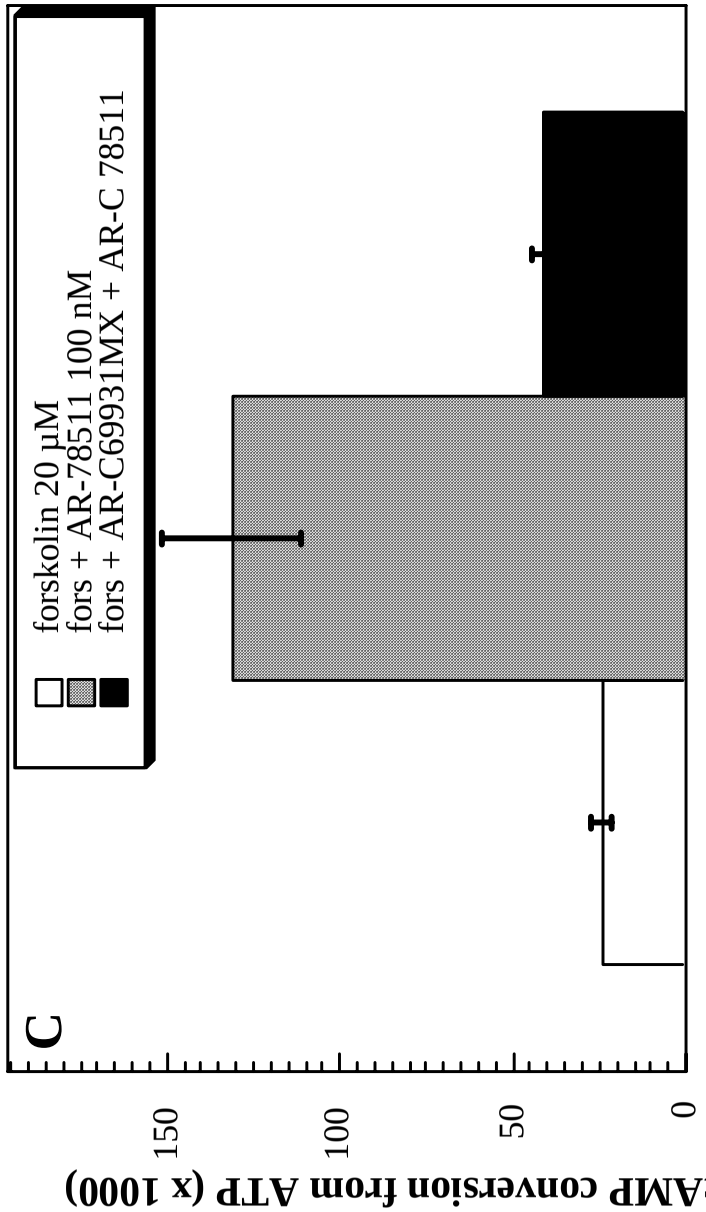

Fig. 5C

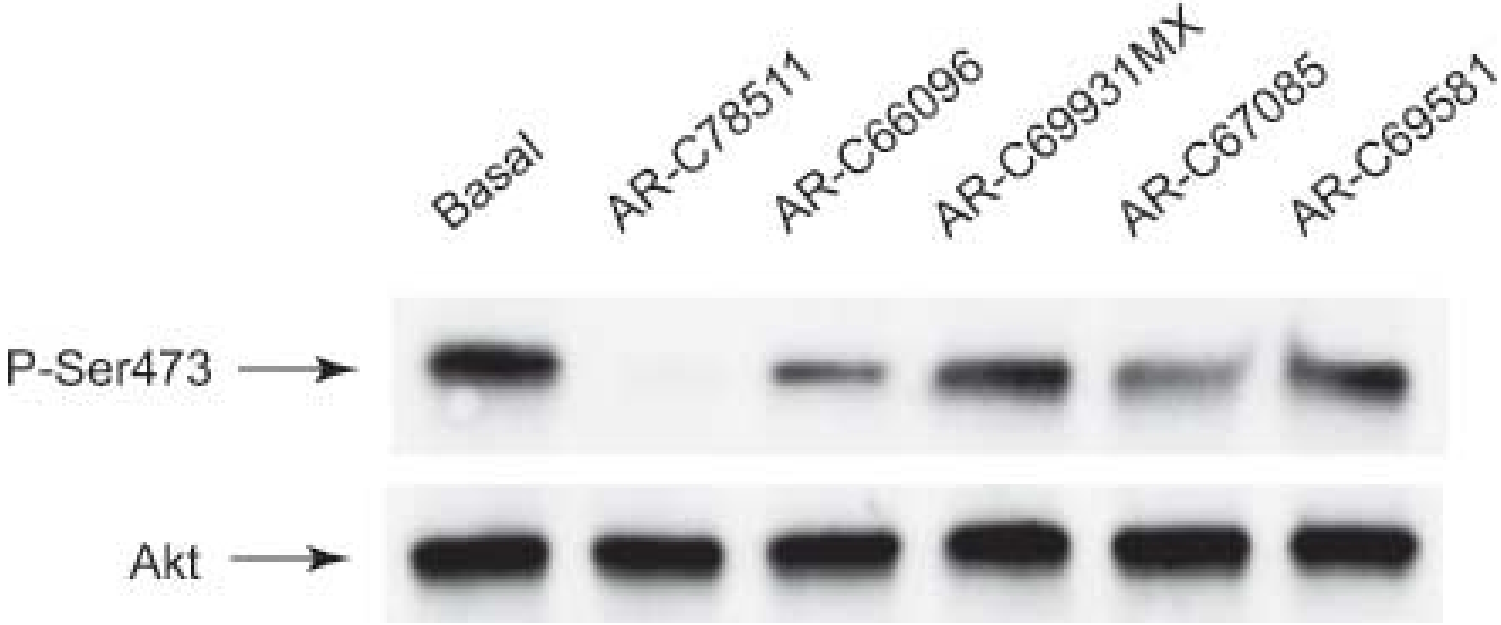

Fig. 6