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DISRUPTION OF LIPID RAFTS INHIBITS $P2X_1$ RECEPTOR MEDIATED CURRENTS AND ARTERIAL VASOCONSTRICTION.

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$P2X_1$ 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) $P2X_1$ receptors are present in cholesterol rich lipid rafts and co-localize with the lipid raft markers flotillin-1 and -2. Lipid rafts are specialised lipid membrane microdomains involved in signalling and trafficking. To determine whether lipid raft association was essential for $P2X_1$ receptor channel function we used the cholesterol depleting agent methyl-β-cyclodextrin (10 mM for 1 hour). This lead to a redistribution of the $P2X_1$ receptor throughout the sucrose gradient and reduced $P2X_1$ 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-cyclodextrin and the inactive analogue α-cyclodextrin had no effect on $P2X_1$ receptor mediated currents or contractions. $P2X_1$ 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 localised signalling domains and play an important role in $P2X_1$ receptor mediated control of arteries.

Introduction

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 vasoconstriction 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 pre-synaptic 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 (7,8)) 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 alpha adrenoceptor and calcium channel antagonists.

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Seven P2X receptor subunits have been identified ($P2X_{1–7}$) and these can assemble as homo- and heterotrimeric receptors with a range of properties (10). The characteristic features of artery smooth muscle P2X receptors; (i) sensitivity to the ATP analogues α,β-methylene and L-β,γ-methylene ATP and (ii) responses that desensitise during agonist application, are consistent with the expression of $P2X_1$ receptor subunits. In arterial smooth muscle the $P2X_1$ receptor is the dominant isoform and P2X receptor mediated responses are abolished in arteries from $P2X_1$ receptor deficient mice (11,12). Studies with these $P2X_1$ receptor deficient mice have established a role for these receptors in sympathetic nerve mediated vasoconstriction (11) and autoregulation of blood flow in the kidney (12,13). The level of $P2X_1$ receptors can be regulated by congestive heart failure (14), cardiomyopathy (15) and shear stress (16). In addition it has been shown recently that P2X receptors are involved in sensitising responses following heart failure (17). $P2X_1$ receptors are also expressed on blood cells, including platelets and $P2X_1$ receptors contribute to platelet activation (18) and aggregation (19–21) and that $P2X_1$ receptor deficiency is protective against thromboembolism (22). Thus evidence is building that $P2X_1$ receptors can play important roles in the cardiovascular system and regulation of blood flow.

In arteries $P2X_1$ receptors do not appear to be randomly distributed throughout the plasma membrane as $P2X_1$ receptor immunoreactivity appears in clusters (23). This clustering of receptors is supported by electrophysioliogical studies on dissociated artery muscle excised membrane patches; some patches had multiple P2X receptor channels whilst channel activity was absent in others (24). Recent studies also indicate that the $P2X_1$ receptors can be regulated by phosphorylation of interacting proteins (25) suggesting that the $P2X_1$ receptor exists in an organised signalling domain. One possible explanation for the clustering of $P2X_1$ receptors could be the inclusion in membrane lipid rafts (26). Lipid rafts are rich in cholesterol and glycosphingolipids that results 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 (27)). A wide range of proteins, including many signalling molecules have been shown to be preferentially associated with rafts (29) including a range of ion channels (for a review see (30)). In this study we have shown that $P2X_1$ receptors are concentrated on lipid rafts and that disruption of rafts reduces $P2X_1$ receptor signalling in arteries.

Materials and methods

Reagents

Adenosine 5’-[α,β-methylene]triphosphate (α,β-meATP), cholesterol, filipin III, KCl, α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), methyl-β-cyclodextrin (Mβ-CD), were obtained from Sigma (Sigma-Aldrich, Poole, UK).

Cell culture and transient transfection—Native human embryonic kidney 293 (HEK293) cells and HEK293 cells subcloned after transfection with the human wild-type $P2X_1$ receptor ($P2X_1$cl-1 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 $P2X_1$ receptor or human wild-type $P2X_1$ receptor tagged with EGFP at its carboxy terminus ($P2X_1$-EGFP cells) using LipofectAMINE™ 2000 Reagent (Invitrogen)/Opti-MEM (Invitrogen, Paisley, UK). Control and β-CD treated cells (10 mM for 1h at 37ºC) were imaged live using a 60X oil immersion lens mounted on a Fluoview FV300 confocal microscope (excitation wavelength 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 Fluoviewver 4.2 software.

Rat tissue collection—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, tails arteries were kept at 4ºC in physiological saline solution (150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2.5mM $CaCl_2$, 1 mM $MgCl_2$, pH to 7.3 with NaOH) for a maximum of 3 hours before use.

Membrane fractionation—Cells and rat tissues were fractionated using a detergent-free method adapted from (31,32). Two 80 cm^2 tissue flasks of HEK293 $P2X_1$cl-1 cells were washed 3 times with PBS and scraped into 2 ml of 500 mM sodium carbonate, pH 11 and left on ice for 20 min. Either seven tail arteries, 3 vas deferens or 3 bladders from rat were crushed in liquid nitrogen using a pestle and a mortar. When reduced to powder, the tissues were then homogenized into 2 ml of 500 mM Na_2CO_3, pH 11 using a tissue grinder and left on ice for 20 min. The lysed cells and rat tissues were 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 (25 mM MES and 150 mM NaCl, pH 6.5) 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 Na_2CO_3 then 4 ml of 5% sucrose (also in MBS/Na_2CO_3). 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 16h at 4ºC. In some studies HEK293 $P2X_1$c1-1cells were lysed in 2 ml MBS containing either 0.1 % Triton-X-100 or 1 % Triton-X-100 and were homogenized using a tissue grinder and left for 20 min on ice. The 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 assessed 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 conditions where repeated 3 times.

Western blots—The 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 $P2X_1$cl-1 cells). After separation of the samples on 10–12% SDS-PAGE gels and transfer onto nitrocellulose, the membrane was processed for staining with the primary antibody (anti-$P2X_1$ receptor antibody (1:2000) (Almoner, Jerusalem, Israel) or anti-Caveolin1 (1:1000), anti-Caveolin-2 (1:250), anti-Flotillin-1 (1:250), anti-Flotillin-2 (1:5000) (BD Biosciences Europe, Erembodegem, Belgium). Protein bands were visualised using an ECL+plus kit and Hyperfilm MP (Amersham Bioscexperiment.

Surface cell expression and immunoprecipitations—Cell surface proteins were biotinylated as previously described (33) and washed with PBS. Cells from two 80cm^2 tissue flasks were processed for membrane fractionation as described above and blotted with Immunopure streptavidin/horseradish peroxidase conjugated (0.5 μg/mL) (Pierce, Perbio Science, Tattenhall, UK). Cells contained in a 35 mm Ø 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) (New England Biolabs, Hitchin, UK)). Each experimental condition was repeated 3 times.

Patch-clamp recordings—Conventional whole-cell and amphotericin permeabilised patch-clamp experiments were performed at a holding potential of 60 mV at room temperature (21ºC) 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 $P2X_1$ receptor responses to α,β-meATP, the cells were incubated with the drugs at a concentration of 10 mM for 1h at 37ºC, for cholesterol repletion cells were subsequently incubated for 1h at 37ºC with 660 μg/ml cholesterol. Filipin (10 μM) was incubated with the cells for 30 min at 37ºC.

Contraction studies—Rat tail artery rings were mounted in a Mulvany myograph and perfused with physiological saline solution at 34ºC and vasoconstrictions to applied drugs measured as described previously (35). Reproducible responses to 10 μM α,β-meATP could be recorded when the agonist was added at 30 min intervals. The superfusate was stopped when the arteries were incubated in presence of cyclodextrin drugs (10 mM for 1h at 34ºC). In the control condition, the superfusate was also stopped for 1h. Before to test subsequent contraction responses to α,β-meATP and KCl (60 mM), the superfusate was reinitiated and the arteries were washed with physiological saline solution.

Data analysis—Data are expressed as mean±s.e.mean 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

$P2X_1$ 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 $P2X_1$ receptors in HEK293 cells provides an ideal model system to investigate the role lipid rafts in receptor function. Cell lysates were extracted under detergent free conditions and ultracentrifuged on a discontinuous sucrose gradient (31). $P2X_1$ receptor protein was predominantly found co-localised with the lipid raft markers flotillin-1 and flotillin-2 in the fractions 3 and 4 (36)(Figure 1a) and was absent from non-transfected cells (Figure 1d). Fractions 3 and 4 contain more than 75% of the total cellular cholesterol (and only ~ 10% of the total protein, Figure 1b) confirming the presence of the $P2X_1$ receptor protein in the cellular enriched-cholesterol fractions. As the $P2X_1$ 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 localisation of the $P2X_1$ 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 kinase was observed at the cell surface although present in the total lysate (Figure 1e). Taken together these data demonstrate that $P2X_1$ receptors are localised to enriched-cholesterol lipid rafts.

$P2X_1$ receptors remain in the lipid raft fraction following activation and desensitisation—Activation of some receptors, for example the β2-adrenoceptor leads to movement out of the lipid rafts and a reduction in signalling (37,38). $P2X_1$ receptors desensitise 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 $P2X_1$ receptor with α,β-meATP (10

μM) for 10 min should fully desensitised the receptor, however it had no effect on the distribution of the receptor in the lipid raft fractions (Figure 1f) indicating that movement out of the lipid rafts is not associated with desensitisation of the $P2X_1$ receptor channel.

Lipid raft disruption regulates $P2X_1$ receptor properties

Depletion of cellular cholesterol leads to dissociation of the lipid rafts. The cholesterol depleting agent Mβ-CD (10 mM for 1h) reduced by 51 ± 28 % the total cellular cholesterol content and induced a redistribution of the $P2X_1$ receptor along the gradient (Figure 2a). Following cholesterol depletion, the $P2X_1$ receptor was now detected readily in fractions 5 to 11. Mβ-CD is membrane impermeable and does not deplete cholesterol from intracellular membranes, this may in part account for the $P2X_1$ 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 through $P2X_1$ receptors from HEK293 $P2X_1$cl-1 cells (Figure 2b). Depletion of cellular cholesterol with either Mβ-CD or β-CD (10 mM for 1h) reduced the amplitude of agonist evoked responses by >90 % with no effect on either the rise time or rate of decay of the response (Figure 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 EC_{50} concentration) was also reduced by >90 % (data not shown). Mβ-CD or β-CD treatment also has no effect on the recovery of $P2X_1$ receptors from the desensitised state as reproducible responses to α,β-meATP were evoked at 5 minute intervals using the amphotericin permeabilised patch technique (as shown previously (34)) from control cells and following Mβ-CD or β-CD treatment (data not shown). The inactive cyclodextrin stereoisomer α-CD (10 mM for 1h)(40) had no effect on agonist-evoked responses (Figure 2b). Following Mβ–CD treatment responses to α,β-meATP (10 μM) were partially restored (~ 4 fold increase in amplitude compared to Mβ–CD, $p<0.05$) by cholesterol repletion (660 μg/ml for l hr)−3222 ± 549, −212 ± 130 and −818 ± 240 p A 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 $P2X_1$ receptor channels at the cell surface as the localisation of EGFP-tagged $P2X_1$ 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)(Figure 2d).

Lipid rafts may also be disrupted by filipin that acts to form multimeric globular complexes with membrane cholesterol (41,42). Filipin (10 μM for 30 mins) reduced by >90% peak currents following $P2X_1$ 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 $P2X_1$ channel function.

What type of lipid rafts are $P2X_1$ receptors associated with?

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 sub-type 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 caveolins –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 later) suggesting that $P2X_1$ 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 while 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 (27)). Thus comparing results from different isolation procedures may give an insight into which sub-population of lipid rafts the $P2X_1$ receptor associates with. We therefore compared rafts isolated by non-detergent methods with extraction with 0.1 and 1% Triton X-100 (Figure 3). At 0.1% Triton X-100 $P2X_1$ receptors were associated with the buoyant fraction, however in addition the $P2X_1$ receptor was also significantly detected in bottom fractions. When 1% Triton X-100 was used the $P2X_1$ receptor was detectable at low levels in the buoyant fractions and predominantly in bottom fractions, this is consistent with a previous report on Triton X-100 (1%) extracted $P2X_1$ receptors (43). The Triton X-100 concentration dependent localisation of the $P2X_1$ receptor was mirrored by the fractions in which the lipid raft marker flotillin-2 was found. This suggests that the association of $P2X_1$ receptors in lipid rafts, like for other proteins, for example the T cell receptor in Jurkat cells (44)(and for a more general review see (27)), are sensitive to Triton X-100 extraction. This raises the possibility that the association of $P2X_1$ receptors with lipid rafts may be regulated by glycerophospholipids or inner-leaflet-membrane lipids as these are depleted by Triton X-100 treatment.

$P2X_1$ receptors in rafts in smooth muscle cells—We have shown that recombinant $P2X_1$ receptors associated with lipid rafts and disruption of these leads to an inhibition of $P2X_1$ receptor mediated currents. In this series of studies we determined whether native $P2X_1$ receptors are expressed in lipid rafts in smooth muscle preparations and whether disruption of the rafts can regulate the functional properties of native $P2X_1$ receptors. Rat tail arteries were lysed under detergent free conditions and ultracentrifuged on a discontinuous sucrose gradient. $P2X_1$ receptor protein was identified in the fractions 4 and 5 of the gradient just as the lipid raft markers, flotillin-1 and flotillin-2. $P2X_1$ receptor protein also co-localised with caveolae (specialised subtype of lipid rafts) proteins caveolin-1 and caveolin-2 (Figure 4a). The enriched-cholesterol fractions represented by fractions 3,4 and 5, contains more than 70% of the total cellular cholesterol (with a peak ~30% for fraction 4) but less than 10% of the total cellular protein (Figure 4b). Taken together, these results confirm that $P2X_1$ receptor protein is present in the cellular enriched-cholesterol fractions of rat tail arteries. $P2X_1$ receptor protein and lipid raft marker caveolin-1 were similarly distributed along rat vas deferens and bladder gradients in fractions 4 and 5 (Figure 4c). It is interesting to note that there were slight differences in the distribution of lipid rafts between HEK293 cells and smooth muscle cells; $P2X_1$ receptor and lipid raft associated proteins were present in fractions 3 and 4 of HEK293 cells while in fractions 4 and 5 of smooth muscle cells. This localisation reflected the distribution of cholesterol (for comparison see figure 1b and 4b). Since HEK293 cells and smooth muscles are distinct cell types, the membrane fractionation divergences are likely to be due to cell membrane composition differences.

Raft disruption reduces $P2X_1$ receptor mediated arterial constriction—The treatment of rat tail arteries with the cholesterol depleting agent Mβ-CD (10 mM for 1h) disrupts lipid rafts and resulted in a redistribution of the $P2X_1$ receptor along the gradient (Figure 5a) similar to its effects observed previously on HEK293 $P2X_1$cl-1 cells. Tail artery constriction amplitudes to α,β-meATP (10 μM for 10 min) were dramatically reduced by

half after Mβ-CD treatment (10 mM for 1h), while the inactive analogue α-CD (10 mM for 1h) had no effect (110.5±5.8%, 52.5±4.1% and 115.4±6.9% of the initial control response, respectively for control, Mβ-CD and α-CD, n=4–7 from 3 and 4 different animals) (Figure 5b, c). In contrast, vasoconstrictions induced by 60 mM KCl were unaffected either by treatment with Mβ-CD or α-CD (97.3±4.2%, 104.2±10.9% and 96.1±2.5% of the initial control response, respectively for control, Mβ-CD and α-CD, n=4–8 from 3 and 5 different animals) demonstrating that the cyclodextrins do not interfere with the ability of the smooth muscle to contract. Taken together these data suggest that $P2X_1$ receptors are associated with lipid rafts in arteries and that disruption of these rafts inhibits responses through the channel.

Discussion

The localisation of the $P2X_1$ receptor to the buoyant cholesterol rich fractions and the co-localisation with lipid raft markers (flotillins 1 and 2, and in addition for the smooth muscle preparations caveolins 1 and 2), combined with the reduction of functional $P2X_1$ receptor mediated responses following interference with lipid raft cholesterol, provides strong evidence that $P2X_1$ receptors are present in lipid rafts in HEK293 cells as well as smooth muscles (arteries, vas deferens and bladder).

Lipid rafts are characterised 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 lipid rafts are the caveolae (46), these membrane invaginations are characterised 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 $P2X_1$ receptor with anti-caveolin antibodies (unpublished observations) suggesting that caveolin is not directly associated with the localisation of the $P2X_1$ receptor to lipid rafts. This is further supported by our work in HEK293 cells where levels of caveolins 1&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 localisation 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 sub-clones of these cells. Myristoylation and palmitoylation of proteins may be involved in associations with cavaeolae (48,49) however the lack of motifs for such forms of protein modification suggests that this is unlikely for the $P2X_1$ receptor. Additional support that caveolin 1 is unlikely to be involved in $P2X_1$ receptor function comes from studies with knockout mice; $P2X_1$ receptor mice show male infertility due to a reduced contractile responses of the vas deferens (50), and in this study we show that disruption of lipid rafts with β-CD reduced $P2X_1$ receptor currents by >90%, however caveolin 1 knockout mice show normal levels of fertility (51). Finally as $P2X_1$ receptors are associated with rapid nerve mediated depolarisation of smooth muscle it seems counterintuitive that the receptor would be localised to membrane invaginations (caveolae) as the ATP would have to diffuse into the caveolae before activating the channel. It therefore seems likely that the $P2X_1$ 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, tween resistant fractions. The findings that the $P2X_1$ 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 $P2X_1$ receptor is associated with class (ii) lipid rafts, however the motif/region of the receptor associated with raft localisation, either directly or through an interacting protein remains to be determined.

Cholesterol depletion with Mβ-CD resulted in a re-distribution of $P2X_1$ 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 and disrupt the lipid rafts (41,42) reduced $P2X_1$ receptor mediated responses. Recombinant human $P2X_1$ receptor currents recorded in HEK293 cells were reduced by >90% (also for recombinant rat $P2X_1$ 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 to patch clamp studies on isolated single cells). It is unlikely that these effects of Mβ-CD result from non-specific effects or a block of the ability of the muscle to contract as responses evoked by depolarisation 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 $P2X_1$ receptor currents or contractions were unaffected by α-CD, the stereoisomer that has no effect on membrane cholesterol (40). In addition cholesterol repletion following Mβ-CD treatment resulted in a significant restoration of $P2X_1$ receptor mediated responses. Taken together these data strongly suggest that the reduction in $P2X_1$ receptor responsiveness is due to 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 $P2X_1$ receptor may be a method for bringing the receptor into an organised signalling domain. We have previously shown that $P2X_1$ receptors are basally phosphorylated, $P2X_1$ 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 signalling cascades (26), for example the Src family of tyrosine kinases are present in rafts (30). Therefore disruption of the localised signalling pathways could result in disruption of ongoing regulatory mechanisms and account for the decrease in $P2X_1$ responses following cholesterol depletion.

The association of $P2X_1$ 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 $P2X_1$ 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 $P2X_1$ receptor at signalling hot-spots. Calcium imaging studies on the rat tail artery showed the initial transient rise in calcium was due to 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, localised, 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 P2X 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 pre-junctionally through α_2-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 localisation of P2X and α-adrenoceptors. This seems likely as in this study we have shown that the $P2X_1$ receptor is localised in lipid rafts and in previous work it has been shown that α_1-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 $P2X_1$ receptors in lipid rafts could be concentrated close to the site of transmitter release whilst α-adrenoceptors would be excluded from these domains and thus be less responsive and require longer periods of nerve stimulation to be activated (57).

In summary the present study shows for the first time that $P2X_1$ receptors are associated with lipid rafts and that disruption of these rafts compromises $P2X_1$ receptor responsiveness probably through interference with organised signalling microdomains. This has significant implications for cardiovascular function where $P2X_1$ receptors have been shown to have important signalling roles, for example in the control of resistance artery vasoconstriction following sympathetic nerve stimulation (6,11), pressure induced autoregulation in the kidney (12) and platelet regulation (18–22).

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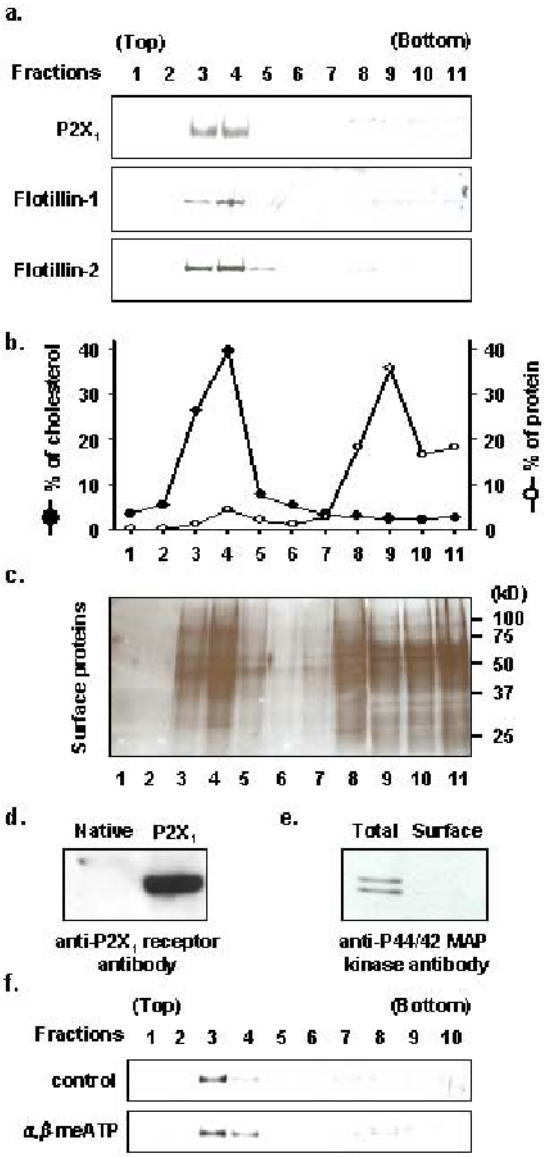

Figure 1. Presence of the $P2X_1$ receptor in the lipid raft-enriched fractions prepared from HEK-293 cells

(a) HEK 293 cells stably expressing $P2X_1$ receptor were lysed and separated on density gradient from 45, 35 and 5% sucrose. Eleven fractions (from top to bottom of gradient) were immunoblotted for $P2X_1$ receptor, Flotillin-1 and Flotillin-2 (representative gels shown for 3 separate experiments). (b) The fractions collected in (a) were also assayed for cholesterol and total protein (representative gels shown from 3 separate experiments). (c) HEK 293 cells stably expressing $P2X_1$ receptor were treated with EZ-Link™Sulfo-NHS-LC-Biotin and then process as described in (a). The fractions were then immunoblotted for streptavidin revealing the cell surface expressed proteins. (d) $P2X_1$ receptor is not expressed in native HEK 293 cells (native lane) but is present in HEK 293 cells stably expressing $P2X_1$ receptor ($P2X_1$ lane) as shown when immunoblotted for $P2X_1$ receptor (cells were lysed with 0.5% Triton X-100 containing buffer and total cell lysate was separated by SDS-PAGE). (e) HEK 293 cells stably expressing $P2X_1$ receptor were treated with EZ-Link™Sulfo-NHS-LC-

Biotin, lysed with 0.5% Triton X-100 containing buffer and immunoprecipitated with streptavidin-agarose. After separation by SDS-PAGE the sample was immunoblotted for p44/42 MAP kinase (surface lane) and compared to an aliquot taken before immunoprecipitation (total lane). Both p44/42 MAP kinases (which are known cytosolic proteins) are absent from the “surface” sample indicating that (i) the process of biotinylation is membrane protein specific and that (ii) the surface expressed protein fraction is not contaminated with cytosolic proteins. (f) HEK 293 cells stably expressing $P2X_1$ receptor were treated or not with α,β-meATP (10 μM for 10 min), processed as described in (a) and immunoblotted for $P2X_1$ receptor. All the gels shown in figure 1 were representative of 3 separate experiments as well as for the cholesterol and protein assays.

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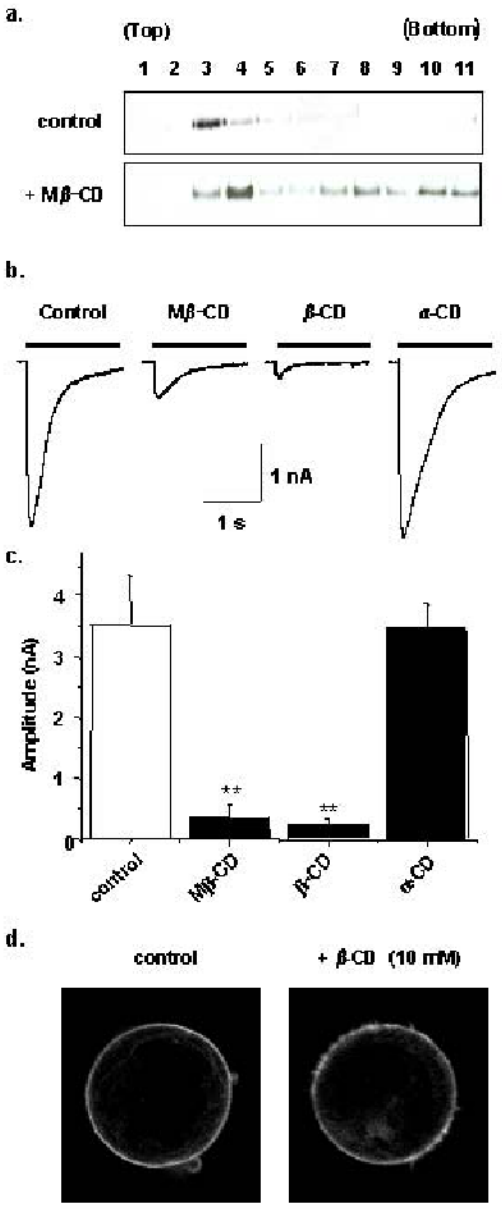

Figure 2. Cholesterol depletion moves the $P2X_1$ receptor out from the lipid raft fraction and inhibited agonist evoked currents but had no effect on the surface localisation of the receptor expressed in HEK293 cells.

(a) Cholesterol depletion with Mβ-CD (10 mM for 1 hr) redistributed the $P2X_1$ receptor from fractions 3 and 4 to a diffuse distribution throughout the sucrose gradient (representative gel shown of 3 separate experiments). (b) Representative traces of $P2X_1$ receptor currents evoked in response to 10 μM α,β-meATP (application indicated by bar). The cholesterol depleting agents Mβ-CD and β-CD (both 10 mM for 1 hr) reduced the amplitude of the current with no obvious effect on the time-course of responses. The inactive analogue α-CD (10 mM for 1 hr) redistributed the $P2X_1$ receptor from fractions 3 and 4 to a diffuse distribution throughout had no effect on $P2X_1$ receptor currents. (c) Summary of the effects of cyclodextrins on $P2X_1$ receptor current amplitude (n=6–9). (d)

EGFP tagged $P2X_1$ receptors are localised to the surface membrane and are unaffected by Mβ-CD treatment (10 mM for 1 hr).

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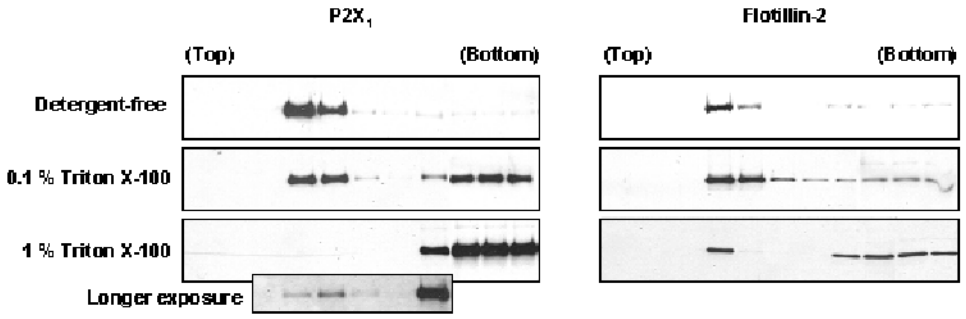

Figure 3. $P2X_1$ receptor and flotillin-2 association with lipid rafts in HEK293 cells is sensitive to the concentration of Triton X-100 detergent

HEK293 cells stably expressing $P2X_1$ receptors were lysed either in detergent-free condition (500 mM Na_2CO_3, pH 11) or in presence of 0.1 or 1% Triton X-100. Under detergent-free condition $P2X_1$ receptors (left panels) and flotillin-2 (right panels) were detected in the buoyant membrane fractions. A different pattern of $P2X_1$ 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 $P2X_1$ receptor along the sucrose gradient while the $P2X_1$ receptor was predominantly in the 45% sucrose bottom fractions following 1 % Triton X-100 treatment (the inset box shows that the $P2X_1$ 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.

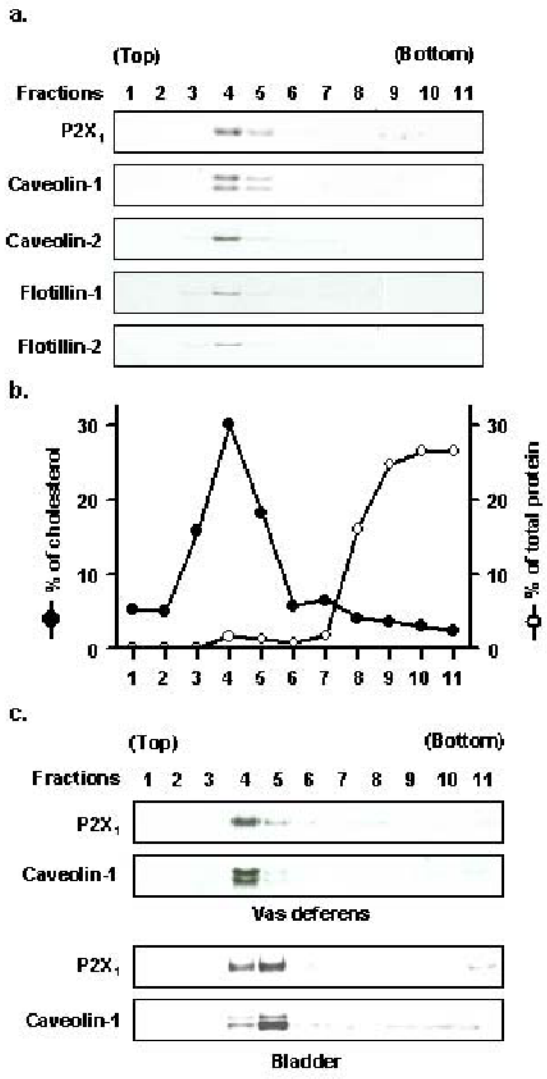

Figure 4. Presence of the $P2X_1$ receptor in the lipid raft-enriched fractions prepared from rat smooth muscles (rat tail artery, vas deferens and bladder)

(a) Rat tail arteries were lysed and separated on density gradient from 45, 35 and 5% sucrose. Eleven fractions (from top to bottom of gradient) were separated on SDS-PAGE gels and immunoblotted for $P2X_1$ receptor, Caveolin-1, Caveolin-2, Flotillin-1 and Flotillin-2. (b) The 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 $P2X_1$ receptor and Caveolin-1. All the gels shown in figure 3 were representative of 3 separate experiments as well as for the cholesterol and protein assays.

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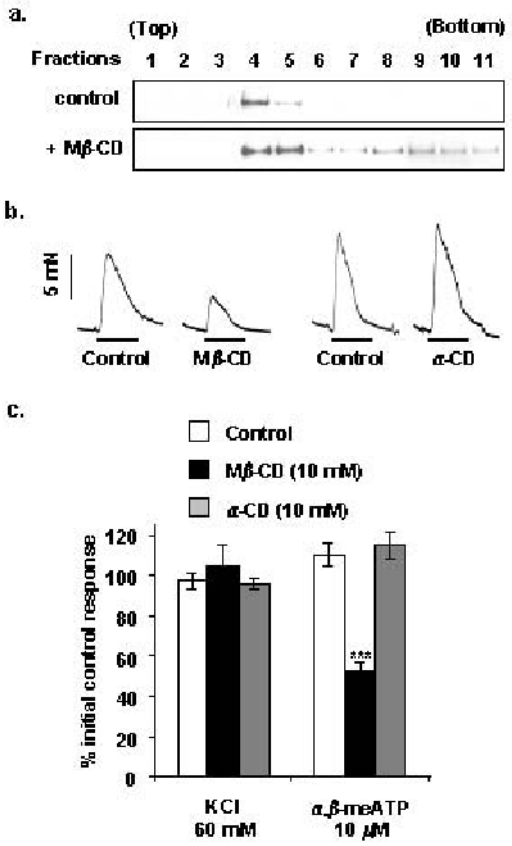

Figure 5. Lipid raft disruption and inhibition of P2X receptor mediated contractions of the rat tail artery.

(a) Cholesterol depletion with Mβ-CD (10 mM for 1 hr) redistributed the $P2X_1$ receptor from the lipid raft fractions to a diffuse distribution throughout the sucrose gradient (representative gel shown of 3 separate experiments). (b) α,β-meATP (10 μM) applied for 10 mins (indicated by bar) mediated transient contractions of the rat tail artery, these were reduced following treatment with Mβ-CD (10 mM, 1 hour) but were unaffected by the inactive analogue α-CD (10 mM, 1 hour). (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) ($p<0.001$).