

# Different patterns of $\text{Ca}^{2+}$ signals are induced by low compared to high concentrations of P2Y agonists in microglia

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**Abstract** Brain-resident macrophages (microglia) are key cellular elements in the preservation of tissue integrity. On the other hand, they can also contribute to the development of pathological events by causing an extensive and inappropriate inflammatory response. A growing number of reports indicate the involvement of nucleotides in the control of microglial functions. With this study on P2Y receptors in rat microglia, we want to contribute to the definition of their expression profile and to the characterisation of their signalling mechanisms leading to  $\text{Ca}^{2+}$  movements. Endogenous nucleotides, when applied at a concentration of 100  $\mu\text{M}$ , elicited robust  $\text{Ca}^{2+}$  transients, thanks to a panel of metabotropic receptors comprising mainly P2Y<sub>2</sub>, P2Y<sub>6</sub> and P2Y<sub>12</sub> subtypes. The involvement of P2Y<sub>12</sub> receptors in  $\text{Ca}^{2+}$  responses induced by adenine nucleotides was confirmed by the pharmacological and pertussis toxin sensitivity of the response induced by adenosine diphosphate (ADP). Beside the G protein involved, Gi and Gq respectively, adenine and uracil nucleotides differed also for induction by the latter of a capacitative  $\text{Ca}^{2+}$  plateau. Moreover, when applied at low (sub-micromolar) concentrations with a long-lasting challenge, uracil nucleotides elicited oscillatory  $\text{Ca}^{2+}$  changes with low frequency of occurrence ( $\leq 1 \text{ min}^{-1}$ ), sometimes superimposed to an extracellular  $\text{Ca}^{2+}$ -dependent sustained  $\text{Ca}^{2+}$  rise. We conclude that different patterns of  $\text{Ca}^{2+}$  transients are induced by low (i.e., oscillatory  $\text{Ca}^{2+}$  activity) compared to high (i.e., fast release followed by sustained

raise) concentrations of nucleotides, which can suggest different roles played by receptor stimulation depending not only on the type but also on the concentration of nucleotides.

**Key words** calcium · capacitative · mitochondria · oscillation

## Introduction

Distributed in the central nervous system (CNS) parenchyma in a ramified morphology, microglial cells play a continuous “resting activity” of surveillance. Signals not well identified as yet seem to be required to keep microglia in such a quiescent but alert state. Similarly, signals resulting from an ongoing acute insult or from a chronic degenerative condition drive toward or maintain microglia in a state of cell activation. Synthesis of pro- and anti-inflammatory cytokines, reactive oxygen species, nitric oxide, prostaglandins, expression of major histocompatibility complex (MHC) molecules and re-arrangement of the ion channel profile are only some of the changes acquired by activated microglial cells [1–3]. In principle, these newly acquired functional capabilities would tend to favour protection and recovery of homeostasis, but in some instances, control on the overall inflammatory response is lost and an exacerbation of the pathology takes place. Participation in the pathogenesis of virtually all acute and chronic diseases has been suggested for microglia. The dichotomy of microglia between guardian and potentially promoter of recovery on one hand and pathogenic cellular entity on the other hand has drawn interest to the search for signals driving microglia to beneficial capabilities and the control of pro-inflammatory and potentially harmful activities [4, 5].

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Adenosine triphosphate (ATP) and other nucleotides have been extensively described as multi-functional agents acting on neuronal and glial cells. ATP acts as a fast synaptic neurotransmitter by binding to post-synaptic P2X receptors in a variety of neuronal cells, in keeping with their wide distribution predominantly on brain neurons [6]. ATP also acts on postsynaptic P2Y receptors, modulating voltage-dependent  $K^+$  and  $Ca^{2+}$  currents [7, 8]. P2Y receptors were also described to interfere with N-methyl-D-aspartate (NMDA) but not with alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors, probably by inducing phosphorylation of the receptor protein [9]. ATP and analogues acting on P2Y receptors regulate the release of neurotransmitters such as acetylcholine, noradrenaline, dopamine, serotonin and glutamate (6 for references) in concert with adenosine.

Also, all glial cell types have been described to react to ATP and other nucleotides. ATP and analogues were shown to induce astrocyte proliferation, stellation and increase of glial fibrillary acidic protein (GFAP) expression, all symptoms of astrogliosis [10–12], by stimulating P2Y receptors.  $Ca^{2+}$  wave propagation in mechanically or pharmacologically stimulated cultured astrocytes are known to depend on the release of ATP and on its effect on adjacent cells [13]. ATP released from stimulated dorsal root ganglia (DRG) cells was also described to modulate the differentiation and proliferation of Schwann cells in a *in vitro* co-culture system [14]. Recently, we identified ATP and adenosine diphosphate (ADP) as agents able to control differentiation and promote migration of oligodendrocyte progenitors by activation of P2Y1 receptors [15]. A number of different effects of nucleotides on microglial cells have also been reported. In microglia, great attention has been dedicated to P2X ionotropic receptors, and thanks to this, their profile of expression and a set of functions clearly linked to each subtype has been delineated. P2X7 receptors, first described for their pro-inflammatory capabilities due to the induction of interleukin (IL)-1 $\beta$  and tumour necrosis factor (TNF)- $\alpha$  release [16, 17], as well as for their cytotoxicity due to the opening of a large conductance pore [18], are now considered also as involved in the fine control of microglial proliferation (Bianco et. al., this issue). A second ionotropic ATP receptor that we tentatively proposed in microglia as the P2X4 subtype on the basis of kinetic and pharmacological properties [19] was then clearly identified in this cell type, and its role in the induction of tactile peripheral allodynia following nerve injury was proposed [20]. A number of studies have addressed the capabilities of nucleotides to mobilise  $Ca^{2+}$  and to induce the opening of store-operated  $Ca^{2+}$  channels by acting on microglial P2Y receptors [21, 22]. Chemotactic activity induced by ATP was described to involve P2Y<sub>12</sub> receptors [23] while the release of pro- and anti-inflammatory cytokines was shown

to be under the control of as yet unidentified P2Y receptors [24–26].

Glial cells are not only targets of nucleotide signalling, they also are more generally responsible of shaping the nucleotide signalling system, being also sources of nucleotides and in charge of their degradation. Regardless of the mechanisms utilised—vesicular, mediated by ATP-binding cassette (ABC) transporters or through connexin emi-channels—during  $Ca^{2+}$  waves propagation, astrocytes release ATP, which acts as an extracellular messenger in homo-typical (i.e., astrocyte to astrocyte) and hetero-typical (i.e., astrocyte to microglia) intercellular communication [13, 27]. When challenged with the pro-inflammatory factor lipopolysaccharide (LPS), microglia was described to release ATP, which, by acting as an autocrine factor, contributed to processing and release of cytokines IL- $\beta$ 1 and IL-10 [16, 25]. Finally, astrocytes and microglial cells are deputed to the degradation of extracellular nucleotides in virtue of the expression of ecto-enzymes ecto-ATPase, ecto-apyrase and ecto-5'-nucleotidase [28]. Interestingly, increase of expression of these enzymes after transient fore-brain ischaemia in the rat was shown to involve mainly microglia [29], which makes these cells capable of reacting to the increase of nucleotide release in the attempt to avoid excessive nucleotide stimulation.

It is known that high doses of nucleotides released by tissue undergoing stress, cell damage or abnormal death might act as “danger signals” and cause release of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6), prostaglandins and reactive oxygen species [30] from immune cells. On the other hand, it was recently suggested that at lower doses, nucleotides might play a regulatory role on inflammatory reactions aimed at the prevention of tissue damage [31].

In this study, we focused on P2 metabotropic receptors in rat microglial cells, and by molecular [reverse transcription polymerase chain reaction (RT-PCR)] and functional ( $Ca^{2+}$  video imaging) approaches, we characterised expression profile, functional capability and some aspects of the pathways involved in receptor activation. By comparing the effects induced by low versus high concentrations of metabotropic agonists, we then aimed to depict dependence on the availability of nucleotides of cytoplasmic  $Ca^{2+}$  signalling.

## Materials and methods

### Cell culture

Microglial cells were obtained from the cerebral cortex of 1- to 2-day-old rats [32]. All experiments were carried out in accordance with directives of the Council of the

European Communities N. 86/609/CEE. Briefly, after pups were rendered hypothermic on an ice-cold surface, they were decapitated. The cerebral cortex was dissected out, and meninges were removed. The tissue was dissociated by a two-step procedure comprising enzymatic digestion followed by mechanical dissociation. Mixed primary cultures were grown on poly-L-lysine-coated culture flasks for 7–10 days in Basal Medium Eagle (BME) supplemented with 10% endotoxin-free foetal calf serum (FCS), 2 mM glutamine and 100  $\mu\text{g ml}^{-1}$  gentamicin (37°C, 5% CO<sub>2</sub>). After mild shaking, microglial cells were harvested and plated on un-coated glass coverslips sealed on home-made silicon wells at a density of  $15 \times 10^4$  cells/cm<sup>2</sup>. To further improve the purity of microglial cultures, non-adhering cells were removed after 20 min by changing the medium.

### RT-PCR

Microglia cells obtained from rat brain at postnatal day 2 were used to extract total ribonucleic acid (RNA) using Trizol reagent according with the data sheet. Two micrograms of total RNA were digested for 30 min with Dnase (Invitrogen) and retro-transcribed using the SuperScript synthesis system (Life Technologies). Specific oligonucleotides with similar annealing temperatures were designed to amplify the following purinergic receptors: P2Y<sub>1</sub> (NM012800.1), P2Y<sub>2</sub> (NM017255.1), P2Y<sub>4</sub> (NM031680.1), P2Y<sub>6</sub> (NM057124.1), P2Y<sub>12</sub> (NM022800.1), P2Y<sub>13</sub> (NM001002853.1) and P2Y<sub>14</sub> (NM133577.1).  $\beta$ -actin (NM031144) was always amplified as control. Samples were analysed on agarose gel containing ethidium bromide.

The following primers were used:

P2Y<sub>1</sub>: forward: 5'-AACAGTACAATCGCCTCGAC-3'; reverse: 5'-TTCGCAGGTACTCATCTGAC-3'. Band size of 559 bp.

P2Y<sub>2</sub>: forward: 5'-AACGGACGTGAGCATCCAA3'; reverse: 5'-AGGTGTCGTGGCAGGTGATT-3'. Band size of 639 bp.

P2Y<sub>4</sub>: forward: 5'-CCATCCTGTGCCATGACACT-3'; reverse: 5'-CATAGCTGCTGGAGCTGGTT-3'. Band size of 418 bp.

P2Y<sub>6</sub>: forward: 5'-CGGCAGCATCCTGTTCCCTCA-3'; reverse 5'- TGGAGCGCACAGCCAAGTAG-3'. Band size of 470 bp.

P2Y<sub>12</sub>: forward: 5'-GGTGTGCCAAGTCACTTCAG; reverse 5'-ATCAAGGCAGGCGTTCAAGG-3'. Band size of 598 bp.

P2Y<sub>13</sub>: forward: 5'-GATGACACAGCTGCTGTTCC-3'; reverse: 5'- TGA CTGCTGTGGTGCTCATC-3'. Band size of 915 bp.

P2Y<sub>14</sub>: forward: 5'-GCACAAGGCGTCTAAC-TATGTC-3'; reverse: 5'-CCACATGCCACTTCTCAT TCG-3'. Band size of 692 bp.

$\beta$ -Actin: forward: 5'-ACCTTCAACACCCCAGCCATGTAC G-3'; reverse: 5'-CTGATCCACATCTGCTGGAAGGTGG-3'. Band size of 698 bp.

### Intracellular Ca<sup>2+</sup> recording

To record intracellular Ca<sup>2+</sup> we employed the video-imaging technique with the Ca<sup>2+</sup>-sensitive probe Fura-2. Acetoxymethyl Fura-2 (Fura-2-AM; Molecular Probes, Leiden, The Netherlands) was dissolved in a solution containing pluronic acid and dimethylsulfoxide (DMSO) (1:4 ratio in weight) and then sonicated for 5 min. Microglial cultures were exposed to a solution containing 2.5  $\mu\text{M}$  Fura-2-AM for 50 min at room temperature to allow cell loading. To achieve a better hydrolysis of Fura-2-AM, the cultures were recorded 30 min after washing of the dye. Silicon wells were removed, and the glass coverslips were placed in a recording chamber on the stage of an Axiovert 35 inverted microscope (Zeiss, Milan, Italy). Fura-2-loaded cells were exposed every 1–5 s to the excitation wavelengths 340 and 380 nm by means of a monochromator (Polychrome II, T.I.L.L. Photonics, Planegg, Germany). The emission light at 510 nm was collected by a digital camera (Sensicam, PCO, Kelheim, Germany) and recorded on the hard disk of a PC computer. Recording and analysis of data were made possible by the Imaging Workbench 5.2 software package (Indec BioSystems, Santa Clara, CA, USA ). Ratio values were converted to free Ca<sup>2+</sup> concentrations by utilising an established equation [33], with  $R_{\text{min}} = 0.28$ ,  $R_{\text{max}} = 6.45$ ,  $\beta = 4.5$ .

Unless otherwise stated, the bulk solution had the following composition (mM): 140 NaCl, 5 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 D-glucose, 10 HEPES/NaOH (room temperature, pH 7.4, 290 mosmol l<sup>-1</sup>). Ca<sup>2+</sup>-free solutions were made by replacing Ca<sup>2+</sup> with an equal amount of Mg<sup>2+</sup>. In parallel with a perfusion system allowing the change of the bulk solution of the bath, a local perfusion system was used (Rapid Solution Changer: RSC 100; Biologic, Grenoble, France). It consisted of a set of reservoirs containing different solutions connected to polyethylene tubings with an internal diameter of 300  $\mu\text{m}$  glued together in a parallel array from which the flow of solution was made possible by operating on solenoid valves. Amplitudes of Ca<sup>2+</sup> signals were calculated as Ca<sup>2+</sup> increase from the pre-application Ca<sup>2+</sup> level. Peak amplitudes were calculated on single cells, and their averages were calculated pooling similar experiments (from a minimum of two experiments for each condition). Not to underestimate an eventual effect on sub-populations of cells, we thought it useful to

represent the data as frequency distributions of the experimental values measured in the single cells. The results obtained with uridine 5'-diphosphate (UDP) were not statistically different from those obtained with uridine 5'-triphosphate (UTP), which we showed to represent uracil nucleotides in all figures following Figure 2.

## Results

### mRNA expression of P2Y receptors

In order to understand which P2Y receptors are expressed in primary microglial cells, we performed RT-PCR analysis on purified messenger ribonucleic acid (mRNA) obtained from primary microglial cells. Specific amplification profiles are shown in Figure 1. High mRNA expression was detected for P2Y<sub>2</sub>, P2Y<sub>6</sub>, P2Y<sub>12</sub> and P2Y<sub>14</sub>. The weak band detected for P2Y<sub>1</sub> and P2Y<sub>4</sub> could not exclude their presence. Negative control (PCR without Retro transcription) was always performed (data not shown).

### Effects of endogenous nucleotides on Ca<sup>2+</sup>

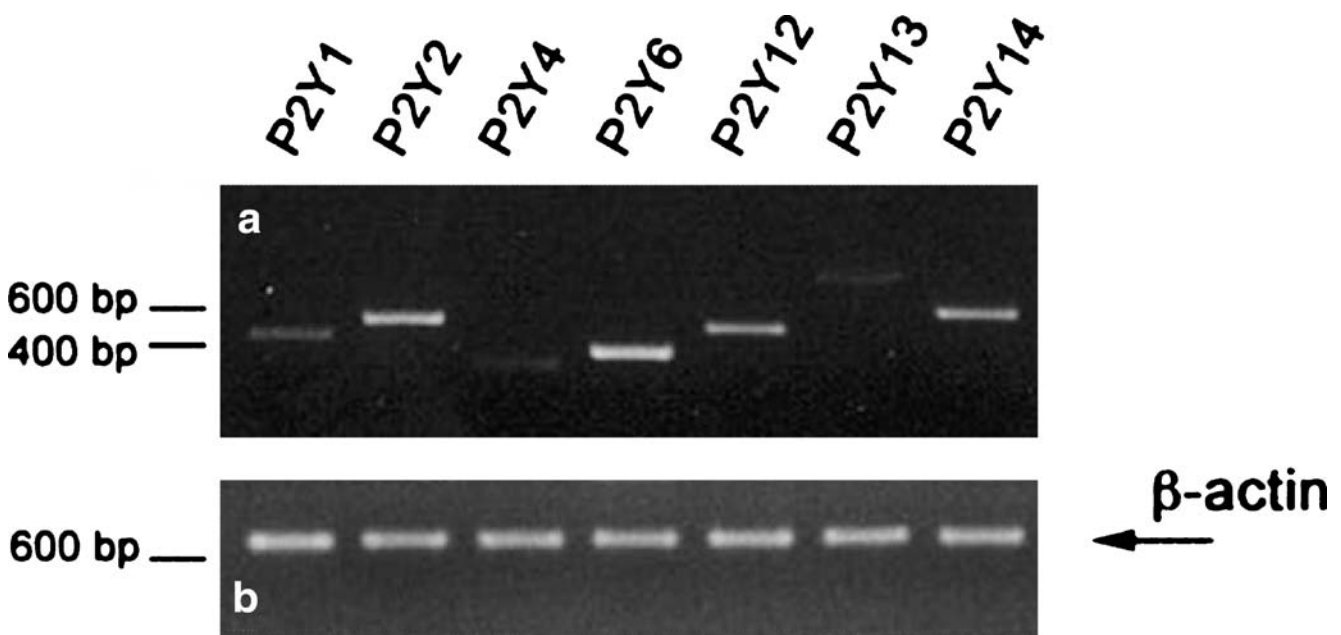
To contribute to the determination of P2Y receptors functionally active in our rat microglial cell cultures, we utilised a number of endogenous nucleotides (ATP, ADP,

UTP, UDP, UDP-glucose). When briefly applied at a high concentration (100 μM for 20 s), all of them, with the exception of UDP-glucose, were able to induce a fast Ca<sup>2+</sup> rise followed by a slower phase of recovery in virtually all microglial cells (Figure 2). The lack of P2Y<sub>14</sub> protein (to be tested when an appropriately validated antibody is available) or the expression of a protein not functionally coupled to Ca<sup>2+</sup> release [15] can explain the apparent incongruence between PCR and Ca<sup>2+</sup> data.

The speed of rise and average Ca<sup>2+</sup> amplitude were generally unaffected by removal of Ca<sup>2+</sup>, with the exception of a slight decrease of the Ca<sup>2+</sup> concentration reached when ATP was used (data not shown). Molecular and functional data are compatible with a P2Y receptor profile in which the P2Y<sub>12</sub> is responsible for adenine nucleotide-induced Ca<sup>2+</sup> signals and P2Y<sub>2</sub> and P2Y<sub>6</sub> for UTP- and UDP-induced Ca<sup>2+</sup> signals. To confirm this description, especially the role of P2Y<sub>12</sub> in Ca<sup>2+</sup> signalling, further studies testing the pharmacological and pertussis toxin sensitivity of the ADP response were carried out.

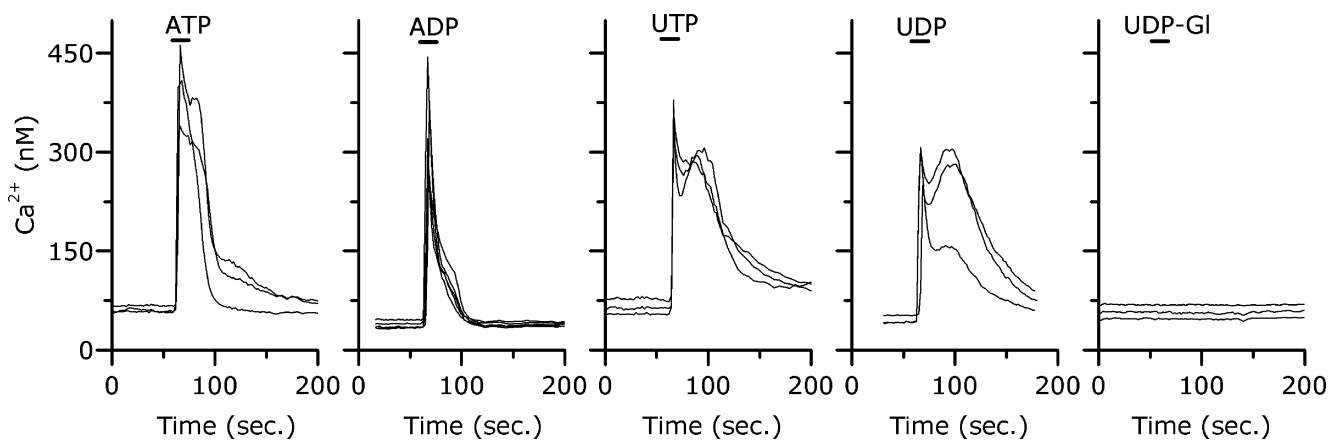
### Pharmacological blockade of P2Y receptors

For a pharmacological characterisation of P2Y receptors, we utilised PPADS and MRS2179. PPADS is a non-selective but non-universal P2 receptor antagonist. It efficiently inhibits P2X receptors, but it discriminates between ADP and UTP responses on P2Y receptors.



**Figure 1** Expression pattern of P2Y receptors analysed by reverse transcription polymerase chain reaction (RT-PCR). (a) In microglial cells, we found the expression of P2Y<sub>2</sub>, P2Y<sub>6</sub>, P2Y<sub>12</sub> and P2Y<sub>14</sub> receptors. Moreover, a weak amplification of the P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors was detected. Ethidium-bromide-stained agarose gel shows

RT-PCR products generated from total ribonucleic acid (RNA) of microglia. (b) In control samples, complementary deoxyribonucleic acid (cDNA) was reverse transcribed and amplified for β-actin. All samples were preincubated with DNase before retro-transcription



**Figure 2** Adenosine triphosphate (ATP), adenosine diphosphate ((ADP), uridine 5'-triphosphate (UTP) and uridine 5'-diphosphate (UDP) but not UDP-glucose (P2Y14 agonist) induce intracellular  $\text{Ca}^{2+}$  release in microglia. Microglial cells were challenged with a panel of naturally occurring nucleotides for 20 s at a concentration of 100  $\mu\text{M}$ .

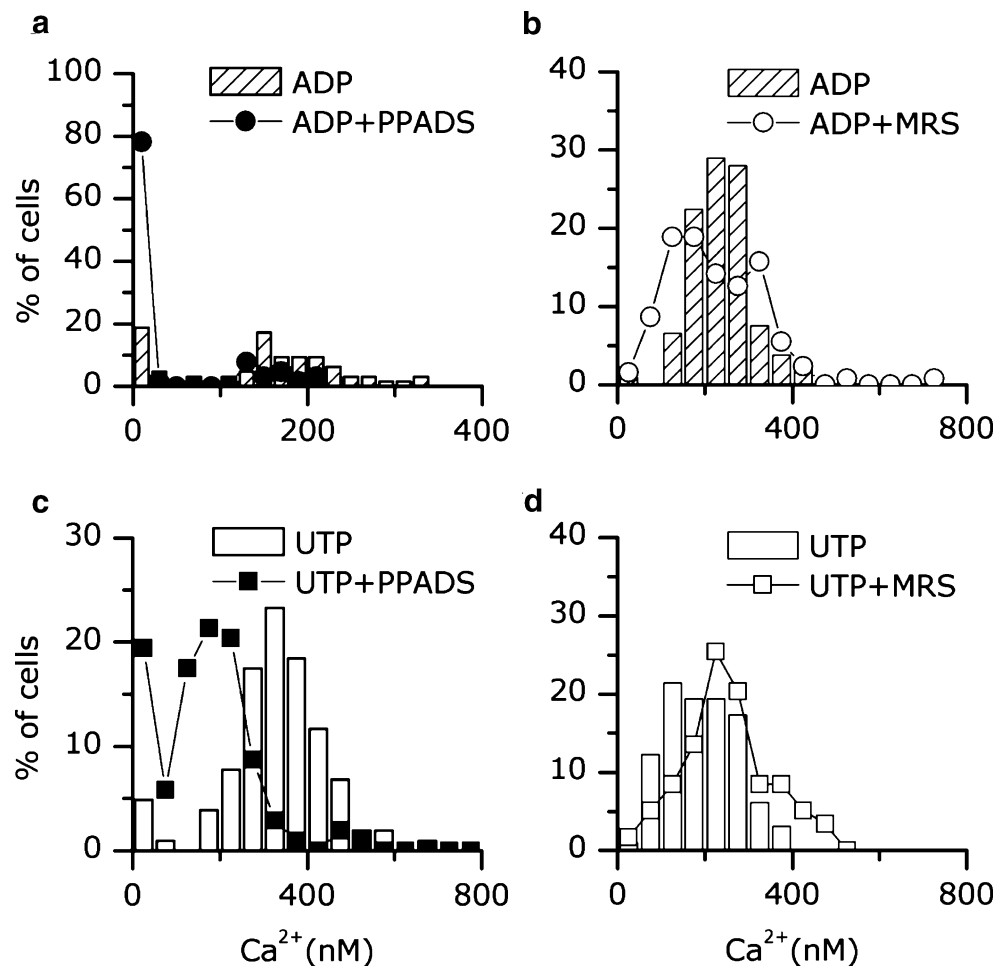
With the exception of UDP-glucose, nucleotide application caused in virtually all cells challenged a fast  $\text{Ca}^{2+}$  rise followed by a variable phase of recovery. The exemplifying traces depict the fact that uracil nucleotides (UTP and UDP) more consistently induced a  $\text{Ca}^{2+}$  plateau following the initial peak

Hence, we used it to confirm the specificity of the effect of ADP and uracil nucleotides. At a concentration of 100  $\mu\text{M}$ , PPADS completely inhibited the response to ADP and UTP in 78% and 18% of the cells (Figure 3a, c), respectively,

and only partially affected the responses induced in the remaining cells.

MRS2179 can be reliably utilised to detect involvement of P2Y<sub>1</sub> receptors, as we recently confirmed in oligoden-

**Figure 3**  $\text{Ca}^{2+}$  release induced by nucleotides is differently affected by pharmacological inhibitors.  $\text{Ca}^{2+}$  transients induced by adenosine diphosphate (ADP) and uridine 5'-triphosphate (UTP) in the absence of  $\text{Ca}^{2+}$  were recorded in the absence or during the application of P2 antagonists (100  $\mu\text{M}$ , with 2 min of pre-treatment). Each panel shows frequency distribution of amplitudes of the  $\text{Ca}^{2+}$  transients recorded in the absence and presence of the antagonist. (a), (c) PPADS more efficiently inhibits the ADP-induced  $\text{Ca}^{2+}$  responses ( $n = 64$ –103). (b), (d) As expected, the P2Y<sub>1</sub> antagonist MRS2179 does not affect the response to UTP but it does not inhibit even that to ADP, outlining the presence of ADP-sensitive receptors other than P2Y<sub>1</sub> ( $n = 59$ –164)



drocyte progenitors [15]. MRS2179, up to a concentration of 100  $\mu\text{M}$ , did not inhibit  $\text{Ca}^{2+}$  responses induced by UTP, as expected considering that UTP acts on receptors other than  $\text{P2Y}_1$  (Figure 3d). However, it was unable to inhibit even ADP-induced responses (Figure 3b), so confirming that  $\text{P2Y}_1$  receptor activation is not appreciably involved in the induction of  $\text{Ca}^{2+}$  movements by ADP in rat microglia. On the other hand, this observation is in keeping with a role played by the  $\text{P2Y}_{12}$  subtype in ADP-induced  $\text{Ca}^{2+}$  responses.

### G protein involvement

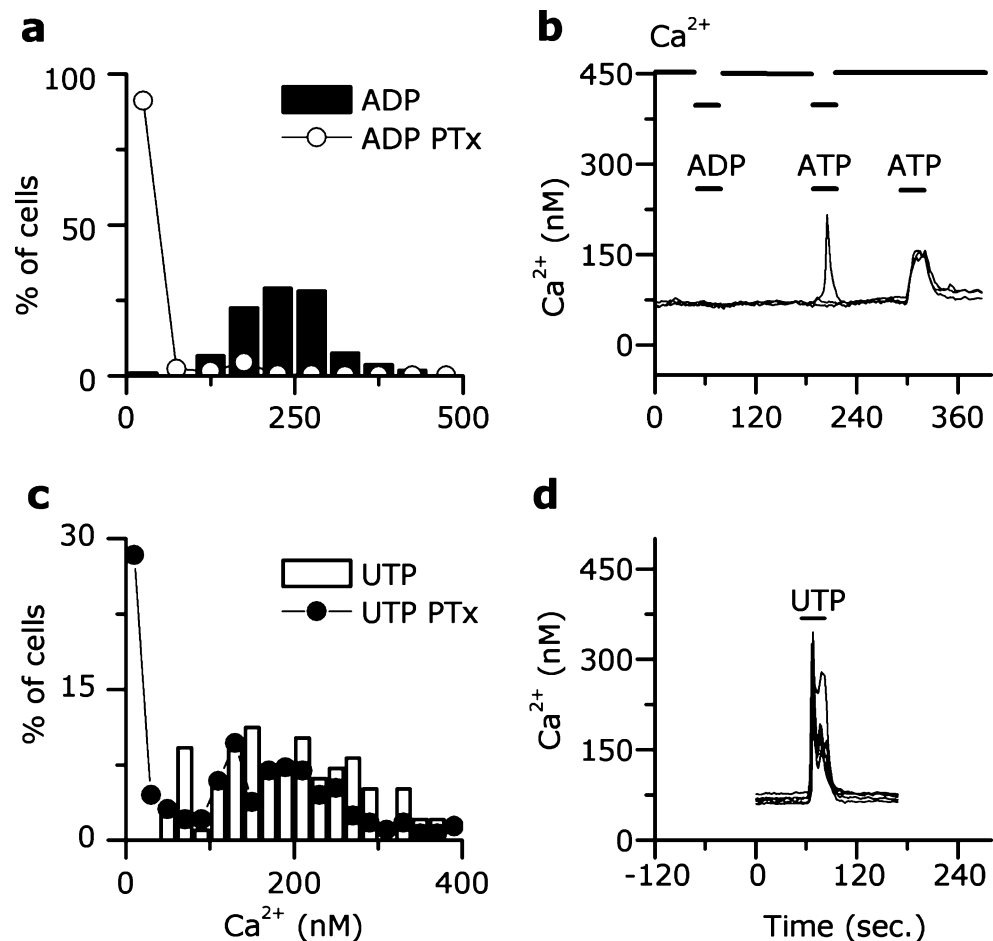
As mentioned above,  $\text{P2Y}$  receptors act by preferentially binding and activating specific hetero-trimeric G proteins. Only 10% of microglial cells treated overnight with PTx were able to show a  $\text{Ca}^{2+}$  response when challenged with ADP (Figure 4a) while 70% of cells maintained a  $\text{Ca}^{2+}$  response to UTP (Figure 4c) though lacking the capacitative plateau (Figure 4d). PTx-treated cells still responding to ADP showed a reduced  $\text{Ca}^{2+}$  signal ( $236 \pm 6$  nM and  $134 \pm 13$  nM in control and treated cultures, respectively) while cells still responding to UTP after PTx-treatment maintained a similar  $\text{Ca}^{2+}$  signal ( $190 \pm 8$  nM versus

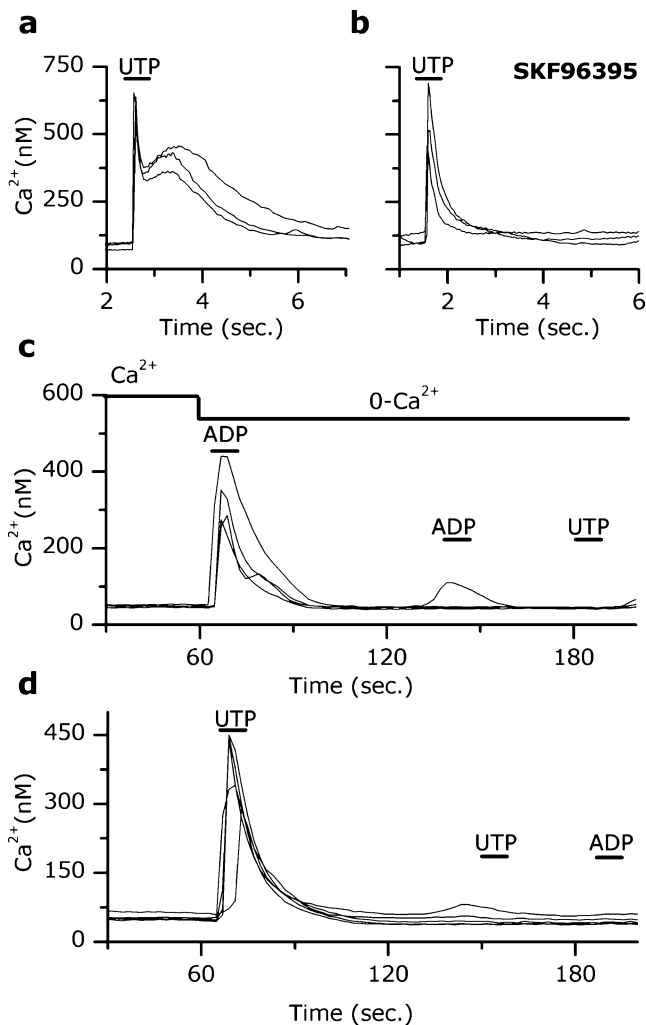
$172 \pm 6$  nM). Involvement of PTx-sensitive  $\text{G}_i$  in the mechanism leading to  $\text{Ca}^{2+}$  rise by ADP further supports the idea that activation of  $\text{P2Y}_{12}$  receptors is responsible for ADP-induced  $\text{Ca}^{2+}$  movements in microglia. When ATP was tested in PTx-treated cultures, it elicited a  $\text{Ca}^{2+}$  transient in only a fraction of cells in the absence of  $\text{Ca}^{2+}$ , and it caused small  $\text{Ca}^{2+}$  rises in most cells when  $\text{Ca}^{2+}$  was present, depicting  $\text{Ca}^{2+}$  influx through ionotropic receptors (Figure 4b).

### Capacitative plateau

According to the above data, a striking difference among the signals triggered by adenine and uracil nucleotides is the main G protein involved. A second difference is the much greater capability of uracil nucleotides to induce, after the rapid  $\text{Ca}^{2+}$  rise, a sustained  $\text{Ca}^{2+}$  plateau sometimes appearing as a second and clearly distinguishable phenomenon (Figure 2). According to extracellular  $\text{Ca}^{2+}$  dependence (data not shown) and blockade by SKF96395 (Figure 5a), it was identified as the capacitative plateau described in a number of cell types. According to the canonical description, its induction is due to the opening of store-operated  $\text{Ca}^{2+}$  (SOC) channels on the plasma

**Figure 4** Pertussis toxin completely inhibits  $\text{Ca}^{2+}$  release induced by adenine nucleotides but not by uridine 5'-triphosphate (UTP). (a), (c)  $\text{Ca}^{2+}$  transients induced by nucleotides (100  $\mu\text{M}$  for 20 s in the absence of  $\text{Ca}^{2+}$ ) were recorded from control microglial cultures and cultures treated overnight with pertussis toxin (PTx) 50 ng/ml. More than 90% of PTx-treated cells were unable to respond to adenosine diphosphate (ADP) ( $n=107-271$ ) while only 30% did not respond to UTP ( $n=98-289$ ). (b) Exemplifying  $\text{Ca}^{2+}$  traces recorded from PTx-treated cultures show the lack of response to ADP, the rare response to ATP in the absence of  $\text{Ca}^{2+}$  and the lower but consistent  $\text{Ca}^{2+}$  transients triggered by ATP in the presence of  $\text{Ca}^{2+}$ , depicting the lack of sensitivity to PTx of the ATP-induced ionotropic response. (d) Exemplifying  $\text{Ca}^{2+}$  traces recorded from PTx-treated cultures showing UTP-induced  $\text{Ca}^{2+}$  signals with amplitudes comparable with those obtained in control conditions but without a sustained plateau





**Figure 5** Nucleotide-induced capacitative Ca<sup>2+</sup> plateau. (a), (b) Uridine 5'-triphosphate (UTP)-induced Ca<sup>2+</sup> transients were recorded from control cells (*left panel*) and cells treated with the store-operated Ca<sup>2+</sup> (SOC) channel blocker SKF96395 (30 μM). The Ca<sup>2+</sup> plateau induced by UTP was abrogated by SKF96395, depicting the nature of the plateau phase, while the Ca<sup>2+</sup> peak (due to Ca<sup>2+</sup> release) was unaffected (*n* = 81). (c), (d) Cross-desensitisation of the adenosine diphosphate (ADP)- and UTP-induced responses. In the absence of Ca<sup>2+</sup>, a series of challenges with the same nucleotide (100 μM for 20 s) was followed by a single challenge with a different nucleotide. Both nucleotides (ADP and UTP) very promptly caused Ca<sup>2+</sup> depletion since in this experiment, after two challenges Ca<sup>2+</sup> release was abolished. Cross-desensitisation of responses elicited by the two nucleotides depicts the fact that the receptors activated by the different nucleotides share the same Ca<sup>2+</sup> stores (*n* = 116–186)

membrane, following (through a not as yet clearly determined mechanism) Ca<sup>2+</sup> depletion in the endoplasmic reticulum. However, this latter mechanism by itself can unlikely account for the induction of SOC opening in microglia since ADP and UTP share the same endoplasmic reticulum (ER) Ca<sup>2+</sup> stores (Figure 5c, d), as depicted by “cross-desensitisation” experiments, and at the dose used both agents are able to efficiently induce TER Ca<sup>2+</sup> depletion. A second mechanism triggered by uracil nucleo-

tides but not by ADP and acting in parallel to store depletion must be involved.

#### Ca<sup>2+</sup> oscillations induced by low doses of nucleotides

In the attempt to describe P2Y signalling in microglia, we have so far depicted some peculiar features of adenine compared with uracil nucleotides, which can cause different functional roles of P2Y subtypes and of their endogenous agonists. Then we wanted to investigate the possibility that differences in the availability of nucleotides for receptor occupancy and activation could cause qualitatively different patterns of signalling and, in turn, functional outcomes. A long-lasting challenge with nucleotides at a concentration of 100 μM (which is a high dose for metabotropic receptors) caused an initial Ca<sup>2+</sup> peak followed by a maintained Ca<sup>2+</sup> rise only slowly declining in the continuous presence of the agonist (Figure 6a,b). On the contrary, when using sub-micromolar concentrations (100–300 nM), a fraction of cells reacted to the challenge with a first Ca<sup>2+</sup> peak. More importantly, a maintained Ca<sup>2+</sup> activity was rarely detected when using ADP (Figure 6c) while a low-frequency ( $\leq 1 \text{ min}^{-1}$ ) oscillatory Ca<sup>2+</sup> activity was detected when cells were challenged with UTP (Figure 6d). In a fraction of cells showing oscillatory Ca<sup>2+</sup> movements, a slow Ca<sup>2+</sup> rise overlapped Ca<sup>2+</sup> oscillations (Figure 7a). When the extracellular Ca<sup>2+</sup> dependence was tested, the slow rise of Ca<sup>2+</sup> disappeared in the absence of extracellular Ca<sup>2+</sup> while the fraction of cells showing oscillatory activity did not change (Figure 7b). These observations are compatible with Ca<sup>2+</sup> movements from intracellular compartments and Ca<sup>2+</sup> entrance as the mechanisms causing oscillatory activity and slow Ca<sup>2+</sup> rise, respectively. Moreover, in the time span evaluated (5–10 min), the frequency of oscillations seemed to be unaffected by Ca<sup>2+</sup> removal, so depicting a mechanism probably independent from extracellular Ca<sup>2+</sup>. The most probable source for Ca<sup>2+</sup> oscillations is the ER. To test this hypothesis, ER were depleted by repeated challenges with high doses of nucleotides or by pre-treatment with the ER Ca<sup>2+</sup>-ATPase inhibitor thapsigargin (Figure 8a, b). In neither of the cases could a subsequent application of a low dose of UTP trigger Ca<sup>2+</sup> oscillations, so confirming that the ER was the source of Ca<sup>2+</sup>.

#### Potential regulatory mechanisms of Ca<sup>2+</sup> oscillations

Once we ascertained the dual nature of the effect of low doses of uracil nucleotides and the ER as the source of Ca<sup>2+</sup> oscillations, we wanted to investigate their possible mechanisms of regulation. According to the previous literature on Ca<sup>2+</sup> oscillations in other cell types, two mechanisms might also apply to microglial cells: an inhibitory mechanisms triggered by capacitative Ca<sup>2+</sup> influx

[34] and a facilitating role played by mitochondria [35]. When a challenge with a low dose of UTP followed previous application of a high dose of the same nucleotide, to induce  $\text{Ca}^{2+}$  influx,  $\text{Ca}^{2+}$  oscillations were not affected (Figure 9a). Neither the percentage of cells showing  $\text{Ca}^{2+}$  oscillations ( $59 \pm 6\%$  and  $51 \pm 12\%$  of the cells in control and after UTP  $100 \mu\text{M}$ , respectively) nor the frequency of  $\text{Ca}^{2+}$  oscillations ( $0.37 \pm 0.04$  and  $0.49 \pm 0.06 \text{ min}^{-1}$ , in control and after UTP  $100 \mu\text{M}$ , respectively) significantly changed after a pre-application of  $100 \mu\text{M}$  UTP. In a further attempt to detect such regulatory mechanism, we used the calcineurin inhibitor cyclosporin A since capacitative  $\text{Ca}^{2+}$  influx was shown to inhibit  $\text{Ca}^{2+}$  oscillation by a mechanism involving calcineurin [34]. Also in this case, neither the percentage of cells showing  $\text{Ca}^{2+}$  oscillations ( $56 \pm 6\%$ ) nor the frequency of  $\text{Ca}^{2+}$  oscillations ( $0.45 \pm 0.06 \text{ min}^{-1}$ ) were affected. We then focused on mitochondria as possible regulators of oscillations. To this aim, we evaluated the effect on oscillatory activity of the mitochondrial uncoupler carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP). After a 2- to 4-min-long pre-treatment with this agent, to cause mitochondrial depolarisation, the percent-

age of cells showing  $\text{Ca}^{2+}$  oscillations dropped to 14%, suggesting a possible role played by mitochondria in the establishment of  $\text{Ca}^{2+}$  oscillations.

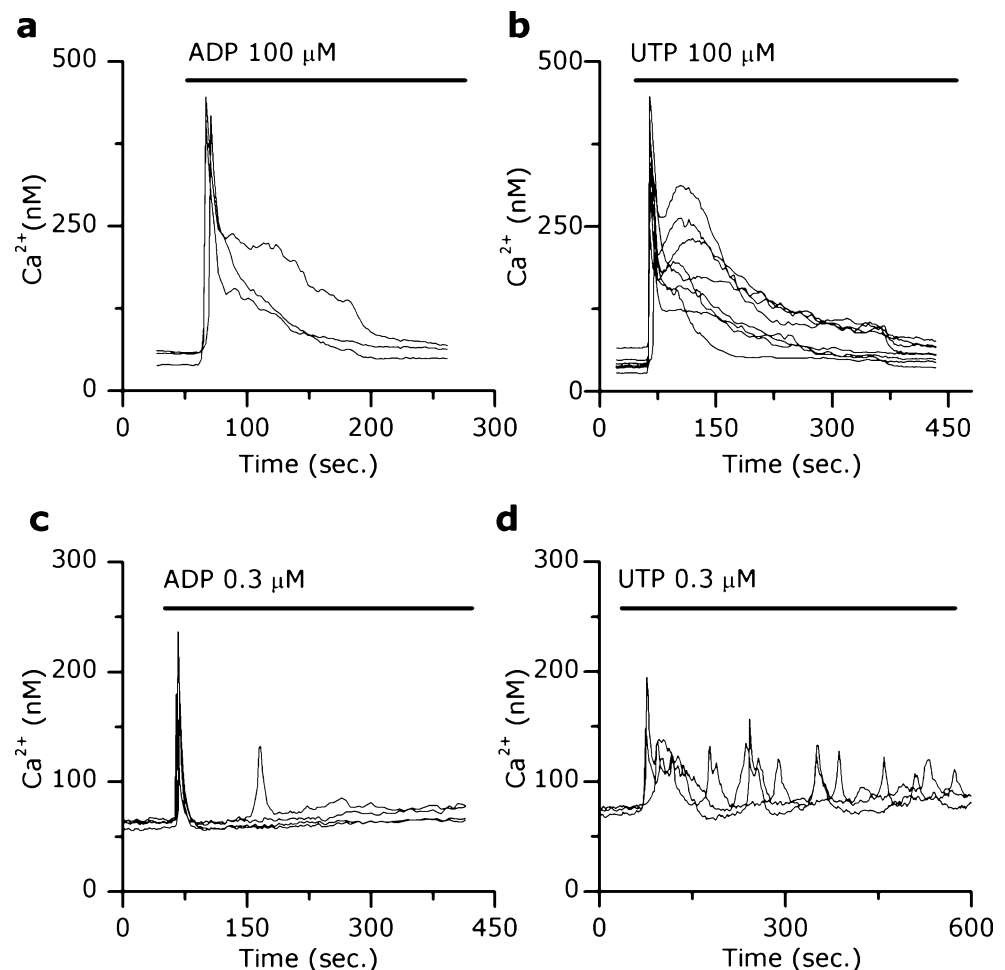
## Discussion

With this study, we wanted to contribute to the understanding of metabotropic P2 receptor signalling in microglia by offering our description of P2Y receptor expression pattern, by depicting some of the features of their signalling and by focusing on the role of agonist availability on the pattern of the  $\text{Ca}^{2+}$  signals.

### Molecular and functional expression profile of P2Y receptors

Since the first electrophysiological reports on microglial cells, nucleotides are known to stimulate both ionotropic and metabotropic receptors. According to these first studies, activation of the former caused inward currents and depolarisation of membrane potential and activation of the latter

**Figure 6** Different patterns of  $\text{Ca}^{2+}$  signals are induced by high compared to low concentrations of nucleotides. (a), (b) Long-lasting challenges with a high dose of nucleotides [adenosine diphosphate (ADP) and uridine 5'-triphosphate (UTP):  $100 \mu\text{M}$  for (2–5 min) were applied to microglial cells. The exemplifying  $\text{Ca}^{2+}$  traces depict the fast  $\text{Ca}^{2+}$  increase followed by a long-lasting and slowly decaying  $\text{Ca}^{2+}$  plateau caused by both nucleotides (left panel: ADP; right panel: UTP). C, D Long-lasting challenges with low doses of nucleotides (ADP and UTP:  $300 \text{ nM}$  for 5–10 min) were applied to microglial cells. As depicted by the exemplifying  $\text{Ca}^{2+}$  traces, ADP triggered a single  $\text{Ca}^{2+}$  peak (c) while UTP caused a long-lasting oscillatory  $\text{Ca}^{2+}$  activity (d)

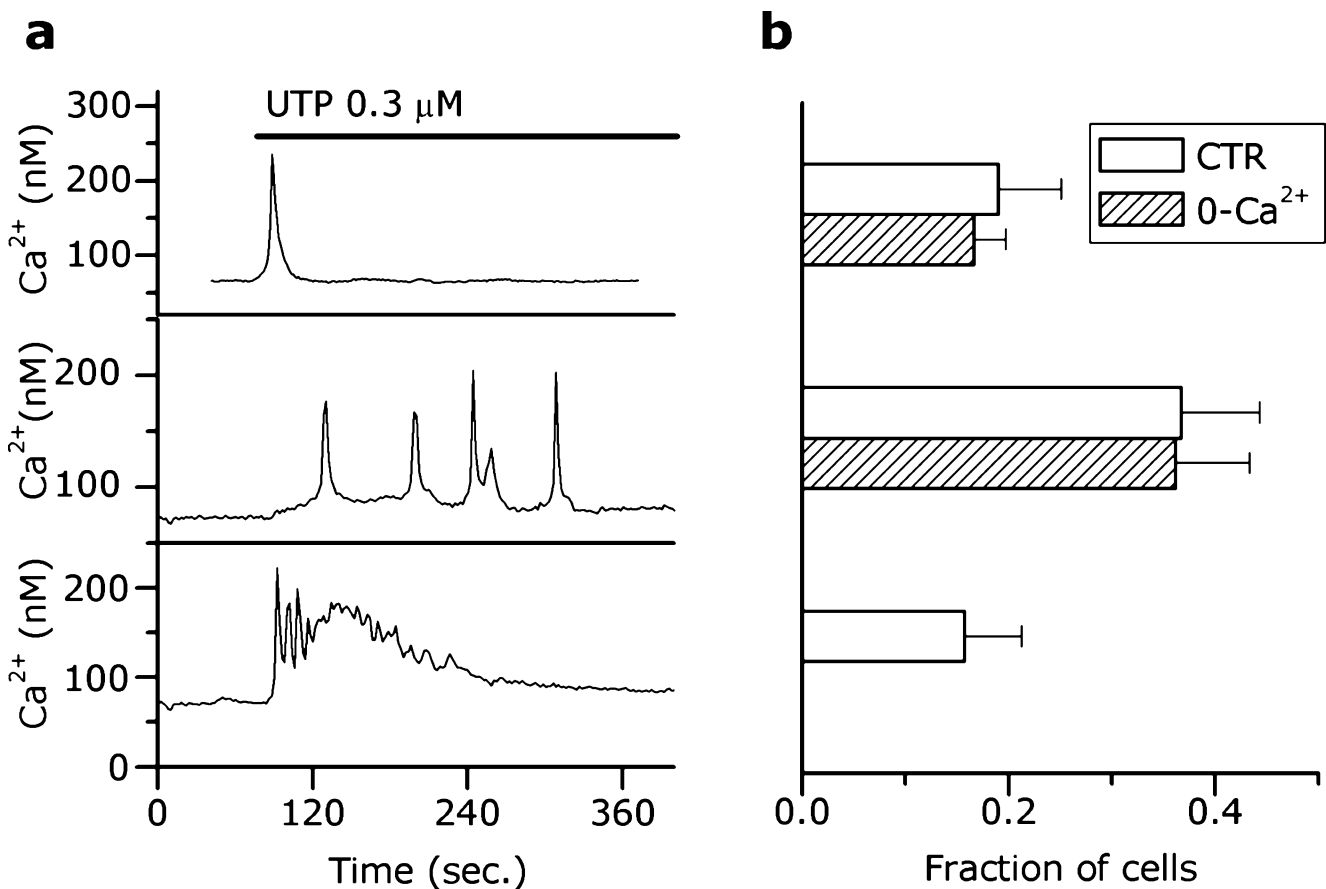




induced a  $K^+$  outward current and  $Ca^{2+}$  release [36–38]. Since then, several insights have been added to the picture of P2 receptor signalling in microglial cells, contributing to delineate a complex array of possible functions. Nevertheless, focused studies on receptor expression, especially of P2Y metabotropic receptors, were missing. Only recently an exhaustive picture of P2 receptor expression in microglia was published, thanks to the work by Verderio and collaborators [39]. Our description of the P2Y receptor profile diverges from the one proposed in the above article for the presence of functional P2Y<sub>1</sub> and P2Y<sub>14</sub> receptors, which we did not detect in our study. P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors were found to co-exist in platelets and both to concur to the control of platelet aggregation [40]. Based on the insensitivity to MRS2179 and on the PTx-induced inhibition of the  $Ca^{2+}$  signal induced by ADP, we depicted P2Y<sub>12</sub> rather than P2Y<sub>1</sub> receptors as the ones responsible for the  $Ca^{2+}$  transients elicited by ADP stimulation. However, we cannot exclude a slight expression of the mRNA coding the P2Y<sub>1</sub> receptor.

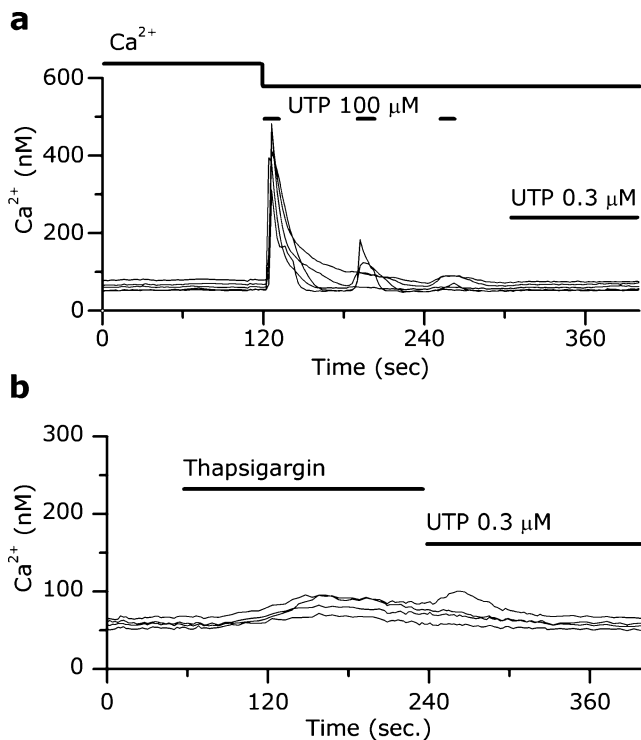
Moreover, as already suggested [39], we depicted the capability of P2Y<sub>12</sub> receptors to move  $Ca^{2+}$  by a mechanism that we describe as PTx sensitive. These aspects of P2Y<sub>12</sub> signalling are particularly interesting if we consider the role these receptors play in microglial chemotaxis induced by adenine nucleotides [23] and the observation that microglia are the only cells belonging to the monocyte/macrophage lineage that express P2Y<sub>12</sub> receptors [41]. If this holds true, P2Y<sub>12</sub> receptors could be the only specific marker distinguishing microglia from peripheral macrophages so far identified.

Regarding P2Y<sub>14</sub> receptors, we found mRNA expression by PCR but did not detect any  $Ca^{2+}$  response when using the endogenous agonist UDP-glucose. Nevertheless, since P2Y<sub>14</sub> receptors are known to activate Gi/o proteins [42], we cannot exclude that the receptor proteins, if expressed, are able to accomplish other functions, such as the control of cyclic adenosine monophosphate (cAMP) accumulation. Interestingly, Bianco et al. [39] described the functional capability of P2Y<sub>14</sub> receptors as highly dependent on cell



**Figure 7**  $Ca^{2+}$  signal induced by low doses of uridine 5'-triphosphate (UTP) only partially depend on the presence of extracellular  $Ca^{2+}$ . (a) When challenged with a low dose of UTP, microglial cells reacted with  $Ca^{2+}$  signals with the following patterns: a single  $Ca^{2+}$  peak, an oscillatory  $Ca^{2+}$  activity and an oscillatory  $Ca^{2+}$  activity superimposed to a slowly raising  $Ca^{2+}$  plateau. (b) Different populations of microglial

cells were challenged with UTP 300 nM in the presence or absence of  $Ca^{2+}$ , and the percentage of cells reacting with the different patterns of  $Ca^{2+}$  signals was calculated ( $n=198-247$ ). The absence of  $Ca^{2+}$  did not affect the percentage of occurrence of the single peak nor of the oscillatory activity while completely abrogated the slow rise of  $Ca^{2+}$



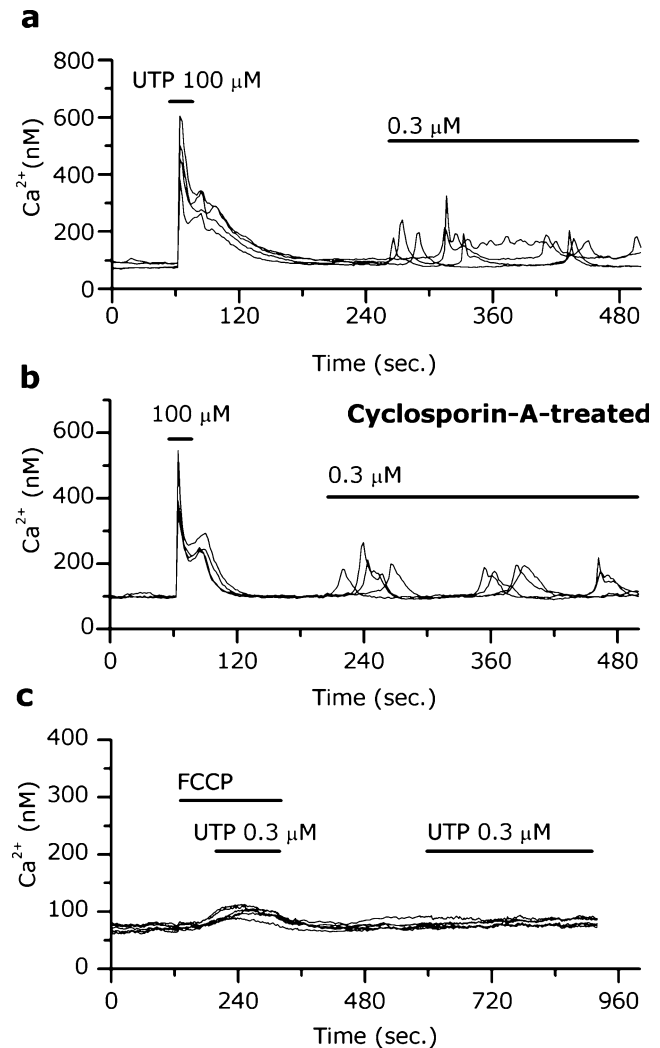
**Figure 8** Oscillatory Ca<sup>2+</sup> activity is abrogated by depleting Ca<sup>2+</sup> from the endothelial reticulum (ER) stores. (a), (b) Ca<sup>2+</sup> depletion from the ER stores was achieved by a repetitive receptor activation (a) or by applying the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitor thapsigargin (b) in the absence of Ca<sup>2+</sup>. Following a series of applications of uridine 5'-triphosphate (UTP) 100 μM or a 2-min-long treatment with thapsigargin (100 nM), 300 nM UTP was applied to microglial cells. The exemplifying Ca<sup>2+</sup> traces show the absence of oscillatory activity following endothelial reticulum (ER) Ca<sup>2+</sup> depletion ( $n=60-94$ )

activation. We can hypothesise that microglial activation promotes P2Y<sub>14</sub> protein expression or coupling of the receptor to Ca<sup>2+</sup> release. We should also consider that culture conditions slightly differing from lab to lab make it illusory to have a completely overlapping picture of a complex signalling system such as the P2Y, even more so in plastic cells such as microglia. Moreover, we found it interesting to consider some other aspects as causes of difference. Among these, the areas of the brain from which cell cultures come (cortex versus hippocampus), the developmental phase (embryonic versus post-natal) and species differences (rat versus murine N9 cell line). All of these aspects should be considered as interesting topics for further, more focused, investigations.

#### Ca<sup>2+</sup> responses induced by adenine versus uracil nucleotides

According to our data, the Ca<sup>2+</sup> signals triggered by adenine and uracil nucleotides differ from two main

aspects: the main G protein involved and the more efficient induction of capacitative influx by uracil nucleotides. We utilised ADP to activate P2Y receptors sensitive to adenine



**Figure 9** Oscillatory Ca<sup>2+</sup> activity is regulated by mitochondrial "energy charge" but not by a previous activation of the capacitative influx. (a) To point out the eventual regulatory role on Ca<sup>2+</sup> oscillations of a high dose of uridine 5'-triphosphate (UTP), an application of 100 μM of the nucleotide was applied prior to an application of 300 nM of the same nucleotide. As depicted by the exemplifying Ca<sup>2+</sup> signals, Ca<sup>2+</sup> oscillatory activity did not differ from that recorded from non-pre-treated cells ( $n=64$ ). (b) To evaluate the regulatory role of calcineurin activated by a previous Ca<sup>2+</sup> influx through capacitative channels, an application of 100 μM UTP was followed by an application of 300 nM of the same nucleotide in the presence of the calcineurin inhibitor cyclosporin-A (1 μM). Also in this case, the oscillatory Ca<sup>2+</sup> activity was not affected ( $n=90$ ). (c) To evaluate the regulatory role of mitochondrial activity on oscillatory Ca<sup>2+</sup> activity, microglial cells were treated with the mitochondrial uncoupler carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (2 μM for 3 min) prior to 300 nM UTP. As shown by the exemplifying Ca<sup>2+</sup> traces, FCCP pre-treatment completely abrogated UTP-induced oscillatory Ca<sup>2+</sup> activity, suggesting the involvement of mitochondria in the regulation of the oscillatory Ca<sup>2+</sup> activity ( $n=273$ )

nucleotides. According to inhibition by PTx, ADP-induced  $\text{Ca}^{2+}$  release occurred by a PTx-dependent mechanism while UTP-induced  $\text{Ca}^{2+}$  signals were almost unaffected by PTx pre-treatment. Involvement of different G proteins determined an early divergence of signal transduction pathways following receptor and G protein activation. In the case of uracil nucleotides, known to act mainly on Gq/11 and here found to be only partially PTx sensitive,  $\text{G}\alpha$  subunit would trigger PLC $\gamma$  activation and  $\text{Ca}^{2+}$  release through IP3 receptor/channels [43]. In the case of P2Y<sub>12</sub> receptors activated by ADP, the  $\text{G}\beta\gamma$  subunit would trigger PLC $\beta$  activation and  $\text{Ca}^{2+}$  release while the  $\text{G}\alpha\text{i}$  subunit would probably cause a concomitant inhibition of adenylyl cyclase and cAMP reduction. However, even if the signalling pathways are so different, they converge to the same ER stores that the two nucleotides, when used at a concentration of 100  $\mu\text{M}$ , deplete with similar efficacy, as depicted by cross-desensitisation experiments. This brings us to the other difference between the  $\text{Ca}^{2+}$  signalling induced by the two receptor types. Depletion of  $\text{Ca}^{2+}$  from the ER stores is known to be the starting signal of induction of the capacitative  $\text{Ca}^{2+}$  influx mainly described in non-excitabile cells, among them microglia [21]. The nature and identity of the agent (or event) following  $\text{Ca}^{2+}$  depletion is still a matter of debate, and it might also be that different mechanisms put in place by ER  $\text{Ca}^{2+}$  depletion act in different cells [44]. In any case, in microglial cells,  $\text{Ca}^{2+}$  depletion might be necessary but certainly is not sufficient to cause capacitative  $\text{Ca}^{2+}$  influx. As already shown [21], uracil nucleotides (UTP and UDP) were clearly more efficient than ADP in inducing the  $\text{Ca}^{2+}$  plateau, which we identified as being due to capacitative influx on the basis of the sensitivity to SKF96395 and extracellular  $\text{Ca}^{2+}$ .

Regardless the mechanisms utilised, the capability to trigger capacitative  $\text{Ca}^{2+}$  influx adds further functional capability to the receptors sensitive to uracil nucleotides, as depicted by the specificity of some functional outcomes linked to capacitative influx besides ER  $\text{Ca}^{2+}$  store replenishment: examples of this specificity are the modulation of some  $\text{Ca}^{2+}$ -dependent adenylyl cyclase isoforms [45] and  $\text{Ca}^{2+}$ -calmodulin-induced activation of nuclear factor of activated T cells (NFAT), which specifically need capacitative  $\text{Ca}^{2+}$  [44].

#### Oscillatory $\text{Ca}^{2+}$ activity induced by low doses of nucleotides

The release of nucleotides can occur not only following cell lysis but also by highly regulated mechanisms in the course of cell activity, such as action potential propagation, synaptic activity and astrocytic  $\text{Ca}^{2+}$  waves [13, 27]. Can the different availability of nucleotides for receptor activation be utilised by microglia as an indicator of the condition

of the host tissue? To address this question, we compared the  $\text{Ca}^{2+}$  signals triggered by P2Y receptors activated by long-lasting applications of low versus high doses of nucleotides. When ADP was applied at a low dose, only a single peak of  $\text{Ca}^{2+}$  was observed in a fraction of cells. On the contrary, when a low dose of UTP was used, we observed a more complex behaviour markedly differing from that induced by a high dose of the nucleotide: while this latter induced a single event of release followed by a long-lasting and slowly decaying phase of capacitative influx, a low dose of UTP (also of UDP: data not shown) caused an oscillatory  $\text{Ca}^{2+}$  activity only rarely superimposed to a slowly rising  $\text{Ca}^{2+}$  influx. Our data do not allow identification of a link between oscillatory release and  $\text{Ca}^{2+}$  influx, but we cannot exclude that oscillatory activity sustained for a time span longer than our experiments would need the contribution of  $\text{Ca}^{2+}$  influx. In recent years, a non-capacitative  $\text{Ca}^{2+}$  influx pathway elicited by low doses of metabotropic agonists, depending on arachidonic acid and responsible for the maintenance of  $\text{Ca}^{2+}$  oscillation, was described in a number of cells [46]. Moreover, calcineurin activation by capacitative  $\text{Ca}^{2+}$  entry was shown to inhibit the non-capacitative entry [34]. However, we did not detect any regulatory role played by a high dose of UTP or by the calcineurin inhibitor cyclosporin-A on the oscillatory  $\text{Ca}^{2+}$  activity.

An increasing body of evidence supports the relationship between cytoplasmic and mitochondrial  $\text{Ca}^{2+}$  changes [47, 48]. Energised mitochondria are known to take up  $\text{Ca}^{2+}$  accumulated in the cytoplasm, mainly thanks to the mitochondrial uniporter, and to release it back by the  $\text{Na}^+/\text{Ca}^{2+}$  exchange system. Not only can mitochondria function as  $\text{Ca}^{2+}$  buffers, they also utilise the ion to regulate their own activity since the  $\text{F}_1\text{F}_0$ -ATP synthase and a number of mitochondrial dehydrogenases are regulated by  $\text{Ca}^{2+}$ . It was suggested that mitochondria could increase the release of  $\text{Ca}^{2+}$  from IP3R channels by rapidly removing  $\text{Ca}^{2+}$  from their mouth, so decreasing  $\text{Ca}^{2+}$ -induced inactivation of the channels. The  $\text{Ca}^{2+}$  taken by mitochondria could be recycled back to the  $\text{Ca}^{2+}$  ATPase on the ER to allow prompt refilling of the stores [49]. This would finally maintain the efficiency of the release process. When we utilised the protonophore FCCP, known to cause mitochondrial depolarisation, we could clearly see a decrease of oscillatory  $\text{Ca}^{2+}$  release induced by low doses of UTP. Interestingly, FCCP did not affect the potent release induced by a high dose of UTP (data not shown), as if for long-lasting oscillatory activity to occur in the presence of low IP3 synthesis a fine regulation of cytoplasmic  $\text{Ca}^{2+}$  was needed to maintain IP3R channels available to open. This is one of the first indications of the involvement of mitochondria in the control of cytoplasmic  $\text{Ca}^{2+}$  changes in microglia, and further investigation is

needed to come to an unambiguous interpretation of this result since FCCP can have a number of outcomes together with the decrease of  $\text{Ca}^{2+}$  uptake by mitochondria, such as ATP depletion and pH changes. In any case, it is noteworthy that during microglia activation, mitochondria change their cellular distribution, morphology and internal organisation, probably to better sustain energy-consuming functions of activated microglia [50].

In this study, we pointed out the capability of P2Y receptors to cause different patterns of cytoplasmic  $\text{Ca}^{2+}$  changes involving capacitative  $\text{Ca}^{2+}$  influx and intracellular  $\text{Ca}^{2+}$  mobilisation, depending not only on the type but also on the concentration of the agonist. Intracellular  $\text{Ca}^{2+}$  changes can independently affect a multitude of functions and events depending on the vicinity of the  $\text{Ca}^{2+}$  sources to specific target proteins, on the concentration reached and also on the time evolution of the  $\text{Ca}^{2+}$  variations. It is known that sustained and oscillatory  $\text{Ca}^{2+}$  changes can differently affect target proteins (for a review, see 51). Protein kinases, phospholipases and transcription factors are examples of the multitude of proteins that can be differently regulated by  $\text{Ca}^{2+}$ .

In conclusion we speculate that the different patterns of  $\text{Ca}^{2+}$  signals elicited by different concentrations of nucleotides would render P2Y receptors capable of signalling to microglia the state of the host tissue and, hopefully, regulate microglial responses in concert with the needs of the tissue.

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