0026-895X/08/7406-1666–1677$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 74:1666–1677, 2008

Vol. 74, No. 6
48496/3409637
Printed in U.S.A.

$P2Y_1$ Receptor Activation Elicits Its Partition out of Membrane Rafts and Its Rapid Internalization from Human Blood Vessels: Implications for Receptor Signaling

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Received May 1, 2008; accepted September 16, 2008

ABSTRACT

The nucleotide $P2Y_1$ receptor ($P2Y_1R$) is expressed in both the endothelial and vascular smooth muscle cells; however, its plasma membrane microregionalization and internalization in human tissues remain unknown. We report on the role of membrane rafts in $P2Y_1R$ signaling by using sodium carbonate or OptiPrep sucrose density gradients, Western blot analysis, reduction of tissue cholesterol content, and vasomotor assays of endothelium-denuded human chorionic arteries. In tissue extracts prepared either in sodium carbonate or OptiPrep, approximately 20 to 30% of the total $P2Y_1R$ mass consistently partitioned into raft fractions and correlated with vasomotor activity. Vessel treatment with methyl β-cyclodextrin reduced the raft partitioning of the $P2Y_1R$ and obliterated the $P2Y_1R$-mediated contractions but not the vasomotor responses elicited by either serotonin or KCl. Perfusion of chorionic artery segments with 100 nM 2-methylthio ADP or 10 nM [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-amino-2-(methylthio)-9*H*-purin-9-yl] 2,3dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS 2365), a selective $P2Y_1R$ agonist, not only displaced within 4 min the $P2Y_1R$ localization out of membrane rafts but also induced its subsequent internalization. 2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS 2179), a specific $P2Y_1R$ antagonist, did not cause a similar displacement but blocked the agonist-induced exit from rafts. Neither adenosine nor uridine triphosphate displaced the $P2Y_1R$ from the membrane raft, further evidencing the pharmacodynamics of the receptor-ligand interaction. Vascular reactivity assays showed fading of the ligand-induced vasoconstrictions, a finding that correlated with the $P2Y_1R$ exit from raft domains and internalization. These results demonstrate in intact human vascular smooth muscle the association of the $P2Y_1R$ to membrane rafts, highlighting the role of this microdomain in $P2Y_1R$ signaling.

Extracellular nucleotides interact with cell surface receptors to produce a broad array of physiological responses. P2 receptors are a large family of plasma membrane (PM) proteins divided into two main subtypes: the ligand-gated ion channels (P2X receptors), and the G protein-coupled receptors (GPCRs; P2Y receptors). The latter is composed of at least eight members (North, 2002), coupled either to $G\alpha_q$ or $G\alpha_i$. The P2Y receptors are promiscuous in terms of ligands because these proteins are activated by either ADP or ATP or pyrimidines, including UTP, UDP, CTP, or UDP-glucose (Burnstock, 2007). Within the past 20 years, the physiology of ATP and related nucleotides has been systematically studied, paving the role of nucleotides as extracellular cell signals. Among other actions, ATP is a sympathetic cotransmit-

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A.N. is a postdoctoral fellow funded by the Centre for Cell Regulation and Pathology; C.S.E. is an undergraduate biochemistry student.

This work was partially funded by grant 13980001 to the Centre for Cell Regulation and Pathology. The Millennium Institute MIFAB also contributed with funds to the Centre.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.108.048496.

ABBREVIATIONS: PM, plasma membrane; $P2Y_1R$, $P2Y_1$ receptor; $P2Y_2R$, $P2Y_2$ receptor; MβCD, methyl β-cyclodextrin; GPCR, G protein-coupled receptor; HCA, human chorionic artery; Tfn-R, transferrin receptor; MRS 2365, [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-amino-2-(methylthio)-9*H*-purin-9-yl] 2,3dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt; MRS 2179, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt; ARC 69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-5′-adenylic acid, monoanhydride with dichloromethylenebis (phosphonic acid); 2-MeSADP, 2-methylthio ADP; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; ADO, adenosine; 5-HT, 5-hydroxytryptamine.

ter and as a transmitter of the pain pathway, a modulator of epithelial functions, bone differentiation, blood coagulation, and has a wide participation in smooth muscle contractility in the vas deferent, intestine, and vascular resistance (Burnstock, 2007).

In a series of classic articles, Ralevic and Burnstock (1988, 1991; Ralevic et al., 1996) first proposed that ATP mediates both relaxant and contractile responses in the rat mesentery, attributed to the activation of P2Y and P2X receptors. The finding that ATP, ADP, UTP, and adenosine are released from endothelial cells and platelets by a variety of mechanisms (Kunapuli and Daniel, 1998; Lazarowski et al., 2003) and that nucleotides dilate conductance and microcirculation vessels, lowering blood pressure (Burnstock, 1990; Lewis and Evans, 2000), further substantiated the role of nucleotides in the regulation of vascular tone. In particular, the P2Y$_1$ receptor (P2Y$_1$R) was identified in several vascular territories, including the rat mesenteric bed and human placental vessels, in which assays provided pharmacological evidence that this receptor is coupled to the NO/cGMP pathway (Buvinic et al., 2006). In an effort to ascertain the role of P2YRs in the regulation of the human placentae wall tone, we described that P2Y$_1$R and the P2Y$_2$R are expressed in the superficial vessels of the human chorionic artery (HCA), in which a gradient of P2YR expression along these vessels and its uneven distribution between the smooth muscles and endothelial cells was established (Buvinic et al., 2006).

The coupling of the P2Y$_1$R to Gα_q protein and the finding that the latter is microregionalized into membrane rafts (Oh and Schnitzer, 2001) incited us to study whether the P2Y$_1$R also partitions into microdomains of the PM. Membrane rafts are a dynamic assembly of cholesterol, glycosphingolipids and structural proteins like caveolins and flotillins that can recruit and exclude specific lipids and proteins involved in signal transduction cascades (Simons and Toomre, 2000). Membrane rafts have been implicated in the regulation of various physiological processes, among others, lipid sorting, protein trafficking, and cell polarization (Jacob et al., 2004; Mayor and Riezman, 2004), human platelet aggregation (Savi et al., 2006), and β-adrenergic signaling (Allen et al., 2007).

We now address whether the P2Y$_1$R is localized in membrane microdomains of human vascular smooth muscle cells and ascertained whether the receptor vasoconstrictive responses are dependent on the integrity of these microdomains. For this purpose, we took advantage of the fact that the superficial arteries of the human placenta essentially lack innervation (Walker and McLean, 1971). In addition, upon endothelium removal, the tissue can be considered a smooth muscle syncytium, a suitable model for cell biology studies and parallel bioassays, avoiding caveats derived from the use of cultured cells. Preparing membrane rafts by either the sodium carbonate or the OptiPrep procedures (Pike, 2004; Macdonald and Pike, 2005), we now demonstrate that a fraction of the total P2Y$_1$R mass is associated to raft domains; upon disruption of the membrane rafts, by lowering tissue cholesterol, we show a reduction of the receptor associated to these microdomains, a procedure that also abolished the P2Y$_1$R-induced vasomotor responses. In addition, agonist-induced receptor activation resulted not only in the partition of the P2Y$_1$R out of raft domains but also, in a subsequent rapid internalization of the receptor, a finding that seems to correlate to the loss of the vasomotor responses. Taken together, these results highlight the relevance of raft domains and the sequestration of the receptor from the cell surface as part of the mechanism P2Y$_1$R signaling. Finally, this study emphasizes the benefits of using human biopsies to study the molecular basis of purinergic signaling correlating the localization of a fraction of the P2Y$_1$R population in the cell surface of human vascular smooth muscle cell endowed with functional vasomotor activity.

Materials and Methods

P2Y$_1$R Ligands and Drug Providers

ATP trisodium salt, 2-MeSADP disodium salt, ADP disodium salt, ADO, UTP trisodium salt, UDP disodium salt, MRS 2179 tetrasodium salt, MβCD, and all antiproteases were purchased from Sigma-Aldrich (St. Louis, MO). MRS 2365 as the trisodium salt was purchased from Tocris Bioscience (Ellisville, MO). ARC 69931MX as the tetrasodium salt was provided by AstraZeneca (Wilmington, DE). Percoll was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Analytical grade reagents for the preparation of buffers were obtained from Merck (Darmstad, Germany).

Obtainment of Human Placentae and Human Vascular Biopses

Full-term placentae from normal pregnancies delivered by vaginal or Caesarean sections were obtained from the maternity associated with the Department of Obstetrics and Gynaecology of the School of Medicine at the P. Catholic University Clinical Hospital (Santiago, Chile). The ethics committees from the School of Medicine and the Faculty of Biology approved the experimental protocols using human tissues; the guidelines for the handling of human materials were strictly adhered to. Appropriate informed consent was obtained as requested by the School of Medicine Ethics Committee. Concerted actions with other investigators in our department allowed a more complete study of this organ. While we dissected chorionic blood vessels, other colleagues used the cord or the body of the placentae for independent protocols. Segments of the human saphenous vein or mammary arteries were likewise obtained after a collaborative effort with cardiovascular surgeons of our clinical hospital. The corresponding ethical regulations were strictly adhered to.

At least 75 placentae were used in the experiments reported in this study. The complete placenta was transported to the laboratory within 5 to 15 min of childbirth; immediately thereafter, segments of superficial chorionic arteries were carefully dissected from the main body to perform the several protocols. In some experiments, the placenta vessel segments, including chorionic veins, conserved an intact endothelium, whereas in other experiments, vessels were manually denuded of the endothelial cell layer by gently rubbing the internal surface of the vessel with a cotton swab; this procedure was described to eliminate the internal endothelial layer without damaging the adjacent smooth muscle layer (Valdecantos et al., 2003). To perform other protocols, as specified separately, the vessel segments were perfused with one of the nucleotide receptor agonists for 1, 2, or 4 min; immediately thereafter, these vessel segments were placed in liquid nitrogen until further tissue processing. Functional assays were also performed to assess the biological activity of the receptor upon challenge with selective nucleotide agonists or the removal of the tissue cholesterol. Each of these protocols is detailed below.

Detergent-Free Purification of Membrane Rafts from HCA

To obtain a smooth muscle cellular extract from HCA segments, the endothelium cell layer was removed as described previously (Valdecantos et al., 2003) and ground in a cold slab in the presence of 1 ml of 500 mM sodium carbonate, pH 11.0, supplemented with an antiprotease mixture (4 mM phenylmethylsulfonyl fluoride; and 4

μg/ml pepstatin, leupeptin, and antipain). Homogenization was carried out sequentially in the following order using a loose-fitting Dounce homogenizer (30 strokes), and a sonicator (four 5-s bursts; Vibra cell, Sonic and Material Inc., Danbury CT). The homogenate (285 μl) was then adjusted to 45% sucrose by the addition of 515 μl of 70% sucrose prepared in modified Barth's solution (25 mM MES, pH 6.5, and 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5 to 35% discontinuous sucrose gradient was formed above (1.6 ml of 5% sucrose and 1.6 ml of 35% sucrose; both in modified Barth's solution containing 250 mM sodium carbonate) and centrifuged at 180,000*g* for 16 h. Eleven fractions of 360 μl each were collected from the top of the centrifuge tube and precipitated by adding 36 μl of a 100% trichloroacetic acid solution. Protein samples were separated in SDS-PAGE, transferred to polyvinylidene difluoride membranes and incubated with antibodies to detect the presence of the blotted proteins. A similar protocol was used to study the association of the $P2Y_1R$ to membrane rafts from human chorionic veins, saphenous veins, and mammary arteries. The OptiPrep procedure to isolate membrane rafts was followed as described previously (Macdonald and Pike, 2005).

Preparations of PM-Enriched Fractions

PMs were obtained according to the methods described by Smart et al. (1995). In brief, segments of HCA were ground in a cold slab in the presence of buffer A (0.25 M sucrose, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.8). Then, the cells were homogenized in a tissue grinder (Wheaton Science Products, Millville, NJ) with 60 strokes followed by centrifugation at 1000*g* for 10 min. The postnuclear supernatant (0.8 ml) was layered on top of 3.2 ml of 30% Percoll in buffer A and centrifuged at 84,000*g* for 30 min in a TST 60.4 rotor (Sorvall, Newton, CT) at 4°C. The PMs were collected and analyzed by immunoblots.

Immunoblotting

Antibodies for the human $P2Y_1R$ were generated and characterized previously (Buvinic et al., 2006). Antibodies against $P2Y_2R$, caveolin 1 and 3, flotillin 1, $G\alpha_q$, and β-subunit of the Na^+/K^+ ATPase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against transferrin receptor (Tfn-R), calnexin, and the Golgi marker p230 were purchased from Zymed Laboratories Inc. (South San Francisco, CA), Sigma-Aldrich, and BD Transduction Laboratories (Lexington, KY), respectively.

Proteins were separated by SDS-PAGE on 10% acrylamide gels and transferred to polyvinylidene difluoride membranes. These membranes were incubated with the specific antibodies to detect the indicated proteins and visualized using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection system (SuperSignal West Femto; Pierce, Rockford, IL). Immunoblots were digitalized in a VISTA-T630 UMax scanner driven by Adobe Photoshop CS (Adobe Systems, Mountain View, CA); quantitative analysis was done with the Image J software (http://rsbweb.nih.gov/ij/).

Protocols Used to Examine the Role of Membrane Raft Association of the $P2Y_1R$

Tissue Perfusion with $P2Y_1R$ Ligands, Including Agonists and Antagonists. A 3- to 5-cm segment of a superficial chorionic artery was carefully dissected from the placental body. The tissue was immediately denuded of the endothelial cell layer as described previously. Immediately thereafter, one end of the vessel was cannulated with PE 190 tubing and perfused with 95% O_2/5% CO_2-gassed Krebs-Ringer buffer maintained at 37°C at a flow of 4 ml/min; the vessels were placed inside a 1.5-ml Eppendorf tube, keeping humid and warm the external surface of the tissue. In this way, the buffer bathed the inside and outside of the vessel during the perfusion procedure. After an equilibrium period of 15 to 20 min, the tissues were perfused with nucleotides such as 100 nM 2-MeSADP for exactly 4 min each. Upon completion of the perfusion procedure, the tissues were rapidly dismounted from the perfusion set and immediately immersed in liquid nitrogen until tissue processing for fractionation and sucrose gradient application as detailed above. Control experiments performed without freezing the tissue showed that these procedures did not alter the membrane raft localization of the $P2Y_1R$ and control proteins.

A separate series of experiments was performed to assess whether tissue preincubation with the $P2Y_1R$ antagonist MRS 2179 blocked the effect of the agonist alone. For this purpose, tissues were prepared in identical experimental conditions as detailed previously, except that 100 nM MRS 2179 was perfuse for 15 min before the 4-min tissue perfusion and that 100 nM 2-MeSADP was now perfused in the presence of 100 nM MRS 2179. Immediately thereafter, the vessels were immersed into liquid nitrogen. As a control experiment, tissues were perfused for 20 min with 100 nM MRS 2179 alone before tissue immersion in liquid nitrogen. Each series of protocols were replicated in three to four separate placentae.

To test the agonist specificity of the putative receptors, we next perfused separated artery segments for 4 min with one of the following nucleotides: 10 nM MRS 2365, 100 nM ADP, 1 μM adenosine (ADO), 1 μM UTP, or 1 μM uridine. Each of these studies was repeated in three to four separate vessels obtained from independent placentae.

To examine whether the receptor shifted its microregionalization upon agonist activation and to assess the timing required for the $P2Y_1R$ to exit the membrane raft domains, additional experiments were performed by perfusing 100 nM 2-MeSADP for either 1, 2, or 4 min; immediately thereafter, blood vessels were immersed into liquid nitrogen for sucrose gradient centrifugation. All of these protocols were repeated using at least three to four independent placentae. An additional set of experiments was designed to examine the reversibility of the receptor distribution out of the raft domain. For this purpose, vessels were perfused with 100 nM 2-MeSADP for 4 min, followed by a second 4-min application of 100 nM 2-MeSADP spaced 120 or 180 min apart. Between the two agonist applications, the tissues were perfused with regular buffer without a $P2Y_1R$ agonist. As a control for this particular set of experiments, and to assess tissue viability after 3 h of tissue perfusion ex vivo, parallel tissues were perfused for 180 min with regular buffer and immediately thereafter applied for an additional 4 min with 100 nM 2-MeSADP. Once the perfusion was completed, the tissues were dismounted and immersed in liquid nitrogen for sucrose gradient processing. Parallel bioassays determined the course of the vasoconstrictive activity ensued by agonist application and the viability of the tissues; these protocols are detailed below.

Removal of Cell Membrane Cholesterol with Methyl β-Cyclodextrin. Because raft domains are enriched in cholesterol, we reason that disruption of these microdomains by the removal of substantial cholesterol should alter the distribution of the $P2Y_1R$ in the sucrose gradients. For this purpose, segments of denuded HCA were first perfused with regular Krebs-Ringer buffer for 15 min to remove blood cells. For the next 90 min, the vessels were perfused with buffer containing 10 mM MβCD, an agent reported to substantially remove membrane cholesterol (Ohtani et al., 1989; Kilsdonk et al., 1995). During the last 4 min of the perfusion with MβCD, the buffer was added with 100 nM 2-MeSADP. Once perfusion was completed, the vessels were immediately processed to examine the membrane raft distribution of the $P2Y_1R$. Results will compare the membrane raft distribution of the $P2Y_1R$ in untreated control vessels versus tissues treated with MβCD.

Vascular Reactivity Assays

Intact segments of HCA were dissected from surrounding tissues; 0.3- to 0.5-cm width rings were prepared carefully as detailed previously (Valdecantos et al., 2003). Rings were placed in buffer maintained at 37°C in a double-jacketed 5-ml organ baths gassed with 95% O_2/5% CO_2. The composition of the Krebs-Ringer solution used

was the same buffer described by Buvinic et al. (2006). Isometric muscular tension from the circular layer was recorded by means of a force-displacement transducer connected to a Grass multichannel polygraph (Grass, Quincy, MA). To record isometric tension, the rings were first challenged with a 70 mM KCl application, which elicited a sustained, nearly maximal contraction within 2 to 3 min (Valdecantos et al., 2003). This value was used as a standard to normalize vasomotor responses of the several bioassays performed. Once the KCl was washed out, the tissue was rested for 15 to 30 min until the vasomotor activities of nucleotides were examined by following the procedure published previously (Buvinic et al., 2006).

Muscular contractions were evoked with 1- to 2-min applications of increasing 2-MeSADP or ADP concentrations; once the maximal contraction was reached, the agonists were rapidly rinsed, avoiding desensitization of the motor response. The agonist EC_{50} value was interpolated from the concentration-response plot adjusted to a sigmoid using GraphPad program (Buvinic et al., 2006) (GraphPad Software, Inc., San Diego, CA). We also used 10 nM MRS 2365, the most selective P2Y$_1$R agonist (Chhatriwala et al., 2004). To confirm 2-MeSADP selectivity of action at the P2Y$_1$R, we next examined whether MRS 2179, a recognized selective P2Y$_1$R antagonist (Boyer et al., 1998), blocked the vasomotor action of 2-MeSADP. For this purpose, tissues were tested with a priming application of 100 nM 2-MeSADP; next, this application was repeated in tissues incubated previously for 5 min with 10 to 100 nM MRS 2179. Reversibility of antagonist was tested by examining the recovery of the contractile response to 2-MeSADP 45 to 250 min after antagonist application. In addition, to discard the influence of P2Y$_{12/13}$ receptors in the vascular reactivity assays, the vasomotor activity of 100 nM 2-MeSADP was examined in the presence of 10 to 100 nM ARC 69931MX, applied 5 min before agonist challenge. ARC 69931MX is a potent P2Y$_{12/13}$ receptor antagonist (Marteau et al., 2003).

To assess the mechanism by which the muscular response elicited in intact HCA rings faded within 4 min by a single application by 100 nM 2-MeSADP, we examined whether the loss of the vasomotor response was related to the agonist-induced shift of the nucleotide receptor out from the smooth muscle membrane raft or the eventual P2Y$_1$R internalization. For this purpose, the next series of experiments recorded continually the vasomotor response evoked by the application of 100 nM 2-MeSADP during 4 min; at this time, a second challenge with the agonist was applied to evaluate its motor response. To examine whether the loss of muscle contraction was extended to another GPCR linked to Gα_q, experiments were conducted challenging tissues with 300 nM 5-HT in rings contracted previously with 100 nM 2-MeSADP. Finally, a next experiment assessed whether P2Y$_1$R receptor desensitization had recovered after a 120- or 180-min agonist washout period.

To assess whether removal of the membrane cholesterol influenced the vasomotor responses elicited by 2-MeSADP or 5-HT, a next series of controlled experiments examined the effect of tissue incu-

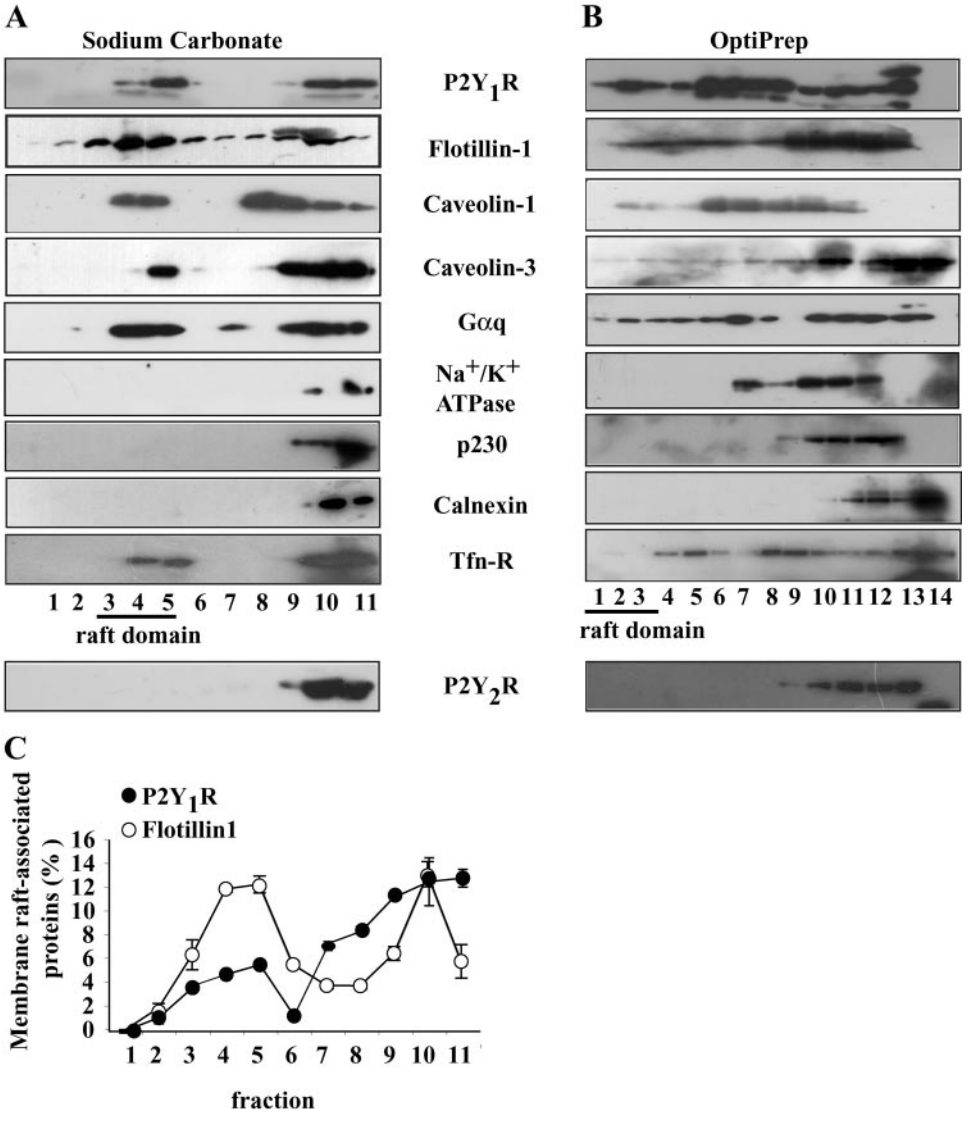

Fig. 1. The P2Y$_1$R associates to membrane rafts in HCA. The association of the P2Y$_1$R to raft domains was assessed in sodium carbonate extracts (A) or the OptiPrep protocol (B) separated in sucrose gradients. Aliquots of each gradient fraction were analyzed by SDS-PAGE followed by Western blotting for the indicated proteins. The raft domains in the sodium carbonate gradients invariably correspond to fractions 3 to 5, which contained 15 to 29% sucrose, whereas in the OptiPrep density gradients, the rafts domains localized in the lighter sucrose fractions, corresponding to tubes 1 to 3. In either procedure, the P2Y$_1$R comigrated with the endogenous membrane raft marker flotillin 1, caveolin 1, caveolin 3, and the receptor signaling partner Gα_q protein; the β-subunit of the Na^+/K^+ ATPase, p230, and calnexin were not found associated to membrane raft domains. A minor fraction of the Tfn-R was only observed in raft domains of the sodium carbonate extracts. Similar results were obtained in three separate experiments. In contrast, the P2Y$_2$R was not detected in raft microdomains (n = 3). For comparative purposes, densitometry of the sodium carbonate immunoblots for P2Y$_1$R and flotillin 1 are shown in C. Symbols indicated the average value; bars show the S.E.M.(n = 3).

bation with 10 mM MβCD. These experiments were performed in tissue precontracted with 16 mM KCl, a concentration of potassium that only partially contracted the vascular smooth muscles and favored the vasomotor effect of 2-MeSADP (Buvinic et al., 2006). Once the rings reached the maximal response evoked by 16 mM KCl, 100 nM 2-MeSADP or 300 nM 5-HT was added to the tissue bath from less than 2 min to avoid tissue desensitization. Next, tissues were exposed to 10 mM MβCD for 90 min, at which time the challenge with either 2-MeSADP or 5-HT was repeated. Results evaluated the contractile responses evoked by either vasomotor agonist before and after treatment with MβCD; additional controls evaluated the influence of desensitization.

Receptor Internalization Assays

In analogy to the observation of the loss of the vasomotor response after 100 nM 2-MeSADP applications, an additional set of experiments analyzed the PM distribution of the $P2Y_1R$ in HCA segments perfused with either 10 to 1000 nM 2-MeSADP or 1 to 10 μM ADP. The procedure for the PM assay of the $P2Y_1R$ was described previously.

Assessment of Tissue Cholesterol Content in Control and MβCD-Treated HCA Segments

Quantification of the total cholesterol was made as described previously (Allain et al., 1974). In brief, tissues were homogenized in H_20; 3 volumes of methanol were added for every 2 volumes of homogenized tissue. The mixture was vortexted for 30 s. Next, 6 volumes of chloroform were added and vortexed for 1 min, left for 1 h at 4°C, and centrifuged at 3000 rpm for 10 min at 4°C. The bottom phase was separated and dried to evaluate the cholesterol content by an enzymatic assay.

Quantification of Vasomotor Responses and Statistical Analysis

The isometric vasomotor responses elicited by the nucleotides in rings of isolated blood vessels were quantified as the tension developed by each agonist in intact vessel rings or vessels denuded of the endothelial cell layer. The motor responses were quantified as the tension force, expressed in Newtons, and were normalized against the standard of 70 mM KCl used at the beginning of each bioassay. At least four to six rings were examined per nucleotide examined in these assays; the rings were derived each time from separate placentae.

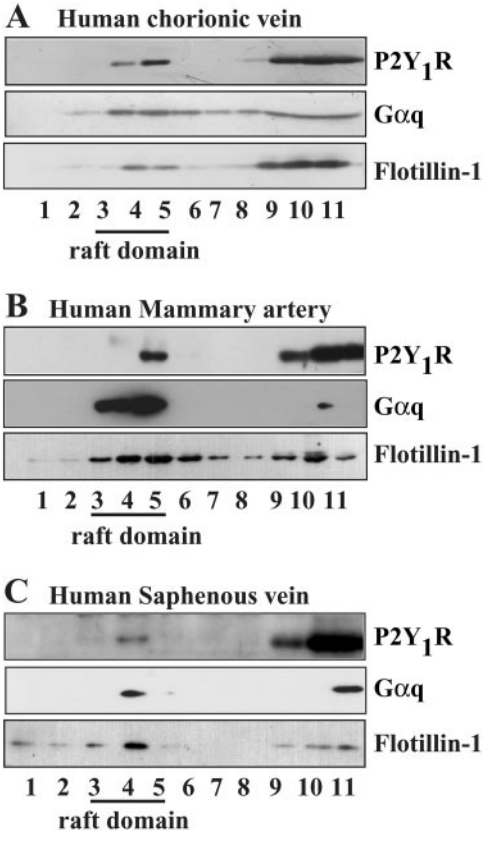

Fig. 2. Membrane rafts association of the $P2Y_1R$ in different human vessels. Membrane rafts from human vessel biopsies were isolated in sodium carbonate extracts and separated in sucrose gradients. Representative immunoblots for the $P2Y_1R$, show that this protein associates to membrane rafts comigrating with the $G\alpha_q$ protein and flotillin 1 in chorionic vein (A), mammary artery (B), and saphenous vein (C) segments. Similar results were obtained in three different vessel biopsies.

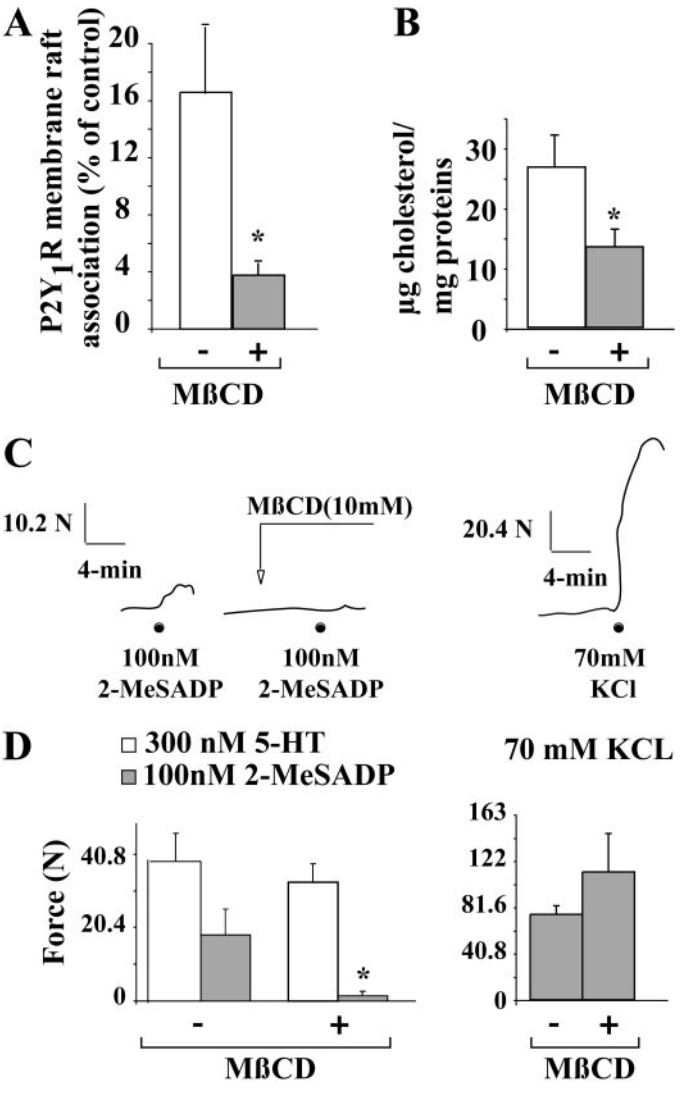

Fig. 3. Cholesterol depletion alters the $P2Y_1R$ distribution in membrane rafts and inhibits agonist-evoked contractions. Perfusion of HCA with buffer containing 10 mM MβCD for 1.5 h caused a 70% reduction in the $P2Y_1R$ mass localized in rafts (A); the dextrin significantly decreased by 50% the tissue cholesterol content (B; $n = 4–6$). MβCD treatment abrogated the $P2Y_1R$ agonist-evoked contractions without affecting the KCl contractures, as shown in representative tracings depicted in C. In addition, cholesterol depletion after MβCD treatment neither affected the serotonin vasomotor responses nor statistically modified the 70 mM KCl-evoked contractions (D). Columns represent mean values; bars, S.E.M. ($n = 3–4$, per assay). $*$, $p < 0.05$ comparing control versus MβCD-treated tissues.

Results

P2Y$_1$R Association to Membrane Rafts in HCA Smooth Muscle Cells. Immunoblots from HCA extracts prepared in sodium carbonate and ultracentrifuged on a discontinuous sucrose gradient showed that the P2Y$_1$R comigrates with flotillin 1, caveolin 1, and caveolin 3, the muscle-caveolin variant (Fig. 1A). The P2Y$_1$R was observed consistently in the gradient fractions containing 15 to 29% sucrose, which in our studies correspond preponderantly to fractions three to five (n = 13). In addition, a population of the protein Gα_q, the signaling partner of the P2Y$_1$R, was also shown to be localized in membrane rafts (Fig. 1A); as a negative control of raft localization, the β-subunit of the Na^+/K^+ ATPase was detected at the bottom of the gradient (Fig. 1A), indicating that this protein is not associated to membrane rafts of these smooth muscle cells. In addition, we observed that p230 and calnexin, markers of Golgi and the endoplasmic reticulum, respectively, were not found in fractions corresponding to raft domains (Fig. 1A). However, Tfn-R, an early endosomal marker, was localized in raft and nonraft fractions of the gradient (Fig. 1A). Densitometry of the immunoblots established that 18 ± 7% (n = 13) of the total P2Y$_1$R mass was associated to membrane rafts (Fig. 1C).

To confirm the association of a fraction of the P2Y$_1$R to raft domains, we performed a parallel series of experiments using tissue extracts prepared in OptiPrep gradients, a method that avoids the high pH of the sodium carbonate procedures. As expected, and consistent with the densitometric analysis of the sodium carbonate protocols, we found that 19 ± 4%

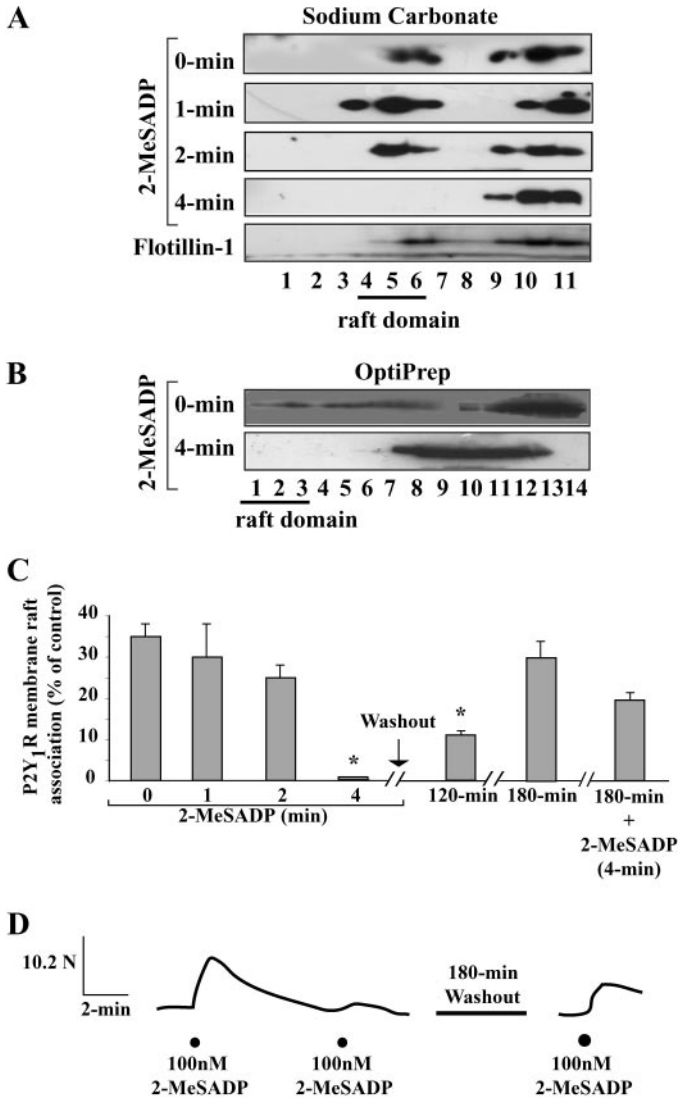

Fig. 4. Kinetics of the P2Y$_1$R translocation from membrane rafts. HCA segments were perfused ex vivo with 100 nM 2-MeSADP for 1, 2, and 4 min (n = 3 each). Immunoblots from sucrose density gradients, from extracts prepared with sodium carbonate, show that 2-MeSADP caused a rapid and complete exit of the P2Y$_1$R from raft domains only after 4 min of perfusion compared with a control protocol perfused for 4 min with agonist-free buffer (A). A similar result was obtained after a 4-min 2-MeSADP perfusion analyzed by the OptiPrep procedure (B). To assess the reversibility of this process, parallel experiments were performed perfusing 2-MeSADP for 4 min followed by agonist washout for 120 and 180 min. Quantitative densitometry of the immunoblots for the P2Y$_1$R associated to membrane rafts are depicted in C. Columns represent mean values; bars, S.E.M. (n = 3 per assay). $*$, $p < 0.05$, compared with the control experiments perfused in the absence of agonist. An additional experiment assessed whether a 4-min challenge with 2-MeSADP 180 min after tissue washout from the initial challenge was able to relocalize the receptor out of membrane rafts (n = 3). D, a typical polygraphic recording of the contractions evoked with 100 nM 2-MeSADP (●) in an isolated HCA ring. The contractile response faded because the contraction was not maintained; a second agonist application performed 6 min after the challenge was nonresponsive at a time concurrent with the P2Y$_1$R exit from the membrane raft. After 180 min of washout, the vasomotor response elicited by a third agonist application showed only 30 to 40% recovery; similar results were obtained in studies from five placentae.

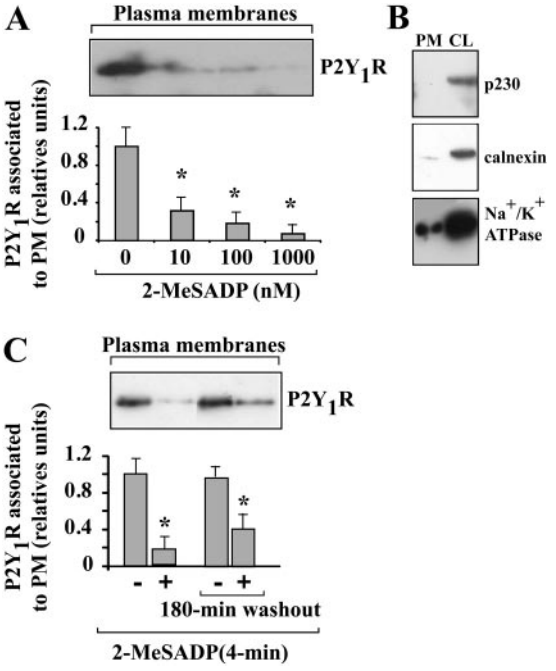

Fig. 5. P2Y$_1$R internalization is induced by 2-MeSADP. HCA segments were perfused with 10, 100, or 1000 nM 2-MeSADP for 4 min; as controls, separated vessels were perfused with agonist-free buffer. Cells were harvested; smooth muscle cell plasma membranes were isolated by centrifugation. These fractions were then subjected to Western blot analysis using an anti-P2Y$_1$R antibody (A). Fifteen micrograms of protein was loaded per lane; representative P2Y$_1$R immunoblots are shown in the top part of the figure. As a control, aimed to analyze the contamination of the PM-enriched fractions, immunoblots for two intracellular markers plus Na^+/K^+ ATPase, a plasma membrane marker from whole-cell lysates are depicted in B. After agonist washout, the P2Y$_1$R reassociates to the plasma membrane. HCA segments were perfused with 100 nM 2-MeSADP during 4 min and then perfused with buffer in the absence of agonist for 180 min. PM fractions and immunoblotting of the receptor showed that the P2Y$_1$R associated to plasma membrane was fully recovered (C). Columns represent mean values; bars, S.E.M. $*$, $p < 0.05$ compared with the controls.

($n = 3$) of the $P2Y_1R$ was localized in the lighter fractions of the gradient comigrating with the raft markers flotillin 1, caveolin 1, and caveolin 3 (Fig. 1B). In addition, the trimeric $G\alpha_q$ protein was also demonstrated to comigrate with the $P2Y_1R$ in the lighter fractions of the OptiPrep gradients. Likewise, the β-subunit of the Na^+/K^+ATPase was not found in raft fractions (Fig. 1B). Moreover, using this procedure, p230, calnexin and the Tfn-R were not found associated to the membrane raft fractions (Fig. 1B).

Moreover we also assessed whether the $P2Y_2R$, a closely related GPCR family member, is associated to membrane rafts. To our surprise, we observed that the $P2Y_2R$ did not partition to these microdomains (Fig. 1, A and B), a finding consistent with observations in stripped membranes used to detect first the $P2Y_1R$ ($n = 3$). This result is consistent with the view that not all GPCRs are localized in membrane rafts, an observation that has vast functional implications for nucleotides receptors, revealing the complexity of the P2YRs distribution in the PM and probably their intracellular signaling mechanisms.

Because caveolin-enriched membranes are insoluble in Triton X-100 at 4°C, we next examined the membrane raft distribution of the $P2Y_1R$ in homogenates prepared with this particular detergent. We observed that the receptor was not detected in raft domains because its localization was restricted to the bottom fractions of the gradient. In contrast, caveolin 3 was detected in raft and nonraft fractions (data not shown).

To examine the relevance of HCA as a biological model for our studies, we performed additional protocols using segments of the human saphenous vein or mammary artery obtained from patients undergoing cardiac revascularization surgery. Immunoblots from sodium carbonate extracts clearly show that the $P2Y_1R$ was detected in all of these vessels, as well as in the chorionic vein, comigrating with flotillin 1 and protein $G\alpha_q$ (Fig. 2). In the saphenous vein, this fraction was relatively less. Altogether, this set of protocols allowed us to generalize that 20 to 30% of the total $P2Y_1R$ mass of human vascular smooth muscles is associated to membrane rafts in arteries and veins from several vascular territories.

MβCD Changed the $P2Y_1R$ Membrane Raft Localization and Obliterated Agonist-Induced Contractions. Consistent with the finding that the $P2Y_1R$ associates to membrane rafts, we next assessed whether removing tissue cholesterol changed the $P2Y_1R$ raft partitioning and the functional properties of the $P2Y_1R$. Tissue perfusion with MβCD, significantly reduced by 70% the $P2Y_1R$ associated to membrane rafts ($n = 3$, $P < 0.05$, Fig. 3A); the $P2Y_1R$ was found displaced toward heavier buoyant fractions (data not shown). MβCD treatment effectively reduced by 50% the total tissue cholesterol content ($n = 4$, $P < 0.05$, Fig. 3B) and abrogated the 2-MeSADP vasomotor responses in tissue-controlled experiments (Fig. 3, C and D) but did not modify the KCl-induced contractions (Fig. 3D) or the vasomotor response elicited by 5-HT, a potent and efficacious vasoconstrictor of placental vessels, used as an internal standard for these studies (Fig. 3D). Additional control experiments demonstrated that MβCD treatment did not modify the total $P2Y_1R$ mass (data not shown). These data demonstrate a functional association of the $P2Y_1R$ with membrane rafts in HCA because disruption of these microdomains reduced its raft domain partitioning and subsequent physiological responses.

$P2Y_1R$ Occupation with Selective Agonists Induced Receptor Partitioning out of Membrane Rafts. We next assessed whether $P2Y_1R$ activation with selective agonists modified its membrane raft distribution. Experiments were performed with tissues extracted in either sodium carbonate or OptiPrep gradients. Perfusion with 2-MeSADP for 1 or 2 min did not elicit a significant change in the membrane raft

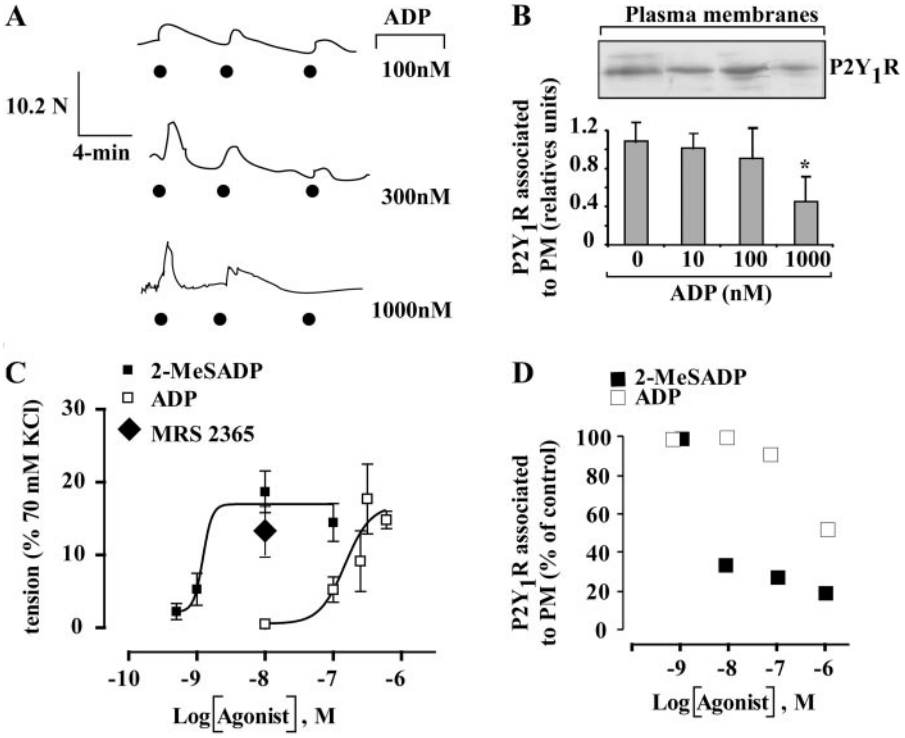

Fig. 6. ADP-induced fading of the vasomotor responses correlate with the $P2Y_1R$ internalization. Vascular reactivity assays on HCA rings perfused with different concentrations of ADP were performed. The contractile response desensitized only at higher concentration of the agonist because the contraction was not maintained, and a second agonist application performed 4 to 6 min after the first was halved (A). To assess $P2Y_1R$ internalization, HCA segments were perfused with different concentration of ADP during 4 min. PMs were prepared and analyzed as described previously. B, 50% of the plasma membrane-associated receptor internalized only with 1 μM ADP. Relative potency and maximal effects of 2-MeSADP and ADP on HCA ring vasoconstrictions. C, concentration-response curves for 2-MeSADP or ADP-induced contractions illustrate that 2-MeSADP is nearly 100-fold more potent than ADP; MRS 2365 has a potency similar to 2-MeSADP. D, concentration-response curves for $P2Y_1R$ internalization induced by 2-MeSADP or ADP indicate that the 2-MeSADP-evoked receptor internalization is almost 100-fold more potent than ADP. Symbols and columns indicate mean values; bars, S.E.M.

distribution of the P2Y$_1$R; however, after 4 min, we observed a complete partitioning of the receptor out of raft domains (Fig. 4, A and C). A similar distribution of the P2Y$_1$R out of raft domains was observed in the gradients prepared in Optiprep (Fig. 4B), confirming that the partitioning of the P2Y$_1$R out of membrane rafts is completed within 4 min. In addition, the agonist-induced shift of the receptor out of membrane rafts seemed to parallel with the fade of the vasomotor effect elicited by 2-MeSADP (Fig. 4D), as evidenced by the gradual loss of the vasomotor effect and the finding that a second 2-MeSADP application at this timing did not elicit the expected full agonist vasomotor response (Fig. 4D).

That the fading of the vasomotor response is not due to tissue metabolism of the ligand can be deduced from the finding that after 6 min of HCA incubation with 1 μM ADP (Fig. 6A), the degradation of the nucleotide did not exceed 12 to 15%. In contrast, 6 min after 2-MeSADP or ADP applications, the vasomotor responses were substantially reduced as shown in a representative tracing for 2-MeSADP or ADP (see recording in Figs. 4D and 6A). Therefore, the fading of the vasoconstriction elicited by P2Y$_1$R occupation and the reduction of the vasoconstriction elicited by a second application seemed to better correlate with the displacement of the receptor from membrane rafts.

Reversible Agonist-Induced Exit of the P2Y$_1$R from Membrane Rafts. We therefore next assessed whether the receptor displacement from raft domains by a short application of 2-MeSADP reversed upon agonist washout and ascertained whether a subsequent 2-MeSADP application again partitioned the receptor out of membrane rafts evoking a consequent vasomotor response. Although 120 min after agonist washout only 32 ± 5% (n = 3) of the P2Y$_1$R associated to raft domains recovered, by 180 min, the P2Y$_1$R recovery to the membrane raft association averaged 85 ± 13% (n = 3; Fig. 4C), an indication that the receptor exit from the raft domains is fully reversible but requires 2 to 3 h for full recovery at the raft domains. Notwithstanding, a new application of 2-MeSADP to these same vessels elicited only a minor displacement of the receptor from membrane rafts, which averaged only 15% of the original shift (Fig. 4C). In parallel, the application of 2-MeSADP after 180 min after the priming challenge attained only 30 to 40% of its original vasomotor response as shown in a representative tracing (Fig. 4D), an indication that the P2Y$_1$R needs additional time to attain full activity in the cell membrane. We carefully assessed the viability of the tissues maintained ex vivo for 3 h. We consistently observed that 28 ± 7% of receptor mass was still associated to membrane raft in tissues perfused with agonist-free buffer for 180 min (n = 4). Furthermore, the distribution of flotillin 1 and Gα_q was unaffected (data not shown), and the 2-MeSADP-evoked contractile responses was 100% conserved (n = 3), demonstrating that the prolonged ex vivo procedures did not affect HCA viability. This conclusion is further reinforced by the finding that the potassium-elicited contracture remained unaltered during the 3-h period, attaining 122 ± 16 N (n = 10) versus 118 ± 17 N (n = 8) at the beginning and at the end of the protocol.

P2Y$_1$R Internalization Correlates with Fading of the Vasomotor Responses Mediated by Receptor Activity. The association of the P2Y$_1$R to the cell surface was monitored by Western blotting of PM-enriched fractions. The PM localization of the P2Y$_1$R was reduced in a concentration-dependent manner after perfusion with either 2-MeSADP or ADP for 4 min. Although 10 nM 2-MeSADP reduced 70% the P2Y$_1$R association to the cell surface, 1 μM reduced the association up to 90% (Fig. 5A). The PM preparations showed scarce contamination with endoplasmic reticulum or Golgi as evidenced by immunoblots with selective organelle markers (Fig. 5B). Moreover, only 60% of the P2Y$_1$R relocalized to the cell surface after 60 min of agonist washout (data not shown); after 180 min, the P2Y$_1$R reached 80% (Fig. 5C). Furthermore, upon a second application of 100 nM 2-MeSADP, the ligand elicited a substantial loss of the receptor from the cell surface, implying that upon ligand application, the P2Y$_1$R may undergo a new cycle of internalization (Fig. 5C). Similar experiments were performed with the endogenous ligand of the P2Y$_1$R. Three repeated applications of 100 nM ADP elicited nonsustained motor responses that reached a similar contractile magnitude. Increasing the ADP concentration elicited proportionally larger vasoconstrictions that faded faster and elicited a gradually diminished motor response upon sequential applications, until the third 1 μM ADP application did not further elicit vasoconstriction (Fig. 6A). Immunoblots also demonstrated an ADP concentration-de-

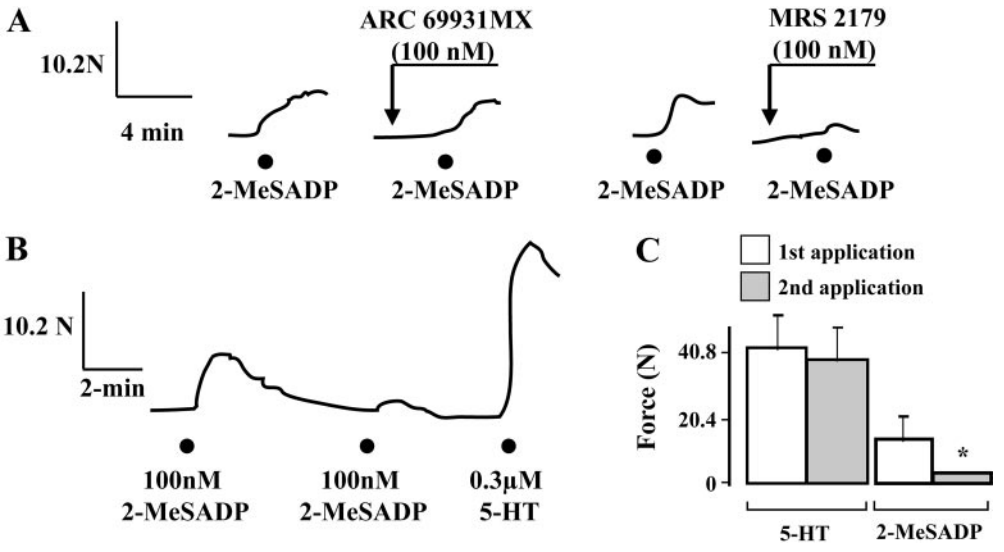

Fig. 7. Selectivity of the vasomotor activity mediated by 2-MeSADP. A, representative tracings of vascular reactivity assays using HCA rings show that the contractile response of 100 nM 2-MeSADP was slightly reduced by 100 nM ARC 69931MX, whereas 100 nM MRS 2179 significantly suppressed the 2-MeSADP-evoked contractions. To assess whether the desensitization of the vasomotor response mediated by 2-MeSADP affected the contractions evoked by 5-HT, a receptor linked to Gα_q, 5-HT applications were performed in tissues desensitized by 2-MeSADP. D, a representative polygraphic recording shows that the 2-MeSADP desensitization did not perturb the 300 nM 5-HT-evoked contractions. C, statistical analysis of six separate experiments. Columns represent the mean average contraction; bars, S.E.M. *, $p < 0.05$.

pendent loss of the receptor form the cell surface (Fig. 6B). Comparisons of the concentration-dependent fraction of the receptor that remained at the cell surface after 4 min of perfusions with 2-MeSADP and ADP are shown in Fig. 6D. Consistent with the potency of these ligands for the $P2Y_1R$, 2-MeSADP is at least 2 orders of magnitude more potent than ADP to sequestrate the receptor from the cell surface, in agreement with the vasomotor potency described in Fig. 6C. Therefore, we conclude that the stimulation of the $P2Y_1R$ and the subsequent fading of the vasomotor activity within a few minutes seem to correlate with the concentration-dependent sequestration of the receptor from the cell surface.

The Vasomotor Responses of 2-MeSADP in HCA are $P2Y_1R$-Selective. To discard the possible involvement of the $P2Y_{12}/_{13}$ receptors in the mechanism of the 2-MeSADP-induced vasoconstrictions, 10 nM ARC 69931MX did not reduce significantly the 2-MeSADP-mediated contractions. Increasing the antagonist concentration to 100 nM, a concentration 25-fold larger than the antagonist K_D, reduced the 2-MeSADP-evoked concentration by 51% ($n = 3$) (Fig. 7A). In contrast, 100 nM MRS 2179, as shown in a representative tracing of Fig. 7A, reduced the 2-MeSADP-evoked vasomotor activity more than 60% ($n = 4$, $p < 0.05$).

The Exit of $P2Y_1R$ from Membrane Rafts Did Not Perturb the 5-HT Contractile Responses. Because $P2Y_1R$ occupancy activates $G\alpha_q$, as a part of the receptor-signaling cascade, it became imperative to examine whether another membrane receptor coupled to $G\alpha_q$ behaved similarly. Although the vasomotor response induced by 2-MeSADP rapidly faded upon repeated agonist applications, fading was not crossed to the 5-HT-evoked muscle contraction, a response probably mediated by $5\text{-}HT_{2A}$ receptors (Fig. 7B). After total loss of the motor action of 2-MeSADP, the 5-HT contractile response was preserved. In fact, the 5-HT-induced contraction was 56 ± 17 N ($n = 6$) before the application of 2-MeSADP and 51 ± 13 N ($n = 7$) after the nucleotide applications. Moreover, the 5-HT-evoked vasomotor response did not elicit a significant homologous desensitization as evidenced by the finding that two consecutives 5-HT applications elicited similar vasoconstrictions (Fig. 7C).

Other Selective $P2Y_1R$ Agonists Also Partition the Receptor out of Membrane Rafts: MRS 2179-Induced Antagonism. Consistent with the previous results, MRS 2365, the most selective $P2Y_1R$ agonist reported (Chhatriwala et al., 2004), also partitioned the receptor out of these microdomains within 4 min (Fig. 8, A and B) and elicited vasoconstriction with a potency comparable with 2-MeADP (Fig. 6D). Moreover, ADP, but neither 1 μM ADO nor 1 μM UTP, partitioned the receptor out of membrane rafts (Fig. 8, A and B). Consonant with the notion that the receptor distribution in raft domains depends on its selective ligand activation, MRS 2179, a selective $P2Y_1R$ antagonist, neither modified per se the receptor distribution in raft domains (Fig. 8, A and B) nor evoked a vasomotor response (Fig. 7A). However, the joint application of MRS 2179 plus 2-MeSADP antagonized the $P2Y_1R$ displacement of raft domains induced by the agonist (Fig. 8, A and B).

Discussion

In this study, we show for the first time the association of the $P2Y_1R$ to membrane rafts of human blood vessels and its coupling with vascular tone control. Four major findings, schematized in Fig. 9, highlight the role of membrane rafts in $P2Y_1R$ signaling. First, we observed that the $P2Y_1R$ is microregionalized into membrane rafts from HCA and systemic conductance vessels such as the mammary artery and the chorionic and saphenous vein. Second, the association of the $P2Y_1R$ to membrane rafts is linked to its vasomotor response, because the contractions elicited by $P2Y_1R$ agonists were selectively abolished by disruption of these microdomains. Third, the receptor association to membrane rafts is regulated by $P2Y_1R$ activation, which results in the partition of receptors out of the rafts. Finally, rapid internalization of the $P2Y_1R$ and its relatively slow cell surface recycling seems to correlate with the prolonged fading of the vasomotor responses elicited by agonist-induced receptor activation.

Membrane rafts are enriched in cholesterol, glycosphingolipids, ganglioside GM1, and in characteristic proteins such as caveolins and flotillins. In view of the limitations raised by the use of detergents in the preparation of membrane rafts (Pike, 2004), we used sodium carbonate and Optiprep meth-

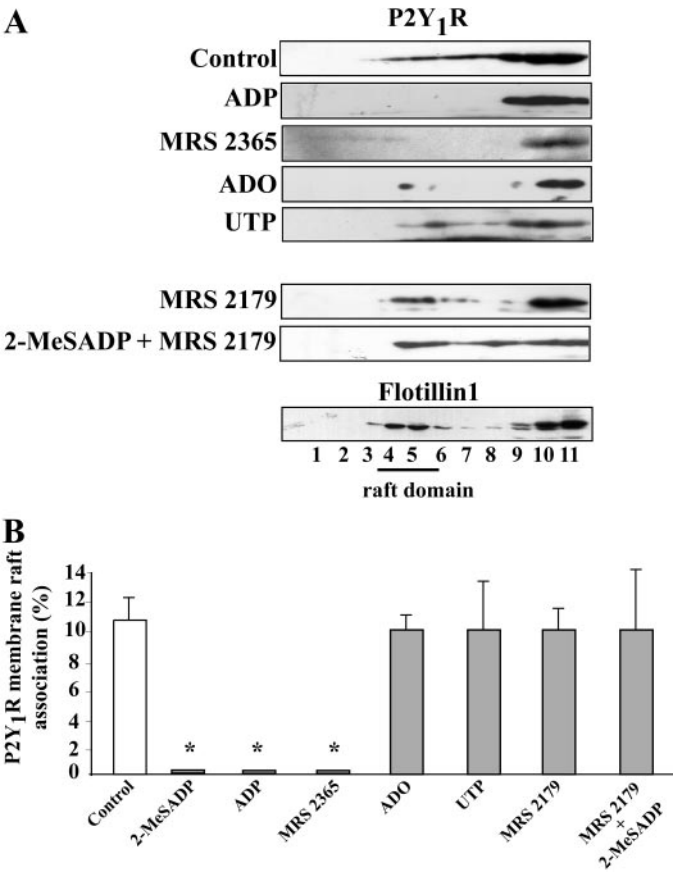

Fig. 8. The $P2Y_1R$ exit of membrane rafts is ligand-selective. To examine whether the $P2Y_1R$ displacement from membrane raft depends on the activation of the receptor, HCA segments were perfused for 4 min with either 1 μM ADP or 10 nM MRS 2365, two selective $P2Y_1R$ ligands. Both nucleotides fully displaced the receptor from the raft domains. In contrast, neither 1 μM ADO nor 1 μM UTP evoked the receptor exit (A). Moreover, 100 nM MRS 2179, a $P2Y_1R$ antagonist, did not mimic per se the agonist action, blocked the receptor displacement elicited by 2-MeSDAP (A). Quantifications of the receptor fraction associated to the membrane raft are shown in B; only selective $P2Y_1R$ ligands completely displaced the $P2Y_1R$ from raft domains. Columns represent mean values; bars, S.E.M. ($n = 3$–4 per assay). As controls, vessels were perfused with buffer-free agonist during 4 min. *, $p < 0.05$ compared with the control group.

ods to prepare HCA extracts avoiding the use of surface active agents. The present results consistently show the co-detection of caveolins 1 and 3, flotillin 1, protein Gα_q, and P2Y$_1$R in the fractions determined to correspond to rafts. In addition, p230, calnexin, and Tfn-R were not found localized in raft domains, demonstrating that our preparations have only minor contamination with intracellular membranes. Therefore, our results allow us to firmly conclude that the P2Y$_1$R is microregionalized in the PM of HCA-derived smooth muscle cells. Consonant with these findings, disruption of these microdomains abrogated the P2Y$_1$R-induced vasoconstrictive responses without affecting the motor responses evoked by either KCl or serotonin. In addition, and consistent with this interpretation, the 5-HT-mediated vasomotor responses did not show heterologous desensitization with the P2Y$_1$R. In contrast, the 5-HT-evoked responses of the rat tail artery are associated to caveolae and its vasomotor response is sensitive to MβCD treatment (Dreja et al., 2002). It is possible that the association of the 5-HT$_{2A}$ to caveolae/rafts is tissue-specific, reflecting a broad diversity in cell signaling linked to specific cells.

The P2Y$_1$R fraction localized in rafts ranged approximately from 20 to 30% of the total receptor mass, an observation independent of the experimental procedures used to isolate these microdomains. Functional assays revealed that this receptor population is of physiological significance. The P2Y$_1$R fraction associated to membrane rafts was abrogated by MβCD treatment, a procedure that disrupted the vasomotor response elicited by P2Y$_1$R agonists. In addition, MRS 2179, the selective P2Y$_1$R antagonist, was inactive per se, but blocked both the exit of the receptor from the raft domains and the vasomotor response induced by the receptor agonist. Among the factors that govern the localization of proteins in raft domains, myristoylation and/or palmitoylation may be determinant (Song et al., 1996). Although neither of these post-translational modifications has been described for the P2YRs as of yet, analysis of the amino acidic sequences of these receptors show only the P2Y$_1$R has a potential site for this lipidic modification (Costanzi et al., 2004). These modifications could encompass a fraction of the total P2Y$_1$R, limiting its location in membrane rafts, helping to account for our finding that only 20 to 30% of P2Y$_1$R mass is localized in membrane microdomains.

Although 2-MeSADP and ADP are agonists for the P2Y$_{12/13}$ receptors (Communi et al., 2001; Zhang et al., 2002), we may reasonably discard the participation of these receptors in the 2-MeSADP-induced contractions of the HCA because ARC 69931MX, a P2Y$_{12/13}$ receptor antagonist (Marteau et al., 2003), did not abrogate the vasoconstrictions induced by 2-MeSADP at concentration up to 100 nM to the same extent that MRS 2179 attenuated the 2-MeSADP-evoked contractions. Moreover, the P2Y$_{12}$R is not expressed in HCA (Buvinic et al., 2006), and the P2Y$_{13}$R is principally expressed in spleen and brain (Communi et al., 2001; Zhang et al., 2002).

Independent of some minor caveats, we repeatedly observed that the receptor partitioned out of the raft domains by a mechanism related to the selective activation of the P2Y$_1$R. Three arguments substantiate this conclusion: 1) 2-MeSADP, MRS 2365, and ADP, the endogenous receptor ligand, were all effective in partitioning the P2Y$_1$R out of the rafts; 2) structurally related compounds, which lack affinity for the P2Y$_1$R, such as ADO, UTP, or UDP, did not mimic the

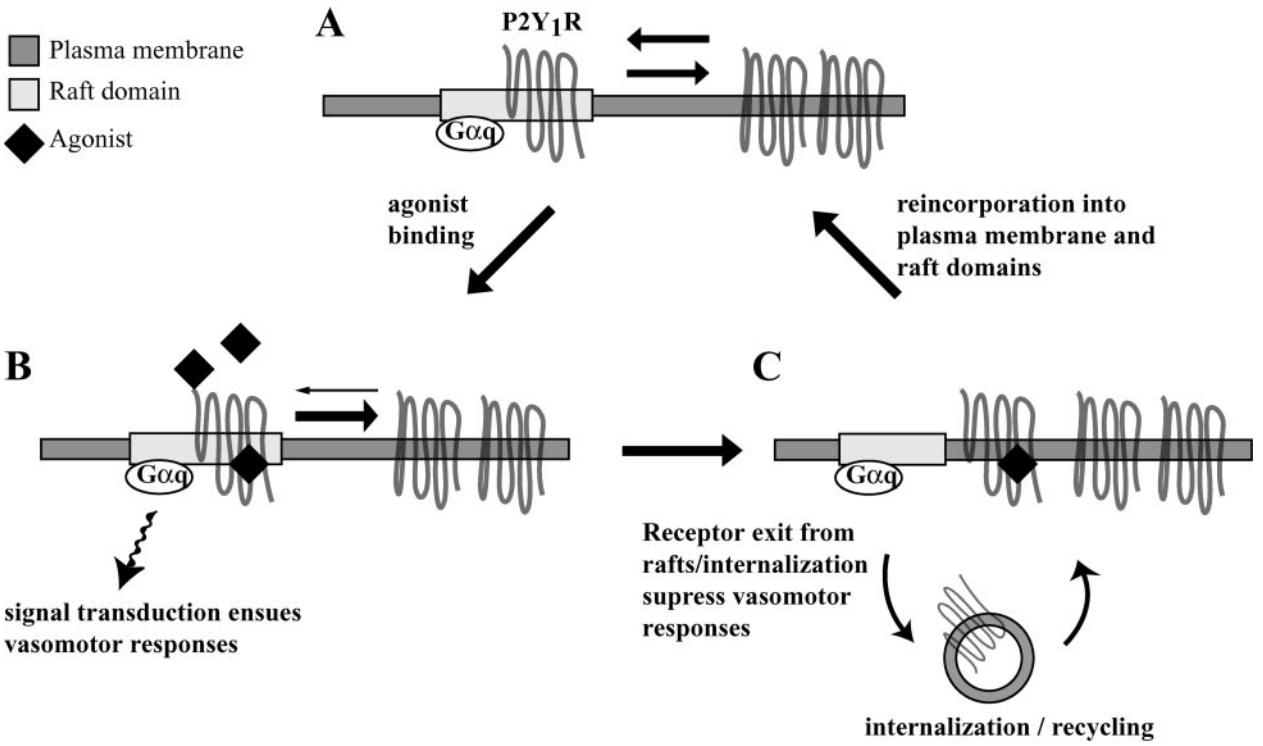

Fig. 9. Schematic model of P2Y$_1$R raft domain association and its regulation by agonists. A, in the absence of extracellular nucleotides, a fraction of the P2Y$_1$R population is found localized in membrane rafts. B, upon exposure to agonists, receptor occupation initiates its association with Gα_q, which results in intracellular signaling and ensues the subsequent vasomotor response. C, within 4 min, the P2Y$_1$R but no the Gα_q exits from raft domains, reducing the vasomotor response probably followed by receptor internalization. The process is reversible because the P2Y$_1$R relocalizes into raft domains, allowing a new cycle of receptor activation and muscular contractions.

receptor exit from the rafts; 3) the $P2Y_1R$-selective antagonist MRS 2179 was ineffective per se but blocked the 2-MeSADP-induced $P2Y_1R$ exit from the membrane raft. It is interesting that the cardiomyocyte β_2-adrenoreceptor also translocates out of raft domains after agonist activation (Ostrom et al., 2001). This is a most interesting observation that may be functionally relevant and related to the concept of sympathetic cotransmission because purines and noradrenaline are costored and coreleased from peripheral sympathetic neuroeffector junctions (Huidobro-Toro and Donoso, 2004).

The present results proposed several possibilities that might be relevant to the role of purines and other ligands in the control of the vascular tone. In our assays, we repeatedly observed that the $P2Y_1R$-induced contractions faded within minutes of ligand application, with a time course and concentration dependence that correlate to the exit of the receptor out from raft domains. Similar exits from membrane rafts, linked with faded responses, have been described for angiotensin II, bradykinin B_2, and endothelin A receptors (Abdellatif et al., 1991; Blaukat et al., 1996). Although the $P2Y_1R$-induced contractions account for up to 20% of the KCl-mediated contractions, the purinergic component must be understood as one of the multiple signals that regulates the blood vessel tone. Finally, the present data support the proposal that the rapid internalization of the activated $P2Y_1R$ and its relatively slowly recovery to the cell surface seems as the most probable explanation for the parallel slow recovery of the vasomotor responses. At present, we have not addressed which is the internalization route (clathrin or caveolae/raft) followed by this receptor, but based on known molecular mechanisms, phosphorylation of $P2Y_1R$ serines is most likely involved (R. Nicholas, personal communication). Similar protein partitioning out of caveolae and internalization has been associated with desensitization of the epidermal growth factor receptor (Mineo et al., 1999), an issue that might have vast physiological implications for cell signaling, because the $P2Y_1R$ was recently found to transactivate the epidermal growth factor receptor (Buvinic et al., 2007).

Although our biochemical methods for the isolation of membrane rafts amply support our conclusions, an ultimate proof of the association of the $P2Y_1R$ to rafts domains will require high-resolution microscopy. Unfortunately, our antibody for the $P2Y_1R$ has limitations in the usage of these kinds of methods. Notwithstanding this caveat, two important consequences of our experimental model reveal the physiological implications of the present study. First, the use of a human tissue as opposed to cultured cells allowed us to study the $P2Y_1R$ in a human smooth muscle cell that expresses this receptor functionally, avoiding the compensatory mechanism that emerges upon the overexpression of the receptor in a cell line. Second, based on the performance of concentration-responses studies, we may now correlate the vasomotor response with the receptor localization in the PM; agonist concentration under the ligand EC_{50} allowed repeated vasomotor responses, although we recognize that the contractions were not sustained. It is possible that the occupation of a minor fraction of the receptors is internalized by these rather low agonist concentrations, without causing a significant deterioration of the functional responses associated to $P2Y_1R$ activation. In contrast, concentrations within or above the ligand EC_{50} value elicited within 4 min the displacement of a significant fraction of the $P2Y_1R$ out of rafts followed by internalization reflected by the fading of the vasomotor responses in addition to a gradual reduction in the vasomotor effects by repeated agonist application. Altogether, these results highlight the significance of the agonist concentration studies in receptor activation and in determining the receptor cycling in the cell membrane of this vascular smooth muscle cells.

In sum, the present study consistently demonstrates the microregionalized distribution of the $P2Y_1R$ into membrane rafts in the smooth muscle cells of human vascular biopsies from placental and systemic conductance vessels. Furthermore, the redistribution of the $P2Y_1R$ out of rafts requires receptor signaling and likely internalization. The possibility that the receptor distribution out of membrane rafts and its interactions/oligomerization with other GPCRs involved in the regulation of the vascular tone needs to be further explored because it was reported recently that the $P2Y_1R$ constitutively forms homooligomers (Choi et al., 2008), whereas the $P2Y_1R$ and the $P2Y_{11}R$ form heteromers (Ecke et al., 2008). In addition, heterodimers with adenosine receptors (Yoshioka et al., 2002) or vasoactive peptide receptors such as that reported between the angiotensin AT-1 and bradykinin B2 receptors (AbdAlla et al., 2000), opens novel opportunities of clinically relevant research. The extension of the present observations and the putative use of vascular biopsies from patients suffering from serious vascular diseases will allow for assessing the role of membrane rafts in cardiovascular diseases, establishing new links between pharmacology and molecular pathology.

Acknowledgments

We appreciate and are greatly indebted to the personnel of our maternity ward and the team of cardiac surgeons from the Clinical Hospital of the P. Catholic University for collaboration in the prompt delivery of the placentae and human blood vessel samples for the operating rooms. We are grateful to Professor J. F. Miquel for help in cholesterol determinations.

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