Disruption of Lipid Rafts Inhibits P2X<sub>1</sub> Receptor-mediated 
Currents and Arterial Vasoconstriction* 

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P2X<sub>1</sub> receptors for ATP are ligand-gated cation channels expressed on a range of smooth muscle preparations and blood platelets. The receptors appear to be clustered close to sympathetic nerve varicosities and mediate the underlying membrane potential changes and constriction following nerve stimulation in a range of arteries and resistance arterioles. In this study we have used discontinuous sucrose density gradients, Western blot analysis, and cholesterol measurements to show that recombinant and smooth muscle (rat tail artery, vas deferens, and bladder) P2X<sub>1</sub> receptors are present in cholesterol-rich lipid rafts and co-localize with the lipid raft markers flotillin-1 and -2. Lipid rafts are specialized lipid membrane microdomains involved in signaling and trafficking. To determine whether lipid raft association was essential for P2X<sub>1</sub> receptor channel function we used the cholesterol-depleting agent methyl-β-cyclohexextrin (10 mM for 1 h). This led to a re-distribution of the P2X<sub>1</sub> receptor throughout the sucrose gradient and reduced P2X<sub>1</sub> receptor-mediated (α,β-methylene ATP, 10 μM) currents in HEK293 cells by >90% and contractions of the rat tail artery by ~50%. However contractions evoked by potassium chloride (60 mM) were unaffected by methyl-β-cyclohexestrin and the inactive analogue α-cyclohexestrin had no effect on P2X<sub>1</sub> receptor-mediated currents or contractions. P2X<sub>1</sub> receptors are subject to ongoing regulation by receptors and kinases, and the present results suggest that lipid rafts are an essential component in the maintenance of these localized signaling domains and play an important role in P2X<sub>1</sub> receptor-mediated control of arteries. 

ATP released from nerves following tissue damage or shear stress acts at P2 receptors to regulate cardiovascular function (1). P2 receptors are divided into ligand-gated P2X receptor channels and G-protein-coupled P2Y receptors. P2X receptor-mediated vasaconstriction has been described in a range of arteries in the periphery (1) and in the brain (2). Sympathetic nerves co-store and co-release ATP and noradrenaline, and the relative contribution of purinergic and noradrenergic mechanisms to vasoconstriction is dependent on the size of the vessel and the parameters of stimulation (3, 4). The P2X receptor-mediated component predominates in small diameter arteries (4, 5) and in submucosal resistance arterioles P2X receptor activation is solely responsible for sympathetic neurogenic vasoconstriction with noradrenaline acting through presynaptic mechanisms to regulate transmitter release (6). In addition P2X receptor channels in smooth muscle are permeant to calcium (~10% of current flowing through the channel under physiological conditions, Refs. 7 and 8), and a substantial component of the calcium required for contraction enters by this route (4, 9). P2X receptors therefore provide a mechanism for sympathetic nerve-mediated regulation of vascular tone that is resistant to α-adrenoceptor and calcium channel antagonists. 

Seven P2X receptor subunits have been identified (P2X<sub>1</sub>–7), and these can assemble as homo- and heterotrimERIC receptors with a range of properties (10). The characteristic features of ATP-regulated smooth muscle P2X receptors; (i) sensitivity to the ATP analogues: α,β-methylene and 1,β,γ-methylene ATP, and (ii) responses that desensitize during agonist application, are consistent with the expression of P2X<sub>1</sub> receptor subunits. In arterial smooth muscle the P2X<sub>1</sub> receptor is the dominant isoform, and P2X receptor-mediated responses are abolished in arteries from P2X<sub>1</sub> receptor-deficient mice (11, 12). Studies with these P2X<sub>1</sub> receptor-deficient mice have established a role for these receptors in sympathetic nerve-mediated vasaconstriction (11) and autoregulation of blood flow in the kidney (12, 13). The level of P2X<sub>1</sub> receptors can be regulated by congestive heart failure (14), cardiomyopathy (15), and shear stress (16). In addition it has been shown recently that P2X<sub>1</sub> receptors are involved in sensitizing responses following heart failure (17). P2X<sub>1</sub> receptors are also expressed on blood cells, including platelets and P2X<sub>1</sub> receptors contribute to platelet activation (18) and aggregation (19–21) and P2X<sub>1</sub> receptor deficiency is protective against thromboembolism (22). Thus evidence is building that P2X<sub>1</sub> receptors can play important roles in the cardiovascular system and regulation of blood flow. 

In arteries P2X<sub>1</sub> receptors do not appear to be randomly distributed throughout the plasma membrane as P2X receptors; receptor immunoactivity appears in clusters (23). This clustering of receptors is supported by electrophysiological studies on dissociated artery muscle-excised membrane patches; some patches had multiple P2X receptor channels whereas channel activity was absent in others (24). Recent studies also indicate that the P2X<sub>1</sub> receptors can be regulated by phosphorylation of interacting proteins (25), suggesting that the P2X<sub>1</sub> receptor exists in an organized signaling domain. One possible explanation for the clustering of P2X<sub>1</sub> receptors could be the inclusion in membrane lipid rafts (26). Lipid rafts are rich in cholesterol and glycosphingolipids that result in liquid-ordered microdomains within the liquid-disordered glycerophospholipid membrane bilayer (27, 28). Recent evidence suggests that there is heterogeneity in lipid rafts and that a range of different domains can be separated based on differences in detergent solubility (for a review see Ref. 27). A wide range of proteins, including many signaling molecules have been shown to be preferentially associated with rafts (29) including a range of ion channels (for a review see Ref. 30). In this study we have shown that P2X<sub>1</sub> receptors are
concentrated in lipid rafts and that disruption of rafts reduces P2X2 receptor signaling in arteries.

**Materials and Methods**

**Reagents**—α,β-meATP,1 cholesterol, filipin III, KCl, α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and methyl-β-cyclodextrin (Mβ-CD) were purchased from Sigma (Sigma-Aldrich). Cell Culture and Transient Transfection—Native human embryonic kidney 293 (HEK293) cells and HEK293 cells subcloned after transfection with the human wild-type P2X2 receptor (P2X2-Cl1 cells), were maintained in culture as previously described (25). For some studies native HEK293 cells were transiently transfected with plasmid cDNA encoding either the rat wild-type P2X2 receptor or human wild-type P2X2 receptor, tagged receptor with EGFP at its C terminus (P2X2-EGFP cells) using Lipofectamine™ 2000 reagent (Invitrogen). Control and β-CD-treated cells (10 mM for 1 h at 37 °C) were imaged live using a >60 oil immersion lens mounted on a Fluoview FV300 confocal microscope (excitation wavelengths of 488 nm for EGFP and filters set to capture emission at wavelengths greater than 510 nm) (Olympus, Tokyo, Japan). Fluorescence was captured using Olympus Fluoview 4.2 software.

**Rat Tissue Culture**—Male Wistar rats (250–350 g) were killed by stunning and cervical dislocation. For membrane fractionation, tail artery, vas deferens, and bladder were excised and processed immediately or frozen in liquid nitrogen for later use. For contraction experiments, tail arteries were kept at 4 °C in physiological saline solution (150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.5 mM CaCl₂, 1 mM MgCl₂, pH 7.3 with NaOH) for a maximum of 3 h before use.

**Membrane Fractionation**—Cells and tissues were fractionated using a detergent-free method adapted from Refs. 31 and 32. Two 80-cm² tissue flasks of HEK293 P2X1Cl-1 cells were washed three times with phosphate-buffered saline and scraped into 2 ml of 500 mM sodium succinate, pH 8.0 containing 0.2% Triton X-100 (MBS/Na succinate) was formed above the homogenates, which were lysed in 2 ml of MBS containing either 0.1% Triton X-100 or 1% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CHAPS), a detergent-free method used to solubilize detergent-sensitive proteins. After the addition of an equal volume of 90% sucrose in MBS/Na succinate (also in MBS/Na succinate), the gradient was centrifuged at 39,000 rpm on a TH-641 rotor in a Sorvall GX-50 ultracentrifuge (Kendro Laboratory Products Plc, Bishop's Stortford, UK) for 16 h at 4 °C. In some studies HEK293 P2X2-Cl1 cells were lysed in 2 ml of MBS containing either 0.1% Triton X-100 or 1% CHAPS, and then subjected to three 20-s bursts of sonication. Homogenates were brought to 45% sucrose by addition of an equal volume of 90% sucrose in MBS/Na succinate and loaded in an ultracentrifuge tube. A discontinuous sucrose gradient was layered on top of the sample by placing 4 ml of 35% sucrose prepared in MBS with 250 mM NaCl, then 4 ml of 5% sucrose (also in MBS/Na succinate). The gradient was centrifuged at 39,000 rpm on a TH-641 rotor in a Sorvall OTD65B ultracentrifuge (Kendro Laboratory Products Plc, Bishop’s Stortford, UK) for 16 h at 4 °C. In some studies HEK293 P2X2-Cl1 cells were lysed in 2 ml of MBS containing either 0.1% Triton X-100 or 1% CHAPS, and then subjected to three 20-s bursts of sonication. Homogenates were brought to 45% sucrose by addition of an equal volume of 90% sucrose in MBS and loaded in an ultracentrifuge tube. A discontinuous sucrose gradient (35 and 5% sucrose in MBS, lacking Triton X-100) was formed above the homogenate and centrifuged as described above for the detergent-free condition. After centrifugation, 11 fractions of 1 ml were collected from the top to the bottom of each tube. Cholesterol measurements were assayed with the Amplex Red cholesterol assay kit (Molecular Probes Europe BV, Leiden, The Netherlands). The cell volume or tissue quantities used for the sample preparations described above where each representative of one individual experiment. Each experimental condition was repeated three times.

**Western Blotting**—Western blotting of P2X2 receptor protein level of fraction 4 was used as a loading reference. To do so, the same volumes of samples (different fractions) were loaded for each gel after normalization for protein quantity in fraction 4 (0.04 μg for tail artery, vas deferens, or bladder and 0.1 μg for HEK293 P2X2-Cl1 cells). After separation of the samples on 10–12% SDS-PAGE gels and transfer onto nitrocellulose, the membrane was processed with the primary antibody (anti-P2X2, receptor antibody 1:2000) (Olympus, Tokyo, Japan) or anti-caveolin-1 (1:250), anti-flotillin-1 (1:250), anti-flotillin-2 (1:250), anti-caveolin-2 (1:250) and anti-caveolin-1 (1:2000) antibodies, followed by incubation with peroxidase-conjugated streptavidin (Pierce). Cells contained in a 35-mm Petri dish were processed for immunoprecipitation (33). The samples were resuspended in 15 μl of gel sample buffer before running on SDS-PAGE gels. In parallel an aliquot of total lysate (0.5 μg of total protein) was blotted with anti-p44/42 MAP kinase antibody (1:1000) (PerkinElmer Life Sciences). Each experimental condition was repeated three times.

**Patch Clamp Recordings**—Conventional whole cell and amphotericin-permeabilized patch-clamp experiments were performed at a holding potential of −60 mV at room temperature (21 °C) as described previously (11, 34). The agonist (α,β-meATP, 10 μM) was rapidly applied via a U-tube. When looking at the effect of cyclodextrins on the P2X2 receptor responses to α,β-meATP, the cells were incubated with the drugs at a concentration of 10 mM for 1 h at 37 °C, for cholesterol repletion cells were subsequently incubated for 1 h at 37 °C with 660 μg/ml cholesterol Mβ-CD. Filipin (10 μM) was incubated with the cells for 30 min at 37 °C.

**Contraction Studies**—Rat tail artery rings were mounted in a M ночта e� yograph and perfused with physiological saline solution at 34 °C and vasoconstrictions to applied drugs measured as described previously (32). The effective response to 10 μM α,β-meATP was determined when the agonist was added at 30-min intervals. The superfusate was stopped when the arteries were incubated in the presence of cyclodextrin drugs (10 mM for 1 h at 34 °C). In the control condition, the superfusate was also stopped for 1 h. Before testing subsequent contraction responses to α,β-meATP and KCl (60 mM), the superfusion was reinitiated, and the arteries were washed with physiological saline solution.

**Data Analysis**—Data analysis was as described previously. The S.E. throughout and n = number of cells, number of arteries. Differences between means were determined by Student’s t test and a p value of < 0.05 was considered statistically significant.

**Results**

**P2X2 Receptors Are Associated with Lipid Rafts**—Membrane proteins can show a variable distribution relating to the lipid composition of the membrane, with a number of proteins being preferentially located in cholesterol-rich lipid rafts. Stable expression of P2X2 receptors in HEK293 cells provides an ideal model system to investigate the role of lipid rafts in receptor function. Cell lysates were extracted under detergent-free conditions and ultracentrifuged on a discontinuous sucrose gradient (31). Supplementary Data Figure S1A). P2X2 receptor protein was predominantly found colocalized with the lipid raft markers flotillin-1 and flotillin-2 in fractions 3 and 4 (36) (Fig. 1a) and was absent from non-transfected cells (Fig. 1d). Fractions 3 and 4 contain more than 75% of the total cellular cholesterol (and only ~10% of the total protein, Fig. 1b) confirming the presence of the P2X2 receptor protein in the cellular enriched-cholesterol fractions. As the P2X2 receptor is a transmembrane protein one possibility was that the fractionation concentrates the plasma membrane in fractions 3 and 4, and this could account for the predominant localization of the P2X2 receptors in these fractions and not lipid raft association. To test this we used membrane-impermeant sulfo-NHS-LC-biotin to label surface proteins and determine their distribution on the gradient. Biotinylated proteins were distributed throughout the gradient, demonstrating that the fractionation does not just concentrate membrane proteins. Moreover, the biotinylation process was specific for surface proteins as no trace of the cytosolic p44/42 MAP kinases was observed at the cell surface although present in the total lysate (Fig. 1e). Taken together these data demonstrate that P2X2 receptors are localized to enriched cholesterol lipid rafts.

**P2X2 Receptors Remain in the Lipid Raft Fraction following Activation and Desensitization**—Activation of some receptors, for example the β2-adrenoreceptor leads to movement out of the lipid rafts and a reduction in signaling (37, 38). P2X2 receptors desensitize rapidly following agonist stimulation, and require several minutes for recovery, raising the possibility...
that the recovery process is dependent on movement between lipid rafts and the rest of bulk membrane. Activation of the P2X<sub>1</sub> receptor with α,β-meATP (10 μM) for 10 min should fully desensitize the receptor; however it had no effect on the distribution of the receptor in the lipid raft fractions (Fig. 1f) indicating that movement out of the lipid rafts is not associated with desensitization of the P2X<sub>1</sub> receptor channel.

Lipid Raft Disruption Regulates P2X<sub>1</sub> Receptor Properties—Depletion of cellular cholesterol leads to dissociation of lipid rafts. The cholesterol-depleting agent Mβ-CD (10 mM for 1 h) reduced by 51 ± 28% the total cellular cholesterol content and induced a redistribution of the P2X<sub>1</sub> receptor along the gradient (Fig. 2a). Following cholesterol depletion, the P2X<sub>1</sub> receptor was now detected readily in fractions 5–11. Mβ-CD is membrane impermeable and does not deplete cholesterol from intracellular membranes, this may in part account for the P2X<sub>1</sub> receptor detected in fractions 3 and 4 that could correspond to receptors on intracellular membranes being trafficked.

In patch-clamp studies, the ATP analogue α,β-meATP (10 μM a maximal concentration) evokes transient inward currents...
P2X₁ Receptors and Lipid Rafts

Fig. 3. P2X₁ receptor and flotillin-2 association with lipid rafts in HEK293 cells is sensitive to the concentration of Triton X-100 detergent. HEK293 cells stably expressing P2X₁ receptors were lysed either in detergent-free condition (50 mM Na₃CO₃, pH 11) or in presence of 0.1 or 1% Triton X-100. Under detergent-free conditions, P2X₁ receptors (left panels) and flotillin-2 (right panels) were detected in the buoyant membrane fractions. A different pattern of P2X₁ and flotillin-2 protein distribution in the gradient was recorded following isolation with Triton X-100, and this was concentration-dependent; 0.1% Triton X-100 redistributed partially the P2X₁ receptor along the sucrose gradient, whereas the P2X₁ receptor was predominantly in the 45% sucrose bottom fractions following 1% Triton X-100 treatment. The inset shows that the P2X₁ receptor was detected in the buoyant fractions on longer exposure of the blot. A similar distribution along the sucrose gradient was also observed for the lipid raft marker flotillin-2.

through P2X₁ receptors from HEK293 P2X₁cl-1 cells (Fig. 2b). Depletion of cellular cholesterol with either Mβ-CD or β-CD (10 mM for 1 h) reduced the amplitude of agonist-evoked responses by >90% with no effect on either the rise time or rate of decay of the response (Fig. 2b). The reduced amplitude of response is unlikely to result from a decrease in agonist potency at the receptor, as suggested for the effects of cholesterol depletion on cyclic nucleotide-gated channels (39), as there was no effect on the time course of the response and a supramaximal concentration of ATP (1 mM, 1,000-fold greater than the EC₅₀ concentration) was also reduced by >90% (data not shown). Mβ-CD or β-CD treatment also has no effect on the recovery of P2X₁ receptors from the desensitized state as reproducible responses to α,β-meATP were evoked at 5-min intervals using the amphotericin-permeabilized patch technique (as shown previously, Ref. 34) from control cells and following Mβ-CD or β-CD treatment (data not shown). The inactive cyclodextrin stereoisomer α-CD (10 mM for 1 h) (40) had no effect on agonist-evoked responses (Fig. 2b). Following Mβ-CD treatment responses to α,β-meATP (10 μM) were partially restored (~4-fold increase in amplitude compared with Mβ-CD, p < 0.05) by cholesterol repletion (660 μg/ml for 1 h)(–3222 ± 549, -212 ± 130, and -818 ± 240 pA for control, Mβ-CD and cholesterol repletion, respectively, n = >8). The reduction in current amplitude in response to Mβ-CD is unlikely to result from a decrease in the number of P2X₁ receptor channels at the cell surface as the localization of EGFP-tagged P2X₁ receptors at the cell membrane (peak currents through these receptors are also reduced by ~85% by Mβ-CD treatment; -5224 ± 1063 and -887 ± 200 pA for control and Mβ-CD respectively, n>6) was unaffected by β-CD treatment (observation of 17 and 14 cells, respectively, for control and β-CD treatment) (Fig. 2d).

Lipid rafts may also be disrupted by filipin, which acts to form multimeric globular complexes with membrane cholesterol (41, 42). Filipin (10 μM for 30 min) reduced by >90% peak currents following P2X₁ receptor activation with no effect on the time course of the response (~5809 ± 790 and 119 ± 70 pA for control and filipin treatment, respectively, n = 12.5). Taken together our results show that lipid raft disruption in HEK293 cells significantly depressed P2X₁ channel function.

With What Type of Lipid Rafts Are P2X₁ Receptors Associated?—Lipid rafts are thought to be heterogeneous with a range of different types of domain dependent on the protein and lipid content of the rafts (27). The only subtype of rafts that can be identified morphologically are caveolae. These are small plasma membrane invaginations associated with the expression of the caveolins (27). In this study caveolin-1 and -2 were below the limit of detection in HEK293 cell lines (data not shown; however caveolins were detected in arterial smooth muscle cells, see below) suggesting that P2X₁ receptors are not associated with caveolae in lipid rafts.

The composition of lipid rafts appears to be dependent on the method of isolation and different types of lipid raft domain have been postulated based on differences in detergent solubility of individual raft proteins. Lipid rafts prepared by non-detergent methods are enriched in glycerophospholipids whereas, in contrast, detergent extraction with Triton X-100 leads to a depletion of these glycerophospholipids and in addition non-detergent methods lead to a greater retention of inner leaflet membrane lipids (for review, see Ref. 27). Thus comparing results from different isolation procedures may give an insight into which subpopulation of lipid rafts the P2X₁ receptor associates with. We therefore compared rafts isolated by non-detergent methods with extraction with 0.1 and 1% Triton X-100 (Fig. 3). At 0.1% Triton X-100 P2X₁ receptors were associated with the buoyant fraction, however in addition the P2X₁ receptor was also significantly detected in bottom fractions. When 1% Triton X-100 was used the P2X₁ receptor was detectable at low levels in the buoyant fractions and predominantly in the bottom fractions, this is consistent with a previous report on Triton X-100 (1%) extracted P2X₁ receptors (43). The Triton X-100 concentration-dependent localization of the P2X₁ receptor was mirrored by the fractions in which the lipid raft marker flotillin-2 was found. This suggests that the association of P2X₁ receptors in lipid rafts, like for other proteins, for example the T cell receptor in Jurkat cells (Ref. 44 and for a more general review, see Ref. 27), are sensitive to Triton X-100 extraction. This raises the possibility that the association of P2X₁ receptors with lipid rafts may be regulated by glycerophospholipids or inner leaflet membrane lipids as these are depleted by Triton X-100 treatment.

P2X₁ Receptors in Rafts in Smooth Muscle Cells—We have shown that recombinant P2X₁ receptors are associated with lipid rafts and disruption of these leads to an inhibition of P2X₁ receptor-mediated currents. In this series of studies we determined whether native P2X₁ receptors are expressed in lipid rafts in smooth muscle preparations and whether disruption of the rafts can regulate the functional properties of native P2X₁ receptors. Rat tail arteries were lysed under detergent-free conditions and ultracentrifuged on a discontinuous sucrose gradient. The P2X₁ receptor protein was identified in the fractions 4 and 5 of the gradient just as the lipid raft markers, flotillin-1 and flotillin-2. P2X₁ receptor protein also co-localized with caveolae (specialized subtype of lipid rafts) proteins caveolin-1 and caveolin-2 (Fig. 4a). The enriched cholesterol fractions represented by fractions 3, 4, and 5, contain more than 70% of the total cellular cholesterol (with a peak ~30% for fraction 4) but less than 10% of the total cellular protein (Fig. 4b). Taken
together, these results confirm that P2X<sub>1</sub> receptor protein is present in the cellular-enriched cholesterol fractions of rat tail arteries. P2X<sub>1</sub> receptor protein and the lipid raft marker caveolin-1 were similarly distributed along rat vas deferens and bladder gradients in fractions 3 and 4 of HEK293 cells whereas in fractions 4 and 5 of smooth muscle cells. This localization reflected the distribution of cholesterol (for comparison see Figs. 1 and 4) and demonstrates that there are likely to be functional P2X<sub>1</sub> receptor mediated responses following interference with lipid raft cholesterol, provides strong evidence that P2X<sub>1</sub> receptors are present in lipid rafts in HEK293 cells and smooth muscles (arteries, vas deferens, and bladder).

**Raft Disruption Reduces P2X<sub>1</sub> Receptor-mediated Arterial Constriction**—The treatment of rat tail arteries with the cholesterol depleting agent Mβ-CD (10 mM for 1 h) redistributed the P2X<sub>1</sub> receptor from the lipid raft fractions to a diffuse distribution throughout the sucrose gradient (representative gel shown of three separate experiments). It is interesting to note that there were slight differences in the distribution of lipid rafts between HEK293 cells and smooth muscle cells; P2X<sub>1</sub> receptor and lipid raft-associated proteins were present in fractions 3 and 4 of HEK293 cells whereas in fractions 4 and 5 of smooth muscle cells. This localization reflected the distribution of cholesterol (for comparison see Figs. 1 and 4b). Because HEK293 cells and smooth muscles are distinct cell types, the membrane fractionation divergences are likely to be caused by cell membrane composition differences.

DISCUSSION

The localization of the P2X<sub>1</sub> receptor to the buoyant cholesterol-rich fractions and the co-localization with lipid raft markers (flotillins 1 and 2, and in addition for the smooth muscle preparations caveolin-1 and -2), combined with the reduction of functional P2X<sub>1</sub> receptor mediated responses following interference with lipid raft cholesterol, provides strong evidence that P2X<sub>1</sub> receptors are present in lipid rafts in HEK293 cells as well as smooth muscles (arteries, vas deferens, and bladder).

Lipid rafts are characterized as cholesterol- and sphingolipid-rich regions of membrane. Density fractionation of cholesterol-rich lipid rafts has shown that there is heterogeneity in the rafts (45) and demonstrates that there are likely to be different populations of microdomains that vary in lipid and protein composition. The only clearly identifiable subtype of

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**FIG. 4.** Presence of the P2X<sub>1</sub> receptor in the lipid raft-enriched fractions prepared from rat smooth muscles (rat tail artery, vas deferens, and bladder). a, rat tail arteries were lysed and separated on a density gradient (45, 35, and 5% sucrose). Eleven fractions (from top to bottom of gradient) were separated on SDS-PAGE gels and blotted for the P2X<sub>1</sub> receptor, caveolin-1, caveolin-2, flotillin-1, and flotillin-2. b, fractions collected in a were also assayed for cholesterol and total protein. Rat vas deferens (c) and bladder (d) were processed as described in a and immunoblotted for P2X<sub>1</sub> receptor and caveolin-1. All the gels shown in Fig. 3 were representative of three separate experiments as well as for the cholesterol and protein assays.

**FIG. 5.** Lipid raft disruption and inhibition of P2X receptor-mediated contractions of the rat tail artery. a, cholesterol depletion with Mβ-CD (10 mM for 1 h) redistributed the P2X<sub>1</sub> receptor from the lipid raft fractions to a diffuse distribution throughout the sucrose gradient (representative gel shown of three separate experiments). b, α,β-meATP (10 μM) applied for 10 min (indicated by bar) -mediated transient contractions of the rat tail artery, these were reduced following treatment with Mβ-CD (10 mM, 1 h) but were unaffected by the inactive analogue α-CD (10 mM, 1 h). c, summary of the effects of Mβ-CD and α-CD on rat tail artery contractions evoked by potassium chloride or α,β-meATP (n = 4–8; p < 0.001).

![Image](http://www.jbc.org/)

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lipid rafts are the caveolae (46), these membrane invaginations are characterized by the presence of caveolins. Many proteins are associated with caveolae through interaction with these caveolins; for example the ATP-sensitive potassium channel pore-forming subunit Kir6.1 co-immunoprecipitates with caveolin in arterial smooth muscle (47). We were unable to co-immunoprecipitate the P2X1 receptor with anti-caveolin antibodies suggesting that caveolin is not directly associated with the localization of the P2X1 receptor to lipid rafts. This is further supported by our work in HEK293 cells where levels of caveolin-1 and -2 were below the limit of detection (data not shown). A similar lack of caveolins has been reported in other HEK293 cell lines and used to suggest that β-adrenoceptor raft localization is not caveolin-dependent (37). However caveolins have been described in some HEK293 cell lines (39) and it seems likely that their expression may vary between different sublines of these cells. Myristylation and palmitylation of proteins may be involved in associations with caveolae (48, 49); however, the lack of motifs for such forms of protein modification suggests that this is unlikely for the P2X1 receptor. Additional support that caveolin-1 is unlikely to be involved in P2X1 receptor function comes from studies with knock-out mice; P2X1 receptor mice show male infertility because of a reduced contractile response of the vas deferens (50), and in this study we show that disruption of lipid rafts with β-CD reduced P2X1 receptor currents by >90%; however caveolin-1 knock-out mice show normal levels of fertility (51). Finally as P2X1 receptors are associated with rapid nerve-mediated depolarization of smooth muscle it seems counterintuitive that the receptor would be localized to membrane invaginations (caveolae), because the ATP would have to diffuse into the caveolae before activating the channel. It therefore seems likely that the P2X1 receptor is associated with non-caveolar lipid rafts.

Pike (27) has recently suggested a model where lipid rafts can be divided into three classes based on their sensitivity to extraction by a variety of methods: (i) Triton X-100- and CHAPS-resistant, (ii) Brij96, Brij98, and non-detergent isolation-resistant, and (iii) lubrol and Tween-resistant fractions. The findings that the P2X1 receptor is found in the buoyant lipid raft fraction when non-detergent methods are used and is sensitive to the concentration of Triton X-100 used suggests that the P2X1 receptor is associated with class (ii) lipid rafts; however, the motif/region of the receptor associated with raft localization, either directly or through an interacting protein remains to be determined.

Cholesterol depletion with Mβ-CD resulted in a redistribution of P2X1 receptors throughout the membrane fractions consistent with a disruption of the lipid rafts. Similar movements have been described for a range of ion channels and raft-associated proteins (e.g. 28, 39, 52). In addition Mβ-CD treatment or the use of filipin to sequester cholesterol in the membrane to disrupt the lipid rafts (41, 42) reduced P2X1 receptor-mediated responses. Recombinant human P2X1 receptors currents recorded in HEK293 cells were reduced by >90% (also for recombinant rat P2X1 receptors, control 799 ± 137 pA and 31 ± 7 following Mβ-CD treatment, p < 0.001, n = 10, 5) and contractions of arteries were reduced by ~50% (this difference most likely results from the reduced access of Mβ-CD in whole artery studies compared with patch clamp studies on isolated single cells). It is unlikely that these effects of Mβ-CD result from nonspecific effects or a block of the ability of the muscle to contract as responses evoked by depolarization with 60 mM KCl were unaffected by Mβ-CD treatment (this study), and Mβ-CD has previously been shown to have no effect on L-type calcium currents expressed in HEK293 cells (53). Similarly P2X1 receptor currents or contractions were unaffected by α-CD, the stereoisomer that has no effect on membrane cholesterol (40). In addition cholesterol depletion following Mβ-CD treatment resulted in a significant restoration of P2X1 receptor-mediated responses. Taken together these data strongly suggest that the reduction in P2X1 receptor responsiveness is caused by depletion of cholesterol and disruption of the lipid rafts. Similar reductions in function following lipid raft disruption have been described for a number of other ion channels for example voltage-dependent potassium channel Kv2.1 (28) and cyclic nucleotide-gated channels (39).

Lipid raft association of the P2X1 receptor may be a method for bringing the receptor into an organized signaling domain. We have previously shown that P2X1 receptors are basally phosphorylated, P2X1 receptor currents are reduced following treatment with the tyrosine kinase inhibitor genistein and can be potentiated by kinase activation or stimulation of Gαq-coupled receptors e.g. mGlur1α (54). Mβ-CD treatment dissociates proteins from rafts and inactivates signaling cascades (26), for example the Src family of tyrosine kinases are present in rafts (30). Therefore disruption of the localized signaling pathways could result in disruption of ongoing regulatory mechanisms and account for the decrease in P2X1 responses following cholesterol depletion.

The association of P2X1 receptors in lipid rafts may not only be important in maintaining the responsiveness of the receptor but may also be a mechanism to achieve clustering of the receptor. Immunohistochemical and single channel recording studies have indicated that the P2X1 receptor is not randomly distributed in the membrane but forms clusters (23, 24) and that in smooth muscle preparations these clusters are often seen close to sympathetic nerve varicosities; the site of transmitter release. Thus lipid rafts may be a way of concentrating the P2X1 receptor at signaling hot spots. Calcium imaging studies on the rat tail artery showed the initial transient rise in calcium was caused by purinergic receptor stimulation, and this was quickly followed by the noradrenergic component that oscillates and propagates as a wave through the vessel (55). Subsequent, higher resolution studies from vas deferens (56), mesenteric arteries (9, 57), and bladder (58) showed discrete, localized, P2X receptor-mediated calcium rises in the smooth muscle cells following nerve stimulation. The sympathetic nerves that innervate arteries and the vas deferens release the co-transmitters ATP and noradrenaline. It is interesting that nerve evoked P2X2 receptor, but not α-adrenoceptor mediated rises in calcium are recorded in response to short trains of stimulation from the vas deferens (56) and mesenteric arteries (9). A similar finding was reported in contractile studies on submucosal arterioles where following sympathetic nerve stimulation the vasoconstriction is mediated solely by P2X receptors, noradrenaline is released from the nerves but acts prejunctionally through α1-adrenoceptors to regulate transmitter release (6). These data suggest that although sympathetic nerves release both ATP and noradrenaline under certain conditions P2X receptors appear to be preferentially activated. One possible reason for this could be the differential membrane localization of P2X and α-adrenoceptors. This seems likely because in this study we have shown that the P2X1 receptor is localized in lipid rafts, and in previous work it has been shown that α1-adrenoceptors are not present in the lipid raft fractions, and noradrenaline-mediated contractions are not affected by cholesterol depletion with Mβ-CD (59). This would provide a mechanism where P2X1 receptors in lipid rafts could be concentrated close to the site of transmitter release while α-adrenoceptors would be excluded from these domains and thus be

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2 C. Vial and R. Evans, unpublished observations.
of these rafts compromises P2X1 receptor responsiveness probably through interference with organized signaling microdomains. This has significant implications for cardiovascular function where P2X1 receptors have been shown to have important signaling roles, for example in the control of resistance artery vasconstriction following sympathetic nerve stimulation (6, 11), pressure-induced autoregulation in the kidney (12), and platelet regulation (18–22).

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