The P2Y$_{12}$ Antagonists, 2-Methylthioadenosine 5’-Monophosphate Triethylammonium Salt and Cangrelor (ARC69931MX), Can Inhibit Human Platelet Aggregation through a G$_{i}$-independent Increase in cAMP Levels*

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ADP plays an integral role in the process of hemostasis by signaling through two platelet G-protein-coupled receptors, P2Y$_4$ and P2Y$_{12}$. The recent use of antagonists against these two receptors has contributed a substantial body of data characterizing the ADP signaling pathways in human platelets. Specifically, the results have indicated that although P2Y$_4$ receptors are involved in the initiation of platelet aggregation, P2Y$_{12}$ receptor activation appears to account for the bulk of the ADP-mediated effects. Based on this consideration, emphasis has been placed on the development of a new class of P2Y$_{12}$ antagonists (separate from clopidogrel and ticlopidine) as an approach to the treatment of thromboembolic disorders. The present work examined the molecular mechanisms by which two of these widely used adenosine-based P2Y$_{12}$ antagonists (2-methylthioadenosine 5’-monophosphate triethylammonium salt (2MeSAMP) and ARC69931MX), inhibit human platelet activation. It was found that both of these compounds raise platelet cAMP to levels that substantially inhibit platelet aggregation. Furthermore, the results demonstrated that this elevation of cAMP did not require G$_i$ signaling or functional P2Y$_{12}$ receptors but was mediated through activation of a separate G protein-coupled pathway, presumably involving G$_i$. However, additional experiments revealed that neither 2MeSAMP nor ARC69931MX (cangrelor) increased cAMP through activation of A2a, IP, DP, or EP$_2$ receptors, which are known to couple to G$_i$. Collectively, these findings indicate that 2MeSAMP and ARC69931MX interact with an unidentified platelet G protein-coupled receptor that stimulates cAMP-mediated inhibition of platelet function. This inhibition is in addition to that derived from antagonism of P2Y$_{12}$ receptors.

Upon damage to the endothelial layer of the blood vessel wall, the underlying subendothelium is exposed to platelets in the blood, initiating a cascade of signaling events resulting in the transformation of “resting” platelets into “activated” platelets (1). One significant characteristic associated with these signaling events is the secretion of ADP from the platelet-dense granules (2). This released ADP acts to further amplify the platelet activation response by interacting with its G-protein-coupled receptors on the platelet surface, namely P2Y$_1$ (coupled to G$_q$) and P2Y$_{12}$ (coupled to G$_i$) (3–5). The consequence of platelet activation through ADP is a conformational change in the platelet membrane glycoprotein aIIb3 (6, 7), which then binds to fibrinogen present in the plasma. The binding of fibrinogen with aIIb3 on the surface of adjacent platelets results in fibrinogen-platelet cross-linking and the formation of a hemostatic plug at the site of vascular injury (8).

Consequently, ADP is thought to play an integral role in the normal process of hemostasis. Of the two ADP-receptor signaling pathways in platelets, evidence has indicated that ADP-mediated P2Y$_{12}$ signaling appears to play a more prominent role in platelet activation than ADP-mediated P2Y$_1$ signaling (9, 10). For the most part, support for this notion derives from the use of the adenosine-based P2Y$_{12}$ antagonists (i.e. 2MeSAMP$^4$ and ARC69931MX$^5$), which have a much broader inhibitory profile than P2Y$_1$ antagonists (e.g. A3P5P (adenosine-3’-phosphate-5’-phosphate) or MRS2179) (9). Thus, 2MeSAMP and ARC69931MX inhibit platelet aggregation in response to multiple agonists, such as thromboxane A$_2$, collagen, thrombin, etc. (11–13), whereas P2Y$_1$ antagonists do not. On the other hand, this general requirement for P2Y$_{12}$ signaling seems to be inconsistent with earlier reports indicating that activation of certain platelet receptors (e.g. thromboxane A$_2$ receptor) can cause aggregation through ADP-independent mechanisms (14, 15). Based on this apparent inconsistency in the contribution of P2Y$_{12}$ signaling to the overall platelet activation response, the present study investigated the possibility that the broad spectrum of inhibitory activity of this new generation of P2Y$_{12}$ antagonists (i.e. MeSAMP and ARC69931MX) may derive from an elevation in platelet cAMP levels.

Our data demonstrated that both 2MeSAMP and ARC69931MX do in fact significantly raise human platelet cAMP. Furthermore, this pharmacological effect is independent of P2Y$_{12}$-G$_i$ signaling and appears to proceed through acti-

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$^4$ The abbreviations used are: 2MeSAMP, 2-methylthioadenosine 5’-monophosphate triethylammonium salt; PTX, pertussis toxin; PRP, platelet-rich plasma; GDP35, guanyl-5’-yl thiophosphate; PGI$_2$, prostacyclin; PGD$_2$, prostaglandin D$_2$; PGE$_2$, prostaglandin E$_2$.
Mechanism of Action for Adenosine-based P2Y12 Antagonists

viation of a separate G_s-coupled platelet receptor. Taken together, the results therefore indicate that these adenosine-based P2Y_12 antagonists can produce their inhibition of platelet function through a cAMP-mediated mechanism.

EXPERIMENTAL PROCEDURES

Reagents—The phosphodiesterase inhibitor Ro20-1724, protein kinase A, protein kinase A inhibitor, Cellosolve, 1321N1 cells, and the anti-rabbit secondary antibody were purchased from Sigma. Cell culture supplies were from Fisher. Human platelet concentrates were purchased from Life Source Blood Services (Glenview, IL) and were used within a few h of being drawn. [3H]cAMP was from Amersham Biosciences, and cold cAMP was from Assay Designs (Ann Arbor, MI). ADP was purchased from Invitrogen. The adenylate cyclase inhibitor, SQ22536, and the P2Y12 antibody were from BIOMOL International (Plymouth Meeting, PA). GDPβS was purchased from Alexis Biologicals (San Diego, CA). ARC69931MX was a generous gift from AstraZeneca.

Platelet Aggregation Analysis—The platelet count in the freshly drawn concentrates was adjusted to 3 × 10^8 platelets/ml with Tyrode’s buffer (pH 7.4), and the platelet-rich plasma (PRP) was incubated with 10 μM indomethacin for 1 min to prevent thromboxane A_2 generation. The PRP was then treated with either vehicle or the specific inhibitor/agonist. The effect of these reagents on platelet aggregation was measured using the turbidimetric method (16) with a model 400 aggregometer (Chrono-Log, Havertown, PA). All of the experiments described have been performed at least three times using PRP from three separate donors.

cAMP Analysis—Human PRP (500 μl) samples were collected in an Eppendorf tube and treated with vehicle or the appropriate concentration of reagents and incubated at room temperature for 1 min. Next, the phosphodiesterase inhibitor Ro20-1724 (100 μM) was added, the PRP was centrifuged, and the platelet pellet was immediately snap-frozen in liquid nitrogen and stored at −70°C. Upon use, the platelet pellet was resuspended in sodium acetate buffer (50 mM; pH 4.0), sonicated, boiled for 4 min, and centrifuged, and the supernatant was transferred to a separate tube. cAMP analysis was performed according to the procedure described originally by Gilman (17). Platelet samples with vehicle treatment were used to evaluate basal cAMP concentrations, and a cAMP standard curve was used to determine cAMP levels in the platelet samples. Separate experiments demonstrated that the negligible amounts of 2MeSAMP and ARC69931MX that were carried over into the cAMP assay had no effect on the measurement of cellular cAMP levels. In addition, other studies revealed that supplementation of the PRP with apyrase (0.5 units/ml) did not affect basal cAMP levels or the ability of 2MeSAMP or ARC69931MX to increase cAMP levels (data not shown).

Platelet Membrane Preparation—Platelet membranes were isolated by modification of a previously described procedure (18). Briefly, 1 unit of PRP was centrifuged at 160 × g for magnus, to remove red blood cells. The platelet-rich plasma was supplemented with EDTA, and the platelets were pelleted by centrifugation at 1600 × g for 20 min. The platelet pellet was suspended in buffer (25 mM Tris-HCl, 5 mM MgCl_2, EDTA, pH 7.4) and recentrifuged. The resulting membrane pellet was resuspended in buffer containing 0.1 mM ATP, 10 μM GTP, 50 mM Tris/HCl, pH 7.5, 2 mM MgCl_2, 1 mM dithiothreitol, and the protein concentration was measured using the BCA protein assay before performing the cAMP assay.

Western Blotting—1321N1 cells were rinsed in phosphate-buffered saline and harvested with 0.1 M HCl. Cells were pelleted by centrifugation at 1000 × g. Cells were resuspended in radioimmune precipitation buffer (with protease-inhibitory mixture) at 4°C and lysed by sonication, and protein content was determined by the BCA protein assay. Samples containing 25–50 μg of protein were resolved on 10% SDS-polyacrylamide gels, electroblotted to nitrocellulose membranes, and probed with the appropriate antibodies. Primary antibody to P2Y_12 was used at a dilution of 1:200, and primary antibody to G_s was used at a dilution of 1:250. Anti-rabbit secondary antibody was used at a dilution of 1:2000.

cAMP Assay in 1321N1 Cells—1321N1 cells were cultured in a tissue culture dish for 48 h in 10% fetal bovine serum-Dulbecco’s modified Eagle’s medium (19). The cells were then counted, and 10^6 cells were plated in a 12-well plate. The cells were allowed to grow for 2 days and were then washed twice with serum-free Dulbecco’s modified Eagle’s medium and starved in the serum-free medium for 1 h before performing the analysis. The cells were stimulated with appropriate reagents for 5 min, in the presence of 100 μM RO-20-1724. Control wells were treated with vehicle to measure the basal CAMP levels in these cells. The medium was then removed, and the cells were lysed in 0.1 M HCl for 1 h at room temperature. The lysates were sonicated and centrifuged, and the supernatant was supplemented with 0.1 mM ATP, 10 μM GTP, 50 mM Tris/HCl, pH 7.5, 2 mM MgCl_2, 1 mM dithiothreitol prior to performing the cAMP assay, as described above.

ADP Desensitization of Human Platelets—Platelet desensitization to ADP was achieved by exposing the platelets to low doses of ADP. The PRP was first exposed to 0.5 μM ADP for 2 min, followed by exposure to 1 μM ADP for 2 min, followed by exposure to 2 μM ADP for 2 min. Platelet treatment with these subaggregatory ADP doses caused complete loss of aggregation in response to 20 μM ADP, whereas 10 μM ADP caused maximal aggregation in control platelets. Furthermore, ADP pretreatment also caused a complete desensitization of P2Y_12 signaling, as evidenced by the inability of ADP to lower CAMP levels in these platelets.

RESULTS

Dose-Response Inhibition of ADP-induced Human Platelet Aggregation by 2MeSAMP and ARC69931MX—Since the primary objective of the present study was to characterize the pharmacological profile of adenosine-based P2Y_12 antagonists in platelets, the initial experiments defined the dose-response relationship for inhibition of ADP-induced platelet aggregation by 2MeSAMP and ARC69931MX. Briefly, human PRP was preincubated with various concentrations of 2MeSAMP (1–50 μM) and ARC69931MX (0.01–0.5 μM) prior to the addition of 10 μM ADP. The results (Fig. 1, A and B) demonstrate that there was a progressive inhibition of ADP-induced platelet aggregation as the concentra-
tions of 2MeSAMP and ARC69931MX were increased. The next experiments determined whether this observed inhibition of platelet aggregation was associated with changes in platelet cAMP levels.

2MeSAMP and ARC69931MX Raise Human Platelet cAMP Levels—It was found that the same doses of 2MeSAMP or ARC69931MX that caused a significant inhibition of aggregation also produced a substantial increase in platelet cAMP levels (Fig. 1, C and D, respectively). These results provide the first documentation that these adenosine-based P2Y12 antagonists have the capacity to markedly raise human platelet cAMP levels.

Relationship between Platelet cAMP Levels and Inhibition of Aggregation—To determine the physiological significance of the 2MeSAMP- and ARC69931MX-mediated cAMP increases, the next experiments used PGI2 to generate a standard curve for cAMP-mediated inhibition of human platelet aggregation. It can be seen (Fig. 1E) that PGI2 caused a dose-dependent inhibition of 10 μM ADP-induced aggregation, which is consistent with previous reports in human platelets (20). In order to measure the amount of cAMP that was associated with these different levels of inhibition, cAMP analysis was next performed. It was found that the same PGI2 doses that caused a progressive inhibition of aggregation also produced a dose-dependent increase in platelet cAMP levels (Fig. 1F). Fig. 2A illustrates the plot of percentage inhibition of ADP-induced aggregation versus cAMP levels. Since the inhibitory effects of PGI2 are known to be limited to activation of Gs (21), this standard curve (\( r^2 = 0.99 \)) defines the dose-response relationship between inhibition of ADP-induced aggregation and elevation of platelet cAMP levels.

Interestingly, when the inhibition of aggregation versus cAMP dose-response curves for 2MeSAMP and ARC69931MX were compared with that seen for PGI2, the results were markedly similar (Fig. 2, B and C). Indeed, the curves for both 2MeSAMP and ARC69931MX were almost superimposable on the PGI2 curve, with only a slight shift to the right in each case. These results therefore indicate that the elevated cAMP levels induced by 2MeSAMP and ARC69931MX are themselves sufficient to account for a substantial portion of the observed inhibition of ADP-induced platelet aggregation. Furthermore, it is also possible that this cAMP elevation may be responsible for the ability of these compounds to inhibit platelet aggregation induced by multiple agonists. This notion was investigated in the following experiments.

Effects of 2MeSAMP and ARC69931MX on Human Platelet Aggregation Induced by Activation of Thromboxane A2 Receptor
and PAR1 Receptors—Fig. 3A illustrates that 2MeSAMP (50 μM) and ARC69931MX (0.5 μM) produced dramatic inhibition of 1 μM U46619-induced aggregation (Fig. 3A, curves b and c) and 15 μM TRAP1-induced aggregation (Fig. 3A, curves e and f). These findings are consistent with previous reports demonstrating a broad spectrum of inhibitory activity for these P2Y₁₂ antagonists (11–13). In order to investigate whether the mechanism of this inhibition involved cAMP, a cell-permeable adenylate cyclase inhibitor (SQ22536) (22) was employed. It was found that pretreatment of human PRP with SQ22536 (300 μM) produced complete reversal of 2MeSAMP- and ARC69931MX-mediated inhibition of U46619- and TRAP1-induced platelet aggregation (Fig. 3B, curves b and c and curves e and f, respectively). Separate experiments confirmed that the same concentration of SQ22536 that reversed the inhibitory effects of 2MeSAMP and ARC69931MX also completely blocked their ability to raise platelet cAMP (Fig. 3C).

These data therefore indicate that the ability of these adenosine-based P2Y₁₂ antagonists to inhibit platelet aggregation induced by agonists other than ADP derives from their capacity to elevate cAMP levels. The next series of experiments investigated the pharmacological mechanism(s) by which these compounds raise cAMP.

2MeSAMP and ARC69931MX Elevate cAMP by a Mechanism Independent of P2Y₁₂-Gi Signaling—It is possible that platelets possess basal P2Y₁₂ activity and that antagonism of this activity might relieve Gi inhibition of adenylate cyclase and thereby increase cAMP production. In order to examine this possibility, P2Y₁₂-Gi signaling was blocked by employing the A protomer of pertussis toxin (PTX), which ADP-ribosylates, and thus uncouples G₂ signaling. Since human platelets are impermeable to PTX, these experiments were performed in an isolated platelet membrane preparation. The results demonstrated (Fig. 4, bar 2) that treatment of the membrane preparation with forskolin (0.5 μM) resulted in a predictable increase in cAMP levels. Furthermore, the addition of ADP (10 μM) substantially reduced this forskolin-induced cAMP production (Fig. 4, bar 3), establishing the functionality of the P2Y₁₂-Gi signaling pathway in this membrane preparation. It was also found (Fig. 4, bar 4) that PTX (3 μg/ml) treatment blocked the ability of ADP to lower forskolin-mediated cAMP increases. This finding demonstrates that this dose of PTX effectively blocked P2Y₁₂-Gi signaling, and because of this inhibition, activation of P2Y₁₂ receptors by ADP did not result in suppression of cAMP production. Taken together, these results therefore established that the P2Y₁₂-Gi signaling pathway in this platelet membrane preparation was both intact and sensitive to inhibition by PTX.

The ability of 2MeSAMP (50 μM) and ARC69931MX (0.5 μM) to alter cAMP levels was next evaluated. It can be seen (Fig. 4, bars 5 and 6) that both compounds did indeed raise cAMP to levels that were comparable with those produced by forskolin (0.5 μM). However, it was also found that treatment of the membranes with PTX alone had no measurable effect on basal cAMP levels (Fig. 4, bar 7); nor did it block increases in cAMP caused by either 2MeSAMP or ARC69931MX treatment (Fig. 4, bars 8 and 9, respectively). These latter results are particularly interesting, because they provide evidence that inhibition of basal G₂ activity is not sufficient to increase cAMP production and that 2MeSAMP/ARC69931MX-mediated increases in cAMP do not derive from inhibition of G₂ sig-
Mechanism of Action for Adenosine-based P2Y12 Antagonists

![Graph A](image1)

**Figure 3.** Effect of SQ22536 on inhibition of aggregation and cAMP elevation by 2MeSAMP and ARC69931MX. A, trace a, PRP was stimulated with U46619 (1 μM). Trace b, PRP was treated with 50 μM 2MeSAMP, prior to stimulation with U46619 (1 μM). Trace c, PRP was treated with ARC69931MX (0.5 μM) prior to stimulation with U46619 (1 μM). Trace d, PRP was treated with TRAP1 (15 μM). Trace e, PRP was treated with 2MeSAMP (50 μM) prior to stimulation with TRAP1 (15 μM). Trace f, PRP was preincubated with ARC69931MX (0.5 μM), prior to stimulation with U46619 (1 μM). Trace d, PRP was preincubated with SQ22536 (300 μM), followed by incubation with 2MeSAMP (50 μM), prior to stimulation with TRAP1 (15 μM). Trace e, PRP was preincubated with SQ22536 (300 μM), followed by incubation with 2MeSAMP (50 μM), prior to stimulation with TRAP1 (15 μM).

![Graph B](image2)

**Figure 4.** Effect of pertussis toxin on cAMP levels in human platelet membranes. Platelet membrane cAMP levels (pmol/mg protein) were measured following treatment with the indicated reagents. Bar 1, basal cAMP levels. Bar 2, cAMP levels upon treatment with forskolin (0.5 μM). Bar 3, cAMP levels in the presence of ADP (10 μM) and forskolin (0.5 μM). Bar 4, cAMP levels in the presence of PTX, a protease (3 μg/ml), ADP (10 μM), and forskolin (0.5 μM). Bar 5, cAMP levels in the presence of 2MeSAMP (50 μM). Bar 6, cAMP levels in the presence of ARC69931MX (0.5 μM). Bar 7, cAMP levels in the presence of PTX (3 μg/ml) alone. Bar 8, co-treatment with 2MeSAMP (50 μM) and PTX (3 μg/ml). Bar 9, co-treatment with ARC69931MX (0.5 μM) and PTX (3 μg/ml). Results are the averages obtained from at least three separate platelet donors.

![Graph C](image3)

**Figure 5.** Effect of SQ22536 on inhibition of aggregation and cAMP elevation by 2MeSAMP and ARC69931MX. A, trace a, PRP was stimulated with U46619 (1 μM). Trace b, PRP was treated with 50 μM 2MeSAMP, prior to stimulation with U46619 (1 μM). Trace c, PRP was treated with ARC69931MX (0.5 μM) prior to stimulation with U46619 (1 μM). Trace d, PRP was stimulated with TRAP1 (15 μM). Trace e, PRP was treated with 2MeSAMP (50 μM) prior to stimulation with TRAP1 (15 μM). Trace f, PRP was preincubated with ARC69931MX (0.5 μM), prior to stimulation with TRAP1 (15 μM). Trace g, PRP was treated with SQ22536 (300 μM) prior to stimulation with U46619 (1 μM). Trace h, PRP was preincubated with SQ22536 (300 μM), followed by incubation with 2MeSAMP (50 μM), prior to stimulation with TRAP1 (15 μM). Trace i, PRP was preincubated with SQ22536 (300 μM), followed by incubation with 2MeSAMP (50 μM), prior to stimulation with TRAP1 (15 μM).

In separate experiments, the functionality of adenylate cyclase in 1321N1 cells was established with forskolin treatment. It was found (Fig. 5B, bar 2) that incubation with 0.5 μM forskolin elevated 1321N1 cell cAMP to levels that were comparable to those obtained with 2MeSAMP/ARC69931MX-induced cAMP elevation.

**2MeSAMP** and **ARC69931MX Elevate cAMP in the Absence of P2Y12 Receptors**—These experiments employed 1321N1 human astrocytoma cells, which are known to be devoid of P2Y12 receptors. The lack of these receptors in this cell line was confirmed by immunoblotting and functional analysis. In the initial studies, the 1321N1 cell lysates were compared with lysates from human platelets. As expected, the platelet lysate revealed intense blotting at 52 kDa and a smaller band at 39 kDa corresponding to P2Y12 receptors (Fig. 5A, lane 1). On the other hand, the 1321N1 cell lysate did not reveal any immunoreactivity with the P2Y12 antibody (Fig. 5A, lane 3), although both preparations revealed staining of Gs (Fig. 5A, lanes 1 and 2).

In separate experiments, the functionality of adenylate cyclase in 1321N1 cells was established with forskolin treatment. It was found (Fig. 5B, bar 2) that incubation with 0.5 μM forskolin elevated 1321N1 cell cAMP to levels that were comparable to those obtained with 2MeSAMP/ARC69931MX-induced cAMP elevation.
found that treatment with either 2MeSAMP (50 μM) or ARC69931MX (0.5 μM) produced marked elevations in 1321N1 cAMP (Fig. 5B, bars 5 and 7). Furthermore, these increases were similar to those produced in platelet membranes (Fig. 4, bars 5 and 6) and were sensitive to adenylate cyclase inhibition by SQ22536 (300 μM) (Fig. 5B, bars 6 and 8).

The next experiments used ADP-desensitized platelets to determine whether these compounds can also produce a P2Y12-independent cAMP elevation in human platelets. In this connection, previous studies have provided conflicting results concerning the ability to desensitize platelet P2Y12 receptors (24, 25). Based on these inconsistencies, the present experiments directly evaluated P2Y12 receptor signaling in desensitized platelets by measuring the ability of ADP to lower PGI2-mediated cAMP elevation. In order to achieve P2Y12 receptor desensitization, platelets in their native plasma were repetitively exposed to subaggregatory doses of ADP (0.5–2 μM). It was found that after repeated ADP exposure, the platelets were unable to aggregate in response to subsequent ADP addition, even at 20 μM (data not shown). The specificity of this desensitization for ADP receptors was revealed by the finding that these platelets aggregated normally to U46619 (data not shown). Furthermore, the desensitization of P2Y12 receptor signaling was confirmed by cAMP analysis (see Fig. 6A for sampling time point). It can be seen that ADP (10 μM) effectively blocked PGI2 (1 nM)-mediated cAMP elevation in control platelets (Fig. 6B, bar 3) but was unable to do so in the desensitized platelets (Fig. 6B, bar 6). These findings therefore demonstrate complete desensitization of P2Y12 receptors. On this basis, subsequent experiments measured the ability of 2MeSAMP and ARC69931MX to elevate platelet cAMP levels in the absence of P2Y12 receptor signaling. Interestingly, it was found that P2Y12 receptor desensitization had no effect on the ability of 2MeSAMP (50 μM) and ARC69931MX (0.5 μM) to elevate platelet cAMP levels relative to nondenitized platelets (Fig. 6C). Collectively, these results therefore provide the first evidence that adenosine-based P2Y12 antagonists stimulate a cAMP-mediated pathway that is independent of both Gs signaling and P2Y12 receptor antagonism. The next experiments examined whether these compounds produce their cAMP effects through a separate G protein-coupled pathway.

**GDPβS Inhibits 2MeSAMP- and ARC69931MX-mediated Increases in Platelet cAMP Levels**—Rather than antagonizing P2Y12 receptors that couple to Gs, 2MeSAMP/ARC69931MX may increase cAMP levels through activation of a separate platelet receptor coupled to Gi. In order to examine the requirement for Gi protein signaling in the observed cAMP increases caused by 2MeSAMP or ARC69931MX, GDPβS was employed. Specifically, GDPβS is known to saturate G proteins with nonhydrolyzable GDP and hence keep them in an inactive state (26). Therefore, if 2MeSAMP and ARC69931MX elevate cAMP through a G-protein coupled receptor, GDPβS would be expected to block this elevation. The results demonstrated that treatment of the membrane preparation with GDPβS (200 μM) neither altered basal cAMP levels (Fig. 8, bar 2) nor lowered cAMP increases caused by forskolin (0.5 μM) treatment (Fig. 7, bars 3 and 4). The forskolin results indicate that adenylate cyclase is functional in this preparation and that
Mechanism of Action for Adenosine-based P2Y12 Antagonists

GDP does not directly interfere with its activity. On the other hand, the results also revealed that the ability of PG1 (2 nM) to increase cAMP (Fig. 7, bar 5) was blocked by GDPβS treatment (Fig. 7, bar 6). This finding is consistent with the fact that PG1 raises cAMP through activation of a Gs-coupled receptor. Finally, it can be seen (Fig. 7, bars 7 and 10) that both 2MeSAMP (50 μM) and ARCh9931MX (0.5 μM) increased cAMP levels and that these increases were abolished by GDPβS treatment (Fig. 7, bars 8 and 11) as well as by treatment with 300 μM SQ22536 (Fig. 7, bars 9 and 12). Therefore, these results provide evidence that 2MeSAMP and ARCh9931MX increase human platelet cAMP levels by G protein-mediated stimulation of adenylate cyclase activity. The next experiments examined whether these reagents produce this effect through stimulation of a known platelet Gs-coupled receptor.

2MeSAMP and ARCh9931MX Do Not Activate Platelet Adenosine A2a Receptors—Since the chemical structures of both 2MeSAMP and ARCh9931MX are adenosine-based, it is possible that they may activate platelet A2a receptors, which are known to couple to Gi. To investigate this possibility, the high-affinity A2a receptor antagonist SCH58261 (Kd = 1.3 nM) (27, 28) was employed. Thus, it was reasoned that if 2MeSAMP and/or ARCh9931MX inhibit platelet aggregation through A2a receptor activation, SCH58261 should block this effect. In order to test this notion, it was first necessary to establish an effective SCH58261 dose in human PRP. This was accomplished by employing the adenosine receptor agonist NECA. Briefly, it was found that NECA produced almost total inhibition of ADP (10 μM)-induced aggregation (Fig. 8, curve b) relative to control (Fig. 8, curve a) and that 50 nM SCH58261 completely blocked this inhibition (Fig. 8, curve c). However, it was also found (Fig. 8, curves e and g) that the same concentration of SCH58261 (50 nM) that was 100% effective in blocking NECA inhibition had no effect on either 2MeSAMP (25 μM) or ARCh9931MX (0.25 μM) inhibition of ADP (10 μM)-induced aggregation (Fig. 8, curves d and f). It should be noted that lower concentrations of 2MeSAMP and ARCh9931MX were used in these experiments to obtain a lesser degree of inhibition of aggregation. This was done primarily to test whether the A2a antagonist has any effect at all on inhibition of platelet aggregation by 2MeSAMP and ARCh9931MX. Taken together, these data therefore indicate that 2MeSAMP and ARCh9931MX do not raise cAMP levels in platelets by the stimulation of adenosine A2a receptors.

Effects of 2MeSAMP and ARCh9931MX on Platelet Prostanoid IP, DP, or EP2 Receptors—The next experiments examined whether 2MeSAMP and ARCh9931MX activate other platelet receptors that are known to couple to Gi (i.e., IP, DP, and EP2 receptors). To test this possibility, antagonists specific to each of these receptors (i.e., CAY10441 (specific to IP), BW231658C (specific to DP), and AH6809 (specific to EP2)) were employed (29–34). In the initial experiments, it was established that 2 nM PG1 (Fig. 9A, curve b), 5 nM PGD2 (Fig. 9B, curve b, and 5 μM PGE2 (Fig. 9C, curve b) produced substantial inhibition of ADP (10 μM)-induced platelet aggregation relative to control (Fig. 9, A–C, curve a). It can also be seen that 100 nM CAY10441 (Fig. 9A, curve a), 150 nM BW231658C (Fig. 9B, curve b), and 5 μM AH6809 (Fig. 9C, curve c) completely reversed the inhibition of ADP-induced aggregation caused by 2 nM PG1, 5 nM PGD2, and 5 μM PGE2, respectively. Finally, the effect of CAY10441, BW231658C, and AH6809 on 2MeSAMP and ARCh9931MX inhibition of ADP-induced aggregation was tested. It was found that the same concentrations of the IP, DP, and EP2 antagonists that were 100% effective in reversing PG1, PGD2, and PGE2 inhibition had no effect on either 2MeSAMP (Fig. 9, A–C, curve e) or ARCh9931MX (Fig. 9, A–C, curve g) inhibition of ADP (10 μM)-induced aggregation. Taken together, these data therefore indicate that 2MeSAMP

FIGURE 6. cAMP analysis in ADP-desensitized human platelets. A, time point for the samples taken for the cAMP assay. PRP was sequentially treated with 0.5, 1, and 2 μM ADP for 2 min, and then the PRP was incubated with PG1 for 1 min, followed by the addition of ADP. After 1 min, the samples were collected for the cAMP assay. B, platelet cAMP levels (pmol/10^8 platelets) were measured following treatment with the indicated reagents. Bar 1, basal cAMP levels in control platelets. Bar 2, cAMP levels upon treatment with PG1 (1 nM) in control platelets. Bar 3, cAMP levels upon incubation with PG1 (1 nM), followed by incubation with ADP (10 μM) in control platelets. Bar 4, basal cAMP levels in ADP-desensitized platelets. Bar 5, cAMP levels upon treatment with PG1 (1 nM) in ADP-desensitized platelets. Bar 6, cAMP levels upon incubation with PG1 (1 nM), followed by incubation with ADP (10 μM) in ADP-desensitized platelets. Results are the averages obtained from at least three separate platelet donors. C, control platelets or ADP-desensitized platelets (see "Experimental Procedures") were treated with 2MeSAMP (50 μM) or ARCh9931MX (0.5 μM) for 1 min and assayed for cAMP, as described under "Experimental Procedures." Tabular data are the average of results obtained with platelets from three separate donors.
and AR69931MX raise cAMP levels in platelets by the stimulation of a Gs-coupled receptor separate from IP, DP, or EP2.

Interestingly, the cAMP elevations in human platelets (Fig. 4) appear to be universal, since stimulation of cAMP production by 2MeSAMP and AR69931MX in C6 gliona cells (35) was only 20% of that seen in 1321N1 cells (7). It is currently believed that platelet aggregation in response to physiologically relevant agonists is primarily dependent on the secretion of ADP (4, 7) and the secondary activation of ADP-P2Y12 receptors (9, 10, 11). In this regard, there are two structurally distinct classes of P2Y12 antagonists, the thiopyridines (clopidogrel and ticlopidine) and the adenosine-based derivatives (i.e. 2MeSAMP and AR69931MX).

Although the thiopyridines have been demonstrated to effectively treat thromboembolic disorders, they require in vivo metabolism to become functionally active, and consequently there is a significant delay in their onset of action (36). To overcome this limitation, the next generation of P2Y12 antagonists, namely the adenosine-based compounds, has recently been developed. One of these derivatives, AR69931MX, is currently being evaluated for its clinical effectiveness as an antithrombotic agent. In addition, AR69931MX, as well as 2MeSAMP, has been extensively employed to identify and characterize P2Y12 signaling pathways in platelets and other cell types. Indeed, the use of AR69931MX and 2MeSAMP has led to the current belief that ADP-P2Y12 signaling is a predominant force driving integrin activation and human platelet aggregation both in vitro and in vivo (9–12, 36–39). However, this notion of a strict dependence on P2Y12 signaling seems to be at odds with previous experiments, which demonstrated that activation of certain platelet receptors (e.g. thromboxane A2 receptors) can cause integrin activation through ADP-independent pathways (14, 15). For example, it was shown (14) that platelets deficient in releasable ADP will nevertheless aggregate in response to added arachidonic acid, even in the presence of creatinine phosphate/creatinine phosphokinase, which enzymatically degrades ADP.

Based on these apparent inconsistencies, the present experiments examined the underlying mechanisms by which 2MeSAMP and AR69931MX produce their inhibition of human platelet function. It was found that the inhibition of platelet aggregation by 2MeSAMP and AR69931MX was
Mechanism of Action for Adenosine-based P2Y12 Antagonists

FIGURE 9. Effect of IP, DP, or EP receptor antagonists on 2MeSAMP- and ARC69931MX-mediated inhibition of ADP-induced human platelet aggregation. A–C, Trace a, PRP was stimulated with ADP (10 μM). Trace b, PRP was treated with PGD2 (2 μM) (A), BWA868C (150 μM) followed by PGD2 (5 μM) (B), or AH6809 (5 μM) (C) prior to stimulation with ADP (10 μM). Trace c, PRP was preincubated with CAY10441 (100 μM), followed by incubation with PGD2 (2 μM) (A), BWA868C (150 μM) (B), or AH6809 (5 μM) (C) prior to stimulation with ADP (10 μM). Trace d, PRP was triggered with 2MeSAMP (25 μM) prior to stimulation with ADP (10 μM). Trace e, PRP was preincubated with CAY10441 (100 μM) (A), BWA868C (150 μM) (B), or AH6809 (5 μM) (C) followed by incubation with 2MeSAMP (25 μM), prior to stimulation with ADP (10 μM). Trace f, PRP was treated with ARC69931MX (0.25 μM) prior to stimulation with ADP (10 μM). Trace g, PRP was preincubated with CAY10441 (100 μM) (A), BWA868C (150 μM) (B), or AH6809 (5 μM) (C) followed by incubation with ARC69931MX (0.25 μM) prior to stimulation with ADP (10 μM). Each aggregation curve is representative of multiple traces obtained from three separate platelet donors.

directly related to their ability to increase platelet cAMP levels, suggesting that cAMP may play an important role in the inhibitory activity of these compounds. This notion was confirmed by using the cell-permeable adenylate cyclase inhibitor SQ22536. Specifically, it was found that SQ22536 not only blocked the cAMP elevation caused by 2MeSAMP and ARC69931MX but also completely blocked their ability to inhibit platelet aggregation induced by two separate agonists (i.e. U46619 and TRAP1). As mentioned, the capacity of 2MeSAMP and ARC69931MX to inhibit platelet aggregation in response to multiple agonists has served as the basis for the current notion that ADP-P2Y12 signaling is required for platelet activation. The present results indicate that the ability of these compounds to inhibit platelet aggregation stimulated by agonists other than ADP largely derives from their capacity to elevate platelet cAMP levels.

The signaling mechanism(s) by which 2MeSAMP and ARC69931MX increase platelet cAMP was next investigated. The first potential mechanism examined was inhibition of P2Y12-coupled Gi signaling. In this regard, it has long been suggested (although never proven) that the ability of ADP to initiate platelet aggregation derives in part from its ability to lower cAMP levels. This suggestion is based on the observation that ADP-mediated Gi signaling reduces cAMP levels that have been raised by prior adenylate cyclase activation (40). On the other hand, it has not been possible to establish that ADP is capable of lowering cAMP levels in resting platelets, and consequently, the contributions of Gi signaling to resting platelet cAMP levels and its involvement in the initiation of platelet aggregation are presently unknown. Nevertheless, in order for 2MeSAMP and ARC69931MX to raise platelet cAMP levels through inhibition of the P2Y12-Gi pathway, one would have to presume that cAMP levels are low in the resting state due to basal P2Y12-Gi signaling. In order to examine this possibility, inhibition of P2Y12-Gi signaling with PTX (18) was employed. It was found that although treatment of platelet membranes with PTX resulted in effective uncoupling of P2Y12-Gi signal transduction, such uncoupling did not result in measurable increases in basal cAMP levels; nor did it block 2MeSAMP- or ARC69931MX-induced increases in cAMP production. Consequently, it can be concluded that the ability of 2MeSAMP and ARC69931MX to increase cAMP levels cannot be ascribed to inhibition of P2Y12-Gi signaling. On the other hand, it is also possible that P2Y12 receptors couple to a separate Gi-independent signaling mechanism. To examine this possibility, experiments were conducted in 1321N1 human astrocytoma cells, which lack P2Y12 receptors. Surprisingly, both 2MeSAMP and ARC69931MX produced substantial increases in cAMP levels even in this P2Y12-deficient cell line. In order to further examine the notion that these compounds can raise cAMP through a P2Y12-independent mechanism, ADP-desensitized human platelets were employed. It was found that although these platelets no longer had functional P2Y12 receptor signaling, 2MeSAMP and ARC69931MX nevertheless increased their production of cAMP. Thus, these findings establish that 2MeSAMP and ARC69931MX activate a cAMP-mediated inhibitory pathway that is both P2Y12- and Gi-independent. Additional studies were next directed at defining the molecular mechanism by which such a signaling pathway may be induced.

Since activation of Gi is known to cause stimulation of adenylate cyclase, we first examined whether the cAMP elevation caused by 2MeSAMP and ARC69931MX is mediated through a G-protein-coupled process. It was found that a concentration of GDPβS that effectively inhibited Gi2 signaling did in fact block the cAMP increases induced by 2MeSAMP and ARC69931MX. Consequently, these results indicate that both 2MeSAMP and ARC69931MX increase platelet cAMP levels through interaction with receptors that are presumably coupled to Gi2.

In this connection, platelet adenosine receptors are known to signal through Gi2 and inhibit platelet function by increasing cAMP production (27, 28). Furthermore, it is also
known that platelets possess other G<sub>a</sub>-coupled receptors, namely IP<sub>3</sub>, DP, and EP<sub>2</sub>. In order to examine the potential interaction of 2MeSAMP and ARC69931MX with these different receptor types, pharmacological inhibitors were employed. It was found, however, that specific antagonists (28–34) against each of these receptor types had no effect on the inhibitory properties of either 2MeSAMP or ARC69931MX, indicating that the unidentified G<sub>a</sub>-coupled receptor is not A2a, IP<sub>3</sub>, DP, or EP<sub>2</sub>.

In conclusion, the present results identify an alternate mechanism by which MeSAMP and ARC69931MX produce inhibition of human platelet function. Interestingly, this cAMP-dependent mechanism derives from activation of a G protein-coupled signaling pathway that does not involve platelet P2Y<sub>12</sub> receptors. Thus, it appears that 2MeSAMP and ARC69931MX produce their pharmacological effects through two distinct mechanisms. Lastly, it should be mentioned that although 2MeSAMP and ARC69931MX have the capacity to activate this cAMP-inhibitory pathway, a similar mechanism of action may not apply to clodigorel or ticlopidine, since they represent a structurally different class of pharmacological agents.

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