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Benzoyl ATP Is an Antagonist of Rat and Human $P2Y_1$ Receptors and of Platelet Aggregation

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The effects of 2′- and 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) on intracellular Ca^{2+} mobilization and cyclic AMP accumulation were investigated using rat brain capillary endothelial cells which express an endogenous $P2Y_1$ receptor, human platelets which are known to express a $P2Y_1$ receptor, and Jurkat cells stably transfected with the human $P2Y_1$ receptor. In endothelial cells, BzATP was a competitive inhibitor of 2-methylthio ADP (2-MeSADP) and ADP induced $[Ca^{2+}]$i responses (Ki = 4.7 μM) and reversed the inhibition by ADP of adenylyl cyclase (Ki = 13 μM). In human platelets, BzATP inhibited ADP-induced aggregation (Ki = 5 μM), mobilization of intracellular Ca^{2+} stores (Ki = 6.3 μM), and inhibition of adenylyl cyclase. In $P2Y_1$-Jurkat cells, BzATP inhibited ADP and 2-MeSADP-induced $[Ca^{2+}]$i responses (Ki = 2.5 μM). It was concluded that BzATP is an antagonist of rat and human $P2Y_1$ receptors and of platelet aggregation. In contrast to other $P2Y_1$ receptor antagonists (A2P5P and A3P5P) which inhibit only ADP-induced Ca^{2+} mobilization, BzATP inhibits both the Ca^{2+}- and the cAMP-dependent intracellular signaling pathways of ADP. These results provide further evidence that $P2Y_1$ receptors contribute to platelet ADP responses. © 1999 Academic Press

Purinergic responses of the P2 type are mediated by two classes of membrane receptor, ionotropic P2X and metabotropic P2Y receptors, which comprise proteins of variable molecular structure (1, 2). $P2Y_1$ receptors from chicken (3), turkey (4), bovine (5), rat, mouse (6), and human (7) species have now been structurally identified.

Several antagonists of $P2Y_1$ receptors have been described to date: pyridoxal phosphate-6-azophenyl-2′,4′ disulfonic acid (PPADS) (8, 9), adenosine-2′-phosphate-5′-phosphate (A2P5P), adenosine-3′-phosphate-5′-phosphate (A3P5P) and their sulfate derivatives (10,11), ATP and its 2- substituted derivatives, 2-chloro ATP (2-ClATP) and 2-methylthio ATP (2-MeSATP) (12, 13) and *N*6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate (14). The $P2Y_1$ receptor is now believed to play a role in ADP induced platelet activation by mediating mobilization of intracellular Ca^{2+} stores (12, 13, 15–17).

This paper describes the effects of 2′- and 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) on purinoceptor responses. BzATP is a selective agonist of the pore forming P2Z receptor now identified as the $P2X_7$ receptor (18) and has also been reported to be a full agonist of the $P2Y_1$ receptor of turkey erythrocytes (10, 19). The present results show that BzATP is on the contrary an antagonist of the endogenous $P2Y_1$ receptor expressed by rat endothelial cells, of the human $P2Y_1$ receptor expressed in Jurkat cells and of platelet aggregation.

MATERIALS AND METHODS

Chemicals. ATP, ADP, BzATP, prostaglandin E_1 (PGE_1), isobutyl methyl xanthine, forskolin and cholera toxin were from Sigma Chemicals (St. Quentin-Fallavier, France). Indo-1/AM and fura-2/AM were from Calbiochem (Meudon, France). 2-MeSADP was from Research Biochemicals International (Natick, USA) and adenosine 5′-*O*-(1-thiotriphosphate) (Sp isomer) (ATPaS) from Boehringer (Mannheim, Germany).

Intracellular Ca^{2+} measurements. Procedures for the preparation of cells of the B7 (20) and B10 clones (21), of Jurkat cells stably transfected with the human $P2Y_1$ receptor ($P2Y_1$-Jurkat cells) (12) and washed human platelets (11) have been described previously. Intracellular Ca^{2+} measurements in intact cells were performed using flow cytometric analysis of indo-1 loaded cells (12, 22). Human platelet rich plasma was centrifuged at 1570*g* for 15 min and the platelet pellet was resuspended in albumin and Ca^{2+} free Tyrode's buffer at a density of about 6×10^5 platelets μl^{-1}. The cells were loaded with 2 μM fura-2/AM for 45 min at 37°C in the dark, washed in Tyrode's buffer containing 0.35% human serum albumin and finally resuspended at 37°C at a density of 3×10^5 platelets μl^{-1} in Tyrode's buffer containing 0.02 units ml^{-1} apyrase, 0.1% essentially fatty acid free human serum albumin and 2 mM Ca^{2+}. Aliquots of fura-2 loaded platelets were transferred to 10 × 10-mm quartz

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cuvettes maintained at 37°C. Fluorescence measurements were performed under continuous stirring, in a PTI Deltascan spectrofluorometer (Photon Technology International Inc, Princeton, NJ). The excitation wavelength was alternately fixed at 340 and 380 nm and fluorescence emission was recorded at 510 nm. Platelets were stimulated with 0.3 μM ADP in the presence or absence of BzATP and the fura-2 fluorescence ratio was determined at the peak of the transient intracellular Ca^{2+} rise.

Adenylyl cyclase assay. B7 cells were incubated at 37°C in Earle's salt solution containing 100 ng ml^{-1} cholera toxin. After 30 min, 1 mM isobutyl methyl xanthine was added, followed by agonists 15 min later and incubation was continued for a final 15 min. The reaction was stopped by addition of 10% (v/v) ice-cold 6.6 N perchloric acid and the incubation solution was eliminated by centrifuging the tubes at 2000*g* for 30 s.

A 450-μl aliquot of washed platelet suspension was stirred at 1100 rpm in an aggregometer cuvette and the following reagents were added at 30-s intervals: 10 μM PGE_1 (or vehicle), 100 μM BzATP or 100 μM ATPaS (or vehicle) and 5 μM ADP (or vehicle), where the vehicle was Ca^{2+} and Mg^{2+} free Tyrode's buffer. One minute later, the reaction was stopped by addition of 50 μl ice-cold 6.6 N perchloric acid.

Perchloric acid extracts were centrifuged at 11,000*g* for 5 min to remove protein precipitate. cAMP was quantified by radioimmunoassay using commercial kits (Immunotech, Luminy, France or Amersham, Les Ulis, France) and cell proteins were determined according to Bradford (23).

Platelet aggregation. Platelet aggregation was measured at 37°C by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). A 450-μl aliquot of washed platelet suspension was stirred at 1100 rpm and activated by addition of agonists to a final volume of 500 μl.

Data analysis. All experiments were carried out in triplicate and were repeated at least three times and results are reported as the overall mean (±SEM). On the figures, which show representative plots, when no error bars are visible these were smaller than the size of the points. Dose-response curves were fitted to a logistic equation using Sigma plot software, while Ki values were determined either by generating Schild plots or through the Cheng-Prüssoff relationship.

RESULTS

Effects of BzATP in endothelial cells. Two clones of endothelial cells were employed, B7 cells which express both $P2Y_1$ and $P2Y_2$ receptors and B10 cells which express only $P2Y_1$ receptors (20, 21, 24). It was first confirmed that BzATP alone (up to 1 mM) had no influence on $[Ca^{2+}]i$ levels in either B7 or B10 cells.

2-MeSADP, a potent and selective agonist of the $P2Y_1$ receptor of B7 cells, produced a half-maximum increase in $[Ca^{2+}]i$ at a concentration (EC_{50}) of 100 ± 10 nM (n = 4). BzATP inhibited the action of 2-MeSADP by shifting its dose–response curve to higher concentrations (Fig. 1A). In three independent experiments, the inhibitory constant (Ki) for BzATP estimated from Schild plots was 3.2 ± 0.7 μM. Identical results were obtained in B10 cells, where EC_{50} values for 2-MeSADP were 30 nM, 0.2 μM and 3 μM in the presence of 0, 10 μM and 100 μM BzATP respectively (data not shown) and the Schild plot indicated a Ki value of 2.8 μM. Dose–response curves were also determined for the inhibition by BzATP of the response to 1 μM 2-MeSADP in B7 cells (Fig. 1B). Half maximum inhibition was observed at 25 μM BzATP. A Ki value of 7.3 ± 2.3 μM was calculated from the results of three independent experiments using the Cheng-Prüsoff relationship.

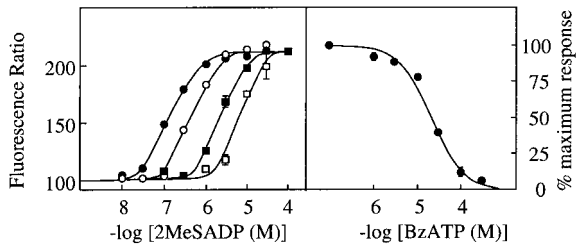

FIG. 1. Inhibitory actions of BzATP in endothelial cells. (Left) Indo-1-loaded B7 cells were stimulated with the indicated concentrations of 2-MeSADP and of BzATP and indo-1 fluorescence was measured after 15 s. EC_{50} values for 2-MeSADP were 0.15 μM (control, ●), 0.5 μM (10 μM BzATP, ○), 3 μM (100 μM BzATP, ■), and 8 μM (300 μM BzATP, □). A Schild plot of the data had a slope close to unity and indicated a Ki value for BzATP of 4.6 μM. (Right) Dose–response curve for the inhibition by BzATP of 2-MeSADP induced $[Ca^{2+}]i$ responses in endothelial cells.

ADP is the natural agonist of $P2Y_1$ receptors. Although high concentrations of ADP were previously found to activate $P2Y_2$ receptors on B7 cells (20), this is now known to be due to trace amounts of ATP contaminating the ADP solutions (25). The effects of BzATP on ADP responses were examined using B10 cells, which express only $P2Y_1$ receptors (24). BzATP inhibited the action of ADP by shifting its dose–response curve to higher concentrations, without modifying its maximum efficacy. EC_{50} values for ADP were 1, 3, and 20 μM in the presence of 0, 10, and 100 μM BzATP, respectively (data not shown) and the Schild plot, of slope close to unity, indicated a Ki of 5.6 μM for BzATP. Dose-response curves for the inhibition by BzATP of the response to 3 μM ADP gave a similar Ki value of 4.8 ± 0.2 μM in three independent experiments.

Thus, BzATP acted as a competitive inhibitor of the effects of ADP and 2-MeSADP on endothelial cells, with an overall Ki value of 4.7 ± 0.8 μM as estimated from five different types of experiment.

ADP inhibits cholera toxin stimulated adenylyl cyclase in endothelial cells and its action is prevented by Pertussis toxin (24). BzATP did not stimulate cAMP formation in B7 cells (Table 1), whereas addition of cholera toxin increased cAMP levels by 60 fold and this effect was partially inhibited by 30 μM ADP. The IC_{50} value for ADP was 7.9 ± 2.5 μM (n = 6). BzATP partially reversed the action of ADP (Table 1) and dose–response curves for the inhibition by BzATP of the response to 30 μM ADP in B7 cells gave an IC_{50} value of 60 μM and a Ki value of 13 μM.

Effects of BzATP on human platelets. BzATP inhibited ADP induced aggregation of human platelets. This inhibition resulted from a shift of the ADP dose–

TABLE 1

BzATP Inhibits the Coupling of ADP Receptors to Adenylyl Cyclase in B7 Cells

Additions	cAMP (nmol/mg protein)
Control	0.11 ± 0.01 (n = 3)
BzATP (0.3 mM)	0.14 ± 0.02 (n = 3)
Cholera toxin	6.13 ± 0.14 (n = 6)
Cholera toxin + ADP (30 μM)	3.23 ± 0.17 (n = 6)
Cholera toxin + ADP (30 μM) + BzATP (0.3 mM)	4.84 ± 0.13 (n = 6)

Note. n denotes the number of experiments.

TABLE 2

BzATP Inhibits the Coupling of ADP Receptors to Adenylyl Cyclase in Human Platelets

Additions	cAMP (pmol/10^8 platelets)
Control	3.45 ± 0.09
BzATP (0.1 mM)	3.36 ± 0.08
PGE_1 (10 μM)	25.39 ± 0.75
PGE_1 (10 μM) + BzATP (0.1 mM)	25.16 ± 0.52
PGE_1 (10 μM) + ADP (5 μM)	7.36 ± 0.34
PGE_1 (10 μM) + ADP (5 μM) + BzATP (0.1 mM)	18.88 ± 0.20
PGE_1 (10 μM) + ADP (5 μM) + ATPαS (0.1 mM)	22.50 ± 0.48

Note. Values are means ± SEM from four independent experiments.

response curve to higher concentrations (Fig. 2A) and a Schild plot of the data indicated a Ki value of 5 μM.

It is now well established that ADP triggers both Ca^{2+} and cAMP dependent signaling pathways in platelets. BzATP inhibited the $[Ca^{2+}]$i rise induced by 0.3 μM ADP in fura-2 loaded platelets (Fig. 2B), with an IC_{50} value of 12.5 μM and a Ki value of 6.3 μM. Although without effect on basal or PGE_1 stimulated formation of cAMP in human platelets, BzATP partially reversed the ADP induced inhibition of cAMP formation in PGE_1 activated platelets (Table 2). The same result was obtained using ATPαS, a well known inhibitor of ADP induced aggregation, intracellular Ca^{2+} increases and adenylyl cyclase inhibition in human platelets (26, 27). Thus, BzATP inhibited ADP induced platelet aggregation by intervening in both Ca^{2+} and cAMP dependent intracellular signaling pathways.

Effects of BzATP on $P2Y_1$-Jurkat cells. ADP and 2-MeSADP induce the mobilization of intracellular Ca^{2+} stores in $P2Y_1$-Jurkat cells (13), with EC_{50} values of 200 ± 60 nM (n = 3) and 10 ± 3 nM (n = 3) respectively. BzATP itself had no influence on $[Ca^{2+}]$i levels but inhibited the effects of ADP and 2-MeSADP (Fig. 3). The Ki value for BzATP was calculated to be 3.8 ± 0.4 μM (n = 3) in experiments using ADP and 1.2 ± 0.5 μM (n = 3) in experiments using 2-MeSADP. As in previous work, no positive or negative coupling of $P2Y_1$ receptors to adenylyl cyclase could be detected in $P2Y_1$-Jurkat cells (11).

Thus, BzATP was an antagonist of the human $P2Y_1$ receptor expressed by $P2Y_1$-Jurkat cells, having a mean Ki value of 2.5 μM as estimated from two types of independent experiment.

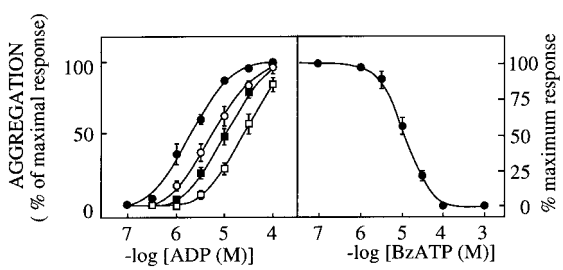

FIG. 2. Inhibitory actions of BzATP human platelets. (Left) Aggregation of washed human platelets was induced by addition of the indicated concentrations of ADP in the presence of BzATP. EC_{50} values for ADP were 1.8 μM (control, ●), 12.4 μM (10 μM BzATP, ○), 26 μM (30 μM BzATP, ■), and 37 μM (100 μM BzATP, □). A Schild plot of the data had a slope of 0.8 and indicated a Ki value for BzATP of 5 μM. (Right) Fura-2 loaded platelets were stimulated with 0.3 μM ADP in the presence of 2 mM extracellular Ca^{2+} and BzATP. Fluorescence ratios were determined at the peak of the transient $[Ca^{2+}]$i rise.

DISCUSSION

Activation by ADP or 2-MeSADP of the endogenous $P2Y_1$ receptor of rat brain capillary endothelial cells or the human $P2Y_1$ receptor stably expressed on Jurkat cells is accompanied by a large increase in $[Ca^{2+}]$i resulting mainly from the mobilization of intracellular Ca^{2+} stores (12, 20). The present study shows that this action of ADP and 2-MeSADP is inhibited by BzATP. All evidence supports the hypothesis that BzATP is a competitive antagonist of ADP and 2-MeSADP actions: (i) BzATP shifts the dose–response curves of these agonists to higher concentrations and (ii) Schild plots

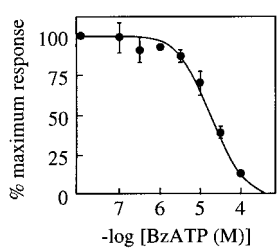

FIG. 3. Inhibitory actions of BzATP in P2Y1-Jurkat cells. Indo-1 loaded $P2Y_1$-Jurkat cells were treated with 0.3 μM ADP and BzATP. Indo-1 fluorescence was measured after 15 s.

of the data have a slope close to unity. BzATP differs from PPADS (22) in that its action is immediate and does not require preequilibration with the cells to be observed. Overall Ki values for BzATP were 4.7 μM in rat endothelial cells and 2.5 μM in $P2Y_1$-Jurkat cells. These values are 5 to 10 times lower than the Ki values that have been previously reported for ATP, 2-ClATP and 2-MeSATP (12, 13). They are similar to the Ki values reported for A2P5P, A3P5P, and their sulfate derivatives (10, 11). They are 20 to 100 times higher than the Ki value for N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate at the turkey $P2Y_1$ receptor (14).

It is noteworthy that BzATP is an agonist rather than an antagonist of turkey $P2Y_1$ receptors (10, 19) and whether this is due to species differences or to receptor expression levels (28) is not yet known. Similar discrepancies have been observed for A3P5P and A3P5PS. These compounds are partial agonists of the turkey $P2Y_1$ receptor on erythrocyte membranes and of the turkey receptor stably expressed in 1321N1 astrocytoma cells (10), but are simple competitive antagonists of the human $P2Y_1$ receptor stably expressed in astrocytoma (10) or Jurkat cells (11) and of the endogenous receptor of rat endothelial cells (11).

In platelets, ADP induces a rapid influx of Ca^{2+} mediated by $P2X_1$ receptors (29, 30), mobilization of intracellular Ca^{2+} stores and inhibition of adenylyl cyclase (26, 27, 31). Similarly, in rat brain capillary endothelial cells of the B10 clone, ADP induces mobilization of intracellular Ca^{2+} stores (21) and inhibition of adenylyl cyclase (24). Recent pharmacological evidence suggests that while ADP induced mobilization of intracellular Ca^{2+} stores is mediated by known $P2Y_1$ receptors, ADP induced inhibition of adenylyl cyclase may be mediated by a yet to be characterized P2TAC receptor (15–17). $P2Y_1$ receptors would be specifically blocked by PPADS, A2P5P and A3P5P (8, 9, 13, 24). P2TAC receptors could be specifically blocked by ARL 66096 (15). This study shows that BzATP differs from other P2Y receptor antagonists (PPADS, A2P5P, A3P5P, and ARL 66096) in that they inhibit both the Ca^{2+} and the cAMP dependent intracellular signaling pathways of ADP in endothelial cells and platelets. This property is shared however by ATP and related analogs such as ATPαS, 2-MeSATP and 2-ClATP (11–13).

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