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(*N*)-methanocarba-2MeSADP (MRS2365) is a subtype-specific agonist that induces rapid desensitization of the $P2Y_1$ receptor of human platelets

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Summary

Adenosine diphosphate (ADP) initiates and maintains sustained aggregation of platelets through simultaneous activation of both the G_q-coupled $P2Y_1$ receptor and the G_i-coupled $P2Y_{12}$ receptor. We recently described the synthesis and $P2Y_1$ receptor-specific agonist activity of (*N*)-methanocarba-2MeSADP (MRS2365). Consequences of selective activation of the $P2Y_1$ receptor by MRS2365 have been further examined in human platelets. Whereas MRS2365 alone only induced shape change, addition of MRS2365 following epinephrine treatment, which activates the $G_{i/z}$-linked, α_{2A}-adrenergic receptor, resulted in sustained aggregation that was indistinguishable from that observed with ADP. Conversely, the platelet shape change promoted by ADP in the presence of the $GP_{IIb/IIIa}$ antagonist eptifibatide was similar to that promoted by MRS2365. Preaddition of the high affinity $P2Y_1$ receptor antagonist MRS2500 inhibited the effect of MRS2365, whereas addition of MRS2500 subsequent to MRS2365 reversed the MRS2365-induced shape change. Preactivation of the $P2Y_1$ receptor with MRS2365 for 2 min resulted in marked loss of capacity of ADP to induce aggregation as evidenced by a greater than 20-fold rightward shift in the concentration effect curve of ADP. This inhibitory effect of $P2Y_1$ receptor activation was dependent on the concentration of MRS2365 (EC_{50} = 34 nM). The inhibitory effect of preincubation with MRS2365 was circumvented by activation of the Gq-coupled 5-HT_{2a} receptor suggesting that MRS2365 induces loss of the ADP response as a consequence of desensitization of the G_q-coupled $P2Y_1$ receptor. The time course of MRS2365-induced loss of aggregation response to epinephrine was similar to that observed with ADP. These results further demonstrate the $P2Y_1$ receptor selectivity of MRS2365 and illustrate the occurrence of agonist-induced desensitization of the $P2Y_1$ receptor of human platelets studied in the absence of $P2Y_{12}$ receptor activation.

Keywords

adenosine diphosphate; desensitization; MRS2365; $P2Y_1$ receptor; $P2Y_{12}$ receptor; platelets

Introduction

Adenosine diphosphate (ADP) was first reported to be a potent activator of platelet aggregation in 1961 [1] and is now known to be the cognate agonist for the $P2Y_1$, $P2Y_{12}$,

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and $P2Y_{13}$ receptors [2–5]. Both $P2Y_1$ and $P2Y_{12}$ receptors are present on platelets and simultaneous activation of both receptors is necessary for ADP-induced platelet aggregation [6–14]. Activation of $P2Y_1$ receptors promotes $G\alpha_q$-mediated activation of phospholipase C, which in turn hydrolyzes membrane PtdIns(4,5)P_2 to yield diacylglycerol and inositol(1,4,5) trisphosphate; these two second messengers activate protein kinase C (PKC) and promote release of Ca^{2+} from intracellular stores, respectively. $P2Y_1$ receptor activation in the presence of a $P2Y_{12}$ receptor antagonist leads to cytoskeletal rearrangement, shape change, and transient aggregation [8,15]. Activation of $P2Y_{12}$ receptors initiates $G\alpha_i$-promoted decreases in intracellular cyclic AMP formation, activation of phosphatidyl inositol-3 kinase via liberated $G\beta\gamma$, and downstream activation of $GP_{IIb/IIIa}$ involved in sustained platelet aggregation.

Because of lack of selective P2Y receptor agonists, examination of the discrete actions of the $P2Y_1$ receptor in platelet biology has required concomitant use of receptor agonists and $P2Y_{12}$ receptor antagonists. We recently synthesized (*N*)-methanocarba-2MeSADP (MRS2365), as a potent $P2Y_1$ receptor agonist [16]. Studies with recombinant $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors illustrated that MRS2365 specifically activates the $P2Y_1$ receptor without either agonist or antagonist activity at the $P2Y_{12}$ or $P2Y_{13}$ receptors [17]. Selective activation of the $P2Y_1$ receptor also was suggested from functional studies of human platelets as MRS2365 potently induced shape change without causing the sustained aggregation that is a hallmark of simultaneous activation of the $P2Y_1$ and $P2Y_{12}$ receptors by ADP or by previously studied ADP analogues. Thus, MRS2365 allows discrete examination of the actions of the $P2Y_1$ receptor. We have further examined the actions of MRS2365 in human platelets, and have applied this $P2Y_1$ receptor-selective agonist to further delineate the interplay of $P2Y_1$ and $P2Y_{12}$ receptor activation in the sustained aggregation response to ADP.

Methods

Materials

Integrilin (2 mg mL^{-1} eptifibatide; Millennium Pharmaceuticals, Cambridge, MA, USA) and heparin (1000 U mL^{-1}; Baxter Healthcare, Deerfield, IL, USA) were obtained from the University of North Carolina Hospital. 2MeSADP, ADP, prostacyclin, fibrinogen, apyrase, bovine serum albumin, and other buffer reagents were purchased from Sigma Chemical Company, St Louis, MO, USA.

Synthesis of (N)-methanocarba-2MeSADP

(*N*)-methanocarba-2MeSADP (MRS2365) was synthesized as previously described [16].

Synthesis of 2-iodo-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate

2-Iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate (MRS2500) was synthesized as previously described [18].

Preparation and assay of washed human platelets

Suspensions of washed human platelets were prepared essentially as described previously [17]. The procedure was a modification of the method of Cazenave *et al.* [19], which was originally derived from Mustard *et al.* [20]. Briefly, one hundred and fifty milliliters of blood was drawn from healthy volunteers into 60 mL syringes containing one-sixth final blood volume of 65 mM citric acid, 85 mM sodium citrate, 110 mM dextrose (ACD), pH 4.5. Immediately after collection, the blood was carefully transferred to 50 mL sterile conical tubes (~30 mL in each) and placed in a water bath at 37 °C for 30 min, mixed by

inversion, and centrifuged at 275 × ***g*** for 16 min. The supernatant (platelet-rich plasma) was collected and further centrifuged (13 min at 2200 × ***g***), and the platelets were gently resuspended in 40 mL of modified Tyrode's buffer containing 137 mM NaCl, 2.7 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES (pH 7.4), and 0.36% bovine serum albumin. Ten units per milliliter heparin and 5 μm prostacyclin (PGI_2) also were included in the first resuspension (to prevent transient platelet activation during the preparation), and the platelets were incubated at 37 °C for 10 min followed by another addition of 5 μm PGI_2 prior to centrifugation (8 min at 1900 × ***g***). This washing step was repeated once using modified Tyrode's buffer without heparin, and the platelets were suspended in the same buffer (without PGI_2) containing 0.05 U mL^{-1} apyrase to a final cell density of 5×10^8 cells mL^{-1} . The platelet suspensions were allowed to rest for 90–120 min at 37 °C prior to experiments [21].

Platelet aggregation was quantified using the optical mode of a two-channel Chrono-Log aggregometer (Model 560-VS; Chrono-Log Corporation, Havertown, PA, USA) at 37 °C using stirred (1000 r.p.m.) washed platelet suspensions (425 μL; ~2.2×10^8 cells) as described previously [17]. Relative platelet aggregation response (maximum extent) was determined by comparison of light transmission in platelet resuspension buffer and is expressed as percentage response. To magnify the signal during measurements of platelet shape change, the reference cuvette was replaced with a platelet suspension diluted to 50% (2.5×10^8 cells mL^{-1}) while the preparation to be studied was maintained at a concentration of 5×10^8 cells mL^{-1}. For all measurements, light scatter through resting platelets was recorded for 1 min prior to addition of drugs as indicated (final volume, 500 μL). Shape change/aggregation was monitored for 6–8 min.

Each experiment was repeated at least three times. The data are presented as representative aggregometer tracings from a typical experiment, or in some cases as mean ± SEM of transformed (i.e. percentage control) data pooled from multiple experiments. Dose-response curves were generated using graphpad prism software (Graphpad Software Inc., San Diego, SC, USA).

Scanning electron microscopy

Scanning electron microscopy was performed at the University of North Carolina Microscopy Services Laboratory. Washed platelets, incubated with drugs as indicated, were fixed by addition of 50 μL of platelet suspension to 500 μL 4% glutaraldehyde in 0.1 m sodium cacodylate [cacodylic acid sodium salt – $Na(CH_3)2AsO_23H_2O$) pH 7.2] followed by rotation for 30 min at room temperature. One hundred microliters of the fixed platelet suspension was spread onto poly-L-lysine-coated glass coverslips and incubated for 30 min. Following adsorption, the coverslips were rinsed once in 0.1 m sodium cacodylate buffer and dehydrated through a graded series of five ethanol washes (30%, 50%, 75%, 100%, and 100%). The coverslips were transferred in 100% ethanol to a critical point dryer (Balzers CPD-020; Bal-Tec AG, Balzers, Liechtenstein) and dried using carbon dioxide as the transition solvent. The coverslips were mounted on aluminum specimen supports with carbon adhesive tabs and coated with a 15 nm thickness of gold–palladium metal (60:40 alloy) using a Hummer × sputter coater (Anatech Ltd, Alexandria, VA, USA). Samples were examined with a Cambridge Stereoscan 200 scanning electron microscope (LEO Electron Microscopy Inc., Thornwood, NY, USA) operating at an acceleration voltage of 20 kV, 20 mm working distance, and 40° tilt.

Results

Our previous studies with recombinant human $P2Y_1$ and $P2Y_{12}$ receptors illustrated that MRS2365 is a selective $P2Y_1$ receptor agonist that neither activates nor blocks the $P2Y_{12}$

receptor [17]. This P2Y receptor selectivity was examined further in functional studies with natively expressed P2Y receptors on washed human platelets. The extent of platelet aggregation was measured over a 6 min period in response to 10 μM ADP as indicated by an increase (downward deflection) in light transmission (Fig. 1A). In contrast, MRS2365 promoted a shape change (decrease in light transmission, upward deflection; Fig. 1B) previously shown to occur with activation of the G_q-coupled $P2Y_1$ receptor [22,23] without inducing the sustained platelet aggregation previously shown to require simultaneous activation of the G_q-coupled $P2Y_1$ receptor and G_i-coupled $P2Y_{12}$ receptor as occurring with ADP. A $G_{i/z}$-coupled α_{2A}-adrenergic receptor also exists on platelets, and whereas addition of 1 μM epinephrine alone had little effect on platelet aggregation, marked and sustained platelet aggregation occurred upon activation of the $P2Y_1$ receptor by MRS2365 in platelets preincubated with 1 μM epinephrine (Fig. 1C).

Because of the rapid aggregation of platelets in response to ADP, it was not possible to directly compare the $P2Y_1$ receptor-dependent shape change elicited by ADP vs. that observed with MRS2365. However, as platelet aggregation requires the binding of fibrinogen to activated $GP_{IIb/IIIa}$, we used eptifibatide, an inhibitor of the binding of fibrinogen to $GP_{IIb/IIIa}$ to block aggregation and to compare the shape change induced by ADP with that promoted by MRS2365 in the absence of platelet–platelet interactions. As illustrated in Fig. 2A,B, the kinetics of occurrence, magnitude, and longevity of the shape change induced by MRS2365 was indistinguishable from that induced by ADP after pretreatment of platelets with eptifibatide. The dynamics of platelet shape change in response to MRS2365 was also examined visually using scanning electron microscopy. MRS2365-induced shape change occurred rapidly with the maximum extent attained within 10 s (Fig. 2C).

MRS2500 was recently synthesized as the highest affinity $P2Y_1$ receptor antagonist reported to date [24]. Moreover, Hechler *et al.* [25] recently reported that MRS2500 potently and selectively inhibited $P2Y_1$ receptor activity in studies of platelets measured *ex vivo* and in several models of experimental thrombosis *in vivo*. Whereas treatment of platelets with 10 μM MRS2500 alone had no effect, preaddition of this high affinity $P2Y_1$ receptor antagonist blocked the effects of MRS2365 on shape change (Fig. 3A,B). The reversibility of the shape change induced by MRS2365 also was examined using MRS2500. Whereas the shape change induced by MRS2365 was sustained for at least 8 min in the presence of drug, addition of MRS2500 2 min after addition of MRS2365 resulted in reversal of the $P2Y_1$ receptor-promoted shape change to resting levels of light transmission over a 5–10 min period with a $t_{1/2}$ of 2.66 min (Fig. 3C).

We previously determined the relative potencies of agonists for purified human $P2Y_1$ [26] and $P2Y_{12}$ [27] receptors using steady-state GTPase assays in model phospholipid vesicles reconstituted with the purified receptors and their cognate heterotrimeric G proteins. Our results indicate that 2MeSADP is approximately 10-fold selective for the $P2Y_{12}$ receptor over the $P2Y_1$ receptor, and therefore, low concentrations of 2MeSADP activate platelet $P2Y_{12}$ receptors with little action at $P2Y_1$ receptors. This selectivity of low concentrations of 2MeSADP for activation of the $P2Y_{12}$ receptor permitted further confirmation of the $P2Y_1$ receptor specificity of MRS2365. Addition of 3 nM 2MeSADP (Fig. 4A) to washed platelets produced no measurable effect, and addition of 1 μM MRS2365 induced an observable shape change without platelet aggregation (Fig. 4B). In contrast, simultaneous addition of 3 nM 2MeSADP with 1 μM MRS2365 resulted in marked platelet aggregation (Fig. 4C). These results also are consistent with selective activation of the $P2Y_1$ receptor by MRS2365.

The availability of a $P2Y_1$ receptor-selective agonist allowed us to examine directly the influence of independent activation of the $P2Y_1$ receptor on the subsequent response of platelets to ADP. The degree of platelet aggregation was proportional to ADP concentration (Fig. 5A; EC_{50} = 4.1 ± 0.6 μM, n = 7). Selective activation of the $P2Y_1$ receptor by preincubation (2 min) with either 1 μM or 3 μM MRS2365 (Fig. 5B,C) resulted in a reduced platelet aggregation response to all submaximal concentrations of ADP tested. As a result, the potency of ADP decreased over 20-fold (EC_{50} = 98 μM) following a 2 min preincubation with 3 μM MRS2365 (Fig. 5D). Results from scanning electron microscopy of platelets support these aggregometer data. When compared with resting platelets (Fig. 6A), incubation of platelets with a maximally effective concentration of MRS2365 (3 μM) resulted in a dramatic shape change including pseudopod projections, but no platelet aggregation was observed (Fig. 6B). In contrast, treatment of platelets with 3 μM ADP caused significant aggregation (Fig. 6C). Although MRS2365-induced pseudopodia formation was still observed, platelets pretreated with MRS2365 for 2 min did not aggregate upon challenge with ADP (Fig. 6D). These results are consistent with the idea that preincubation with MRS2365 leads to functional desensitization of the $P2Y_1$ receptor-promoted pathway that synergizes with a $P2Y_{12}$ receptor-promoted signaling pathway to confer the sustained aggregation response to ADP.

The hypothesis that loss of the aggregation response to ADP after preincubation with MRS2365 occurs as a consequence of diminution of a $P2Y_1$ receptor-promoted G_q-signaling response was tested by examining the capacity of another G_q-coupled receptor to overcome the MRS2365-induced loss of response to ADP. Thus, serotonin, which activates the G_q-coupled 5-HT_{2A} receptor on platelets, caused a shape change but no sustained platelet aggregation (Fig. 7A). As illustrated above, preincubation of platelets with MRS2365 resulted in a marked decrease in the capacity of ADP to induce aggregation (Fig. 7B,C). However, co-addition of serotonin with ADP to MRS2365-preincubated platelets produced an aggregation response similar to that observed with ADP in platelets in the absence of preincubation with MRS2365 (Fig. 7D). Thus, activation of another G_q-activating receptor largely overcomes the loss of response to ADP observed as a consequence of preactivation of the $P2Y_1$ receptor.

The concentration dependence of MRS2365 for inducing desensitization of the $P2Y_1$ receptor was examined by preincubating washed platelets with various concentrations of MRS2365 for 2 min prior to challenge with 10 μM ADP. Preincubation of platelets with increasing concentrations of MRS2365 resulted in a concentration of MRS2365-dependent decrease in ADP-promoted platelet aggregation (Fig. 8A). When plotted as a percentage of the control response (ADP only, no preincubation with MRS2365), the calculated potency of MRS2365 for inducing loss of ADP-stimulated aggregation as a consequence of a 2 min preincubation with MRS2365 was 34 nM (Fig. 8B). These data support the notion that occupancy of the human platelet $P2Y_1$ receptor by MRS2365 concurrently induces shape change and desensitization of a $P2Y_1$ receptor-promoted signaling pathway that is necessary in the sustained aggregation response of platelets to ADP.

The time course of induction of desensitization of the $P2Y_1$ receptor by MRS2365 was examined by preincubating washed platelets with a maximally effective concentration (3 μM) of MRS2365 for various times prior to challenge with 10 μM ADP. Significant loss of ADP-induced aggregation response was observed within 15 s of incubation with MRS2365, and little to no response to ADP was observed after 1 min of MRS2365 preincubation (Fig. 9A). The $t_{1/2}$ for occurrence of loss of capacity of ADP to promote platelet aggregation following 3 μM MRS2365 was approximately 18 s (Fig. 9B). To verify that the observed MRS2365-induced loss of the aggregation response was independent of the $P2Y_{12}$ receptor, a similar time course experiment was carried out using epinephrine to activate the $G_{i/z}$-

coupled α_{2A}-adrenergic receptor. The extent of loss of epinephrine-induced aggregation was similar to that observed with ADP in MRS2365-pretreated platelets (Fig. 9C). Moreover, the time course of loss of aggregation response to epinephrine ($t_{1/2}$ = 13 s; Fig. 9D) was in close agreement with that observed with ADP (Fig. 9B). Thus, desensitization of the $P2Y_1$ receptor with a receptor-specific agonist has inhibitory consequences on two different receptors that promote aggregation through a G_i-mediated signaling response.

Discussion

The results presented here further illustrate the $P2Y_1$ receptor selectivity of MRS2365 and introduce a potentially highly useful pharmacological agent for the study of the role of this receptor in platelet function. Capacity to independently activate the $P2Y_1$ receptor in the absence of a $P2Y_{12}$ receptor antagonist has allowed us to study ADP-promoted aggregation in real time in platelets subjected to selective preactivation of the $P2Y_1$ receptor. Previous studies with selective receptor antagonists [6–9,12,14] and platelets from mice with targeted gene disruption [10,11,13] have established that both the $P2Y_1$ and $P2Y_{12}$ receptors are necessary for the sustained aggregation response of human and mouse platelets to ADP. The results presented here illustrate that the aggregation response to ADP is rapidly lost as a consequence of selective preactivation of the $P2Y_1$ receptor. That this effect of MRS2365 is a consequence of receptor-specific desensitization of the $P2Y_1$ receptor/G_q-promoted signaling response is supported by the observation that it is circumvented by independent activation of the G_q-coupled 5-HT_{2A} receptor. That the loss of aggregation response is independent of the $P2Y_{12}$ receptor is supported by observation of a similar MRS2365-induced loss of aggregation response to epinephrine, which activates the $G_{i/z}$-coupled α_{2A}-adrenergic receptor.

The development of MRS2365 as a selective agonist of the $P2Y_1$ receptor complements the earlier discovery of adenosine 3′-,5′-bisphosphate as a selective competitive antagonist of the $P2Y_1$ receptor [28]. Adenosine 3′-,5′-bisphosphate and several higher affinity analogues (e.g. MRS2179) were instrumental in pharmacologically resolving the responses of platelets to ADP into the $P2Y_1$ receptor/G_q- and $P2Y_{12}$ receptor/G_i-mediated signaling events [8,29–31]. Replacement of the ribose group with a cyclopentane ring fused in the Northern conformation with a cyclopropane ring [i.e. an (*N*)-methanocarba moiety] led to the development of high affinity non-nucleotide antagonists of the $P2Y_1$ receptor [32,33]. Moreover, this (*N*)-methanocarba substitution also was well tolerated in adenine and uridine nucleotides and in certain other agonist analogues including (*N*)-methanocarba-2MeSADP (MRS2365) [16,34]. Three ADP-selective P2Y receptors exist (i.e. the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors), and the specificity of MRS2365 as an agonist for the $P2Y_1$ receptor was illustrated with recombinant P2Y receptors [17].

That MRS2365 alone does not promote sustained aggregation but rather induces the shape change known to occur as a consequence of G_q-dependent signaling provides strong indication that the G_q-coupled $P2Y_1$ receptor is activated by this agonist without simultaneous activation of the G_i-linked $P2Y_{12}$ receptor. We recently illustrated the high selectivity of MRS2500 as an antagonist for the human $P2Y_1$ receptor [24,35], and Hechler *et al.* [25] confirmed this selectivity in studies of platelets *ex vivo* and *in vivo*. The observation that MRS2500 completely blocks the effects of MRS2365 further establishes the $P2Y_1$ receptor-selective agonist action of MRS2365. Other functional studies also are consistent with this conclusion. For example, whereas neither MRS2365 nor epinephrine alone caused sustained aggregation, marked and sustained aggregation was observed with addition of MRS2365 following epinephrine, which activates a $G_{i/z}$-linked α_{2A}-adrenergic receptor. Similarly, selective activation of the $P2Y_{12}$ receptor with 3 nM 2MeSADP caused little aggregation and no shape change, and a maximally effective concentration of

MRS2365 only produced shape change. However, concurrent addition of 3 nM 2MeSADP and MRS2365 induced marked and sustained aggregation, again supporting the idea that the agonist action of MRS2365 is $P2Y_1$ receptor specific.

Platelets rapidly lose their capacity to aggregate in response to ADP [36–38]. The recent studies of Baurand *et al.* [21,39] illustrated that preincubation of platelets with ADP or ADPβS results in rapid loss of $P2Y_1$ receptor-promoted elevation of intracellular Ca^{2+} and loss of the required function of the $P2Y_1$ receptor in ADP-induced aggregation. In contrast, the capacity of the ADP-activated $P2Y_{12}$ receptor to inhibit adenylyl cyclase or promote aggregation (in the presence of serotonin which activates the G_q-linked 5-HT_{2A} receptor) was largely retained in ADP- or ADPβS-preincubated platelets. Thus, these investigators proposed that the $P2Y_1$ receptor of platelets rapidly desensitizes in the presence of ADP whereas the $P2Y_{12}$ receptor does not. The results of the current study in which the responses of platelets have been studied after the selective activation of the $P2Y_1$ receptor confirm the conclusions of Baurand *et al.* [21,39]. Thus, we show under conditions in which only the $P2Y_1$ receptor is activated that responsiveness of this receptor is rapidly lost, that desensitization is apparently receptor-specific as it can be circumvented by activation of another G_q-linked receptor, and that loss of the aggregation response occurs heterologously, as neither ADP by activation of the G_i-linked $P2Y_{12}$ receptor nor epinephrine by activation of the $G_{i/z}$-linked α_{2A}-adrenergic receptor promoted aggregation after preincubation with MRS2365. Given the synergy observed between the $P2Y_1$ and $P2Y_{12}$ receptors in the aggregation response to ADP, it is important to point out that the remarkably rapid desensitization of the $P2Y_1$ receptor observed here occurs in the absence of coactivation of both the $P2Y_1$ and $P2Y_{12}$ receptors as was the case in previous studies with the non-selective agonists, ADP or ADPβS. That is, remarkably rapid desensitization of the $P2Y_1$ receptor occurs irrespective of whether the $P2Y_1$ and $P2Y_{12}$ receptors are activated simultaneously or the $P2Y_1$ receptor is activated independently.

The molecular basis of P2Y receptor desensitization in platelets is unknown. However, Baurand *et al.* [39] illustrated with receptor-specific antibodies that incubation of platelets with ADP results in rapid internalization of the $P2Y_1$receptor to an intracellular compartment, whereas little change in the distribution of the $P2Y_{12}$ receptor occurs. In contrast to the conclusions of Baurand *et al.*, Hardy *et al.* [40] recently reported that preincubation of platelets with ADP results in rapid desensitization of both the $P2Y_1$ and $P2Y_{12}$ receptors. Moreover, these investigators provide evidence that the $P2Y_1$ receptor is subject to regulation by protein kinase C isozymes whereas $P2Y_{12}$ receptor desensitization involves a G protein receptor kinase(s). Interestingly, Fam *et al.* [41] have identified Thr^{339} in the carboxy terminus of the human $P2Y_1$ receptor as a phosphorylation site necessary for protein kinase C-mediated attenuation of $P2Y_1$ receptor promoted signaling. Again, capacity to independently activate the $P2Y_1$ receptor with MRS2365 in the absence of activation or inhibition of the $P2Y_{12}$ receptor should aide in developing the molecular understanding of the regulation of $P2Y_1$ receptor-dependent signaling.

One of the well-recognized effects of $P2Y_1$ receptor activation during ADP-induced aggregation is the promotion of the shape change of platelets, and although the change of shape is undoubtedly involved in the process of aggregation, it is neither sufficient nor the only process triggered by activation of the $P2Y_1$ receptor. Our results illustrate that the platelet shape change induced by $P2Y_1$ receptor activation is sustained for up to 10 min in the presence of agonist and reverses over a 10 min time period upon $P2Y_1$ receptor blockade. In contrast, the $P2Y_1$ receptor signaling response that synergizes with the $P2Y_{12}$ receptor-promoted signaling to produce sustained aggregation fully desensitizes within 1 min of receptor activation. Targeted gene disruption in mice [10,11] suggests that $P2Y_1$ receptor signaling entirely emanates from activation of G_q, but loss of function could occur

at this or multiple downstream steps not restricted to initial mobilization of Ca^{2+}, activation of protein kinase C, or phospholipase C-catalyzed changes secondary to alteration in membrane phosphoinositide levels. That activation of a G_q-linked receptor for 5-HT circumvents MRS2365-induced loss of ADP-induced aggregation is consistent with, but does not prove, that MRS2365-induced desensitization occurs at the level of the $P2Y_1$ receptor *per se*. A complex and still unclear set of signaling pathways underlie the formation of pseudopodia, and the molecular machinery necessary to reverse the platelet shape change once it occurs remains largely undefined.

In summary, the studies outlined above have demonstrated that MRS2365 activates the $P2Y_1$ receptor with high potency and selectivity as observed by hallmark features (i.e. shape change/pseudopod formation), leads to rapid $P2Y_1$ receptor desensitization with no observable action at the $P2Y_{12}$ receptor, and attenuates ADP-induced aggregation upon subsequent agonist challenge. MRS2365 is a novel pharmacological tool that should prove highly useful in discrete analysis of the bifurcating ADP-induced signaling pathways in platelets.

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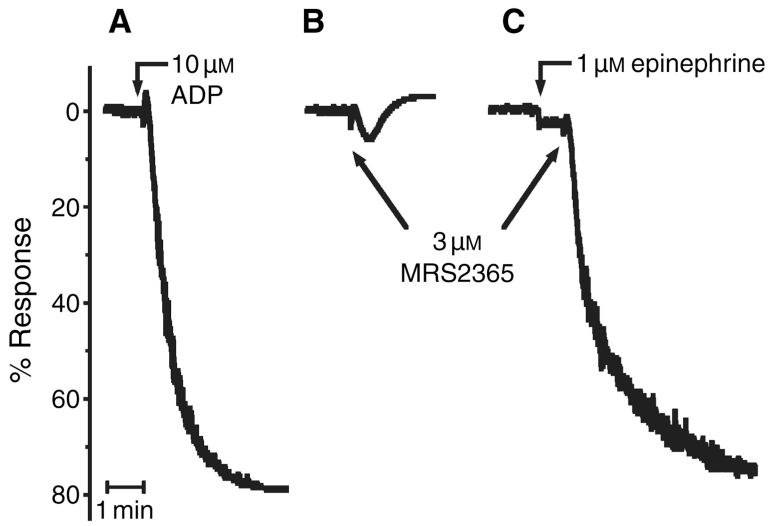

Fig. 1.

Agonist-induced responses of human platelets. Platelets were prepared as described in Methods. Ten micromolar adenosine diphosphate (ADP) was added after a 1 min (A) while 3 μM MRS2365 was added either alone (B) or following 1 μM epinephrine (C). Aggregometer traces are from a single experiment and are representative of at least three experiments using different platelet preparations.

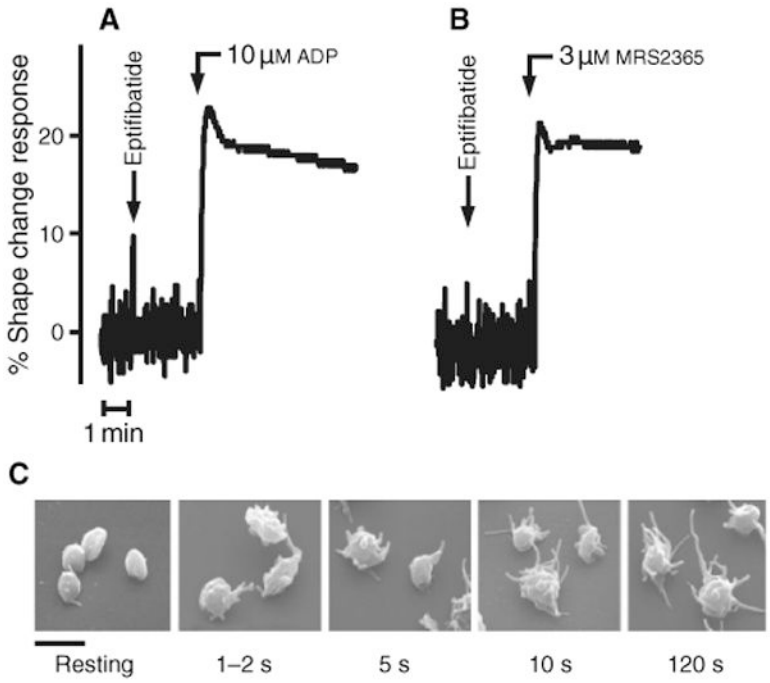

Fig. 2.

Comparison of ADP and MRS2365-induced platelet shape changes. Agonist-induced shape change of washed platelets was measured as described in Methods. One hundred twenty-five micromolar eptifibatide ($GP_{IIb/IIIa}$ antagonist) was added 2 min prior to agonist treatment. Either 10 μM ADP (A) or 3 μM MRS2365 (B) was added and the resulting shape changes recorded. Aggregometer traces are from a single experiment and are representative of at least three experiments. Scanning electron micrographs of platelet shape change at time points following selective activation of the $P2Y_1$ receptor with 3 μM MRS2365 (C). The maximum extent of shape change was observed at 15 s and was maintained through the measurement period of 2 min. Bar = 2 μm, 10 000×.

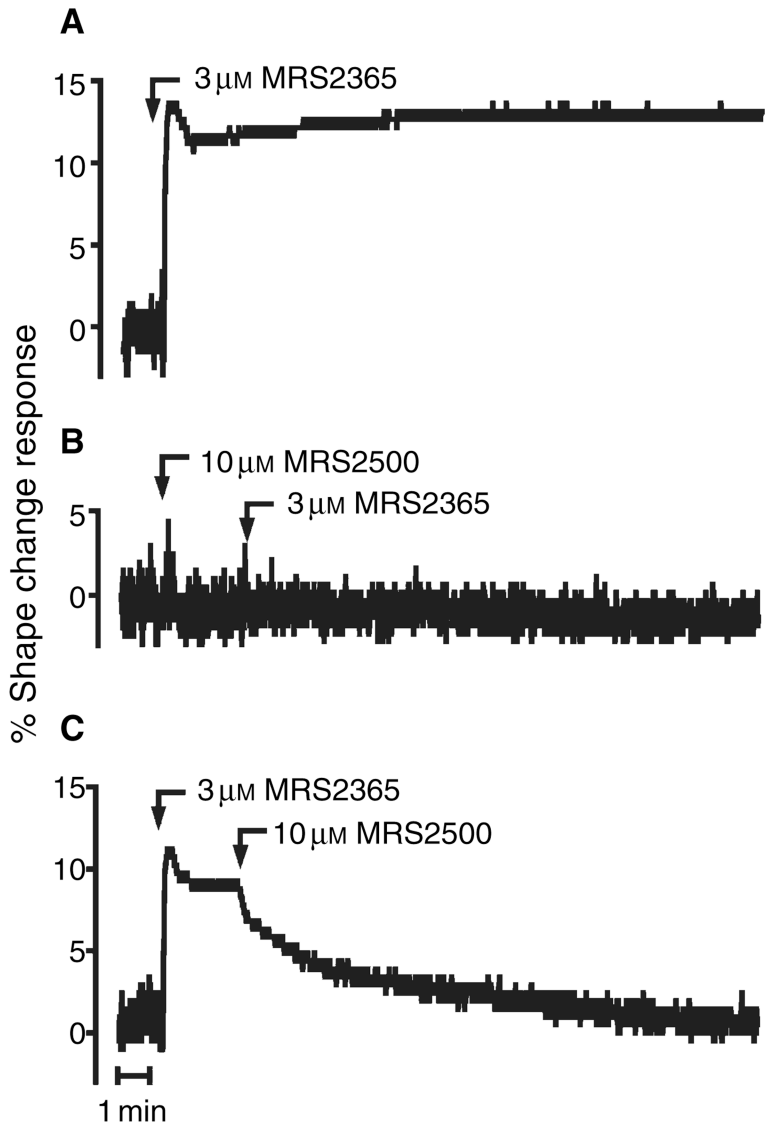

Fig. 3.

Reversal of MRS2365-promoted platelet shape change with MRS2500. Platelet shape change was measured following the addition of 3 μM MRS2365 alone (A), 10 μM MRS2500 followed by 3 μM MRS2365 (B), or 3 μM MRS2365 followed by 10 μM MRS2500 (C). The $t_{1/2}$ for reversal of MRS2365-induced shape change following the addition of MRS2500 was 2.7 min ($r^2 = 0.89$).

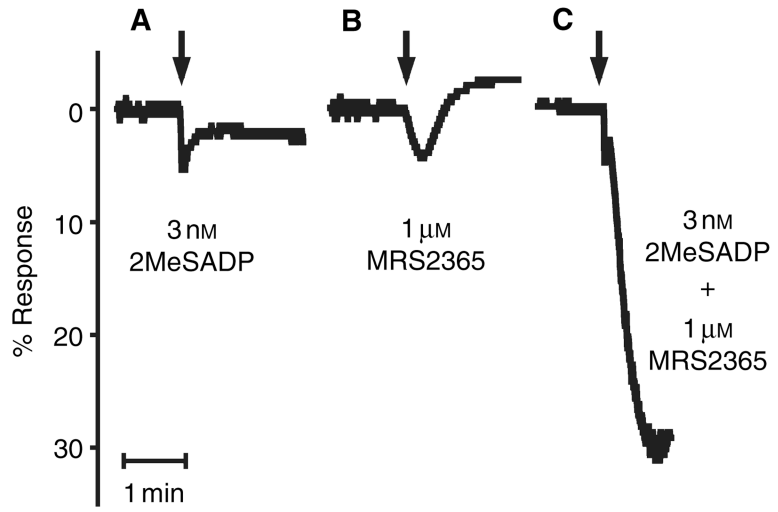

Fig. 4.

Requirement of simultaneous addition of 2MeSADP and MRS2365 to elicit platelet aggregation. After 1 min of recording, compounds were added to the platelet suspensions (arrow) and shape change or aggregation was recorded for 6–8 min. (A) 3 nM 2MeSADP, (B) 1 μM MRS2365, (C) 3 nM 2MeSADP and 1 μM MRS2365 added simultaneously. Aggregometer traces are from a single experiment and are representative of at least three experiments using different washed platelet preparations.

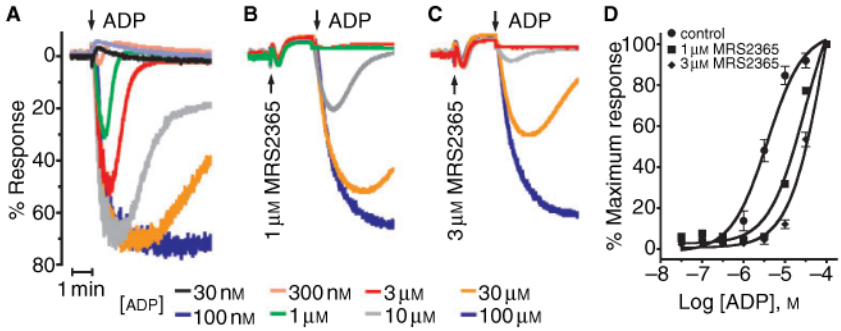

Fig. 5.

Decreased ADP-induced aggregation following treatment with MRS2365. Platelet aggregation was measured in response to the indicated concentrations of ADP added (arrow) after a 1 min recording period (A) or an additional 2 min pretreatment with either 1 μM MRS2365 (B) or 3 μM MRS2365 (C). Maximal aggregation values were used to generate concentration–response curves (D). Concentration–response curves under control conditions (panel A) were identical for agonist added at either 1 min or 3 min. Aggregometer traces are from a single experiment and are representative of at least three experiments using different platelet preparations.

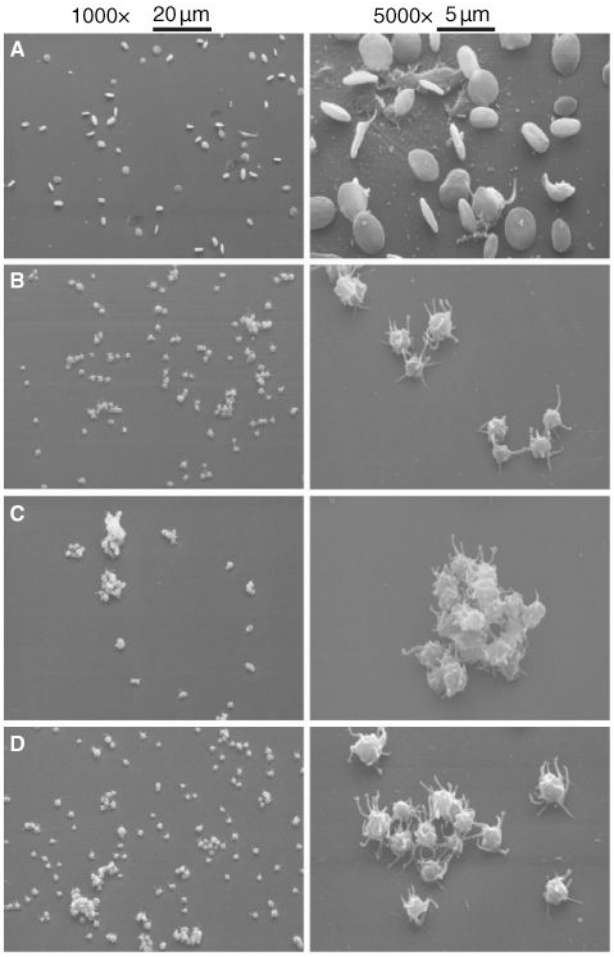

Fig. 6.

Scanning electron microscopy of human platelets following exposure to (*N*)-methanocarba-2MeSADP (MRS2365). Electron microscopy was performed as described in Methods. (A) resting platelets, (B) 3 μM MRS2365 alone (2 min), (C) 3 μM ADP for 30 s, (D) 3 μM MRS2365 (2 min) followed by 3 μM ADP for an additional 2 min.

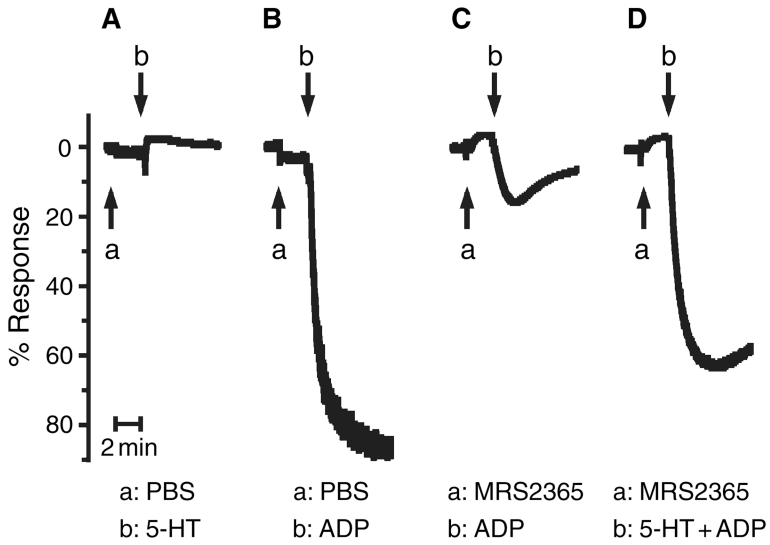

Fig. 7.

Restoration of MRS2365-promoted inhibition of ADP-induced platelet aggregation by independent stimulation of a G_q signaling pathway. Suspensions of human washed platelets were treated with vehicle (PBS; A,B) or 3 μM MRS2365 (C,D) two minutes after 1 μM 5-HT (A), 10 μM ADP (B,C), or 5 μM 5-HT + 10 μM ADP (D) were added and platelet aggregation was recorded for a total of 8 min. Aggregometer traces are from a single experiment and are representative of results from at least three experiments.

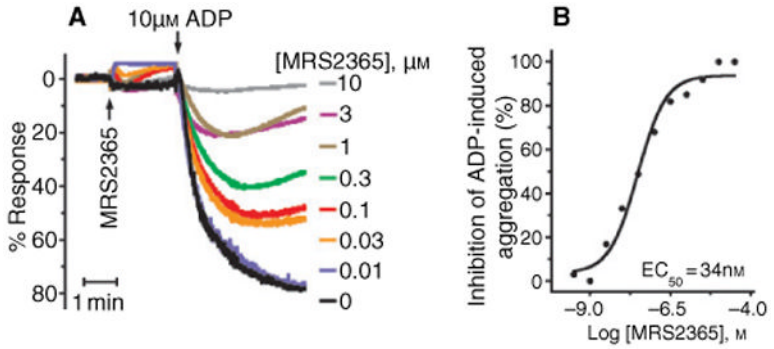

Fig. 8.

Concentration dependence of MRS2365 for induction of loss of the ADP-induced platelet aggregation response. Platelets were preincu-bated with the indicated concentrations of MRS2365 for 2 min followed by challenge with 10 μM ADP (A). Tracing amplitudes for each condition were transformed with respect to control (no MRS2365) to produce a concentration–response curve (B). Aggregometer traces are from a single experiment and are representative of results from at least two experiments.

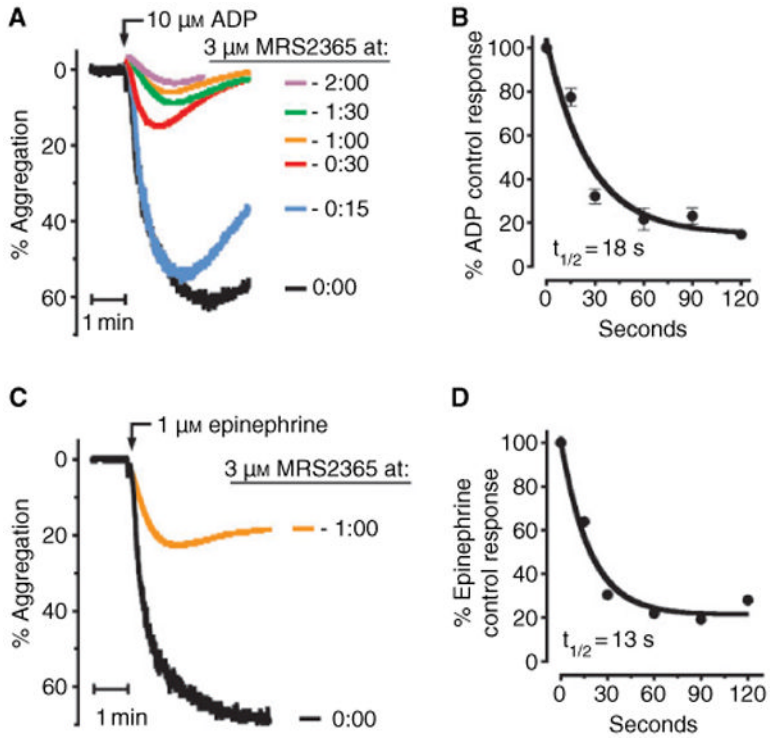

Fig. 9.

Time dependence of MRS2365-promoted reduction in ADP- or epinephrine-induced platelet aggregation. Platelets were pretreated for the indicated times with 3 μM MRS2365 followed by challenge with 10 μM ADP (A) or 1 μM epinephrine (C). The maximum amplitude measured in each tracing was compared with control [0:00; (A), 10 μM ADP; (C), simultaneous addition of 1 μM epinephrine and 3 μM MRS2365] to generate time course curves in which the $t_{1/2}$ for loss of response was calculated. Aggregometer traces are from a single experiment and are representative of results from four (B) or two (D) experiments.