The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts

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P2Y12, a G protein-coupled receptor that plays a central role in platelet activation has been recently identified as the receptor targeted by the antithrombotic drug, clopidogrel. In this study, we further deciphered the mechanism of action of clopidogrel and of its active metabolite (Act-Met) on P2Y12 receptors. Using biochemical approaches, we demonstrated the existence of homooligomeric complexes of P2Y12 receptors at the surface of mammalian cells and in freshly isolated platelets. *In vitro* treatment with Act-Met or *in vivo* oral administration to rats with clopidogrel induced the breakdown of these oligomers into dimeric and monomeric entities in P2Y12 expressing HEK293 and platelets respectively. In addition, we showed the predominant association of P2Y12 oligomers to cell membrane lipid rafts and the partitioning of P2Y12 out of rafts in response to clopidogrel and Act-Met. The raft-associated P2Y12 oligomers represented the functional form of the receptor, as demonstrated by binding and signal transduction studies. Finally, using a series of receptors individually mutated at each cysteine residue and a chimeric P2Y12/P2Y13 receptor, we pointed out the involvement of cysteine 97 within the first extracellular loop of P2Y12 in the mechanism of action of Act-Met.

mechanism of action | platelet | antiaggregant

Many G protein-coupled receptors (GPCRs) have been shown to assemble as homodimers, heterodimers, as well as larger oligomers (1, 2). The existence of such oligomeric entities raises questions as to their functional consequences as well as their physiological relevance. Heterologous expression systems have provided a variety of answers concerning ligand-dependent regulation of GPCR oligomeric states. Ligand binding, depending on the GPCR studied, can promote (3–10) or inhibit (11–13) dimer formation, as well as having no effect on preexisting constitutive homo- or heterodimers (14–25). The fact that heterodimerization may alter the pharmacological properties of a GPCR along with its internalization and signal transduction behavior is of critical importance (26, 27).

Clustering, even for nonheptahelical receptors, now appears as a common feature of cell signaling. Specialized structures such as clathrin-coated pits, caveolae, and lipid rafts contain high concentrations of signaling molecules. Rafts represent dynamic assemblies of proteins and lipids, mostly sphingolipids and cholesterol (28, 29). Proteins such as glycophosphatidylinositol-anchored proteins, nonreceptor tyrosine kinases, Gα subunits of heterotrimeric G proteins, and palmitoylated proteins appear to localize to these microdomains (30). In addition, recent studies have shown that partitioning of proteins in and out of rafts can depend on their state of activation or dimerization (31–33). A variety of GPCR have also been identified in caveolae or rafts. These include α and β-adrenergic receptors (34, 35), adenosine A1 receptor (36), angiotensin II type 1 receptor (37), muscarinic receptor (38), EDG1 receptor (39), bradykinin B1 and B2 receptors (33, 40, 41), endothelin receptor (43), rhodopsin (44), and *N*-formyl peptide receptor (45). In the majority of cases, this location was found to be sensitive to ligand stimulation, clustering in raft being either increased or decreased.

Platelets are key elements in hemostasis and thrombosis. Diverse agonists are known to activate platelet aggregation and fibrinogen binding to the subsequently activated integrin GPIIb-IIIa complex. In this process, ADP is of particular importance, because it is released by damaged cells and activated platelets, thus enhancing the action of many platelet activators. ADP mediates platelet aggregation through its binding to two GPCRs P2Y1 and P2Y12, acting together to achieve complete aggregation (46). P2Y12 is expressed in platelets, megakaryocytes, and neuronal cells (47). Upon activation, P2Y12 triggers a cascade of signaling events including adenylyl cyclase inhibition and PI3K activation (48). P2Y12 knockout mice are particularly protected against thrombosis (49). In humans, two genetic P2Y12 deficiencies have been described, associated with a hemorrhagic phenotype and a pronounced impairment of ADP-induced platelet aggregation (50, 51).

P2Y12 is the target of clopidogrel, a well known antithrombotic compound that has demonstrated its efficacy and favorable safety profile in an extensive clinical program, by preventing ischemic events such as cardiovascular death, myocardial infarction, or stroke in atherothrombotic patients, on top of standard treatment (52). Clopidogrel does not, by itself, exhibit direct antiaggregant activity *in vitro*. Indeed, *in vivo* studies have demonstrated that clopidogrel has to undergo hepatic metabolization to obtain an active metabolite (53). This active metabolite (Act-Met) has been isolated, and its structure has been elucidated (54). It contains a free thiol function, and its activity is lost when the thiol is derivatized (55), suggesting its possible interaction with cysteine-containing sequences. *In vitro*, Act-Met inhibits the binding of 2MeS-ADP to platelets and ADP-induced aggregation of platelets. In a recent study, Act-Met was found to inhibit the binding of 2MeS-ADP to P2Y12 (56). This inhibition was shown to be irreversible and selective for P2Y12 (57, 58).

Here, we have further determined the mechanism of action of clopidogrel and of its active metabolite on the P2Y12 receptor. We have found that these compounds act on this receptor by an original mechanism, by interfering with P2Y12 assembly and its localization in lipid rafts. This allowed us to demonstrate the importance of oligomerization and membrane localization on the function of this

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Abbreviations: GPCR, G protein-coupled receptor; HA, hemagglutinin.

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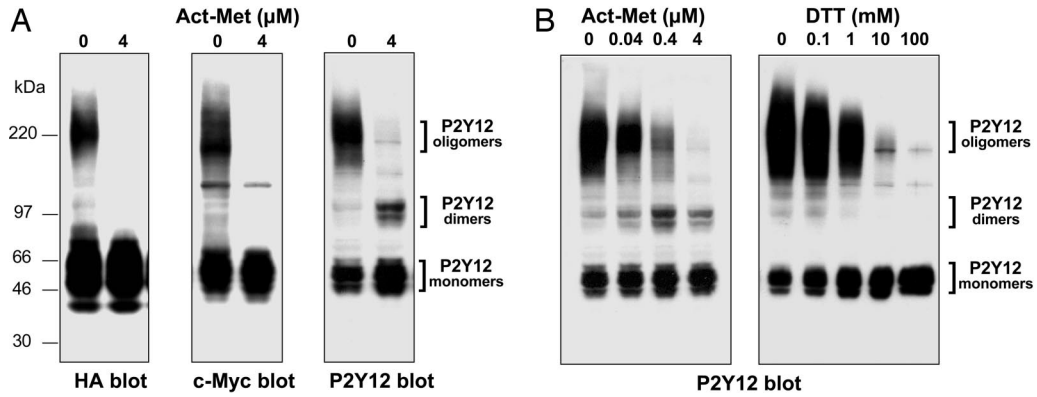

Fig. 1. Disruption by Act-Met of oligomeric forms of P2Y12 expressed in HEK293 cells. HEK293 cells coexpressing HA- and cMyc-tagged P2Y12 receptors were incubated for 1 h at room temperature with the indicated concentration of Act-Met, and whole-cell extracts were prepared in octyl-glucoside buffer, as described in *Materials and Methods*. (*A*) Cell extracts were subjected to SDS-PAGE under nonreducing conditions followed by anti-HA, anti-cMyc or anti-Cterm P2Y12 immunoblotting. (*B*) Extracts from Act-Met-treated cells (*Left*) were subjected SDS/PAGE under nonreducing conditions, whereas extracts from untreated cells were subjected to SDS/PAGE under reducing conditions (*Right*, increasing concentrations of DTT). Anti-P2Y12 immunoblotting was then performed.

receptor. Finally, we provide evidence for the molecular interaction between Act-Met and P2Y12.

Results

Effect of the Active Metabolite of Clopidogrel on the Oligomeric Forms of P2Y12. P2Y12 receptors tagged at their NH_2 termini with either hemagglutinin (HA) or the c-myc epitope were transiently expressed in HEK293T cells to investigate their state of multimerization by using immunoblotting analyses. As illustrated in Fig. 1*A* (left lanes), anti-HA, anti-cMyc and anti-P2Y12 antibodies all identified monomeric as well as oligomeric receptor species in whole cell lysates. It should be noted that dimers were only detected with the anti-P2Y12 antibody, and not with the anti-tag antibodies. This may be due to a lesser accessibility of NH_2-located tags by anti-tag antibodies in dimeric structures, as opposed to the accessibility by the anti P2Y12 antiserum to the COOH-located peptidic sequence used to raise the antibody. Upon treatment of the cells with the active metabolite of clopidogrel (Act-Met), the bands corresponding to the oligomeric state of P2Y12 receptors were no longer detected by all three antibodies (Fig. 1*A*, right lanes). The use of an anti-P2Y12 antiserum recognizing the COOH terminus of this receptor allowed for the immunodetection of dimers in whole cell lysates of Act-Met-treated cells. Using this antiserum, an increase in the intensity of the bands corresponding to dimeric and monomeric forms of P2Y12 was also observed, potentially reflecting a compensation for the loss of the oligomeric forms of P2Y12. As for the inability of the other two anti-tag antibodies to detect the P2Y12 dimers after Act-Met treatment, conformational changes at the NH_2 terminus of the receptors may eliminate recognition by the anti-HA and anti-c-Myc antibodies.

Because Act-Met exhibits a free thiol-reactive function (55), we compared the Act-Met-induced changes in oligomerization of P2Y12 with those induced by DTT, a commonly used thiol-reducing reagent (Fig. 1*B*). Both compounds strongly affected the high-molecular-mass species of P2Y12 in a concentration-dependent manner, Act-Met being at least three orders of magnitude more potent than DTT. However, the two compounds induced somewhat different effects on P2Y12 oligomeric organization. Act-Met appeared to preferentially disrupt oligomers into dimers, whereas treatment with DTT only generated monomers. The activity of Act-Met on P2Y12, when compared to DTT activity, seems to be restricted to a limited class of thiol-sensitive chemical functions.

Effect of Act-Met on the Localization of P2Y12 in Lipid Rafts. In the course of the biochemical characterization of P2Y12 receptors, we found that greater amounts of P2Y12 oligomeric forms were obtained when octyl-glucoside was used for cell lysis instead of Triton X-100 (data not shown). This observation led us to suggest that P2Y12 oligomers could be located in particular microdomains within the plasma membrane. These microdomains, e.g., lipid rafts (28), are known to be insoluble in Triton X-100 due to their lipid composition, rich in cholesterol and sphingolipids. To ascertain the presence of P2Y12 oligomers in lipid rafts, Triton X-100 cell lysates were fractionated by sucrose gradient centrifugation and gradient fractions were analyzed for the presence of P2Y12 by immunoblotting. Lipid raft-containing fractions were monitored by caveolin immunoblotting, these proteins serving as a convenient marker for caveolae, a subpopulation of lipid rafts (59). P2Y12 detection using the anti-P2Y12 antibody (Fig. 2*A Upper Left*) showed that oligomers were mainly located in the microdomain-rich fractions (fractions 4 and 5), whereas dimers and monomers were predominantly found in the microdomain-free fraction 6. This fraction contained the monomeric, dimeric, as well as the oligomeric form of P2Y12.

After treatment with Act-Met, oligomers were no longer detected in any of the gradient fractions (Fig. 2*A Upper Right*). This loss/disruption of the P2Y12 oligomeric entities was accompanied by an increase in monomers and dimers in fractions 5 and 6 of the sucrose gradient. The vast majority of monomers and dimers were redistributed outside microdomains as attested by their strong enrichment in the gradient fraction 6 that has a very poor content of caveolin.

We next wanted to verify the selectivity of the observed disruption and redistribution of P2Y12 complexes induced by Act-Met, and rule out any nonspecific effect on membrane microdomains that might result in artefactual partitioning of proteins out of lipid rafts. Microdomains prepared from HEK293T cells coexpressing c-Myc-P2Y12 and HA-P2Y13 were analyzed by anti-HA immunoblotting. In this heterologous expression system, P2Y13 existed as monomeric and oligomeric species, the latter in far lower amounts than P2Y12 expressed in those same cells (Fig. 2*A Lower Left*). Both monomeric and oligomeric P2Y13 species were mostly associated with lipid rafts (fractions 4 and 5). In nontreated HEK293T cells, P2Y13 monomers were targeted to cellular lipid microdomains, as opposed to P2Y12 monomers that were mainly localized outside such microdomains. When cells were treated with Act-Met, P2Y13 localization remained unchanged (Fig. 2*A Lower Right*) in contrast to P2Y12, thus demonstrating the selective action of the active metabolite of clopidogrel on P2Y12 oligomers.

To test the functionality of the various species, we performed binding of [^{33}P]2MeS-ADP to the proteins present in the sucrose gradient fractions 3–7. The P2Y12 protein content was evaluated by measuring the intensity on Western blots of bands migrated under reducing conditions (Fig. 2*B Upper*). In this experiment, significant specific binding was detected in fractions 3–5, identified as caveolin-containing fractions (Fig. 2*B Lower Left*), measured in the same amount of proteins deposited. In fraction 6, no binding was detected despite its high content in P2Y12. This was particularly obvious

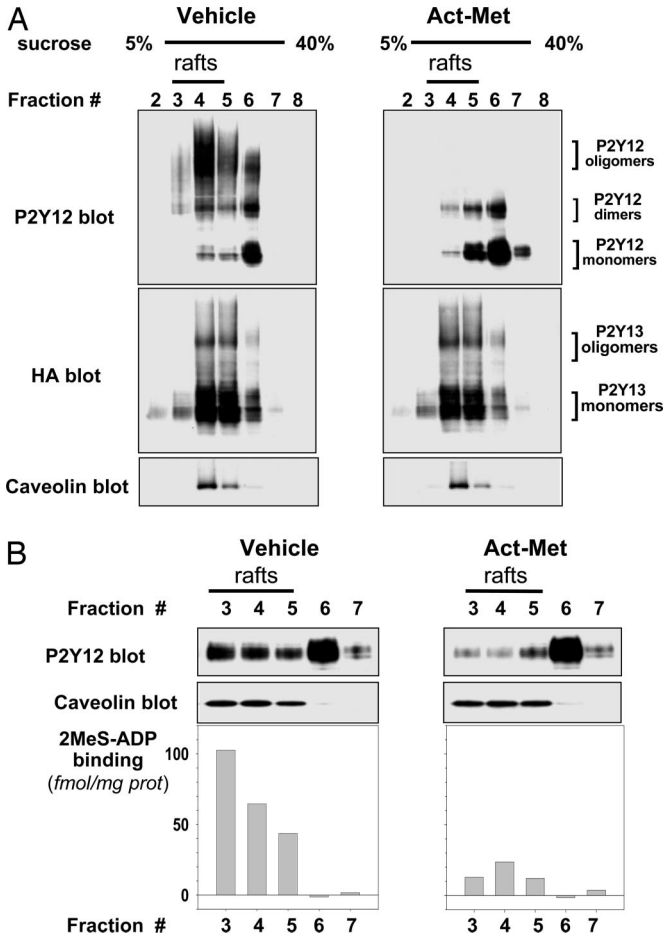

Fig. 2. Localization of functional P2Y12 oligomeric complexes in rafts and redistribution of the receptor as dimers and monomers outside membrane microdomains on treatment of P2Y12 expressing HEK293 cells with Act-Met. (*A*) HEK293T cells coexpressing cMyc-P2Y12 and HA-P2Y13 were incubated for 1 h at 37°C in the presence of 4 μM Act-Met or vehicle. Then, cells were lysed in 0.5% Triton X-100 containing buffer. Lysates were fractionated on a sucrose step gradient (5–40%). The centrifuged gradient was divided into eight equal fractions, and protein concentrations were determined for each fraction. Aliquots containing 2 μg of protein (fraction 2), 4 μg of protein (fraction 3), and 20 μg of protein (fractions 4–8) were alkylated with 10 mM iodoacetamide and subjected to SDS/PAGE under nonreducing conditions followed by anti-P2Y12, anti-HA or anti-caveolin immunoblotting. Gradient fraction 1 was not analyzed because it did not contain any detectable protein. (*B*) HEK293T cells expressing cMyc-P2Y12 were incubated for 1 h at 37°C in the presence of 4 μM Act-Met or vehicle. Cell lysate density fractionation was performed as in *A*. To quantify P2Y12 amount in each gradient fraction, 4-μg aliquots of protein (fractions 3–7) were subjected to SDS/PAGE under reducing conditions followed by anti-P2Y12 immunoblotting. P2Y12 monomer band intensity was measured by using a GS-800 densitometer (Kodak). Anti-caveolin immunoblotting was performed in parallel to monitor the raft-enriched fractions. Binding of [^{33}P]2MeS-ADP was measured on 1 μg of protein as described in *Materials and Methods*. Bar graphs represent the specific binding reported to the protein amount.

when the data were expressed as ratios (specific binding/P2Y12 content). In fractions 3–7, these ratios were 142, 37, 57, 0, and 1, respectively. Only minimal binding was detected in fractions 3–5 of the gradient fractions prepared from Act-Met treated cells (Fig. 2*B Lower Right*). Sensitivity to Act-Met of P2Y12 oligomers present in lipid raft-enriched fractions was assessed by immunoblotting (Fig. 2*B Upper Right*), showing a strong reduction in P2Y12 detection in fractions 3–5.

Effect of Clopidogrel on P2Y12 Oligomers in Rat Platelets. Because the conclusions of the *in vitro* experiments performed on cells expressing recombinant proteins may not be relevant *in vivo*, we examined the effects of an *in vivo* treatment with clopidogrel on endogenous P2Y12 in platelets, the physiologically targeted cell (see Fig. 4, which is published as supporting information on the PNAS web site).

In this set of experiments, rats were treated orally for 2 h with various doses of clopidogrel, then their blood was taken and platelets were prepared. Platelet proteins were resolved by SDS-/PAGE under nonreducing conditions. P2Y12 receptors were detected by Western blotting using anti-P2Y12 rabbit antiserum (Fig. 4*A*). The main P2Y12 species present in resting platelets corresponded to oligomers that were diffusely resolved on the acrylamide gel. Monomeric and dimeric species were hardly detected under the experimental conditions used. When animals were treated with a single dose of clopidogrel, and their platelets prepared 2 h later, no expression of P2Y12 oligomers was detected in platelet lysates. This loss was accompanied with a dramatic increase in detection of P2Y12 dimers. This increase depended on the dose of clopidogrel administered to the animals and closely correlated with the antiaggregating activity of clopidogrel (42), as verified on the same platelet preparation (Fig. 4*B*). Platelet microdomains were then studied for their P2Y12 content. Because platelet rafts are devoid of caveolin, CD36, a membrane glycoprotein strongly enriched in platelet rafts (60), was used to monitor the raft enrichment in platelet extracts separated on sucrose gradients. In resting platelets, P2Y12 receptors appeared to be predominantly expressed as oligomers located in lipid microdomains (Fig. 4*C Left*). Isolation of such microdomains led to a much better detection of P2Y12 oligomer bands by immunoblotting than that observed with whole platelet extracts. The observation that oligomers represent the main P2Y12 species in platelets (*i*) reinforces the hypothesis that the raft-associated P2Y12 oligomers actually represent the functional form of the receptor and (*ii*) emphasizes the critical role of membrane microdomains in ADP-mediated platelet activation (61).

After *in vivo* clopidogrel treatment, platelet P2Y12 oligomers were completely converted into dimeric forms and to a lesser extent into monomeric forms of the receptor; then, dimers and monomers were partitioned outside the platelet lipid rafts. Thus, the situation previously observed in the HEK293 heterologous expression system was found in a more physiologically relevant model.

Effect of Act-Met on P2Y12 Mutants. Act-Met contains a free thiol functional group that is necessary for its activity, suggesting that an interaction with cysteine residues on P2Y12. We decided to investigate whether a mutation of one of these cysteines could modify the activity of Act-Met on 2MeS-ADP binding as well as P2Y12 oligomerization. P2Y12 contains 10 cysteines. According to seven-transmembrane (7TM) receptor modeling, only four cysteines are predicted to be exposed at the surface of the cell (Fig. 3*A*). However, because the cell permeability of Act-Met is not currently known, and to study the possible involvement of cysteine residues in the mechanism of action of clopidogrel, 10 P2Y12 receptor mutants were generated in which a cysteine was replaced by an alanine. Mutated receptors were transiently expressed in Cos7 cells and binding of [^{33}P]2MeS-ADP was performed. Specific binding was detected with all mutant P2Y12 receptors, except C97A and C175A (Table 1). On cells expressing the eight other mutants, Act-Met was able to inhibit [^{33}P]2MeS-ADP binding with efficacies comparable to that measured on cells expressing the wild-type P2Y12 receptor. Because Ding *et al.* (62) suggested that C17 and C270 were the targets of the active metabolite of clopidogrel, we tested the double mutant C17A/C270A receptor. Affinity for 2MeS-ADP dropped 10-fold, but sensitivity to Act-Met was not affected (Table 1).

Although neither C97A nor C175A mutated receptors were able to bind 2MeS-ADP, we nevertheless tested their sensitivity to

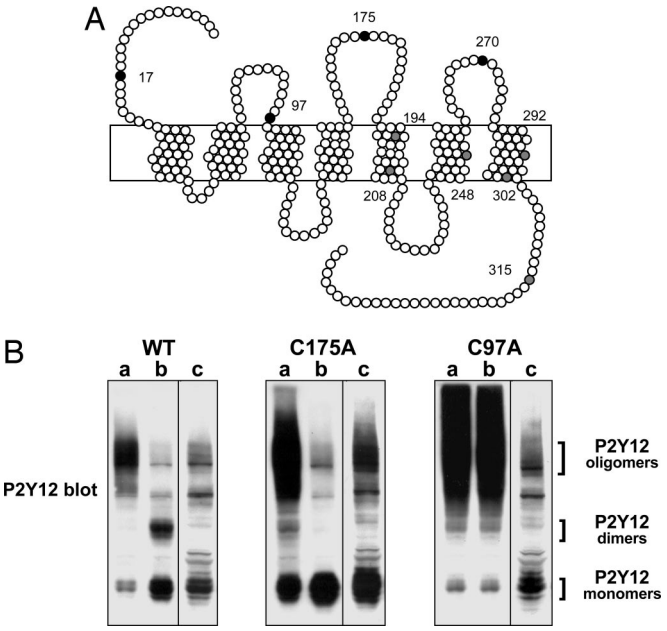

Fig. 3. Expression at the cell surface, oligomerization, and sensitivity to Act-Met of P2Y12 receptors mutated at Cys 97 or Cys 175, as compared to wild-type receptors. (*A*) The human P2Y12 amino acid sequence contains 10 cysteine residues, four extracellular cysteine at position 17, 97, 175, and 270 (black filled circles), five cysteine residues at position 194, 208, 248, 292, and 302 within the transmembrane domains, and one intracellular cysteine residue at position 315 (gray filled circles). (*B*) HEK293 expressing cMyc-tagged P2Y12 receptors, either wild type (WT) or mutated at Cys 97 (C97A) or Cys 175 (C175A), were incubated for 1 h at 37°C with 4 μM Act-Met. Cells were directly lysed in octyl-glucoside containing buffer.

Act-Met by measuring P2Y12 oligomer disruption. Fig. 3*B* illustrates the fact that both mutants were normally expressed in HEK293T cells, as shown by Western blot detection with anti-P2Y12 antiserum (lanes a). Both mutant oligomers were reduced by the treatment with DTT (lanes c), showing that, as in wild-type P2Y12, disulfide bonds are involved in these oligomeric complex formations. However, the treatment with Act-Met revealed some differences (lanes b). Oligomers of C175A mutants were strongly disrupted, concurrent with an increase in the monomer bands, indicating that the clopidogrel-sensitive disulfide bond was still present in this mutant. Surprisingly, no dimers were detected in this case. With regards to the C97A mutant, Act-Met failed to reduce the amount of oligomers and to increase those of monomers, showing that the mutant oligomers were totally insensitive to Act-Met.

This investigation of P2Y12 receptors individually mutated on cysteine residues led us to pinpoint C97 as the amino acid targeted by Act-Met.

Table 1. [^{33}P]2MeS-ADP binding characteristics and Act-Met astagonism on mutant P2Y12 receptors

	Binding 2MeS-ADP		
	KD, nM	B_{max}, (10^3 sites per cell)	Effect of ActMet, IC_{50}, nM
P2Y12 WT	0.34 ± 0.09	486.7 ± 86.1	154.9 ± 23.8
C17A-P2Y12	0.23 ± 0.04	275.9 ± 25.9	191.5 ± 102.4
C97A-P2Y12	No binding	No binding	ND
C175A-P2Y12	No binding	No binding	ND
C194A-P2Y12	0.42 ± 0.07	411.4 ± 44.0	147.9 ± 60.6
C208A-P2Y12	0.39 ± 0.05	1,365.4 ± 97.6	159.2 ± 18.3
C248A-P2Y12	0.33 ± 0.04	718.5 ± 51.8	164.8 ± 74.6
C270A-P2Y12	0.51 ± 0.13	539.7 ± 86.7	70.4 ± 11.3
C292A-P2Y12	0.37 ± 0.06	464.4 ± 44.0	280.3 ± 78.1
C302A-P2Y12	0.63 ± 0.23	495.7 ± 138.5	85.9 ± 18.3
C315A-P2Y12	0.48 ± 0.15	453.5 ± 107.8	154.9 ± 58.8
C17A/C270A-P2Y12	3.15 ± 2.03	445.7 ± 236.7	137.9 ± 7.4

Cos7 cells transfected with plasmids encoding for P2Y12 cystein–alanine mutants were studied for their ability to bind to 2MeS-ADP and the sensitivity of this binding to Act-Met. ND, not determined.

Discussion

The main finding in the present investigation is that P2Y12 receptors exist predominantly as homooligomers situated in lipid rafts and that this state is essential for their functionality. Upon treatment with Act-Met, the active metabolite of clopidogrel (see *Supporting Text*, which is published as supporting information on the PNAS web site), the homooligomers are disrupted into nonfunctional dimers and monomers that are sequestered outside the lipid rafts. This original mechanism accounts for the *in vivo* irreversible antiplatelet activity of clopidogrel.

Our demonstration that P2Y12 receptors in freshly isolated platelets or expressed heterologously exist at the plasma membrane principally in an oligomeric form is in line with recent biochemical and biophysical studies that have provided evidence for the existence of GPCR dimers/oligomers both *in vitro* and in living cells. Evidence for the existence of GPCR oligomeric species using denaturing SDS/PAGE has suffered criticism based on technical shortcomings. However, a recent study (63) of the β2-adrenergic receptor (β2AR) elegantly demonstrated the clear existence of dimers and oligomers at the plasma membrane and convincingly showed that the detection of such species, after receptor solubilization and resolution by SDS/PAGE, was not simply the result of the formation of spurious disulfide bonds during cell lysis. Therefore, we followed a similar biochemical approach with P2Y12 expressed in HEK293T cells and have shown that P2Y12 receptors are present as homooligomers as well as dimers and monomers (Fig. 1 and Figs. 5 and 6, which are published as supporting information on the PNAS web site). In contrast, we found only very low levels of monomers in freshly isolated resting platelets compared to oligomers. This finding suggested that the large amount of monomers detected in the HEK293T heterologous mammalian expression system most probably resulted from an overload of the cell capability to fully process the overexpressed receptors. The oligomeric association of P2Y12 receptors at the plasma membrane was shown to depend on disulfide bond formation as demonstrated by the sensitivity of these complexes to DTT reduction after cell solubilization. This finding indicated that the P2Y12 receptor appears to behave in a similar way as β2AR in response to thiol-reducing agents, unlike P2Y1 receptors, which oligomers are resistant to reducing reagents (64). In contrast to some other GPCRs (3–13), the oligomerization of P2Y12 does not seem to be modulated by agonist binding, 2MeS-ADP being ineffective in changing the P2Y12 size distribution (data not shown).

In resting cells, P2Y12 oligomers were located in lipid microdomains, and these corresponded to the species capable of binding 2MeS-ADP (Fig. 2 *A* and *B*). Lipid microdomains are known to contain a variety of proteins (30), and although several GPCR have already been shown to be present in rafts and caveolae, we have discovered this location for a receptor of the P2Y class. The raft localization was not affected by a stimulation of cells with 2MeS-ADP (0.1 μM) (Fig. 7, which is published as supporting information on the PNAS web site). Additionally, 2MeS-ADP dependent stimulation of raft-associated P2Y12 was clearly demonstrated by local mitogen-activated protein kinase (MAPK) activation and β-arrestin 1 and 2 recruitment to these microdomains. The raft localization of active P2Y12 receptors is also supported in a recently published article (65) describing that P2Y12 coupling to adenylyl

cyclase down-regulation and platelet aggregation depended on cholesterol content of platelet membrane.

Incubation of P2Y12 expressing cells with Act-Met resulted in a dramatic decrease in oligomeric structures, as also seen with DTT (Fig. 1). However, in contrast to DTT, a dimeric structure was generated under these conditions; the exact nature of this dimer remains to be determined. The fact that the overall increase in dimers was only obtained with Act-Met and not with DTT, even at moderate concentrations, suggests that disulfide bonds are involved in the constitution of the oligomers, of which a fraction is resistant to Act-Met.

As a consequence of the disruption of the oligomeric structure by Act-Met, we found that P2Y12 was translocated outside of Triton X-100 insoluble lipid rafts toward membrane structures that are almost devoid of the lipid raft marker caveolin (Fig. 2). This finding correlated with the loss of 2MeS-ADP binding in lipid raft-containing fractions, binding still being absent from the other fractions. Binding to GPCR has been demonstrated to depend on G protein coupling in some cases (66, 67). Because G proteins seem to be located mainly in rafts, the absence of coupling between P2Y12 and its G protein may account for this loss in binding capacity. However, G protein uncoupling by GTPγS or p[NH]ppG has been shown to moderately decrease the affinity on receptors but not to totally impair binding, as Act-Met does, and therefore this hypothesis seems unlikely. Another possibility is that changes from an oligomeric to a dimeric organization modify the binding site of ADP in each individual monomer. To our knowledge, this has never been described. Finally, one cannot exclude that bound Act-Met may impair the accessibility of ADP or that the conformation of monomeric subunits could be modified by disulfide bond reduction. The effect of thiol-reducing agents on ligand binding to GPCR has already been observed, but not explained for AngIIR (68), MC1R (69), GABAbR (70), and 5HT1AR (71).

These results from *in vitro* studies were confirmed by *in vivo* experiments. In rat platelets, the presence of the oligomers, their loss and the P2Y12 translocation from microdomains were also observed when the animals were treated orally with clopidogrel (Fig. 4). This strengthens the finding that the observations made *in vitro* with Act-Met on P2Y12 expressing cells are relevant for the *in vivo* activity of clopidogrel.

Act-Met irreversibly affects the binding of 2MeS-ADP to P2Y12, which we presumed to be a result of a thiol-reductive action on one of ten cysteines of this receptor (55). Evidence of covalent binding of Act-Met through a disulfide bond was obtained showing that after treatment with 2-mercaptoethanol, the resulting adduct could be eluted from P2Y12 isolated by immunoprecipitation from Act-Met treated HEK293 (Fig. 8, which is published as supporting information on the PNAS web site). To identify the cysteine residue targeted by Act-Met, we have studied its activity on cysteine-mutated P2Y12 ectopically expressed in mammalian cells. Eight of 10 cysteines of the P2Y12 receptor were mutated without change in 2MeS-ADP binding affinity. In these mutants, Act-Met was as efficient as in the wild-type P2Y12 receptor, indicating that none of these amino acids were a target for the active metabolite of clopidogrel (Table 1). The two mutants (C97A and C175A) were unable to bind 2MeS-ADP, so we could not conclude on the activity of Act-Met on these mutants by using this methodology.

These results are not in accordance with those published by Ding *et al.*, who measured the adenylyl cyclase inhibition induced by ADP and 2MeS-ADP (62). From an observation showing that P2Y12 doubly mutated on C17 and C270 was less sensitive to pCMPS, a thiol reagent, the authors suggested that a similar result would be observed with clopidogrel. However, in our hands, binding of 2MeS-ADP was equally affected by Act-Met in the C17A/C270A double mutant and in the wild-type control, eliminating this hypothesis. Because 2MeS-ADP binding could not be used to explore the activity of Act-Met on the C97 and C175 mutants, we decided to investigate these receptors by structural analysis.

Even if they could not bind 2MeS-ADP, the C97A and C175A mutant receptors were both expressed at the cell surface (Fig. 9, which is published as supporting information on the PNAS web site) in oligomeric forms (Fig. 3*B*). These results differ from those obtained by Ding *et al.* (62), who did not obtain expression of C97S and C175S P2Y12 mutants. The use of a transient expression system instead of stable clones could explain this difference. The fact that neither C97 or C175 mutations affected oligomer production does not indicate that these residues are not required for the constitution of oligomers in wild-type receptor, because oligomers could assemble differently in P2Y12 mutants. However, unlike the C175A receptor, the C97A mutated receptor formed oligomers that were not sensitive to Act-Met. In contrast, DTT efficiently disrupted the C97A mutant oligomers, suggesting that these oligomers might adopt a conformation inaccessible to the reducing activity of Act-Met. C97 is located at the junction between the first extracellular loop and the third transmembrane domain. GPCR modeling predicts that C97 should bond with C175 in the second extracellular loop. The fact that the C175 mutant does not bind 2MeS-ADP supports the hypothesis that this cysteine is important for the structure of the receptor and may be involved in an intrareceptor disulfide bond. However, because Act-Met decreased the oligomers of C175 mutated P2Y12, an intraprotein C97–C175 bond does not seem to be the target of clopidogrel. We would rather suggest that C97 has a free thiol function regulating the oligomeric forms or participates in an interprotein link between two adjacent P2Y12 receptors. Moreover, Act-Met reduced the C175 mutated P2Y12 oligomers to monomeric species, and did not generate dimers as observed with the wild-type P2Y12 receptor. Replacing the amino acids surrounding C97 in P2Y12 by those from P2Y13 in a chimeric receptor was sufficient to decrease P2Y12 sensitivity to Act-Met, both by affecting binding and by reducing the cleavage of the oligomeric form, thereby pointing to the role of the whole first extracellular loop of P2Y12 in its specific recognition by Act-Met (Fig. 10, which is published as supporting information on the PNAS web site).

In conclusion, we have demonstrated that P2Y12 receptors preferentially associate as functional oligomeric complexes within microdomains at the cell surface. The active metabolite of clopidogrel couples through a disulfide bridge to the P2Y12 receptor, presumably to the cysteine residue in the first extracellular loop; this results in oligomers dissociating into dimeric receptors that are partitioned out of lipid rafts, thereby losing the ability to bind their endogenous ligands. Because P2Y12 receptors play a key role in platelet aggregation, we conclude from the present study that this mechanism accounts for the *in vivo* antiaggregating and antithrombotic activities of clopidogrel. To our knowledge, this is the first report describing such a mechanism of action for a pharmacologically active compound.

Materials and Methods

Reagents. Clopidogrel [SR25990C, 2-(2-chlorophenyl)-2-(2,4,5,6,7,7a hexahydrothieno [3,2c] pyridine-5yl-acetic acid methyl ester hydrogen sulfate 7S] was from Sanofi-Aventis (Toulouse, France). The active metabolite of clopidogrel (Act-Met) was obtained by biotransformation and purified as described (54, 55).

Plasmid Constructions. Human P2Y1, P2Y12, and P2Y13 cDNAs were obtained from the Origene Technologies' Trueclone Collection (Rockville, MD), subcloned into the eukaryotic expression vector p7055 (72) to provide N-terminal c-Myc epitope-tagged mammalian expression plasmids, which have been validated by DNA sequencing. Point mutations and P2Y12/P2Y13 chimera construction are described in *Supporting Text*.

Lipid Raft Isolation Procedure. Plasma membrane lipid microdomains were prepared by density gradient fractionation of Triton X-100 insoluble cell material, essentially as described (73). Briefly, rat platelets or HEK293T cells were lysed in ice-cold Triton X-100 buffer (0.5% Triton X-100/150 mM NaCl/25 mM Hepes, pH 7.0, final concentrations) containing protease and phosphatase inhibitors. Lysates were adjusted to 40% (wt/vol) sucrose by addition of an equal volume of 80% sucrose in Hepes-buffered saline (25 mM Hepes, pH 7.0/150 mM NaCl). A step-gradient of 5–30% (5% steps) sucrose was layered on top of the 40% homogenates in an ultracentrifuge tube. After centrifugation at 200,000 × *g*, 4°C, for 18 h in an SW55Ti rotor (Beckman, Villepinte, France), eight equal-volume fractions were taken, starting from the top of the gradient, and the hard pellet at the bottom was discarded. Quantitative distribution of protein across the density gradient was then monitored by using BCA assay (Pierce).

Binding on P2Y Receptors. Experiments on the specific binding of [^{33}P]2MeS-ADP (PerkinElmer) to Cos7 cells or sucrose gradient fractions were performed with a filtration technique to separate the free from bound [^{33}P]2MeS-ADP. The methodological procedure and calculations are detailed in *Supporting Text*.

Cell culture and transfection, cell extract preparations, rat platelet preparation, antibody generation, immunoprecipitation and protein electrophoresis are detailed in *Supporting Text*.

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