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ORIGINAL ARTICLE

Fluvastatin suppresses native and recombinant human $P2X_4$ receptor function

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Abstract Statins have both cholesterol lowering and anti-inflammatory activities, whether mechanisms underlying their activities are independent remains unclear. The ATP-gated $P2X_4$ receptor is a pro-inflammatory mediator. Here, we investigate the action of fluvastatin and other cholesterol depleting agents on native and recombinant human $P2X_4$ receptor. Fluvastatin and mβCD suppressed $P2X_4$-dependent calcium influx in THP-1 monocytes, without affecting P2Y receptor responses. mβCD or filipin III suppressed the current density of recombinant human $P2X_4$ receptors. Human $P2X_2$ was insensitive to cholesterol depletion. Cholesterol depletion had no effect on intrinsic $P2X_4$ receptor properties as judged by ATP concentration–response relationship, receptor rundown or current decay during agonist occupancy. These data suggest fluvastatin suppresses $P2X_4$ activity in monocytes through cholesterol depletion and not by modulating intrinsic channel properties.

Keywords ATP · P2X receptor · Statin · Inflammation · Cholesterol

Abbreviations
MβCD Methyl-β-cyclodextrin

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Introduction

Statins are the most effective treatment for artherosclerosis and lowering cardiovascular risk [1]. Statins reduce cellular cholesterol through the inhibition of HMG-CoA reductase [1], though a second anti-inflammatory action is thought to contribute to their effectiveness as anti-atherosclerosis agents. Mechanisms of anti-inflammatory action by statins remains poorly described [2, 3]. Monocytes are major participants in the progression of atherosclerosis [4]. Monocyte recruitment and retention at sites of lesion contribute to plague development by direct secretion of pro-inflammatory molecules and monocyte maturation into macrophage and dendritic cells.

Signalling via extracellular ATP is important in the induction and resolution of inflammation. P2X receptors are a family of ATP-activated calcium channel, of which seven human receptor subunits ($P2X_{1–7}$) have been cloned and characterised [5]. $P2X_4$ is highly expressed in peripheral myeloid cells [6–8]. $P2X_4$ expression is up-regulated in microglia following nerve damage [9, 10]. More recently, activation of $P2X_4$ was demonstrated to mediate PGE2 release in macrophage [10]. In light of the pro-inflammatory action of $P2X_4$, this study sought to investigate whether $P2X_4$ is a target for fluvastatin and other cholesterol-depleting agents.

Materials and methods

Cell culture and transfection

Human THP-1 cells were cultured in RPMI medium containing 10% foetal calf serum. HEK293 cells were cultured in DMEM medium with 10% foetal calf serum at 37°C with 5% CO_2 in a humidified incubator. For electrophysiology, cells were transfected with 1 μg plasmid-encoded human

$P2X_4$ with a C-terminal glu-glu tag or human $P2X_2$ (both kind gifts from Prof. R. Alan North FRS, University of Manchester). Cells were co-transfected with 0.1 μg plasmid expressing GFP alone to allow identification of transfected cells.

Intracellular calcium measurements

Cells were pre-incubated with fura-2 AM for 1 h at 37°C followed by a 0.5-h wash at room temperature. Human THP-1cells were centrifuged in the plate at 2×10^5 cells/well. Measurements were made at room temperature on a 96-well plate reader (FlexStation, Molecular Devices). The change (Δ) in intracellular calcium (Ca_i^{2+}) concentration is indicated as the ratio of fura-2 emission intensities for 340- and 380-nm excitation (F ratio). Wells within columns of the 96-well plate were loaded alternately for test and control conditions. The recording solution contained (millimolars): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES and 1.2 MgCl2, titrated to pH 7.4 with NaOH. Ca^{2+}-free extracellular solution (0 Ca^{2+}) was prepared by excluding $CaCl_2$. Cells were pretreated with the reagents for 0.5 h during the washing period of the fura-2 before Ca^{2+} measurements at room temperature.

Electrophysiology

Whole-cell or perforated patch recordings were made 48 following plasmid transfection. HEK293 cells were seeded onto glass covers and allowed to adhere for at least 1 h prior to recording. The extracellular solution contained (millimolars): 145 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 13 D-glucose and 10 HEPES, pH 7.3. The intracellular pipette solution contained (millimolars): 145 NaCl, 10 EGTA and 10 HEPES, pH 7.3. The pipette solution was supplemented with 120 μg/mL amphotericin for perforated patch recordings; perforation typically occurred 5 mins after gigaseal formation. Pipettes had resistance of 3–5 MΩ. ATP was applied using an RSC 160 rapid solution changer (Biological Science Instruments, Intracell). ATP was applied for 2 s at 2-min intervals and cells were clamped at −60 mV. HEK293 cell treatment with cholesterol depleting agents was at 37°C.

Quantitative mRNA analysis

Total RNA was extracted from control and fluvastatin-treated cells using TriReagent (Sigma). First-strand cDNA was synthesised using Maxima Reverse Transcriptase (Fermentas) and Oligo$(dT)_{18}$ priming. cDNA was amplified using sense and anti-sense oligonucleotide pairs selective for human $P2X_4$ and GAPDH using an ABI Biosystems PCR Instrument. $P2X_4$ mRNA abundance was expressed relative to GAPDH housekeeping gene.

Drugs and treatments

Fluvastatin sodium salt (Tocris) stock solutions were prepared to 10 mM in DMSO. For chronic treatment of monocytes, THP-1 cultures (<1×10^6 cells/mL) were supplemented with

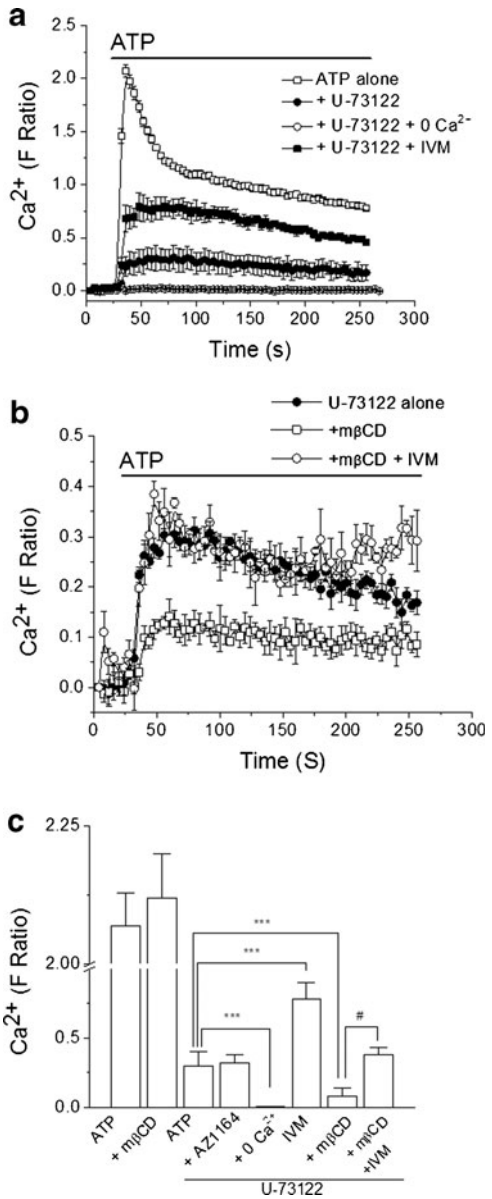

Fig. 1 Cholesterol depletion suppresses $P2X_4$-dependent calcium entry in human monocytes. **a** Pharmacological isolation of $P2X_4$-dependent calcium entry in THP-1 cells. Elevation in $[Ca^{2+}]_i$ in response to 100 μM ATP is significantly inhibited by U-73122 though a resistant component remains. (N=6). The resistant $P2X_4$ component is abolished in the absence of extracellular calcium (0 Ca^{2+}) and potentiated by ivermectin (*IVM*). **b** Treatment with the cholesterol depleting agent mβCD (10 mM, 1 h) reduces $P2X_4$-dependent calcium entry (N=6). **c** Mean peak calcium response data for 100 μM ATP summarising effect of cholesterol depletion of P2Y and $P2X_4$-dependent calcium entry. (N=4–6; ***p<0.01 vs ATP with U-71322; #p<0.01 vs ATP with U-71322 and mβCD) Note that the U-71322-resistant component is not sensitive to AZ11645373 (1 μM, 30 min), a selective $P2X_7$ antagonist

10 μM fluvastatin or an equivalent volume of DMSO for 48 h prior to experimentation. For shorter treatments, 10 μM fluvastatin was applied 1 h prior to experimentation. Methyl-β-cyclodextrin (Sigma) was prepared in water and applied at 10 mM 1 h prior to experimentation.

Statistical analysis

Numerical data are expressed as mean±S.E. Statistical significance was tested by Student's *t* test and analysis of variance.

Results and discussion

Application of ATP (100 μM) to fura-2 loaded THP-1 cells robustly increased $[Ca^{2+}]_i$ (Fig. 1a). Intracellular calcium rapidly peaked then decayed to a sustained elevated phase (Fig. 1a). Pre-incubation with the phospholipase C inhibitor U-73122 (10 μM, 30 min) abolished the rapid peak in ATP-evoked in $[Ca^{2+}]_i$ though a residual sustained calcium rise persisted (Fig. 1a). The U-73122-resistant calcium rise was not inhibited by the selective $P2X_7$ antagonist AZ11645373 (1 μM, 30 min) (Fig. 1c). Moreover 100 μM ATP is sub-threshold for human $P2X_7$ receptor activation. The peak residual calcium component was potentiated ~2.5-fold by ivermectin (10 μM, 30 min) (Fig. 1a), a positive allosteric modulator of $P2X_4$ [11, 12]. These data strongly suggest that $P2X_4$ mediates the U-73122-resistant ATP-evoked calcium influx in THP-1 monocytes. $P2X_1$, $P2X_4$ and $P2X_7$ mRNA transcripts have been detected in THP-1 monocytes though it is most unlikely that $P2X_1$ would mediate a sustained calcium influx due to its rapid desensitisation. Functional $P2X_1$ has been detected electrophysiologically in other inflammatory cell types [8, 13]. Pre-incubating monocytes with the cholesterol depleting agent mβCD suppressed the magnitude of $P2X_4$-dependent calcium influx threefold (Fig. 1b). The suppressed $P2X_4$-dependent calcium entry could be potentiated ~2.5-fold by ivermectin (Fig. 1b and c). α-Cyclodextran, an inactive analogue, had no effect (data not shown). mβCD did not suppress the ATP stimulated calcium rise observed in the absence of U-73122 (Fig. 1c) suggesting that P2Y receptor-dependent calcium signalling is unaffected by cholesterol depletion.

Next, we sought to determine whether clinically relevant cholesterol lowering drugs had a similar effect on $P2X_4$-mediated calcium entry as acute chemical sequestration of cholesterol. THP-1 monocytes were cultured for 48 h with 10 μM fluvastatin to mimic clinical doses. As for mβCD treatment, fluvastatin treatment also had no effect on P2Y receptor-mediated calcium rises compared to vehicle controls (Fig. 2a). However, fluvastatin treatment suppressed peak $P2X_4$-dependent calcium response by 2-fold compared to their vehicle control counterparts (Fig. 2b). Ivermectin was capable of potentiating suppressed $P2X_4$-mediated

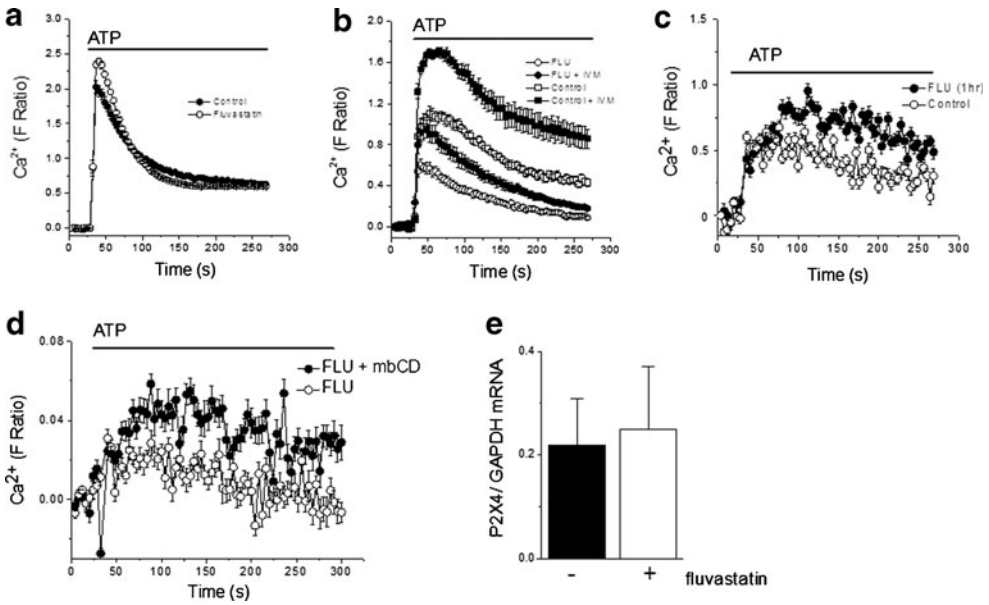

Fig. 2 Fluvastatin treatment inhibits $P2X_4$-dependent calcium entry in human monocytes. **a** Comparison of P2Y receptor-mediated Ca^{2+} responses evoked by 100 μM ATP in control and fluvastatin-treated THP-1 cells (10 μM, 48 h) (*N*=8). **b** Effect of fluvastatin (FLU) on the U-73122-resistant $P2X_4$ component. Control cells are untreated. Ivermectin (IVM; 10 μM, 30 min) potentiates $P2X_4$-dependent calcium entry in control and FLU-treated cells (*N*=8). **c** No acute effect of FLU (10 μM, 1 hr) on P2X4-dependent calcium entry (*N=4*). **d** No effect of mβCD (10 mM, 1 h) on $P2X_4$-dependent Ca2+-dependent entry in FLU-treated cells (10 μM, 48 h) (*N*=3). **e** qRT-PCR analysis of $P2X_4$ mRNA in naïve and fluvastatin-treated (10 μM, 48 h) monocytes. mRNA abundance is expressed relative to GAPDH housekeeper gene (*N*=4)

calcium entry (Fig. 2b). The cholesterol depleting action of statins through HMG-CoA reductase inhibition takes many hours to manifest. To this end, we tested the effect of acute treatments of fluvastatin to rule out the possibility that fluvastatin inhibits $P2X_4$-dependent Ca^{2+} entry via a different mechanism. Unlike 48-h treatment, 1-h treatment with 10 μM fluvastatin had no significant effect on $P2X_4$-dependent Ca^{2+} entry (Fig. 2c) (N=4; p<0.05). Moreover, application of mβCD to cells treated with fluvastatin for 48 h showed no significant difference in $P2X_4$-dependent Ca^{2+} entry compared to cells treated with fluvastatin alone (Fig. 2d). These data indicate that fluvastatin and mβCD suppress $P2X_4$ receptor activity by the same mechanism. Furthermore, no significant difference was observed for the abundance of $P2X_4$ mRNA in control monocytes or monocytes treated with fluvastatin for 48 h (Fig. 2e), suggesting fluvastatin does not reduce $P2X_4$ activity through a transcriptional influence. These data suggest $P2X_4$ activity is suppressed following depletion of cellular cholesterol.

To investigate the mechanism further, we explored the effects of cholesterol depletion on human $P2X_4$ heterologously expressed in HEK293T cells [12]. ATP (100 μM) evoked robust inward currents that moderately desensitised during agonist application (Fig. 3a). The mean whole-cell current density was 82±8 pA/pF (N=10 cells). To examine the influence of membrane cholesterol on human $P2X_4$ activity, we employed mβCD and filipin III, two chemically unrelated cholesterol sequestering agents. Treatment of HEK293T cells with 10 mM mβCD for 1 h significantly reduced $P2X_4$ current density compared to untreated cells (18±4 pA/pF; N=10, p<0.01 vs control) (Fig. 3a). The effect was not mimicked by 10 mM mαCD (95±10pA/pF; N=8, p>0.05 vs control) (Fig. 3b). Treatment with 10 μM filipin III for 30 min also significantly reduced $P2X_4$ current density (30±5 pA/pF; N=10, p<0.01 vs control) (Fig. 3b). Previous studies have revealed some selectivity of cholesterol depletion on P2X receptor function. As for THP-1 monocytes, ivermectin was able to potentiate P2X4 suppressed by mβCD (35±8 pA/pF;

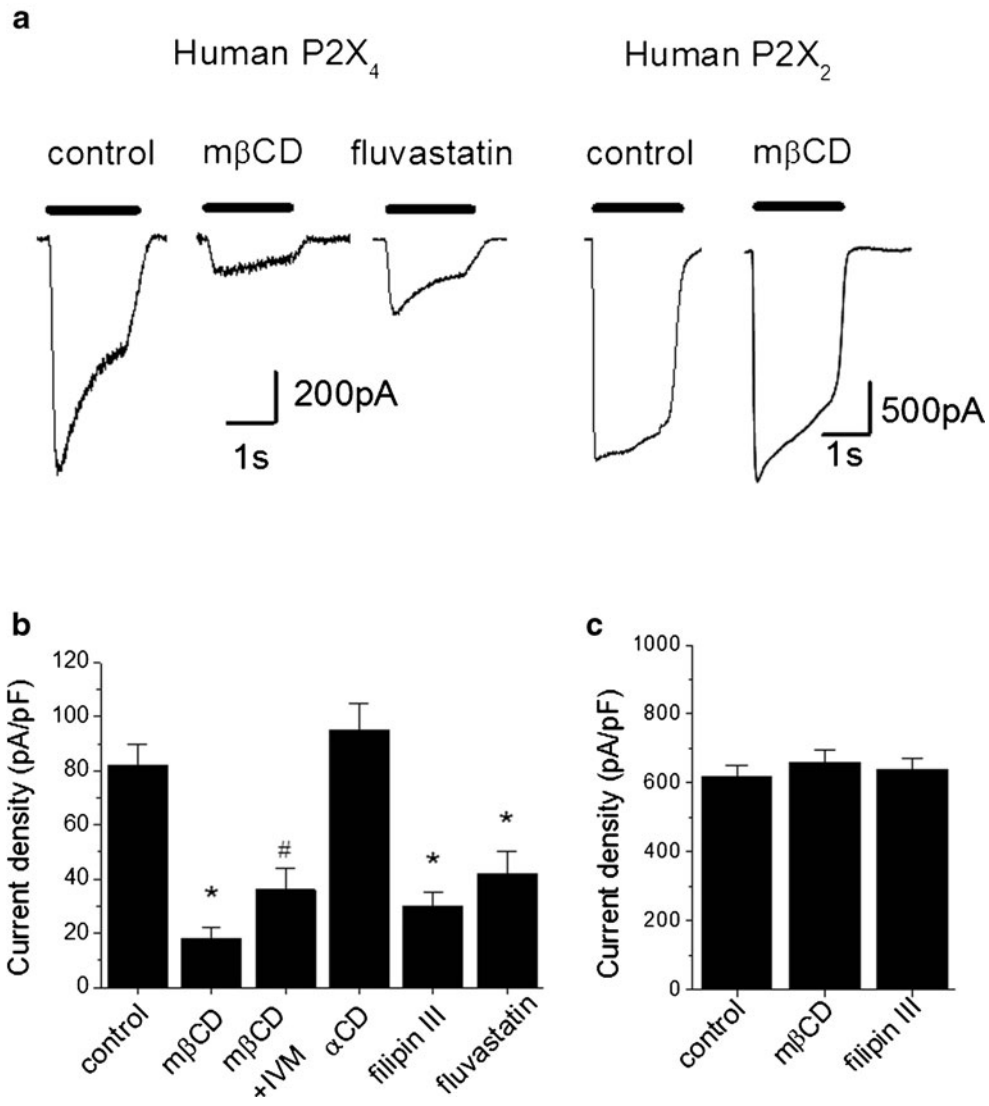

Fig. 3 Effect of cholesterol-depleting agents on human $P2X_4$ and $P2X_2$ receptors in HEK293T cells. **a** Representative traces showing effect of mβCD treatment of $P2X_2$ and $P2X_4$ currents in HEK293 cells, and the effect of fluvastatin (10 μM, 48 h) on $P2X_4$ currents. **b** Pooled averages demonstrated reduction in $P2X_4$ current density following cholesterol depletion by methyl-β-cyclodextrin or filipin III, but not by αCD. Ivermectin (IVM; 10 μM, 30 min). Fluvastatin (10 μM, 48 h). N=8; *p<0.01 vs control, #p<0.01 vs mβCD treatment. **c** $P2X_2$ currents are unaffected cholesterol depletion, N=8. All currents evoked by 100 μM ATP

$N=5$, $p<0.01$ vs control) (Fig. 3b). Forty-eight-hour treatment with fluvastatin (10 μM) significantly reduced $P2X_4$ peak current density (Fig. 3a and b).

Cholesterol depletion suppresses $P2X_1$ function when expressed in HEK293 cells [14, 15] but not $P2X_2$ or $P2X_3$ [15]. Native $P2X_3$ currents are also unaffected by cholesterol depletion [16]. Consistent with previous studies [15], ATP-evoked currents were unaffected by cholesterol depletion in HEK293T cells expressing human $P2X_2$ receptor (Fig. 3a). ATP (100 μM) evoked an average current density of 620±30 pA/pF ($N=9$ cells) in control cells which was unaffected by mβCD (661±30pA/pF, $N=8$cells) or filipin III (640±30pA/pF) (Fig. 3c). Our observations of the effect of cholesterol depletion on native and transiently expressed $P2X_4$ receptor are in contrast to those of Allsopp et al. (2010) who employed stable $P2X_4$ HEK293 cells. A main difference between this study and that of Allsopp et al. (2010) is that electrophysiological recordings have been made using the perforated patch configuration. Statins may therefore impinge on intracellular factors that regulate P2X4R function and/or trafficking which are dialysed out during whole-cell recordings. Indeed, patch perforation minimises the extensive rundown of P2X4R activity observed in whole-cell recordings [12]. The data presented here suggests that the effect of cholesterol depletion is consistent between native and recombinant human $P2X_4$ receptors.

P2X receptor current density is governed by multiple factors including responsiveness to ATP, receptor desensitisation and receptor number. ATP concentration–response curves were not significantly different between control and cholesterol depleted cells (Fig. 4a). The EC_{50} value for control cells was 2.5±1.2 μM ($N=10$ cells), comparable to previous studies [17], and 2.1±2.0 μM and 2.2±1.4 μM for mβCD and filipin III-treated cells, respectively (both $N=10$ cells; $p>0.05$ vs control). Responses were recorded using the perforated patch configuration to minimise receptor rundown [12]. The effects of cholesterol depletion on two modes of receptor desensitisation were also tested. The human $P2X_4$ receptor runs down with repeated agonist application [12]. In control cells, repeated application of 100 μM ATP at 2 min intervals was marked with an exponential decay in peak whole-cell current ($\tau=1.19\pm0.05$; $N=8$). The rate of rundown was unaffected by cholesterol depletion; $\tau=1.19\pm0.08$ and 1.10±0.08 ($N=8$ cells each, $p>0.05$ vs control) for mβCD and filipin III-treated cells, respectively (Fig. 4b). Human $P2X_4$ currents decayed by 51.5±1.5% ($N=8$ cells) during application of 100 μM ATP (Fig. 4c). Again, cholesterol depletion had no effect on the magnitude of current decay; 46.9±4.3% and 50.2±2.5% ($N=8$ cells each; $p>0.05$ vs control) for mβCD and filipin III-treated cells, respectively (Fig. 4c and d). These data support the notion that $P2X_4$ current density and receptor desensitisation during agonist occupancy are independent [12].

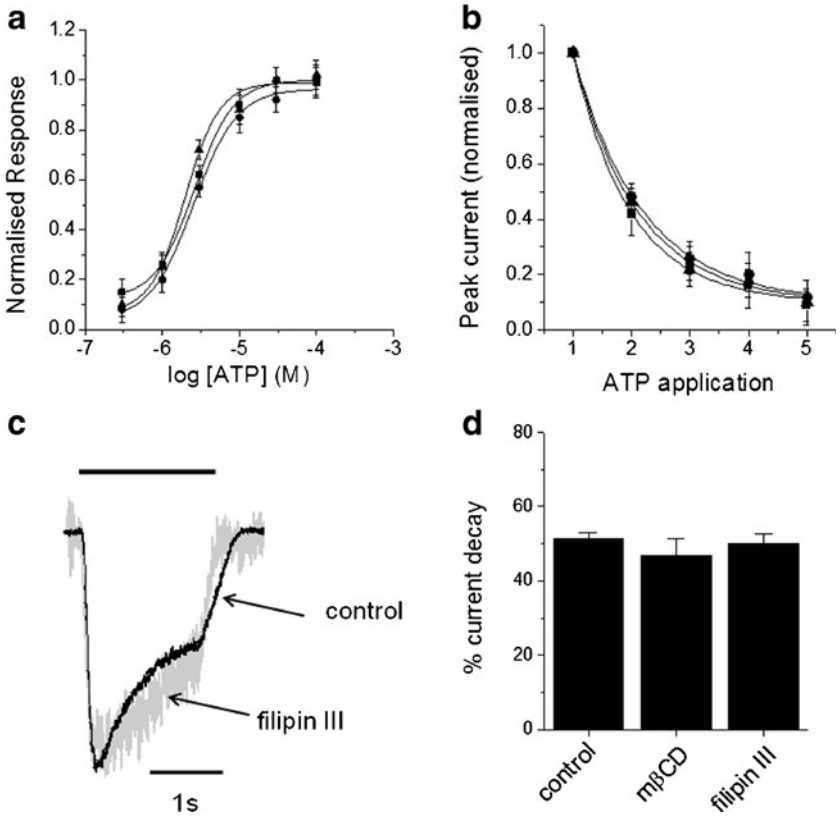

Fig. 4 Influence of cholesterol depletion of intrinsic human $P2X_4$ receptor properties. **a** Concentration–response relationships for ATP in control cells (*squares*), methyl-β-cyclodextrin (*triangles*) or filipin III (*circles*) treated cells ($N=10$ cells each). Whole-cell currents in perforated patch configuration normalised to maximum response. **b** Run-down of peak current with repeated ATP application in control (*squares*), methyl-β-cyclodextrin (*triangles*) or filipin III (*circles*) treated cells ($N=8$ cells each). Peak currents are normalised to peak after first application. **c** Representative current traces showing current decay in control and fillipin III-treated cells. Currents are normalised to peak response to 100 μM ATP. **d** Pooled average demonstrating current decay during agonist occupancy is independent of membrane cholesterol ($N=8$ cells each)

In summary, this study suggests that fluvastatin inhibits $P2X_4$ through its cholesterol depleting activity, as fluvastatin, mβCD and filipin III produce the same outcome. The mechanism underlying suppression of P2X4 does not involve modulation of intrinsic channel properties and is therefore likely to be a consequence of interfering in mechanisms controlling receptor trafficking or regulation by signal transduction or protein–protein interaction. Disruption of lipid raft-dependent signalling is a possible mechanism of action of fluvastatin. The existence of $P2X_4$ in lipid rafts has been demonstrated in epithelia [18] and HEK293 cells [15]. Indeed, fluvastatin inhibits raft-dependent signalling in monocytes [19].

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References

1. Scandinavian Simvastatin Survival Study Group (1994) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 344:1383–1389
2. Scalia R et al (2001) Simvastatin exerts both anti-inflammatory and cardioprotective effects in apolipoprotein E-deficient mice. Circulation 103:2598–2603
3. Pruefer D et al (2002) Simvastatin inhibits inflammatory properties of *Staphylococcus aureus* α-toxin. Circulation 106:2104–2110
4. Østerud B, BjÈ-Rklid E (2003) Role of monocytes in atherogenesis. Physiol Rev 83:1069–1112
5. North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067
6. Wang L, Jacobsen SE, Bengtsson A, Erlinge D (2004) P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. BMC Immunol 5:16
7. Qureshi OS, Paramasivam A, Yu JCH, Murrell-Lagnado RD (2007) Regulation of P2X4 receptors by lysosomal targeting, glycan protection and exocytosis. J Cell Sci 120:3838–3849
8. Sim JA, Park CK, Oh SB, Evans RJ, North RA (2007) P2X1 and P2X4 receptor currents in mouse macrophages. Br J Pharmacol 152:1283–1290
9. Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K (2003) P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424:778–783
10. Ulmann L, Hirbec H, Rassendren F (2010) P2X4 receptors mediate PGE2 release by tissue-resident macrophages and initiate inflammatory pain. EMBO J 29:2290–2300
11. Priel A, Silberberg SD (2004) Mechanism of ivermectin facilitation of human P2X4 receptor channels. J Gen Physiol 123:281–293
12. Fountain SJ, North RA (2006) A C-terminal lysine that controls human P2X4 receptor desensitization. J Biol Chem 281:15044–15049
13. Wareham K, Vial C, Wykes RCE, Bradding P, Seward EP (2009) Functional evidence for the expression of P2X1, P2X4 and P2X7 receptors in human lung mast cells. Br J Pharmacol 157:1215–1224
14. Vial C, Evans RJ (2005) Disruption of lipid rafts inhibits P2X1 receptor-mediated currents and arterial vasoconstriction. J Biol Chem 280:30705–30711
15. Allsopp RC, Lalo U, Evans RJ (2010) Lipid raft association and cholesterol sensitivity of P2X1-4 receptors for ATP. J Biol Chem 285:32770–32777
16. Liu M, Huang W, Wu D, Priestley JV (2006) TRPV1, but not P2X3, requires cholesterol for its function and membrane expression in rat nociceptors. Eur J Neurosci 24:1–6
17. Jones CA, Chessell IP, Simon J, Barnard EA, Miller KJ, Michel AD, Humphrey PPA (2000) Functional characterization of the P2X4 receptor orthologues. Br J Pharmacol 129:388–394
18. Barth K, Weinhold K, Guenther A, Linge A, Gereke M, Kasper M (2008) Characterization of the molecular interaction between caveolin-1 and the P2X receptors 4 and 7 in E10 mouse lung alveolar epithelial cells. Int J Biochem Cell Biol 40:2230–2239
19. Hillyard DZ, Jardine AG, McDonald KJ, Cameron AJM (2004) Fluvastatin inhibits raft dependent Fc[gamma] receptor signalling in human monocytes. Atherosclerosis 172:219–228