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ORIGINAL ARTICLE

P2Y receptor subtypes evoke different Ca^{2+} signals in cultured aortic smooth muscle cells

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Abstract Adenine and uridine nucleotides evoke Ca^{2+} signals via four subtypes of P2Y receptor in cultured aortic smooth muscle cells, but the mechanisms underlying the different patterns of these Ca^{2+} signals are unresolved. Cytosolic Ca^{2+} signals were recorded from single cells and populations of cultured rat aortic smooth muscle cells, loaded with a fluorescent Ca^{2+} indicator and stimulated with agonists that allow subtype-selective activation of P2Y1, P2Y2, P2Y4, or P2Y6 receptors. Activation of P2Y1, P2Y2, and P2Y6 receptors caused homologous desensitisation, while activation of P2Y2 receptors also caused heterologous desensitisation of the other subtypes. The Ca^{2+} signals evoked by each P2Y receptor subtype required activation of phospholipase C and release of Ca^{2+} from intracellular stores via inositol 1,4,5-trisphosphate (IP_3) receptors, but they were unaffected by inhibition of ryanodine or nicotinic acid adenine dinucleotide phosphate (NAADP) receptors. Sustained Ca^{2+} signals were independent of the Na^+/Ca^{2+} exchanger and were probably mediated by store-operated Ca^{2+} entry. Analyses of single cells established that most cells express P2Y2 receptors and at least two other P2Y receptor subtypes. We conclude that four P2Y receptor subtypes evoke Ca^{2+} signals in cultured aortic smooth muscle cells using the same intracellular (IP_3 receptors) and Ca^{2+} entry pathways (store-operated Ca^{2+} entry). Different rates of homologous desensitisation and different levels of receptor expression account for the different patterns of Ca^{2+} signal evoked by each P2Y receptor subtype.

Keywords Aortic smooth muscle cells · Ca^{2+} signalling · Desensitisation · P2Y receptors

Introduction

The primary function of vascular smooth muscle cells (VSMC) is to contract and thereby to regulate vascular tone and blood pressure [1]. However, in response to local environmental cues such as interactions with other cells, the extracellular matrix or growth factors, and particularly during vascular injury, VSMC can change phenotype. They then lose their ability to contract and become able to proliferate. This switch from a contractile to a proliferative phenotype may be necessary for vascular repair, but it also plays a role in the development and progression of such vascular diseases as atherosclerosis, restenosis, and hypertension [2]. In culture, VSMC undergo similar phenotypic changes, losing some proteins that are typical of the contractile state, like L-type Ca^{2+} channels and ryanodine receptors (RyR) [1, 3]. Other proteins that are up-regulated in VSMC in vivo after vascular injury, like TRPC (transient receptor potential canonical) channels and the STIM1 proteins that regulate store-operated Ca^{2+} entry (SOCE), are also expressed at increased levels in cultured VSMC [1, 4]. Cultured VSMC

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are, therefore, a useful model of VSMC in diseased states [5, 6].

P2Y receptors are a family of G protein-coupled receptors that are activated by adenine and uridine nucleotides [7]. In VSMC, many functional responses are regulated by the Ca^{2+} signals evoked by purinoceptors [8, 9]. Analysis of these responses in native tissues is, however, made complicated by the diversity of P2Y receptors and the paucity of ligands with adequate selectivity for P2Y receptor subtypes. We recently established, using a combination of subtype-selective ligands and quantification of receptor expression, that four different P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, and P2Y6) evoke Ca^{2+} signals in cultured rat aortic smooth muscle cells (ASMC) [10]. Two of these receptors (P2Y2 and P2Y4) probably respond to the same endogenous agonists (ATP and UTP), while the others respond to ADP (P2Y1) and UDP (P2Y6) [7]. Because all four P2Y receptors evoke Ca^{2+} signals and they are stimulated by interrelated agonists, it is worth considering why ASMC should express such a diversity of purinoceptors linked to Ca^{2+} signalling. The present study addresses this issue by characterizing the increases in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by each P2Y receptor subtype in cultured ASMC, the roles of plasma membrane and intracellular Ca^{2+} channels in generating these Ca^{2+} signals, and the contribution of desensitisation to the very different Ca^{2+} signals evoked by each receptor subtype.

Materials and methods

Materials

Cell culture materials were from Gibco (Paisley, UK), except for foetal bovine serum (Sigma, Poole, UK). Fluo-4 AM and fura-2 AM were from Invitrogen (Paisley, UK). MRS2365 ((*N*)-methanocarba-2-methylthio-adenosine-5'-diphosphate), UDP, U73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), U73343 (1-[6-[[(17*β*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione) and KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea) were from Tocris (Bristol, UK). 2'-amino-UTP (2'-amino-2'-deoxyuridine-5'-triphosphate) and 2'-azido-UTP (2'-azido-2'-deoxyuridine-5'-triphosphate) were from Trilink Biotechnologies (San Diego, CA). Ryanodine was from Ascent Scientific (Avonmouth, UK). Thapsigargin was from Alomone Labs (Jerusalem, Israel). Collagenase B and hexokinase were from Roche Diagnostics Ltd. (Burgess Hill, UK). *Trans*-Ned-19 (1-(3-((4-(2-fluorophenyl)piperazin-1-yl)methyl)-4-methoxyphenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylic acid) was from Enzo Life Sciences (Exeter, UK). All other reagents, including 2-aminoethoxydiphenyl borate (2-APB), cyclopiazonic acid (CPA), nimodipine, elastase, $GdCl_3$ and probenecid, were from Sigma. Stocks of UDP (10 mM) were treated (1 h, 37 °C) with hexokinase (50 UmL^{-1}, pH 7.0 in 0.1 M phosphate buffer containing 6.5 mM $MgCl_2$ and 110 mM glucose) to remove contaminating UTP before use [10].

Isolation and culture of rat aortic smooth muscle cells

Methods to isolate and culture rat ASMC were described previously [10]. All animal care and experimental procedures complied with UK Home Office policy. Briefly, adult male rats were humanely killed by cervical dislocation. The aorta was isolated under sterile conditions in Hank's balanced salt solution (HBSS) supplemented with penicillin (100 UmL^{-1}) and streptomycin (100 $\mu g\ mL^{-1}$), and cleared of adhering fat, connective tissue and the inner endothelial layer. After digestion (37 °C, 12 min) in HBSS containing antibiotics and collagenase B (0.015 UmL^{-1}), the adventitia were removed, and the tissue was further digested (37 °C, 90 min) in HBSS containing antibiotics, collagenase B (0.075 UmL^{-1}) and elastase (8 UmL^{-1}). Single cells were then dispersed by trituration with a sterile Pasteur pipette. The cells were washed with Dulbecco's modified Eagle's medium (DMEM) (650 g, 5 min), resuspended in DMEM with antibiotics and foetal calf serum (10 %) and grown at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. ASMC were either passaged or frozen when they reached confluence.

Measurement of $[Ca^{2+}]_i$ in cell populations

Confluent cultures of ASMC grown in 96-well plates were loaded with fluo-4 by incubation with fluo-4 acetoxymethyl ester (fluo-4 AM, 4 μM in fresh dimethyl sulfoxide (DMSO); final DMSO concentration, 0.4 %) in HEPES-buffered saline (HBS) supplemented with probenecid (2.5 mM). After 1 h at 20 °C, cells were washed with HBS, and after a further 15 min, they were used for experiments. HBS had the following composition: 135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11.5 mM glucose, 11.6 mM HEPES, pH 7.3, at 20 °C. All experiments were performed in HBS (or nominally Ca^{2+}-free HBS) at 20 °C. We confirmed that incubating cells in nominally Ca^{2+}-free HBS was as effective as incubation in Ca^{2+}-free HBS with 1 mM EGTA in preventing both Ca^{2+} entry evoked by activation of P2Y2 receptors and refilling of intracellular stores with Ca^{2+} (Online Resource, Supplementary Fig. S1). Fluorescence (excitation at 485 nm, emission at 525 nm) was recorded from the 96-well plate using a FlexStation III (MDS Analytical Technologies Wokingham, Berks, UK) [10]. At the end of each experiment, fluorescence of Ca^{2+}-saturated indicator (F_{max}) was determined for each well by addition of Triton X-100 (0.05 %) and $CaCl_2$

(10 mM). Background fluorescence, measured in parallel from cells treated with Triton X-100 (0.05 %) and BAPTA (10 mM), was subtracted from all measurements. The corrected fluorescence (F) was then calibrated to $[Ca^{2+}]_i$: $[Ca^{2+}]_i = K_D.F/(F_{max}-F)$, where the K_D of fluo-4 for Ca^{2+} is 345 nM [10].

Because it is difficult to wash ASMC in 96-well plates without causing some detachment of cells (usually <10 %), comparisons between treatments that required changes of media were always performed using controls matched to ensure identical washing procedures.

Single-cell measurement of $[Ca^{2+}]_i$

ASMC grown to ~75 % confluence on 22-mm round glass coverslips coated with poly-L-lysine (0.01 %) were loaded with fura-2 exactly as described for fluo-4. Fluorescence from single cells at 20 °C was recorded (emission >510 nm) using an Olympus IX71 inverted fluorescence microscope and Luca EMCCD camera (Andor Technology, Belfast, UK) at 5-s intervals with alternating excitation at 340 and 380 nm. All recordings were corrected for autofluorescence determined at the end of each experiment by addition of $MnCl_2$ (10 mM) and ionomycin (1 μM). The corrected fluorescence readings (F_{340} and F_{380}) were calibrated to $[Ca^{2+}]_i$ from: $[Ca^{2+}]_i = K_D \frac{(R-R_f)F_f}{(R_b-R)F_b}$ where K_D is the equilibrium dissociation constant of fura 2 for Ca^{2+} (220 nM), R is the fluorescence ratio (F_{340}/F_{380}) from the cells. R_f and R_b were determined in vitro using Ca^{2+} calibration buffer kit#1 (Invitrogen) in Ca^{2+}-free and Ca^{2+}-saturating conditions, respectively; and F_f and F_b are the fluorescence intensities determined after excitation at 380 nm for Ca^{2+}-free and Ca^{2+}-saturated indicator, respectively [11].

Data analysis

Concentration–effect relationships were fitted to Hill equations (GraphPad Prism, version 5, La Jolla, CA) to allow estimation of the maximal effects, Hill coefficients and $-\log EC_{50}$ (pEC_{50}) (or pIC_{50}) values. Results are presented as means±SEM from n independent experiments (n=3, unless otherwise stated). Statistical comparisons used one-tailed paired or unpaired Student's t test, as appropriate, with $P<0.05$ considered significant.

Results

Ca^{2+} signals evoked by P2Y receptor subtypes

We used the conditions established in our previous work to stimulate selectively the four P2Y receptor subtypes expressed in cultured rat ASMC that evoke Ca^{2+} signals [10]. That study and previous work had shown that cultured ASMC do not express functional P2X receptors [10, 12]. MRS2365 was used selectively to activate P2Y1 receptors [13], 2'-amino-UTP for P2Y2 receptors, 2'-azido-UTP for P2Y4 receptors [14] and UDP for P2Y6 receptors [15]. For simplicity, we refer in the text only to the subtype of P2Y receptor that the agonist selectively activates, rather than to the agonists themselves. Details of the stimuli used are defined in the figure legends.

Within a single experiment using populations of ASMC, the amplitude of the peak Ca^{2+} signal evoked by activation of each P2Y receptor was similar between replicates, but there was greater variability between experiments, which included cells from both different animals and cell passages (Fig. 1a). We have not explored this variability further, although differences in the expression levels of P2Y receptors and/or the proteins that link them to Ca^{2+} signals are likely causes. Despite this variability in the amplitudes of the Ca^{2+} signals, there are clear and consistent differences in the relative amplitudes and time courses of the Ca^{2+} signals evoked by the four P2Y receptor subtypes. P2Y2 receptors invariably evoked the largest Ca^{2+} signals, and P2Y2, P2Y4, P2Y6, though not P2Y1, receptors evoked responses that were sustained by Ca^{2+} entry (Fig. 1, a–e). The Ca^{2+} signals evoked by activation of each of the four P2Y receptor subtypes were abolished by U73122 (20 μM), an inhibitor of phospholipase C (PLC), but unaffected by its inactive analogue, U73343 (20 μM) (Fig. 1f). These results establish that each of the four P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, and P2Y6) evoke Ca^{2+} signals via activation of PLC, but the patterns of response are subtype-selective. Subsequent experiments examine the mechanisms responsible for the different Ca^{2+} signals evoked by each P2Y receptor subtype.

Homologous desensitisation of P2Y receptor signalling

Figure 2a shows the protocol used to determine whether stimulation of each P2Y receptor subtype affected the Ca^{2+} signal evoked by subsequent stimulation of the same receptor. This comprised an initial 60-s period of stimulation, after which the agonist was removed by washing and then restored after an interval of between 5 and 60 min. Because washing invariably caused some loss of cells (making it difficult to compare responses to sequential stimuli directly, see "Materials and methods" section), we measured desensitisation by comparing responses to the second stimulus after an initial incubation with or without the first stimulus. The results are shown in Fig. 2b, c.

Within the minimal period of 5 min required to wash the cells and re-apply the stimulus, the response to P2Y4 receptors had fully recovered, suggesting either that there was no desensitisation or that it fully reversed within 5 min (Fig. 2b, c). Responses from P2Y1, P2Y2, and P2Y6 receptors were

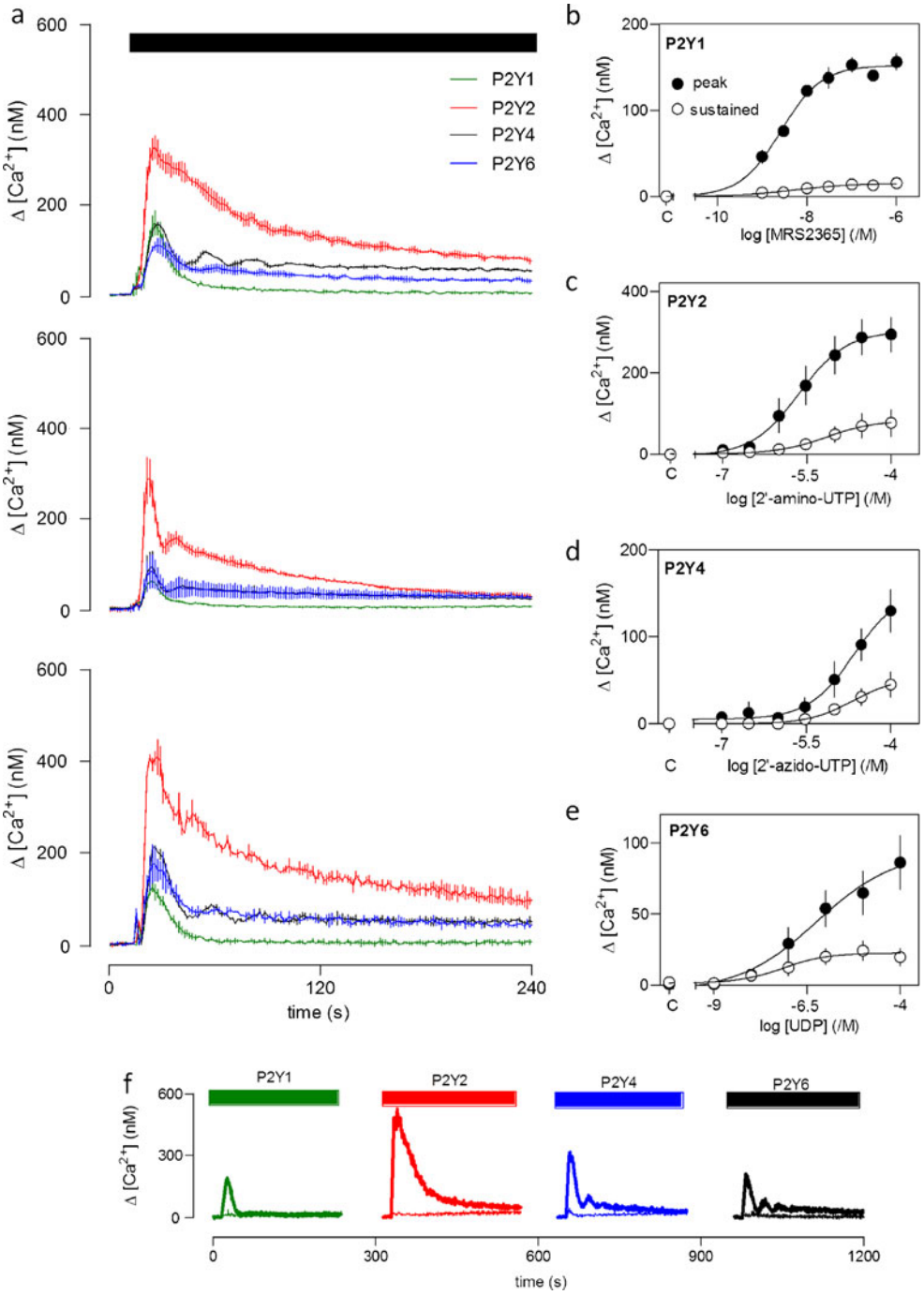

Fig. 1 Ca^{2+} signals evoked by different P2Y receptors in populations of cultured rat ASMC. **a** Typical results (each showing mean±range from two wells) show the Ca^{2+} signals evoked by activation of each of the four P2Y receptors subtypes. Three examples are shown from different experiments using cells from different passages and aorta preparations. The stimuli used to achieve maximal activation of each P2Y receptor subtype here and in all subsequent figures were: P2Y1, MRS2365 (1 μM); P2Y2, 2'-amino-UTP (100 μM); P2Y4, 2'-azido-UTP (100 μM), and P2Y6, UDP (100 μM). **b–e** Concentration-dependent effects of the subtype-selective agonists on peak Ca^{2+} signals, which result largely from release of Ca^{2+} from intracellular stores [10], and the sustained signal measured 260–300 s after agonist addition. Results are means± SEM from three independent experiments. **f** Ca^{2+} signals evoked by maximal activation of the four P2Y receptors subtypes in the presence of the inhibitor of PLC, U73122 (20 μM, thin traces), or its inactive analogue, U73343 (20 μM, thick traces), each added 5 min before and then during the stimulation. Results are typical of those from three independent plates

reduced by prior stimulation of the same receptor. The desensitisation detected after a 5-min period of recovery was greatest for P2Y1 (72±3 %), but significant ($P<0.05$) also for P2Y2 (21±7 %) and P2Y6 (30±10 %) receptors. P2Y6 receptors recovered fully within 10 min. The lesser desensitisation of P2Y2 receptors recovered rather slowly, being almost complete (to 90±2 % of control) within 60 min. Recovery of P2Y1 receptors was very slow and still incomplete after 60 min (to 66±11 % of control) (Fig. 2b, c). Others have suggested, from analyses of inositol phosphate accumulation in response to activation of heterologously expressed human receptors, that P2Y6 receptors desensitise only very slowly (hours, rather than minutes), while activation of P2Y4 receptors causes their rapid desensitisation and internalisation [16, 17]. The latter differs from our observations, where P2Y4 receptors evoked prolonged Ca^{2+} signals (Figs. 1a, d and 8c) with no evidence of sustained desensitisation (Table 1). Different levels of expression of P2Y4 receptors in native cells and after heterologous expression or species differences [18] may contribute to the different patterns of desensitisation. The behaviours of P2Y6 receptors in ASMC and after heterologous expression [16, 17] are more easily reconciled. In

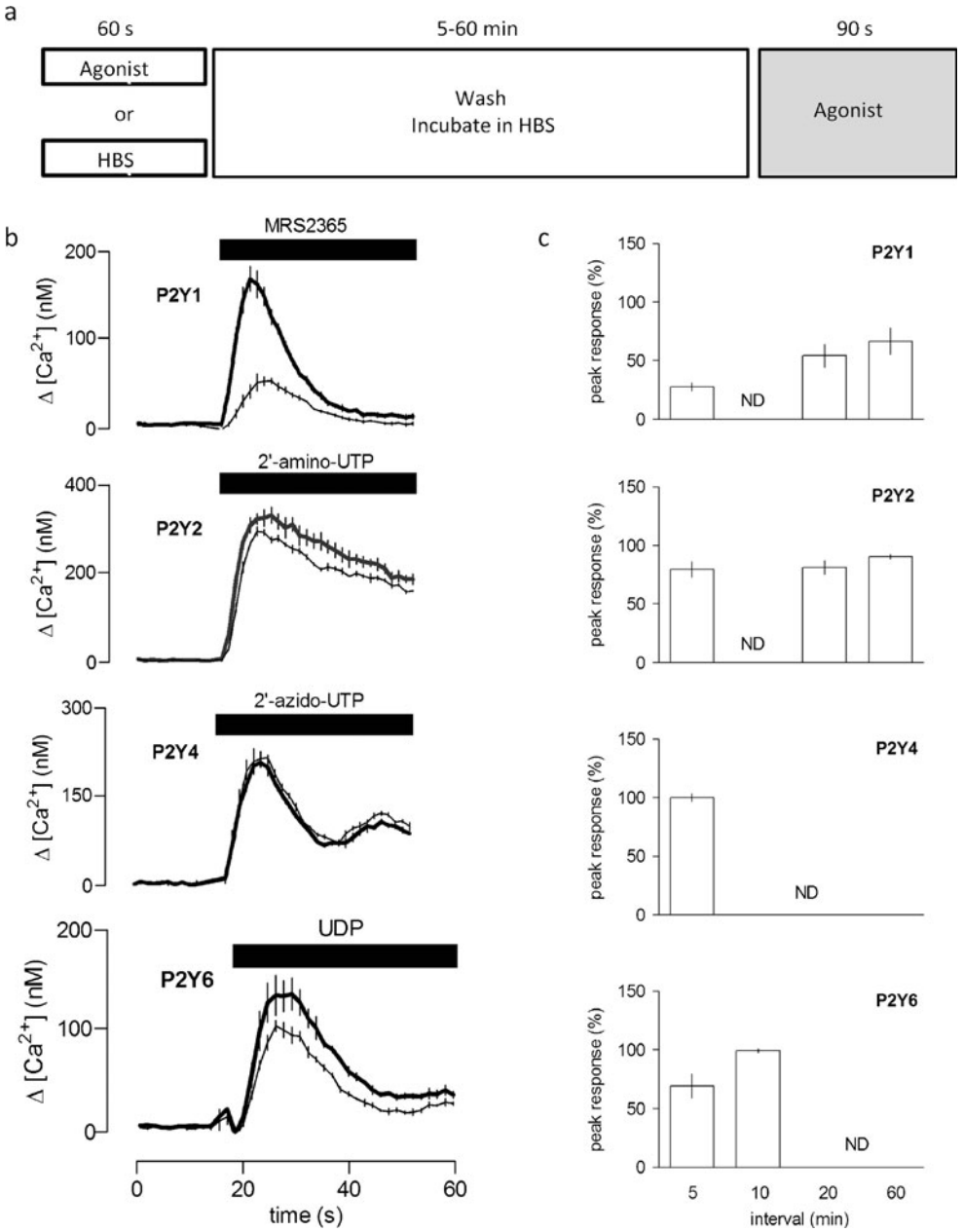

Fig. 2 Homologous desensitisation of the Ca^{2+} signals evoked by P2Y receptors. **a** The protocol is designed to avoid errors arising from the small loss of cells during washing of plates between stimuli (see "Materials and methods" section). Cell populations were first stimulated for 60 s with either a maximally effective concentration of the subtype-selective agonist (as described in the legend to Fig. 1a) or HBS alone. The wells were then washed and incubated in HBS for a further 5–60 min before addition of the same agonist and assessment of the Ca^{2+} responses. The relative amplitudes of the Ca^{2+} signals evoked during the second addition (shaded area) provide the measure of desensitisation. **b** Typical traces (means±SEM of three replicates from a single experiment, each repeated at least three times) show the Ca^{2+} signals evoked by each P2Y receptor subtype in control cells (pre-treated with HBS, thick traces) and in cells pre-treated with the homologous agonist (thin traces). The interval between the pre-treatments and measurement of the Ca^{2+} signals was 5 min. **c** Summary data show the peak Ca^{2+} signals for cells pre-treated with the homologous agonist, expressed as percentages of the response from control cells (pre-treated with HBS). The interval between the end of the pre-treatment and measurement of the Ca^{2+} signals is indicated. Results are means± SEM from three independent experiments. *ND* not determined

ASMC, P2Y6 receptors evoked sustained Ca^{2+} signals (Figs. 1a, e and 8d) and our evidence suggests a modest, but rapidly reversed (Fig. 2b, c; Table 1), homologous desensitisation. The rapid onset and reversal of this desensitisation is likely to cause a modest acute 'steady-state' desensitisation of P2Y6 receptors that would be difficult to resolve with assays of inositol phosphate accumulation. The time courses of the experiments we describe would not have detected the much slower and more complete desensitisation of P2Y6 receptors that others have observed [16, 17].

Table 1 Desensitisation of P2Y receptor signalling

	Second stimulus			
	Peak $[Ca^{2+}]_i$ (%)			
	P2Y1	P2Y2	P2Y4	P2Y6
P2Y1	16±1*	99±5	95±8	99±9
P2Y2	48±1*	77±4*	61±12*	62±13*
P2Y4	95±8	97±12	107±14	111±12
P2Y6	84±8	91±6	99±6	78±4*

From experiments similar to those shown in Figs. 2 and 3, cells were first stimulated (60 s) with an agonist that allowed selective activation of each of the P2Y receptor subtypes (*left column*) or with HBS alone (control). The ligands used are defined in the legend to Fig. 1. After washing and a further 5-min incubation in HBS, cell populations were stimulated with the second stimulus as shown. Peak Ca^{2+} signals evoked by the second stimulus are expressed as a percentage (mean± SEM, n=3) of that recorded with the same second stimulus after pre-treatment with HBS alone. * P<0.05, one-tailed paired Student's t test. The absolute values reported here for pairs of homologous stimuli are different (though not statistically) from those shown in Fig. 2c because each refers to similar, but independent experiments

Slow recovery of the ability of P2Y1 receptors to evoke Ca^{2+} signals might result from either slow refilling of intracellular stores after the first response and/or sustained attenuation of the coupling between extracellular stimuli and activation of signalling pathways. Two lines of evidence establish that the rate at which stores refill with Ca^{2+} is more rapid than recovery of the responses to P2Y1 receptors. First, P2Y2 receptors are expressed in almost all cells (the evidence is presented later) and they trigger the largest Ca^{2+} responses (Fig. 1a, f). Their ability to evoke Ca^{2+} signals that were only minimally reduced by prior stimulation (Fig. 2b, c) argues that slow refilling of stores is an unlikely explanation for the reduced responses observed for other P2Y receptor subtypes. Additional evidence is provided by examining the recovery of responses to P2Y2 receptors after restoration of extracellular Ca^{2+} to cells in which the Ca^{2+} stores had been emptied by reversible inhibition of the endoplasmic reticulum Ca^{2+} pump (SR/ER Ca^{2+}-ATPase, SERCA) using cyclopiazonic acid (CPA, 10 μM, 35 min in Ca^{2+}-free HBS). After a 2-min period of store refilling, the peak Ca^{2+} signal evoked by activation of P2Y2 receptors had recovered by 72±6 % and to 81±4 % after 5 min (data not shown). We conclude that the substantial homologous desensitisation observed for P2Y1 and P2Y6 receptors is not due to slow refilling of intracellular Ca^{2+} stores.

Heterologous desensitisation of P2Y receptor signalling

Using the same protocol applied to examine homologous desensitisation in cell populations, we assessed the effects of first stimulating one P2Y receptor subtype and then another (Fig. 3a). The P2Y2 receptor was the only subtype to cause significant attenuation of the global Ca^{2+} signals evoked by subsequent activation of a different subtype in populations of ASMC (Fig. 3b and Table 1). Indeed stimulation of P2Y2 receptors caused a greater reduction in the responses evoked by the other three subtypes (~40–50 %) than it caused to the response evoked by a second stimulation of P2Y2 receptors (23±4 %) (Table 1).

Distribution of P2Y receptor subtypes between aortic smooth muscle cells

Analyses of homologous desensitisation in cell populations are straightforward because the sequential stimuli are likely to stimulate the same cells (Fig. 2). But for analyses of heterologous agonists, there is an additional complexity because individual cells may differ in the stimuli to which they respond. The apparent lack of heterologous desensitisation among P2Y1, P2Y4 and P2Y6 receptors (Table 1) might then simply reflect the presence of each receptor subtype in a different population of cells. Immunocytochemistry using subtype-selective P2Y receptor antibodies [19] or single-cell analysis of mRNA might reveal whether cells co-express different P2Y receptor subtypes, but it would be impracticable to examine all four subtypes simultaneously and nor could it prove that the receptors are functional. We, therefore, used the following two methods to assess whether different functional P2Y receptor subtypes co-exist in the same cell.

In the first approach, we sequentially stimulated populations of cells with two stimuli in Ca^{2+}-free medium, arguing that depletion of a finite Ca^{2+} store by one stimulus should diminish the response to a second stimulus if they share the same intracellular Ca^{2+} stores. The results to these experiments reveal two important features (Fig. 4a and Table 2). As expected, the response to a second challenge with each homologous agonist was generally reduced, but it was not abolished (Table 2). This suggests either that even relatively sustained maximal stimulation fails to empty entirely the intracellular Ca^{2+} stores to which each receptor subtype has access, or that the relevant stores can partially refill after agonist removal despite the absence of extracellular Ca^{2+}. The second point is that prior stimulation of P2Y2 receptors substantially attenuates responses to all other P2Y receptor subtypes, suggesting that cells expressing P2Y1, P2Y4 or P2Y6 receptors usually co-express P2Y2 receptors. Responses to P2Y1 receptors were attenuated by prior stimulation of each of the other P2Y receptor subtypes, suggesting that many cells that express P2Y1 receptors co-express at least one other P2Y receptor subtype.

A second approach used single-cell Ca^{2+} imaging to examine the responses of individual cells to activation of each of the four P2Y receptor subtypes. Almost all cells (95

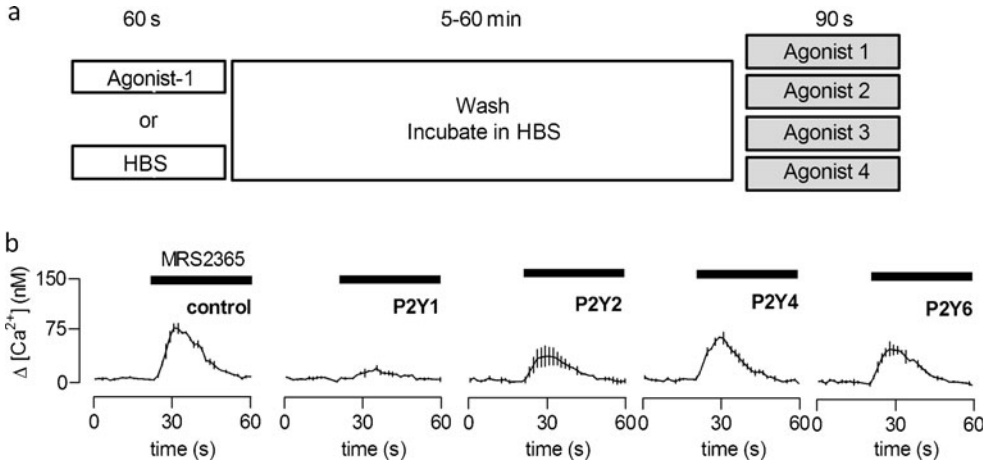

Fig. 3 Heterologous desensitisation of responses to P2Y receptor subtypes. **a** The protocol is similar to that described in Fig. 2a but with the second stimulus different from that used for the first stimulation. The interval between stimuli (each applied for 60 s) was 5 min. **b** Typical results (means±range of duplicates from a single experiment) are shown for cell populations in which the second stimulus activated P2Y1 receptors and the first stimulus activated the receptors shown. Summary data are presented in Table. 1

±2 %, n=92 cells from 5 coverslips) responded to activation of P2Y2 receptors with an increase in $[Ca^{2+}]_i$. This is consistent with their ability both to evoke the largest Ca^{2+} signals in cell populations (Fig. 1) and to deplete the Ca^{2+} stores to which other P2Y receptors have access (Table 2). We therefore restricted our subsequent single-cell analyses

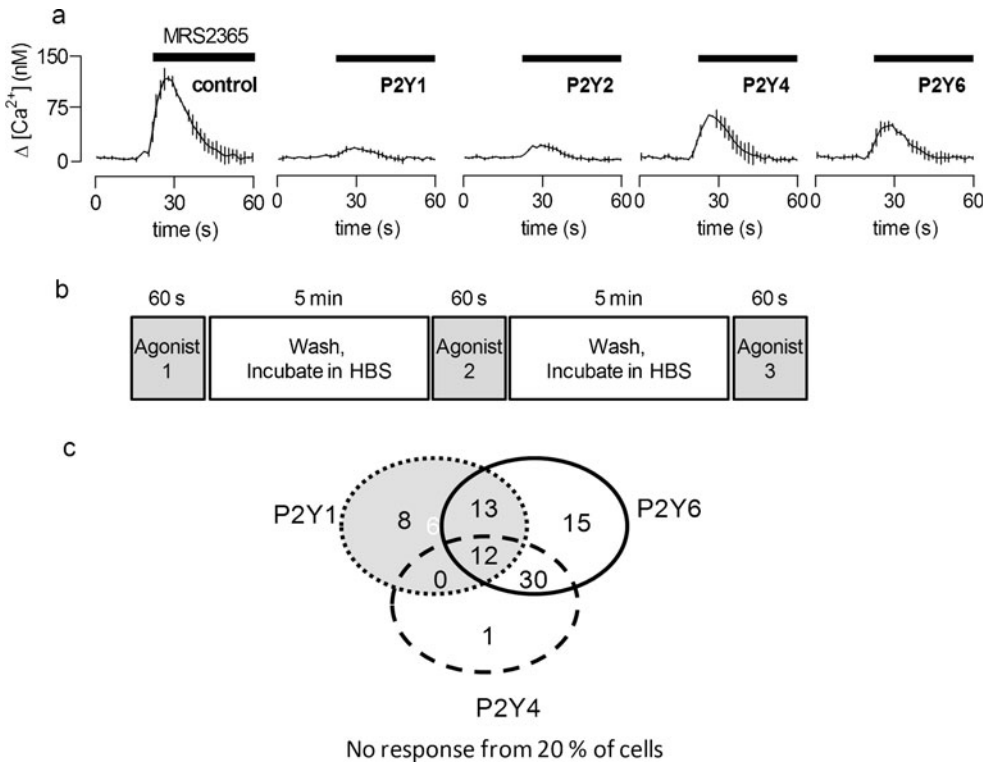

Fig. 4 Distribution of functional P2Y receptor subtypes between individual ASMC. **a** Populations of cells were stimulated in Ca^{2+}-free HBS using a protocol similar to that shown in Fig. 2a but with different subtype-selective agonists used for the first and second stimulation. Typical results (means±range of duplicates from a single experiment) are shown for cells in which the second stimulus activated P2Y1 receptors and the first stimulus activated the receptors shown. The interval between stimuli (each applied for 60 s) was 5 min. The data are summarised in Table 2. **b** In a second series of experiments, Ca^{2+} signals were recorded from fields of single cells in normal HBS according to the stimulus regime shown. **c** The diagram shows the distribution of cells (percent, derived from analysis of 158 cells from 7 fields) that responded to activation of the indicated P2Y receptor subtypes. P2Y2 receptors are omitted from the diagram because most cells (95±2 %) responded to activation of P2Y2 receptors

Table 2 Shared access to intracellular Ca^{2+} stores by P2Y receptor subtypes

	Second stimulus			
	Peak $[Ca^{2+}]_i$ (%)			
	P2Y1	P2Y2	P2Y4	P2Y6
P2Y1	13±3*	98±4	83±7	83±4
P2Y2	10±3*	51±7*	18±5*	24±6*
P2Y4	47±3*	101±2	90±5	83±10
P2Y6	58±12*	93±13	80±14	68±12

From experiments similar to those shown in Fig. 3a, cell populations in Ca^{2+}-free HBS throughout were first stimulated with the stimuli indicated on the left, then washed, and after a further 5 min in Ca^{2+}-free HBS stimulated with the second stimulus. Results (means±SEM, from at least 3 independent experiments) show the peak Ca^{2+} signals evoked by the second stimulus expressed as a percentage of that for cells for which the first stimulation period included only Ca^{2+}-free HBS. * $P<0.05$, one-tailed paired Student's *t* test

of sequential stimuli to P2Y1, P2Y4 and P2Y6 receptors. Cells in normal HBS were stimulated briefly (60 s) with a maximally effective concentration of a subtype-selective agonist, washed and after a 5-min period of recovery, stimulated with the second agonist, and then, after a further wash and recovery, stimulated with the third agonist (Fig. 4b). From these results, where we varied the sequence in which the agonists were presented to avoid bias, we established the distribution of responsiveness among 158 cells. The results show that 33 %, 43 % and 70 % of cells responded to activation of P2Y1, P2Y4 and P2Y6 receptors, respectively (Fig. 4c), and from the preceding analysis, 95±2 % of cells responded to activation of P2Y2 receptors. These single-cell analyses of P2Y receptor distribution are consistent with the observation that P2Y2 receptors cause heterologous desensitisation of the other subtypes (Table 1) and depletion of the Ca^{2+} stores from which each of the other P2Y receptor subtypes evokes Ca^{2+} release (Table 2). Furthermore, the ability of P2Y4 and P2Y6 receptors to cause ~50 % loss of Ca^{2+} from the stores released by P2Y1 receptors is consistent with the overlapping expression of these three receptor subtypes (Fig. 4c and Table 2). The substantially overlapping expression of P2Y receptor subtypes in individual ASMC (Fig. 4c) suggests also that the lack of heterologous desensitisation among P2Y1, P2Y4 and P2Y6 receptors is not due to their expression in distinct populations of cells.

All P2Y receptors stimulate Ca^{2+} release via inositol 1,4,5-trisphosphate (IP_3) receptors

Evidence that each P2Y receptor subtype evokes Ca^{2+} signals in the absence of extracellular Ca^{2+} (Fig. 1b–e) [10] and that each requires PLC activity (Fig. 1f) suggests that IP_3 and IP_3 receptors (IP_3R) are likely to be essential components of the signalling pathway recruited by each P2Y receptor subtype. But the universal requirement for IP_3 need not exclude the involvement of additional intracellular Ca^{2+} channels, or the possibility that any such involvement might be specific to particular P2Y receptor subtypes. The most likely candidates are RyR and two pore channels (TPC), which are activated by nicotinic acid adenine dinucleotide phosphate (NAADP). Each of these channels is expressed in many cell types including VSMC [20, 21].

Caffeine stimulates RyR [22], but at concentrations (1–10 mM) known to activate RyR maximally, caffeine had no effect on $[Ca^{2+}]_i$ in cultured ASMC (Fig. 5a). Furthermore, ryanodine under conditions that block the activity of RyR (100 μM, pretreatment for 30 min) [23] had no effect on either the peak (Fig. 5b) or sustained Ca^{2+} signals (data not shown) evoked by activation of any of the P2Y receptor subtypes. These results are consistent with previous reports showing that expression of RyR in VSMC is repressed in cells with a proliferative phenotype [3].

Trans Ned-19 is an antagonist of NAADP-evoked Ca^{2+} signals. It interacts with TPC, although its mode of action is incompletely defined [24]. A concentration of *trans* Ned-19 (100 nM) that effectively blocks NAADP-evoked responses in many other cells had no effect on the Ca^{2+} signals evoked by maximal concentrations of the subtype-selective agonists of P2Y receptors (Fig. 5c). The effectiveness of *trans* Ned-19 was confirmed in parallel analyses of SKBR3 cells where the same concentration (100 nM) inhibited Ca^{2+} signals evoked by micro-injected NAADP [25]. This suggests that NAADP plays no significant part in the Ca^{2+} signals evoked by stimulation of any P2Y receptors in cultured ASMC.

There are no selective membrane-permeant antagonists of IP_3R [26]. 2-aminoethoxydiphenylborane (2-APB) inhibits IP_3R, but at higher concentrations, it inhibits SERCA [27] and enhances a non-specific leak of Ca^{2+} from intracellular stores [28]. 2-APB also has complex effects on store-operated Ca^{2+} entry (SOCE, discussed below) and other Ca^{2+} entry pathways [27, 29]. The peak Ca^{2+} signals evoked by maximal activation of P2Y1, P2Y4 and P2Y6 receptors were almost abolished by 2-APB (300 μM, pretreated for 3 min) (Fig. 5d), whereas for P2Y2 receptors, the response was substantially attenuated (by 61±10 %), but abolished only after submaximal stimulation (Fig. 5d). Although the actions of 2-APB at IP_3R are ill-defined, it does not compete with IP_3 for its binding site [27] and it more effectively inhibits submaximally stimulated IP_3R [30]. The latter observation probably explains the greater effectiveness of 2-APB against those P2Y receptors that evoke the smallest Ca^{2+} signals (P2Y1, P2Y4 and P2Y6, Fig. 1a) and against submaximally, rather than maximally, activated P2Y2 receptors (Fig. 5d).

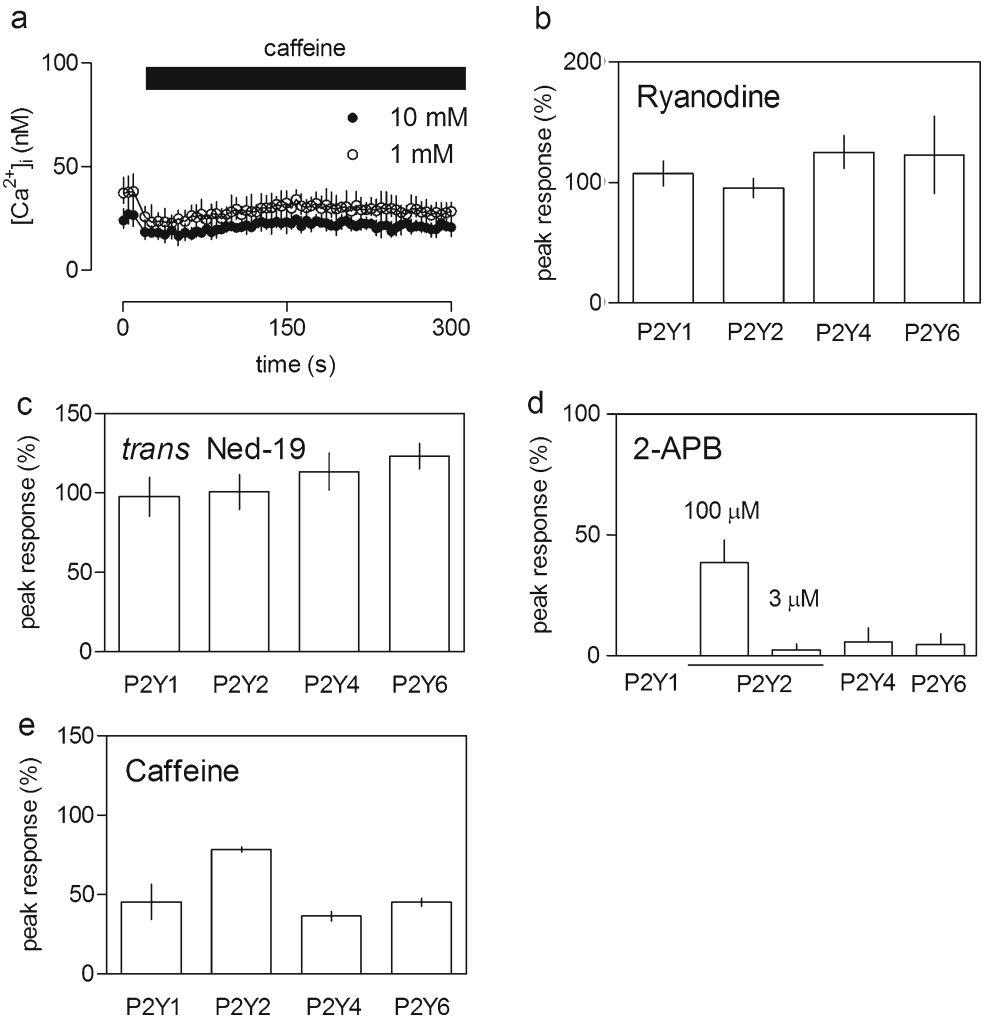

Fig. 5 IP_3 receptors are alone responsible for the Ca^{2+} release evoked by P2Y receptors. **a** Populations of cells were stimulated with caffeine in HBS (1 or 10 mM) for the period shown. Results are means± SEM for triplicate determinations from a single experiment, which was repeated three times. **b**–**e** Peak increases in $[Ca^{2+}]_i$ evoked by maximal activation of the indicated P2Y receptor subtypes (details in legend to Fig. 1a) are shown as percentages of control responses for cell populations treated with ryanodine (**b** 100 μM, pretreatment for 30 min), *trans* Ned-19 (**c** 100 nM, pretreatment for 3 min), 2-APB (**d** 300 μM, pretreatment for 3 min) and caffeine (**e** 10 mM, pretreatment for 3 min). Results (**b**–**e**) are means±SEM from three independent experiments. In **d**, P2Y2 receptors were stimulated either sub-maximally or maximally with 3 μM or 100 μM 2'-amino-UTP, respectively

Caffeine is a low-affinity antagonist of IP_3R [22], though it too has many additional actions. A high concentration of caffeine (100 mM, pretreated for 1 min) substantially inhibited the Ca^{2+} signals evoked by each of the four P2Y receptor subtypes (by ~75 %, not shown), but at this concentration, it is impossible to conclude that this results from a specific interaction of caffeine with IP_3R. A lower concentration of caffeine (10 mM, pre-treated for 3 min), caused lesser (at least 22 %), but significant ($P<0.05$), inhibition of the Ca^{2+} release evoked by each of the four P2Y receptor subtypes (Fig. 5e). Xestospongin C is another poorly characterised antagonist of IP_3R [22]. We have not succeeded in achieving substantial and selective inhibition of IP_3R in other cell types using xestospongin C and nor, in limited experiments (constrained by the cost of xestospongin C), did xestospongin C (10 μM) inhibit Ca^{2+} signals evoked by P2Y receptors in ASMC (data not shown).

The Ca^{2+} release evoked by all four P2Y receptors requires activation of PLC (Fig. 1f) and it is independent of both RyR (Fig. 5b) and TPC (Fig. 5c). This evidence, together with that provided by two, albeit imperfect, antagonists of IP_3R (2-APB and caffeine) (Fig. 5d, e) strongly suggest that IP_3R are entirely responsible for the Ca^{2+} release evoked by each P2Y receptor subtype in cultured ASMC.

Mechanisms of Ca^{2+} entry

In the absence of extracellular Ca^{2+}, the Ca^{2+} signals evoked by activation of each of the P2Y receptor subtypes were transient and complete within about 5 min (Fig. 1b–e) [10]. This indicates that any sustained responses require Ca^{2+} entry across the plasma membrane. Figure 1a shows that P2Y2, P2Y4 and P2Y6 receptors stimulate Ca^{2+} entry, while there is no detectable sustained response to activation of P2Y1 receptors. The latter probably results from the rapid homologous desensitisation of P2Y1 receptors (Fig. 2b, c). Subsequent experiments examine the mechanisms responsible for the Ca^{2+} entry evoked by P2Y2, P2Y4 and P2Y6 receptors.

Depolarisation of the plasma membrane by addition of HBS containing 53 mM KCl in place of NaCl had no effect

on $[Ca^{2+}]_i$ in ASMC (data not shown). Nimodipine (100 nM), an inhibitor of L-type Ca^{2+} channels, had no effect on either the peak or sustained Ca^{2+} signals evoked by activation of any of the P2Y receptor subtypes (Fig. 6a). In parallel experiments with A7r5 cells, KCl did evoke an increase in $[Ca^{2+}]_i$ that was blocked by nimodipine, confirming the effectiveness of the treatments in cells that do express functional L-type Ca^{2+} channels. These results, suggesting that L-type Ca^{2+} channels do not contribute to the Ca^{2+} signals evoked by P2Y receptors in ASMC, are consistent with evidence that L-type Ca^{2+} channels are down-regulated in the proliferative phenotype of VSMC [1].

Ca^{2+} can also enter cells across the plasma membrane in exchange for Na^+ extrusion via the Na^+/Ca^{2+} exchange (NCX) operating in 'reverse mode'. It has been suggested that ATP-evoked Ca^{2+} entry in cultured ASMC is entirely mediated by the NCX working in reverse mode [31]. The diacylglycerol produced by PLC is proposed to open a non-selective cation channel formed by transient receptor potential canonical 6 (TRPC6) proteins allowing Na^+ entry. The local increase in cytosolic Na^+ concentration is then suggested to allow the NCX to operate in reverse mode, and so mediate Ca^{2+} entry [31, 32]. KB-R7943, at a concentration (10 μM) known to be selective for inhibition of NCX working in reverse mode [33], had no effect on either the peak (data not shown) or sustained Ca^{2+} signals evoked by activation of any of the P2Y receptor subtypes (Fig. 6b). Furthermore, complete replacement of extracellular Na^+ (NaCl replaced by *N*-methyl-D-glucamine in HBS) had no effect on the Ca^{2+} signals evoked by P2Y receptors (Fig. 6c). These results establish that in our preparations of cultured rat ASMC, the NCX operating in reverse mode does not contribute to Ca^{2+} entry. We cannot easily explain the disparity with work from van Breemen and colleagues [31, 32], although we note that they used rat ASMC from rather later passages (passages 8–12) than ours (passages 2–8).

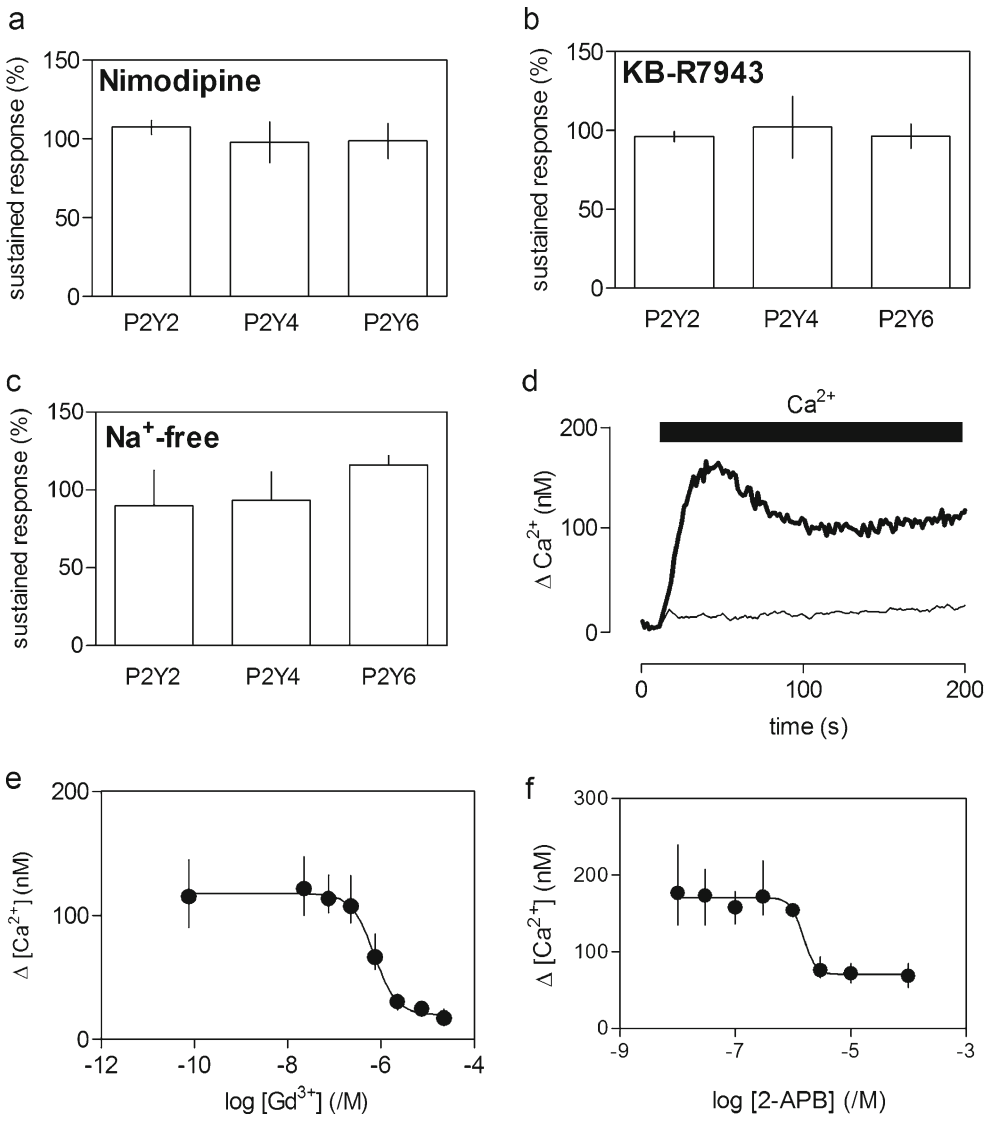

Fig. 6 Ca^{2+} entry evoked by P2Y2, P2Y4 and P2Y6 receptors. **a**–**c** Effects of nimodipine (100 nM, applied 40 s before the stimulus, **a**, KB-R7943 (10 μM, applied) 15 min before the stimulus), **b** or replacement of extracellular Na^+ with *N*-methyl-D-glucamine (**c**) on the sustained Ca^{2+} signals (measured 260-300 s after agonist addition) evoked by maximal activation of each of the P2Y receptor subtypes. Results show means±SEM from three independent experiments. The agonists used and their concentrations are defined in the legend to Fig. 1a. **d** Restoration of extracellular Ca^{2+} (1.5 mM) to cells incubated in Ca^{2+}-free HBS alone (thin trace) or after pre-incubation with thapsigargin (1 μM, 30 min in Ca^{2+}-free HBS) to empty intracellular stores (thick trace). Results are from a single experiment, which was repeated three times. **e**–**f** Concentration-dependent effects of Gd^{3+} (**e** applied 40 s before) and 2-APB (**f** applied 3 min before) on Ca^{2+} signals evoked by restoration of extracellular Ca^{2+} to cells incubated with thapsigargin (1 μM, 30 min) in Ca^{2+}-free HBS. Results (**e** and **f**) from populations of ASMC are means± SEM from three independent experiments

Treatment of cultured ASMC with thapsigargin (an inhibitor of SERCA) in Ca^{2+}-free HBS (1 μM, 30 min) emptied intracellular Ca^{2+} stores. Restoring extracellular Ca^{2+} then evoked a modest increase in $[Ca^{2+}]_i$ (152±7 nM), whereas in cells treated similarly, but without thapsigargin, the increase was very small (18±2 nM) (Fig. 6d). These results, which confirm the presence of SOCE in ASMC, are consistent with many studies [4, 34]. SOCE is typically sensitive to block by low concentrations of Gd^{3+} [35, 36]. $GdCl_3$ caused a concentration-dependent inhibition of the thapsigargin-evoked SOCE in ASMC (pIC_{50}=6.13±0.1) (Fig. 6e). $GdCl_3$ also caused a concentration-dependent inhibition of the Ca^{2+} entry evoked by P2Y2, P2Y4 and P2Y6 receptors, and for each the sensitivity (pIC_{50} ~6) was similar to that for inhibition of thapsigargin-evoked SOCE (Table 3). Whereas the maximal inhibition by Gd^{3+} was similar for thapsigargin-evoked SOCE and Ca^{2+} entry evoked by P2Y2 receptors, the lesser Ca^{2+} entry evoked by P2Y4 and P2Y6 receptors was less completely inhibited (Table 3). It is unclear whether this reflects a small SOCE-independent component of Ca^{2+} entry or the experimental difficulty of quantitatively analysing inhibition of the very small Ca^{2+} entry signals evoked by P2Y4 and P2Y6 receptors (Fig. 1). The peak increases in $[Ca^{2+}]_i$, which are almost entirely due to release of Ca^{2+} from intracellular stores [10], were unaffected by Gd^{3+} (data not shown).

SOCE is also sensitive to inhibition by low concentrations of 2-APB [29, 37]. 2-APB independently inhibits IP_3R and SOCE, and its effects on the latter are often biphasic, causing stimulation at low concentrations (<5 μM) and inhibition at higher concentrations (>10 μM) [37, 38]. 2-APB caused a concentration-dependent inhibition of thapsigargin-evoked SOCE (pIC_{50}=5.83±0.04), but the inhibition, although clearly maximal, was incomplete (57±12 % inhibition at 100 μM) (Fig. 6f and Table 4). Inhibition of SOCE by relatively low concentrations of 2-APB, with no evidence of it causing stimulation, is consistent with another study of ASMC [34]. The effects of 2-APB on Ca^{2+} entry evoked by P2Y receptors are more difficult to interpret because 2-APB also inhibits the IP_3R, which we have shown to be necessary for all P2Y receptors to empty intracellular Ca^{2+} stores and thereby activate SOCE. For each P2Y receptor, the sensitivity to inhibition (pIC_{50} ~6) and the maximal effect of 2-APB on Ca^{2+} entry were similar to its effects on thapsigargin-evoked Ca^{2+} entry (Table 4 and Fig. 7). Furthermore, the peak Ca^{2+} signals evoked by P2Y receptors were less sensitive than Ca^{2+} entry to inhibition by 2-APB, although the difference was statistically significant for only P2Y2 receptors (Table 4). These results are consistent with each receptor evoking Ca^{2+} entry primarily via SOCE. The evidence is most compelling for P2Y2 receptors, where maximal inhibition of Ca^{2+} entry by 2-APB occurred with no detectable inhibition of Ca^{2+} release from intracellular stores (Table 4). Our results with low concentrations of Gd^{3+} and 2-APB suggest, although not conclusively, that SOCE mediates the Ca^{2+} entry evoked by P2Y2 receptors, and perhaps also by P2Y4 and P2Y6 receptors. Similar results have been reported for the Ca^{2+} entry evoked by platelet-derived growth factor (PDGF) in cultured rat ASMC [39].

Table 3 Effects of $GdCl_3$ on Ca^{2+} entry evoked by thapsigargin and P2Y receptors

Ca^{2+} entry	pIC_{50} (/M)	Inhibition (%)
SOCE	6.13±0.06	78±1
P2Y2	5.80±0.32	81±4
P2Y4	5.49±0.19	54±16
P2Y6	6.10±0.04	46±14

Ca^{2+} entry, in ASMC populations, was measured 250–400 s after addition of maximally effective concentrations of the subtype-selective agonists (details in legend to Fig. 1) or, for SOCE, measured 10–20 s after restoration of extracellular Ca^{2+} to cells preincubated with thapsigargin (1 μM, 30 min) in Ca^{2+}-free HBS. $GdCl_3$ was present for 40 s before and then during the stimulation. Results (means±SEM from three independent experiments) show pIC_{50} values and the inhibition caused by 7.5 μM Gd^{3+}

Table 4 Effects of 2-APB on Ca^{2+} entry evoked by thapsigargin and P2Y receptors

	Ca^{2+} entry		Ca^{2+} release	
Ca^{2+} entry	pIC_{50} (/M)	Inhibition (%)	pIC_{50} (/M)	Inhibition (%)
SOCE	5.83±0.04	51±4	NA	NA
P2Y2	6.17±0.30	77±9	4.88±0.11	0
P2Y4	5.83±0.12	64±6	5.65±0.10	19±6
P2Y6	5.74±0.12	65±5	5.29±0.29	26±9

Experiments similar to those shown in Table 3 were used to examine the effects of 2-APB. Results (means±SEM from three independent experiments) show pIC_{50} values for inhibition of Ca^{2+} release from intracellular stores (peak Ca^{2+} signal) and Ca^{2+} entry (sustained response, measured 250–400 s after addition of the agonist) and the inhibition caused by 3 μM 2-APB. *NA*, not applicable

Discussion

Four subtypes of P2Y receptor evoke Ca^{2+} signals in cultured ASMC [10] and while each requires activation of PLC (Fig. 1f), the responses differ considerably in their amplitudes, durations and requirement for Ca^{2+} entry across the plasma membrane (Fig. 1). In populations of cultured ASMC, P2Y2 receptors evoke the largest Ca^{2+} signals (Fig. 1). This results from both expression of functional P2Y2 receptors in most cells and their ability to evoke the

largest Ca^{2+} signals in single cells (Fig. 8b). Activation of the other P2Y receptor subtypes (P2Y1, P2Y4 and P2Y6) evokes smaller Ca^{2+} signals (Fig. 1), consistent with their expression in only a subset of the cells that express P2Y2 receptors (Fig. 4 and Table 2), and with the smaller amplitudes of the Ca^{2+} signals they evoke in single cells (Fig. 8). Expression of the four P2Y receptor subtypes is apparently randomly distributed between individual ASMC, with most cells expressing several functional P2Y receptor subtypes (Fig. 4c). This suggests that the four P2Y subtypes, each evoking Ca^{2+} signals in response to inter-related nucleotides, are unlikely simply to provide redundant means of achieving similar responses in different cells.

Despite the diversity of Ca^{2+} signals, each of the four P2Y receptor subtypes uses the same signalling machinery to increase $[Ca^{2+}]_i$. Each activates PLC (Fig. 1f) and thereby release of Ca^{2+} from intracellular stores via IP_3R, without involvement of ryanodine or NAADP receptors (Fig. 5). Others have also concluded that IP_3R are the predominant means of Ca^{2+} release evoked by growth factors in proliferating VSMC [1, 3].

The mechanisms underlying Ca^{2+} entry in cultured ASMC are more contentious. Rapid and substantial desensitisation of P2Y1 receptors prevents them from evoking sustained Ca^{2+} signals, but P2Y2, P2Y4 and P2Y6 receptors do stimulate Ca^{2+} entry (Figs. 1 and 2 and Table 1) [10]. Depletion of intracellular Ca^{2+} stores using thapsigargin activates SOCE in cultured rat ASMC (Fig. 6d), and others have shown that SOCE is attenuated by knockdown of Orai1 and STIM1 in ASMC [34, 40]. STIM1 is the sensor of luminal Ca^{2+} within the endoplasmic reticulum and Orai1 is a pore-forming subunit of a SOCE pathway. Whether, under physiological conditions, STIM1 activates Orai directly [41] or via an intervening biochemical sequence [42]

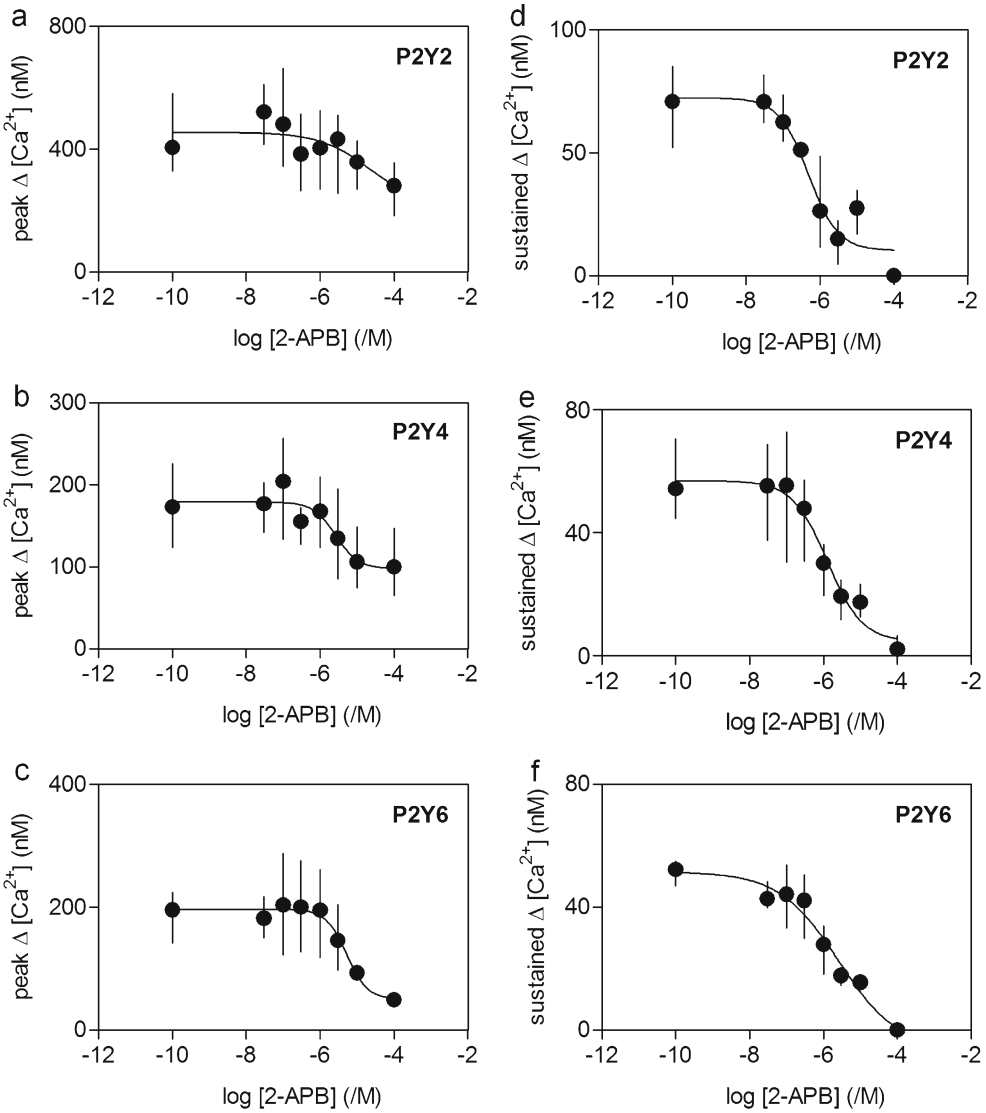

Fig. 7 Effects of 2-APB on the Ca^{2+} signals evoked by P2Y receptors. **a**–**f** Concentration-dependent effects of 2-APB on peak (**a**–**c**) and sustained (**d**–**f**) Ca^{2+} signals evoked by maximally effective concentrations of subtype-selective agonists of P2Y receptors (details in Fig. 1). Sustained responses were measured 250–400 s after addition of agonists. 2-APB was present for 3 min before and then during the stimulation. Results from cell populations are means±SEM from three independent experiments

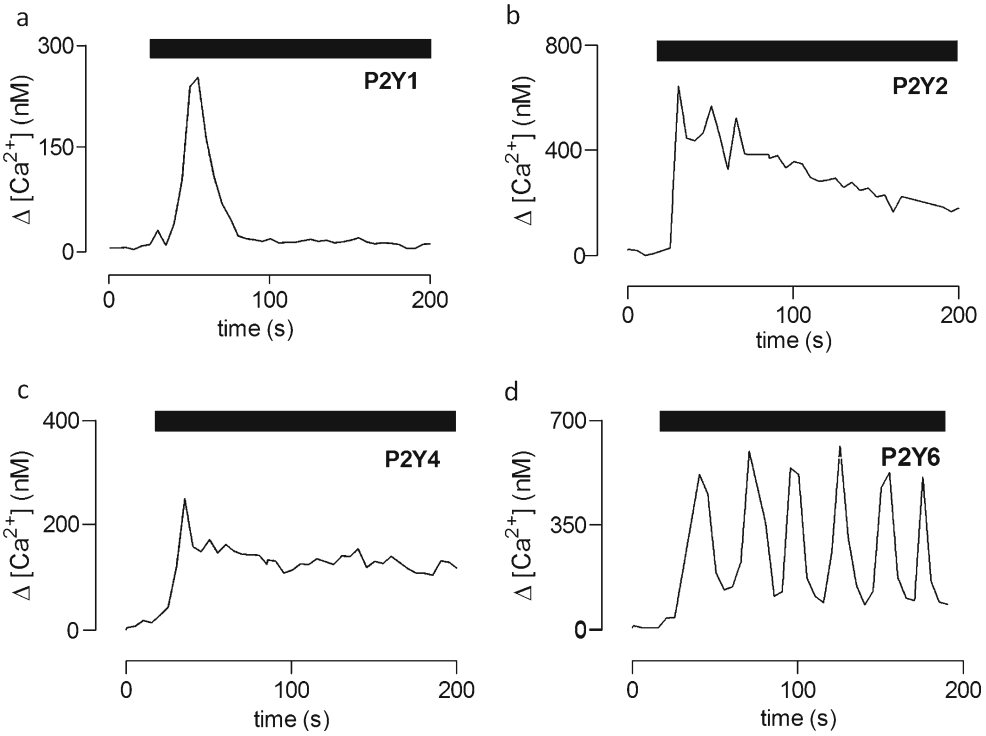

Fig. 8 Ca^{2+} signals evoked by activation of different P2Y receptor subtypes in single cells. **a**–**d** Traces show typical Ca^{2+} signals recorded from single cells after maximal activation of each of the four P2Y receptor subtypes. Details of the agonists used and their concentrations are given in the legend to Fig. 1. Results are typical of responses measured from 7 (**a**, **c** and **d**) or 4 (**b**) different coverslips. Only P2Y6 receptors (72±3 % of cells from 7 coverslips) reproducibly evoked Ca^{2+} oscillations

is not entirely resolved. TRPC channels, which may also be activated by STIM1 and associate with Orai proteins, may also contribute to SOCE [43, 44], although this suggestion is disputed [36]. Several studies have suggested a role for TRPC1 in mediating SOCE in VSMC [45], but this is also contentious. Others suggest that in cultured ASMC, knockdown of Orai1 attenuates SOCE current, while loss of TRPC1, TRPC4 or TRPC6 does not [34]. Furthermore, Ca^{2+} entry evoked by PDGF in ASMC is mediated by STIM1 and Orai1, and resembles SOCE in its inhibition by low concentrations of Gd^{3+} and 2-APB [39].

An alternative role for TRPC proteins in contributing to Ca^{2+} entry suggests that ATP-evoked Ca^{2+} entry is mediated by TRPC6 and the NCX working in reverse mode [31, 32]. Our results suggest that NCX does not contribute to the Ca^{2+} entry evoked by P2Y receptors (Fig. 6b, c) and that SOCE alone is probably sufficient to account for the Ca^{2+} entry evoked by P2Y receptors (Figs. 6 and 7 and Tables 3 and 4), although we cannot entirely exclude a minor role for stimulation of an additional Ca^{2+} entry pathway by P2Y4 and P2Y6 receptors.

Despite the shared signalling pathways, the four P2Y receptors evoke Ca^{2+} signals with different characteristics (Fig. 1) that appear to be substantially due to differing patterns of homologous desensitisation (Fig. 2). Homologous desensitisation of P2Y1 receptors, which is mediated by protein kinase C and by G protein receptor kinases in other cells [46, 47], is most pronounced (Fig. 2) and causes the Ca^{2+} signals in ASMC to terminate before there is detectable Ca^{2+} entry (Figs. 1a and 8a). There is no evidence for homologous desensitisation of P2Y4 receptors (Fig. 2), consistent with their ability to evoke sustained responses (Figs. 1a and 8c) and considerable Ca^{2+} entry (Fig. 1d). P2Y2 and P2Y6 receptors show intermediate levels of homologous desensitisation, with the latter recovering most quickly (Fig. 2). This is consistent with the ability of both receptor subtypes to stimulate sustained Ca^{2+} entry (Fig. 1). It is tempting to speculate that the consistent triggering of Ca^{2+} oscillations by P2Y6 receptors (Fig. 8d) may be driven by their substantial, but rapidly reversed, homologous desensitisation (Fig. 2c). Within a physiological setting, where endogenous nucleotides are likely to cause parallel activation of several P2Y receptor subtypes in the same cell (Fig. 4c), the temporal pattern of Ca^{2+} signals is likely to be further shaped by the ability of P2Y2 receptors to cause heterologous desensitisation of each of the other subtypes (Table 1).

We conclude that four P2Y receptor subtypes (P2Y1, P2Y2, P2Y4 and P2Y6) evoke Ca^{2+} signals in ASMC via signalling pathways that require activation of PLC, release of Ca^{2+} from IP_3-sensitive Ca^{2+} stores and consequent activation of SOCE. Despite the shared signalling machinery, the patterns of Ca^{2+} signalling evoked by different P2Y receptors differ profoundly. We suggest that the differences are due largely to different patterns of homologous desensitisation.

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