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The C6-2B glioma cell $P2Y_{AC}$ receptor is pharmacologically and molecularly identical to the platelet $P2Y_{12}$ receptor

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1 P2Y receptor activation in many cell types leads to phospholipase C activation and accumulation of inositol phosphates, while in blood platelets, C6-2B glioma cells, and in B10 microvascular endothelial cells a P2Y receptor subtype, which couples to inhibition of adenylyl cyclase, historically termed $P2Y_{AC}$, ($P2T_{AC}$ or P_{2T} in platelets) has been identified. Recently, this receptor has been cloned and designated $P2Y_{12}$ in keeping with current P2 receptor nomenclature.

2 Three selective P_{2T} receptor antagonists, with a range of affinities, inhibited ADP-induced aggregation of washed human or rat platelets, in a concentration-dependent manner, with a rank order of antagonist potency (pIC_{50}, human: rat) of AR-C78511 (8.5 : 9.1) > AR-C69581 (6.2 : 6.0) > AR-C70300 (5.4 : 5.1). However, these compounds had no effect on ADP-induced platelet shape change.

3 All three antagonists had no significant effect on the ADP-induced inositol phosphate formation in 1321N1 astrocytoma cells stably expressing the $P2Y_1$ receptor, when used at concentrations that inhibit platelet aggregation.

4 These antagonists also blocked ADP-induced inhibition of adenylyl cyclase in rat platelets and C6-2B cells with identical rank orders of potency and overlapping concentration–response curves.

5 RT–PCR and nucleotide sequence analyses revealed that the C6-2B cells express the $P2Y_{12}$ mRNA.

6 These data demonstrate that the $P2Y_{AC}$ receptor in C6-2B cells is pharmacologically identical to the $P2T_{AC}$ receptor in rat platelets.

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Keywords: $P2T_{AC}$ receptor; P_{2T} receptor; $P2Y_{AC}$ receptor; $P2Y_{12}$ receptor, platelets; C6-2B glioma cells

Abbreviations: $P2T_{AC}$, P_{2T}, or $P2Y_{12}$, platelet ADP receptor coupled to inhibition of adenylyl cyclase; $P2Y_1$, platelet ADP receptor coupled to stimulation of phospholipase C; PLC, phospholipase C; InsP, inositol phosphates

Introduction

ATP and ADP are released from several sources in the body, including purinergic nerve endings, platelets, chromaffin cells, and endothelial cells (Gordon, 1986). The nucleotides elicit several physiological responses through activation of specific cell surface receptors known as P2 receptors (Burnstock, 1978). The P2 receptors are divided into P2X ligand gated channels, and P2Y receptors coupled to heterotrimeric G proteins (Fredholm *et al*., 1997). To date, several members of the P2X family and the P2Y family have been cloned (Fredholm *et al*., 1997). Several other P2 receptors have been characterized, but not cloned, including the P2Y receptors coupled to adenylyl cyclase in platelets (Kunapuli, 1998), C6 rat glioma cells (Boyer *et al*., 1993; Planet *et al*., 1989), and B10 microvascular endothelial cells (Feolde *et al*., 1995). In C6 cells and B10 cells, P2Y receptor stimulation does not lead to phospholipase C (PLC) activation (Boyer *et al*., 1993; Feolde *et al*., 1995).

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In the platelet, we proposed a three-receptor model for activation of platelets by ADP, that includes a receptor coupled to inhibition of adenylyl cyclase ($P2T_{AC}$), a receptor coupled to activation of phospholipase C ($P2Y_1$), and a ligand gated channel ($P2X_1$) (Daniel *et al*., 1998; Jin *et al*., 1998). Several laboratories have independently confirmed this model, but the ADP receptor coupled to adenylyl cyclase has been designated as P_{2T} (Fagura *et al*., 1998) P2cyc (Hechler *et al*., 1998a), $P2Y_{ADP}$ (Jantzen *et al*., 1999), $P2Y_{AC}$ (Fabre *et al*., 1999; Geiger *et al*., 1998), and P2Y? (Cattaneo & Gachet, 1999). We have demonstrated that ADP-induced platelet shape change is exclusively mediated by the $P2Y_1$ receptor and that $P2T_{AC}$ receptor antagonists have no effect on this event. Furthermore, ADP-induced platelet aggregation is mediated by concomitant signalling from both the $P2Y_1$ and the $P2T_{AC}$ receptors and hence either $P2Y_1$ receptor antagonists or the $P2T_{AC}$ receptor antagonists can abolish ADP-induced platelet aggregation (Jin & Kunapuli, 1998). Recently, the ADP receptor coupled to adenylyl cyclase has been cloned and designated $P2Y_{12}$ (Hollopeter *et al*., 2001). In addition, an orphan receptor in the brain was recently shown

to be an ADP receptor that couples to Gi signalling (Zhang *et al*., 2001) and to have a primary sequence identical to that of the $P2Y_{12}$ receptor. We have also designated the C6-2B cell P2Y receptor coupled to the inhibition of adenylyl cyclase, $P2Y_{AC}$, and speculated that the platelet and C6-2B cell receptors will be identified as the same subtype (Jin *et al*., 1998). In this manuscript, $P2T_{AC}$ indicates the adenylyl cyclase coupled platelet ADP receptor subtype, and $P2Y_{AC}$ is used for the C6-cell P2Y receptor coupled to the inhibition of adenylyl cyclase. Previous studies have shown that AR-C66096 and related compounds are competitive antagonists at the P2 receptor subtype, which mediates ADP-induced inhibition of adenylyl cyclase in platelets (Humphries *et al*., 1995). Since the discovery of these compounds pre-dates description of the 3-receptor model, the term P_{2T} receptor antagonist remains in common usage for these potent, selective antagonists at the $P2T_{AC}$ receptor.

Selective antagonists are preferable to agonist potency orders in pharmacologically defining a receptor subtype. This is particularly relevant in the P2 receptor area given the complexities in analysing such data, which can be confounded by the presence of impurities (Leon *et al*., 1997). In addition, the agonist potencies and agonist/antagonist profiles appear to depend on the receptor number in a heterologous expression system. For example, when P2Y1 receptor is expressed in astrocytoma cells, ATP was found to be a partial agonist (Palmer *et al*., 1998), whereas ATP is an antagonist at the P2Y1 receptor in platelets (Mills, 1996), where it is constitutively expressed, and in Jurkat cells, upon heterologous stable expression (Hechler *et al*., 1998b). This difference could be due to the variations in the receptor number expressed in different cells. Furthermore, any ectonucleotidases present on a cell could potentially complicate the agonist/antagonist profile. In the present study we have investigated the effects of three selective P_{2T} receptor antagonists on both aggregation of rat platelets and on ADP-mediated inhibition of adenylyl cyclase in rat platelets and C6-2B cells. In addition, we demonstrate that the C6-2B cells express the $P2Y_{12}$ mRNA. The findings indicate that the $P2Y_{AC}$ receptor is pharmacologically similar to the $P2Y_{12}$ receptor on platelets.

Methods

Materials

Myo-[2-^{3}H] inositol, [^{3}H]-adenine, and [^{14}C]-cyclic AMP were from NEN Life Science Products (Boston, MA, U.S.A.). Fibrinogen, PGI_2, ADP, A2P5P, A3P5P and A3P5PS were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). AR-C70300, AR-C69581 and AR-C78511 were all synthesized by the Department of Medicinal Chemistry, AstraZeneca R&D Charnwood, Loughborough, U.K.

Cell culture

1321N1 astrocytoma cells and C6-2B rat glioma cells were a gift from T.K. Harden, Department of Pharmacology, University of North Carolina, Chapel Hill, NC, U.S.A. and were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum, 1% penicillin/streptomycin at 37°C with 5% CO_2. The $P2Y_1$ receptor transfected cells were grown in the same medium supplemented with 500 μg ml^{-1} G418. Experiments were carried out in confluent cultures 2 days after plating out in 12-well plates.

Stable expression of human $P2Y_1$ receptor in 1321N1 astrocytoma cells

The expression construct (pcDNA3–HP2Y1) containing the short form of the $P2Y_1$ receptor cDNA (Ayyanathan *et al*., 1996) was used to transfect 1321N1 astrocytoma cells using lipofectamine as described previously (Akbar *et al*., 1996). 1321N1 astrocytoma cells were also transfected with pcDNA3 to serve as a control. The medium was replaced after 6 h with fresh medium containing 0.5 mg ml^{-1} G418. Stable transfectants were selected on medium containing 0.5 mg ml^{-1} G418 and screened for the expression of the $P2Y_1$ receptor by second messenger (inositol phosphate; InsP) generation. One clone (HP2Y1-1), out of six clones selected, was chosen for further characterization.

Measurements of inositol phosphates (InsP)

The inositol phosphates were measured essentially as described (Filtz *et al*., 1994; Kunapuli *et al*., 1997). Confluent cultures of HP2Y1-1 cells in 12-well plates were labelled with 1 μCi ml^{-1} of myo-[2-^{3}H]-inositol in inositol-free DMEM for 24 h. Labelled cells were washed once, the medium was replaced with 890 μl of [^{3}H]-inositol-free 20 mM HEPES-buffered Eagle's medium, pH 7.4, and the cells were incubated at 37°C for 30 min before proceeding. This step helps to reduce background levels of [^{3}H]-inositol phosphates, so that agonist-stimulated accumulation could be detected more easily. After incubation for 30 min at 37°C, 10 μl of 1 M LiCl were added to a final concentration of 10 mM and the incubation continued for an additional 10 min. The cells were stimulated with 100 μl of ADP at a final concentration of 10 μM for 15 min and the reaction was terminated by aspiration of the medium, addition of 0.75 ml of 10 mM formic acid and incubation at room temperature for 30 min. The solution containing the extracted InsP was neutralized and diluted with 3 ml of 10 mM NH_4OH (yielding a final pH of 8–9) and then applied directly to a column containing 0.7 ml of the anion exchange resin, AG 1-X8. The column was washed with 4 ml of 40 mM ammonium formate, pH 5.0, to remove the free inositol and the glyceroinositol. Total InsPs were eluted with 4 ml of 2 M ammonium formate, pH 5.0. One ml of the eluate was counted with 9 ml of scintillation fluid. Results presented are an average of three–five independent experiments.

Platelet preparation

Cyclic AMP measurements and aggregation on chronologue Rats (Sprague-Dawley) were anaesthetized with Isoflurane, an inhalation anaesthetic. Entry was gained into the abdominal cavity through a transverse incision inferior to the diaphragm. The heart was exposed by cutting through the sternum to the clavicle. Syringes (1 ml) prepared with 100 μl of ACD (acid/citrate/dextrose) buffer were used to draw blood from the heart. Platelet rich plasma was acquired by stepped centrifugation of the whole blood, for maximal

platelet content. Platelet rich plasma (PRP) was aspirinated (1 mM) for 1 h at 37°C. Platelets were isolated from plasma by centrifugation at $900 \times g$ for 10 min, then resuspended in Tyrode's buffer with 20 μg ml^{-1} apyrase.

96-well plate method Suspensions of washed platelets were prepared by differential centrifugation of human blood obtained from either healthy male or female volunteers by venepuncture using 1/10 volume 3.2% trisodium citrate as anti-coagulant or from Isoflurane anaesthetized rats from the dorsal aorta using 1/10 volume 3.2% trisodium citrate as anti-coagulant. A two-stage washing procedure was used with prostacyclin (PGI_2, 300 ng ml^{-1}) included at each stage to prevent platelet activation (Humphries *et al*., 1994). The final suspension in calcium-free Tyrode's buffer was adjusted to a platelet count of 2×10^5 μl^{-1} and stored in a capped syringe at 8°C for at least 2 h prior to use in aggregation experiments.

Platelet aggregation

Chronolog Platelet aggregation (0.5 ml sample volume) was measured in a Chronolog lumi-aggregometer with stirring at 37°C.

96-well plate method Aliquots (150 μl) of platelet suspensions containing $CaCl_2$ (1 mM) and human fibrinogen (0.2 mg ml^{-1}) were added to individual wells of 96-well plates. The plate was read (R1) at 650 nm to establish baseline. Saline or the appropriate solution of test compound was added to each well and the plate was then shaken for 5 min on an orbital shaker on setting 10 and read (R2) at 650 nm. Saline or ADP (3 or 30 μM) was then added to each well and the plate shaken for a further 5 min before reading (R3) again at 650 nm.

Responses were measured as extent of aggregation calculated as follows after subtraction of baseline values:-

$$\%\ \text{aggregation for each well } ((R2 - R3)/R2) * 100$$

Effects on ADP-induced platelet aggregation were calculated as % inhibition. The concentration of compound producing 50% inhibition (pIC_{50}) was derived by graphical interpolation.

Measurement of cyclic AMP

Cells were incubated with 2 μCi ml^{-1} [^{3}H]-adenine (25 Ci mmol^{-1}) for 2 h at 37°C. The cells were washed once with DMEM. The medium was replaced by medium containing 100 μM forskolin. Various concentrations of agonist and antagonist were added after 1 min 30 s. The reactions were terminated by addition of 1 M HCl containing 2000 c.p.m. of [^{14}C]-cyclic AMP (2 GBq mmol^{-1}) as a recovery standard. Cyclic AMP levels were determined as described earlier (Daniel *et al*., 1998) and expressed as percentage of total [^{3}H]-adenine nucleotides.

Reverse transcription-coupled polymerase chain reaction (RT–PCR)

The total RNA was isolated from C6-2B cells by RNAzol procedure (Tel-Test Inc., Friendswood, TX, U.S.A.) and the cDNA was prepared from 5 μg of total RNA using the first strand synthesis kit (Gibco-BRL, Gaithersburg, MD, U.S.A.). As a control, total RNA without reverse transcription was used to eliminate the possible contamination of genomic DNA in the RNA preparation. The PCR was carried out using forward and reverse primers specific for rat $P2Y_{12}$ receptor cDNA [GenBank Accession No. AF313450] (Hollopeter *et al*., 2001). The forward primer was 5′-CAGGTTCTCTTCCCATTGCT-3′ (corresponds to 214–233 nt) and the reverse primer was 5′-CAGCAATGATGAT-GAAAACC-3′ (852–871 nt). After initial denaturation for 5 min at 94°C the amplifications were carried out for 35 cycles using 5.0 units of *pfu* DNA polymerase as follows: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The final cycle was followed by an additional extension for 7 min at 72°C.

Results

Effects of three P_{2T} receptor antagonists on ADP-induced platelet aggregation

Subsequent to the characterization of AR-C66096 as an antagonist at the human platelet $P2T_{AC}$ receptor (Humphries *et al*., 1994; Daniel *et al*., 1998; Jin *et al*., 1998), a number of other selective P_{2T} receptor antagonists have been synthesized. The structures of the compounds used in the present study are shown in Figure 1. The compounds were chosen because of the non-overlapping potency for inhibition of ADP-induced platelet aggregation. All these compounds inhibited ADP (30 μM)-induced aggregation of human washed platelets in a concentration-dependent manner (Figure 2A). The rank order of antagonist potency was AR-C78511 > AR-C69581 > AR-C70300 (Table 1). Since the C6-2B cells are of rat origin, we investigated the effect of these compounds on ADP-induced aggregation of rat washed platelets. As shown in Figure 2B and Table 1, all these compounds inhibited ADP (3 μM) -induced aggregation in a concentration-dependent manner, with an identical rank order of antagonist potency (AR-C78511 > AR-C69581 > AR-C70300) to that seen in human platelets. Since platelet aggregation can be blocked either byantagonizing the $P2Y_1$ receptor or the $P2T_{AC}$ receptor (Jin & Kunapuli, 1998), we investigated the effect of these compounds on ADP-induced platelet shape change. When platelets are stimulated with agonists they first undergo change in their shape from smooth discs to spiculated spheres, resulting in a decrease in light transmission in an aggregometer. Upon aggregation, when platelets are cross-linked through fibrinogen bound to activated integrin αIIbβ3, and settle to the bottom of the cuvette, the light transmission increases. As shown in Figure 3, all three compounds inhibited ADP (3 μM)-induced rat platelet aggregation but not shape change, suggesting that they inhibit only the $P2T_{AC}$ receptor but not the $P2Y_1$ receptor.

Effects of three P_{2T} receptor antagonists at the $P2Y_1$ receptor

In order to investigate the effect of these compounds at the $P2Y_1$ receptor, we developed a 1321N1 astrocytoma cell line expressing the human $P2Y_1$ receptor. ADP caused inositol

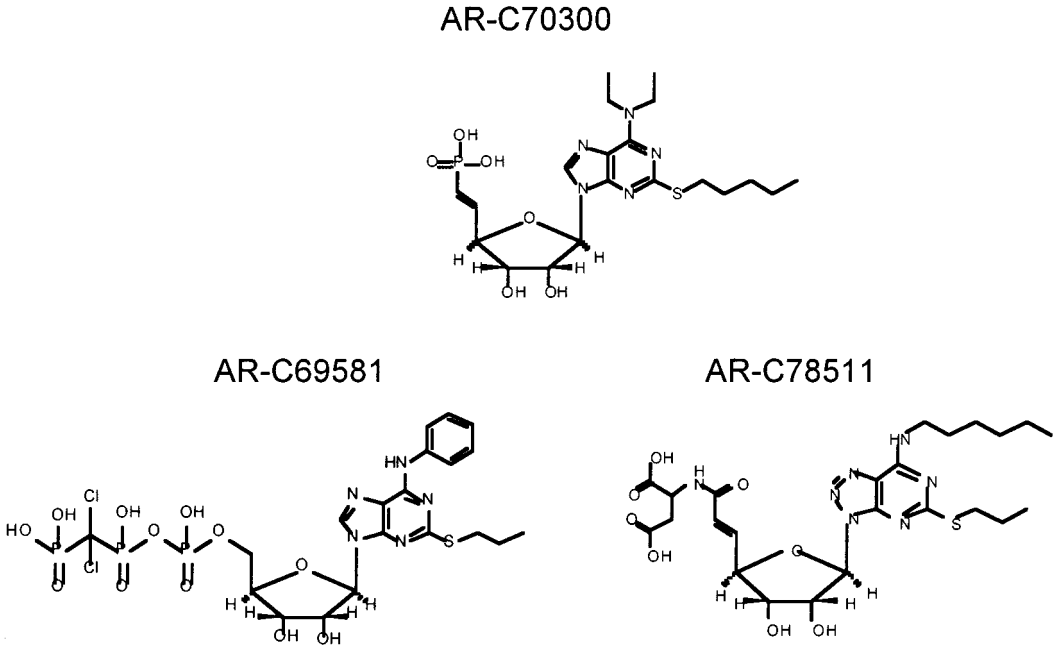

Figure 1 Chemical structures of the P_{2T} receptor antagonists used in the present study.

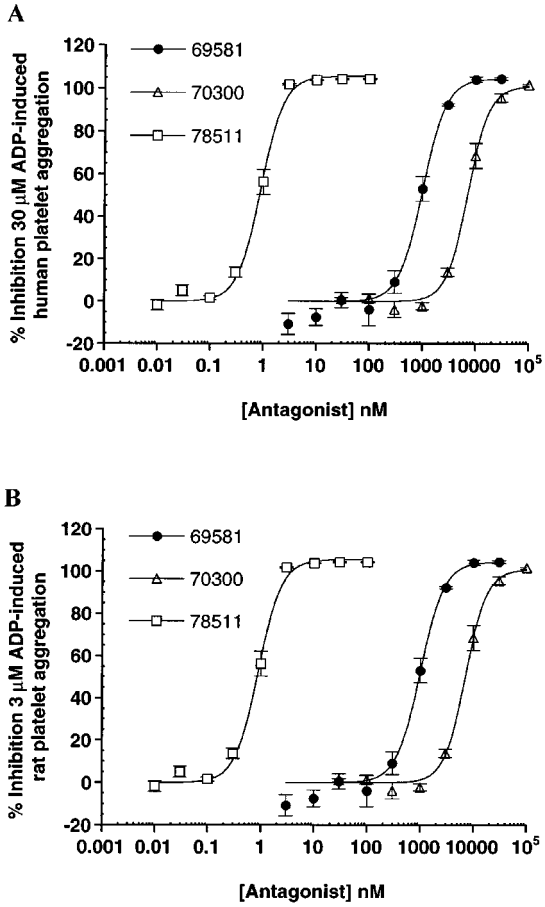

Figure 2 Effect of three AR-C compounds on ADP-induced (A) human and (B) rat platelet aggregation. Platelet aggregation studies were performed as described in the Methods in the presence or absence of different concentrations of AR-C78511, AR-C69581 or AR-C70300 (as marked) with varying concentrations of ADP.

Table 1 Comparison of pIC_{50} values for inhibition of ADP-induced platelet aggregation

Compound	*Human platelets*	*Rat platelets*
AR-C78511	8.5±0.0	9.1±0.0
AR-C69581	6.2±0.1	6.0±0.0
AR-C70300	5.4±0.0	5.1±0.0

Values are mean±s.e.mean ($n=4$). The values are derived from data presented in Figure 2.

phosphate formation in these cells and the $P2Y_1$ selective antagonists, A3P5PS, A3P5P and A2P5P, significantly blocked ADP-induced inositol phosphate formation (not shown). The $P2Y_1$ receptor selective antagonist, A3P5PS, caused a rightward shift of the ADP concentration–dependence curve, consistent with the competitive nature of A3P5PS at the $P2Y_1$ receptor (Figure 4). As shown in Figure 5, the P_{2T} receptor antagonists, AR-C78511, AR-C69581 and AR-C70300, had no effect on ADP-stimulated inositol phosphate formation in $P2Y_1$ receptor-expressing astrocytoma cells. These data clearly demonstrate that these three compounds do not antagonize the stimulation of the $P2Y_1$ receptor by ADP in the concentration range (AR-C78511 and AR-C69581 up to 1 μM, and AR-C 70300 up to 300 μM) tested.

Effects of three P_{2T} receptor antagonists on ADP-induced reduction in [cyclic AMP] in rat platelets and C6-2B glioma cells

To determine if the platelet $P2T_{AC}$ receptor is the same as the P2Y receptor in C6-2B cells coupled to inhibition of adenylyl

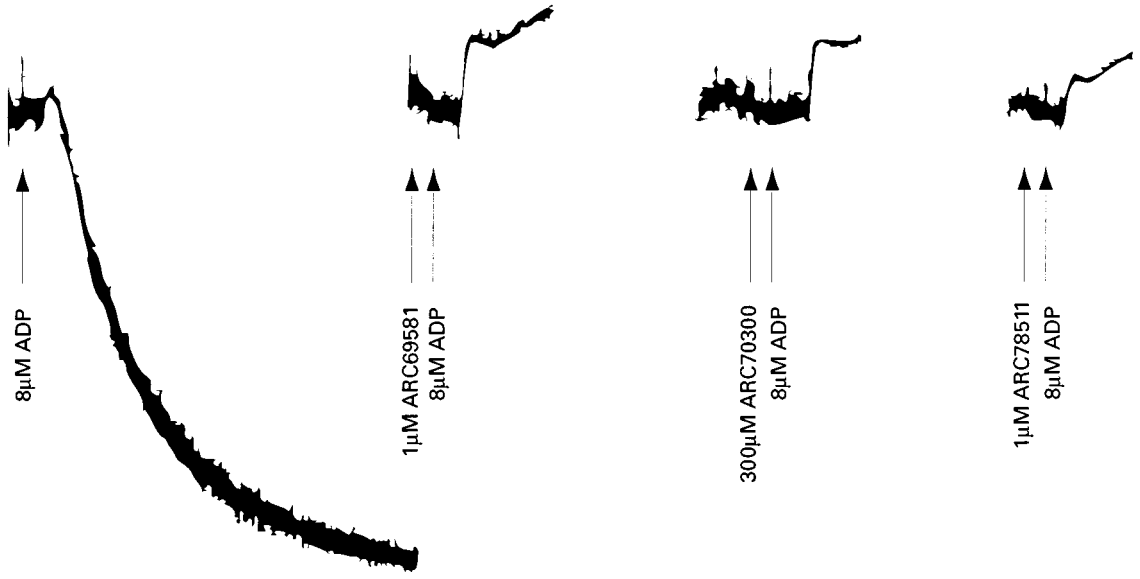

Figure 3 Effect of P_{2T} receptor antagonists on ADP-induced aggregation of rat platelets. Rat blood was collected by cardiac puncture. Aspirin-treated washed platelets were stimulated with ADP in the presence or absence of AR-C78511, AR-C69581 or AR-70300 in a Chronolog lumi-aggregometer with stirring at 37°C. The additions are indicated by arrows.

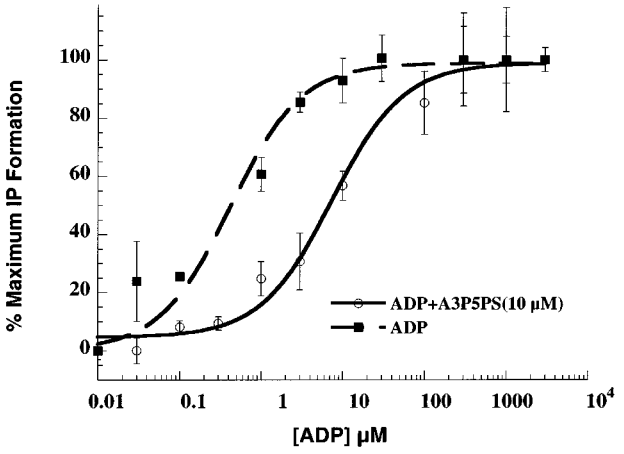

Figure 4 Effect of A3P5PS on ADP-induced inositol phosphate formation in astrocytoma cells stably expressing the human $P2Y_1$ receptor. Effect of A3P5PS on ADP-induced inositol phosphate formation was determined at various ADP concentrations in the presence and absence of a single dose of 10 μM A3P5PS. The data are normalized to response by maximum concentration of ADP in the absence of the antagonist (taken as 100%).

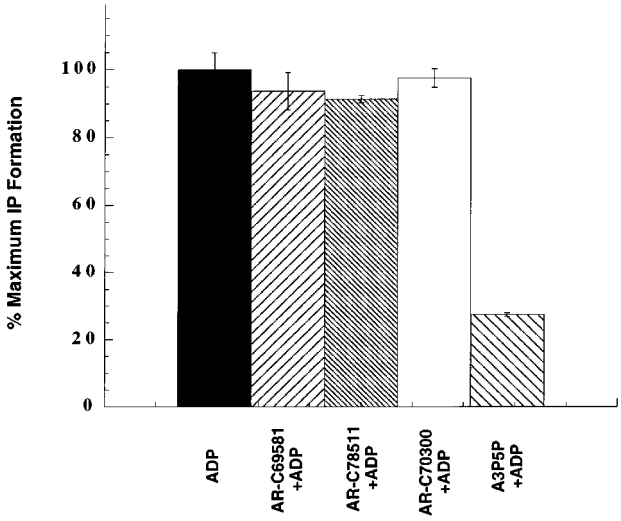

Figure 5 Effect of $P2Y_1$ and P_{2T} receptor selective antagonists on ADP-induced inositol phosphate formation in astrocytoma cells stably expressing the human $P2Y_1$ receptor. Human $P2Y_1$ receptor was stably expressed in 1321N1 astrocytoma cells and ADP-induced total inositol phosphate formation was measured as described in the methods section. The measurements were made in confluent monolayers of cells in 24-well plates in triplicate. The antagonists, AR-C 78511 (1 μM), AR-C 69581 (1 μM), AR-C 70300 (300 μM), or A3P5P (1 mM), were added to the cells immediately before the addition of 10 μM ADP and incubated for 10 min at 37°C. The results were normalized to total inositol phosphate formation with 10 μM ADP (taken as 100%).

cyclase, we used the three AR-C compounds to inhibit ADP-induced responses in rat platelets and C6-2B cells. We used rat platelets, instead of human platelets, since C6-2B cells are derived from rat brain. These antagonists blocked ADP-induced inhibition of forskolin-stimulated adenylyl cyclase in rat platelets (Figure 6A) and C6-2B cells (Figure 6B) with similar antagonist profiles. The rank order of antagonist potency to block ADP-induced adenylyl cyclase in rat platelets is identical to that in C6-2B cells (AR-C78511 > AR-C69581 > AR-C70300; Table 2).

Detection of the $P2Y_{12}$ receptor mRNA in C6-2B cells

Since pharmacological evidence indicated that the C6-2B cell $P2Y_{AC}$ receptor is similar to the $P2Y_{12}$ receptor, and the rat $P2Y_{12}$ receptor is cloned from platelets, we have investigated

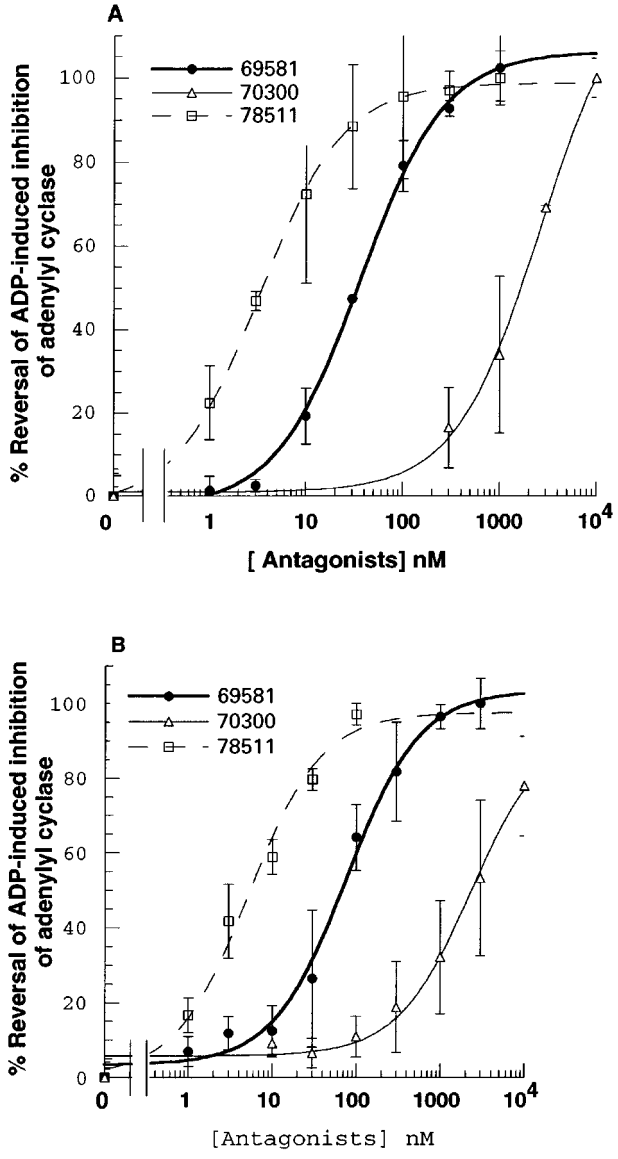

Figure 6 Effect of P_{2T} receptor antagonists on ADP-induced inhibition of adenylyl cyclase in (A) rat platelets and (B) C6-2B cells. Effect of varying concentrations of AR-C78511, AR-C69581 or AR-C70300 (as marked) on ADP (10 μM)-induced inhibition of forskolin (100 μM)-stimulated adenylyl cyclase activity in rat platelets and C6-2B cells was determined as described in the Methods section. The data are normalized to the maximum response obtained with each antagonist (taken as 100%).

Table 2 Comparison of pIC_{50} values for reversal of ADP-induced inhibition of adenylyl cyclase in rat platelets and C6 cells

Compound	*C6 cells*	*Rat platelets*
AR-C78511	8.6±0.1	8.2±0.0
AR-C69581	7.1±0.2	7.4±0.0
AR-C70300	5.3±0.5	5.3±0.2

Values are mean±s.e.mean (n=3). The values are derived from data presented in Figure 6.

the expression of the $P2Y_{12}$ receptors in C6-2B cells. The RNA (5 μg) from C6-2B cells was analysed for the $P2Y_{12}$ receptor by RT–PCR analysis. As shown in Figure 7, ethidium bromide staining of the 657 bp PCR product reveals that the C6-2B cells express the $P2Y_{12}$ receptor mRNA. Nucleotide sequence analysis of the PCR product revealed its identity with the rat $P2Y_{12}$ receptor (GenBank Accession No. AF313450) (Hollopeter *et al*., 2001). The PCR product could have originated from the contaminating genomic DNA. To rule out this possibility we carried out PCR using RNA without reverse transcription which did not amplify any PCR product (Figure 7).

Discussion

Platelet responses to ADP are mediated by at least three P2 receptor subtypes: $P2X_1$; $P2Y_1$; $P2T_{AC}$. The $P2T_{AC}$ receptor has been demonstrated pharmacologically (Kunapuli, 1998) and a recent report indicated that this receptor has been cloned and designated $P2Y_{12}$ (Hollopeter *et al*., 2001). Most of the G protein-coupled P2 receptors activate phospholipase C whereas stimulation of the P2Y receptors in C6 glioma cells and B10 microvascular endothelial cells causes inhibition of adenylyl cyclase (Feolde *et al*., 1995; Planet *et al*., 1989). The purpose of this study was: (a) to further characterize the $P2Y_{AC}$ receptor in the C6-2B cells; (b) to compare the pharmacological profiles of the C6 cell $P2Y_{AC}$ receptor and the platelet $P2Y_{12}$ receptor.

We propose that the $P2Y_{AC}$ receptor in C6-2B cells, is the same receptor subtype as the $P2Y_{12}$ receptor in platelets based on the following observations. The agonist profiles of these two receptors are very similar (Boyer *et al*., 1993; Mills, 1996) and ATP has been shown to be an antagonist at the $P2Y_{AC}$ receptor on C6-2B cells (Planet *et al*., 1989) and the $P2Y_{12}$ ($P2T_{AC}$) receptor (Daniel *et al*., 1998). Several selective P_{2T} antagonists, tested in this study, inhibited ADP-induced rat platelet aggregation ($P2T_{AC}$-mediated), but not shape change ($P2Y_1$-mediated). Furthermore, these P_{2T} receptor antagonists blocked ADP-induced inhibition of stimulated adenylyl cyclase in rat platelets and in C6-2B cells, with identical potencies and rank order (Table 2). The possibility

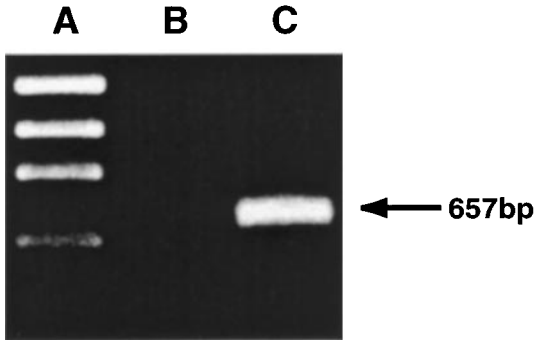

Figure 7 RT–PCR analysis of RNA from C6-2B cells: PCR product electrophoresed on a 1.0% agarose gel and stained with ethidium bromide. PCR was carried out with C6-2B cell total RNA as described in the methods section. A: ϕX174 DNA/*Hae*III marker; B: C6-2B cell RNA–PCR (control for Genomic DNA contamination); C: C6-2B cell RT–PCR.

of confounding influence of effects at the $P2X_1$ receptors can be discounted since, under the assay conditions used the $P2X_1$ receptors would not be activated (Jin & Kunapuli, 1998). These observations indicate that the C6-2B cell $P2Y_{AC}$ receptor is pharmacologically similar to the $P2Y_{12}$ receptor.

Webb *et al*. (1996) identified abundant $P2Y_1$ receptor mRNA in both C6-2B glioma cells and B10 microvascular endothelial cells, and proposed that the P2Y receptor in these cells is the $P2Y_1$ receptor. It was suggested that the $P2Y_1$ receptor could couple to different G proteins in different cells (Webb *et al*., 1996). Since the pharmacological agents used in the study of Webb *et al*. (1996) are not specific for the $P2Y_1$ receptor, we believe that C6-2B cells and B10 cells express mRNAs for at least two P2Y receptor subtypes: the $P2Y_1$ receptor, that was detected by RT–PCR and at least one other P2Y receptor coupled to inhibition of adenylyl cyclase. Boyer *et al*. (1994) demonstrated differential effects of a non specific P2 receptor antagonist, PPADS, on the turkey erythrocyte P2Y receptor, coupled to phospholipase C, and the C6-2B $P2Y_{AC}$ receptor. PPADS inhibited nucleotide-induced phospholipase C activation in turkey erythrocytes, while even at 100 μM it had no effect on agonist-induced adenylyl cyclase inhibition in C6-2B cells (Boyer *et al*., 1994). It was argued that the substitution of 41-lysine by arginine in the rat $P2Y_1$ receptor might prevent a Schiff's base formation with PPADS, an essential step in antagonism by PPADS. However, Schachter *et al*. (1997) demonstrated that PPADS antagonizes the rat $P2Y_1$ receptor.

Based on the studies of Webb *et al*. (1996), Gachet *et al*. (1997) suggested that the $P2Y_1$ receptor in platelets couples to inhibition of adenylyl cyclase. However, we have shown that in human platelets AR-C66096 (previously FPL or ARL 66096) failed to inhibit ADP-induced mobilization of calcium from intracellular stores, while blocking the ADP-induced inhibition of adenylyl cyclase (Daniel *et al*., 1998; Jin *et al*., 1998). Even at high concentrations, A3P5PS, A3P5P, and A2P5P did not block ADP-induced inhibition of adenylyl cyclase in platelets (Jin *et al*., 1998). Furthermore, in platelets of mice deficient in the $P2Y_1$ receptor, ADP causes inhibition of adenylyl cyclase (Leon *et al*., 1999, Fabre *et al*., 1999) confirming that the receptor coupled to Gi signalling in platelets is distinct from the $P2Y_1$ receptor. The recent cloning of the Gi coupled brain and platelet P2Y receptors (Zhang *et al*., 2001; Hollopeter *et al*., 2001), further demonstrate that the Gi coupled receptors are molecularly distinct from the $P2Y_1$ receptor.

It was recently shown that stem cells could be differentiated into glial cells (the origin of C6 cells) when treated with hematopoietic growth factors and cytokines (Reyes & Verfaillie, 1999). In addition, brain cells have been shown to differentiate into hematopoietic cells (Bjornson *et al*., 1999). Hence, it is conceivable that C6 cells, derived from glial cells, and platelets, a hematopoietic cell, have at least some common signal transduction mechanisms, including the P2 receptor subtype coupled to Gi. Although pharmacological evidence suggests that the $P2Y_{12}$ and the $P2Y_{AC}$ receptors are similar, only molecular cloning of these receptors could provide conclusive evidence. To achieve this goal, we used RT–PCR of the C6-2B cell mRNA using primers specific for the rat $P2Y_{12}$ receptor and confirmed the identity of the RT–PCR product by nucleotide sequence analysis. Our data demonstrate that the $P2Y_{12}$ mRNA is expressed in C6-2B cells. This molecular evidence, coupled with the pharmacological data, indicates that the C6-2B cell $P2Y_{AC}$ receptor is identical to the platelet $P2Y_{12}$ receptor.

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