British Journal of Pharmacology (2004) 143, 525–532
www.nature.com/bjp

Evidence for $P2Y_1$, $P2Y_2$, $P2Y_6$ and atypical UTP-sensitive receptors coupled to rises in intracellular calcium in mouse cultured superior cervical ganglion neurons and glia

[1]Jennifer A. Calvert, [1]Amelia E. Atterbury-Thomas, [2]Catherine Leon, [1]Ian D. Forsythe, [2]Christian Gachet & *,[1]Richard J. Evans

[1]Department of Cell Physiology & Pharmacology, University of Leicester, Medical Sciences Building, University Road, Leicester LE1 9HN and [2]INSERM, U311, Establissment Francais du Sang (EFS) Alsace, 67065 Strasbourg Cedex, France

1 P2Y receptors are expressed in the nervous system and are involved in calcium signalling in neurons and glia. In the superior cervical ganglion (SCG), RT–PCR analysis indicated the presence of $P2Y_{1,2\&6}$ receptors. Rises in intracellular calcium in response to P2Y receptor stimulation were determined from adult mouse cultured SCG neurons and glia.

2 ADP evoked suramin (100 μM)- and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 30 μM)-sensitive rises in intracellular calcium in $\sim$80% of SCG neurons ($EC_{50}\sim 20\,\mu$M). ADP-evoked responses were abolished in neurons from $P2Y_1$ receptor-deficient mice (responses to UTP were unaffected).

3 The pyrimidines UTP ($EC_{50}\sim 85\,\mu$M) and UDP ($EC_{50}>90\,\mu$M) evoked PPADS- and suramin-sensitive responses in $\sim$70 and $\sim$20% of SCG neurons, respectively.

4 In SCG glial cells, ADP ($EC_{50}\sim 30\,\mu$M) evoked calcium responses in $\sim$50% of glia. These were suramin and PPADS sensitive and essentially abolished in SCG glial cells cultured from adult $P2Y_1$ receptor-deficient mice.

5 UTP ($EC_{50}\sim 25\,\mu$M) and UDP ($EC_{50}>200\,\mu$M) evoked suramin- and pyridoxalphosphate-6-azophenyl-2′,5′-disulphonate-sensitive rises in calcium in $\sim$60 and 20% SCG glial cells, respectively.

6 These results indicate the presence of several P2Y receptors coupled to an increase in intracellular calcium in the SCG: ADP-sensitive $P2Y_1$ receptors and UDP-sensitive $P2Y_6$ receptors in SCG neurons and glial cells, a novel UTP-sensitive P2Y receptor in SCG neurons and UTP- and ATP-sensitive $P2Y_2$ receptors in SCG glia.
British Journal of Pharmacology (2004) **143,** 525–532. doi:10.1038/sj.bjp.0705959

Keywords: SCG; purinergic; P2Y; ADP; UTP; calcium; neurons; glia; knockouts

Abbreviations: DRG, dorsal root ganglia; HBSS, Hank's buffered salt solution; L-15, Leibovitz's-15; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; iso-PPADS, pyridoxalphosphate-6-azophenyl-2′,5′-disulphonate; SCG, superior cervical ganglion

Introduction

P2 receptors for nucleotides are expressed widely in the nervous system and are involved in a range of signalling events. They can be divided into ionotropic P2X receptors and metabotropic P2Y receptors. G-protein-coupled P2Y receptors have been shown to be involved in the regulation of neuronal excitability (Boehm, 2003), mediating calcium waves in glial cells (Fam *et al*., 2003) and signalling between neurons and glia (Fields & Stevens, 2000). Genes encoding seven nucleotide-sensitive P2Y receptors have been identified. An additional member of the family ($P2Y_{14}$) has been identified based on sequence homology and this receptor is activated by UDP-glucose (Abbracchio *et al*., 2003). $P2Y_{1,2,6,12\&13}$ receptors are thought to be expressed in the nervous system, whereas $P2Y_4$ and $P2Y_{11}$ have to date not been detected in neuronal tissues (Boehm, 2003). A putative $P2Y_{15}$ receptor that is adenosine and AMP sensitive has been suggested (Inbe *et al*., 2004); however this G-protein-coupled receptor has also been shown to be activated by citric acid cycle intermediates (He *et al*., 2004). The properties of P2Y receptors have been determined in recombinant systems and the different subtypes can be distinguished based on their pharmacology and signalling characteristics (see Table 1).

The superior cervical ganglion has been used as a model of a peripheral sympathetic ganglion. These ganglia receive innervation from the spinal cord and send projections to the lacrimal gland, salivary gland and dilator pupillae muscles in the eye. Studies on rat superior cervical ganglion (SCG) have looked at the role of P2Y receptors in mediating changes in membrane potential, transmitter release and regulation of membrane currents. These approaches have documented the presence of neuronal P2Y receptors for ADP, UTP and UDP (Connolly *et al*., 1993; Boehm *et al*., 1995; Bofill-Cardona *et al*., 2000) on rat SCG neurons. The SCG glial cells are also likely to express P2Y receptors. For example, in other glial preparations, functional $P2Y_1$ and $P2Y_2$ receptors have been reported (e.g.

*Author for correspondence; E-mail: RJE6@le.ac.uk
Advance online publication: 4 October 2004

Table 1 Summary of the properties of P2Y receptors in comparison to the characteristics seen in the SCG

	G-protein coupling	*Agonist sensitivity*	*Antagonist sensitivity*	*Neuronal expression*	*RT–PCR in SCG (this study)*	*Tissue correlates in this study*
$P2Y_1$	$G\alpha_q$	ADP	Suramin PPADS	Detected	Present	SCG and DRG neurons SCG and DRG glia
$mP2Y_2$	$G\alpha_q$, $G\alpha_o$	ATP = UTP	Suramin PPADS	Detected	Present	SCG glia
$mP2Y_4$	$G\alpha_q$	ATP = UTP	PPADS	Not detected	Undetectable	
$mP2Y_6$	$G\alpha_q$, $G\alpha_o$	UDP > ADP	Suramin PPADS	Detected	Present	SCG and DRG neurons SCG and DRG glia
$hP2Y_{11}$	$G\alpha_q$, $G\alpha_o$, $G\alpha_s$	ATP = UTP > ADP	Suramin	Not detected	ND	
$P2Y_{12}$	$G\alpha_{i/o}$	ADP	Suramin Clopidogrel Ticlopidine	Detected	Present	
$mP2Y_{13}$	$G\alpha_{i/o}$	ADP > ATP	Suramin PPADS	Detected	Present	
$mP2Y_{14}$	$G\alpha_{i/o}$	UDP-glucose > UDP-galactose	ND	ND	ND	

Where possible, information is given for the murine homologue (m). Where these are unavailable, information is either for human receptors (h) or a combination of species (no prefix). ND = not determined. For references on pharmacology and signalling of P2Y receptors, see Ralveic & Burnstock (1998) and Dubyak (2003).

James & Butt, 2002; Fam *et al*., 2003). We have recently shown that SCG neurons express a heterogeneous population of P2X receptors (Calvert & Evans, 2004); however the complement of P2Y receptors in mouse SCG neurons and glia, and whether this also shows heterogeneity, remains to be determined.

Calcium homeostasis/signalling is important in both neurons and glia. $P2Y_{1,2,4,6\&11}$ receptors couple to phospholipase C and generation of IP_3 (Dubyak, 2003). In turn, IP_3 stimulates calcium release from intracellular stores. Our RT–PCR studies suggest that $P2Y_{1,2\&6}$ receptors are expressed in the SCG. In this study, we have used fluorescent calcium imaging to determine the functional complement of P2Y receptors in both SCG neurons and glia from the mouse. P2Y receptors were heterogeneously expressed in the SCG and characterization of the pharmacological properties and utilization of tissues from $P2Y_1$ receptor-deficient mice established roles for $P2Y_1$ and $P2Y_6$ receptors in neurons and glia, $P2Y_2$ receptors in glia and a novel UTP-sensitive P2Y receptor in neurons.

Methods

Primary cell culture

The primary cell cultures were performed as described previously (Calvert & Evans, 2004). Briefly, MF1 (majority of studies) or C57BL/6 mice (studies with ADP and $P2Y_1$ receptor-deficient mice) of either sex were given a lethal interperitoneal injection of pentobarbitone, and death was confirmed by femoral exsanguination. $P2Y_1$ receptor-deficient mice were as described previously (Leon *et al*., 1999). SCG or dorsal root ganglia (DRG) were removed immediately, cut in half and placed in ice-cold Hank's buffered salt solution (HBSS). The tissue was placed in 0.1% L-cysteine and 0.05% papain in HBSS and digested for 15 min at 37°C. It was then transferred to 0.05% collagenase and 4% dispase in HBSS for 40 min for SCG or 20 min for DRG at 37°C. The pieces of ganglia were washed with Leibovitz's-15 (L-15) media before being placed in 2.5 ml feeding media (L-15 media supplemented with 1% fetal calf serum, 0.22% sodium bicarbonate, 0.54% glucose, 0.01 ng ml^{-1} nerve growth factor, 30 U ml^{-1} penicillin and 30 μg ml^{-1} streptomycin). Mechanical trituration was used to separate the cells, which were seeded onto poly-D-lysine-coated 16 mm coverslips and incubated for 1.30 h before a further 1.5 ml of feeding media was added.

Fluorescent calcium imaging

Cells were kept in culture for up to 3 days, and the calcium imaging protocol described previously was used (Calvert & Evans, 2004). Cells were ester loaded with Fluo-3-AM (final concentration 1 μM) for 30 min at 37°C. The coverslips were washed for 30 min at 37°C in extracellular perfusion solution containing (mM): NaCl, 150; HEPES, 10; glucose, 11; KCl, 2.5; $CaCl_2$, 2.5; $MgCl_2$, 1; pH 7.3 (NaOH). Argon laser excitation of the dye was at 488 nm and emission was captured at wavelengths greater than 510 nm. Neurons were imaged using a Fluoview FV300 confocal microscope at room temperature. Real-time fluorescence was captured by Olympus Fluoview v4.2 software at a frequency of 0.5 Hz, for a continuous time period of ~360 s. Agonist application was *via* fast-flow U-tube (Evans & Kennedy, 1994) for 40 s after 30 s of baseline fluorescence was acquired. Agonist applications occurred at an interval of 15 min. We have shown previously that concentration–response curves to ATP at P2X receptors were identical whether generated from patch clamp studies to measure directly P2X receptor currents or in Fluo-3 calcium imaging studies (Calvert & Evans, 2004); this indicates that in our studies the calcium indicator Fluo-3 can be used to give an accurate estimate of drug potency. Antagonists were applied for 8 min prior to test solution application and had no effect on basal calcium levels. Extracellular solution changes were performed 2 min prior to agonist application to allow complete exchange of the bath solution.

Neurons were identified visually. Neuronal viability was confirmed by Ca^{2+} increase in response to 60 mM K^+ perfusion solution and only cells that gave an increase in calcium were included in the analysis (~85% of neurons). Glial cells were identified visually. Cells that had artefacts due to mechanical stimulation (as described previously; Calvert & Evans, 2004) were discounted from the analysis. Average background fluorescence was measured from a cell-free area of the field of view. Baseline measurements were calculated as a mean of the 10 s of recording prior to agonist application. Neuronal and

glial traces were corrected for background, and then expressed as a ratio of the baseline, to compensate for differential loading of the cells (increase in fluorescence are expressed as self-ratio). Comparisons were made between the normalized peak calcium changes under different agonist conditions.

RT–PCR

Total RNA was isolated from SCG and DRG using the RNeasy Mini Kit (Qiagen, U.K.). The RNA was treated with DNase I (Sigma-Aldrich, Dorset, U.K.) to remove any DNA contamination. Half of the purified RNA was reverse transcribed to cDNA using Superscript II-reverse transcriptase (Invitrogen, Paisley, U.K.), and the remainder of the RNA was used as a negative control to ensure there was no genomic contamination. The primers used were as follows: $mP2Y_1$: forward: TGG CGT GGT GTA CCC TCT CAA GTC; reverse: ACC GTG CTC GCA AAT TCA TCG TT; expected size: 410 bp; $mP2Y_2$: forward: ACC AGC GTG CGG GGA ACC; reverse: GCA TCT CGG GCA AAG CGG ACA AGT; expected size: 440 bp; $mP2Y_4$: forward: TGC CTC GTG CCC AAC CTC TTC TTT; reverse: CAG TTG TTC GGC GCT TAG GTG TGC; expected size: 499 bp; $mP2Y_6$: forward: CCT GGC ACT GGC GGA CCT GAT; reverse: GGC GGG CCA TAC TGG; expected size: 425 bp; $hP2Y_{11}$: forward: GGG ACT TCC TGT GGC CCA TAC TGG; reverse: CGT GGT CTG CTG TCC CCA GAC AC; expected size: 510 bp. PCR reactions were carried out with 2 μl of cDNA and a final concentration of 500 nM of each primer. Amplification took place in a Techne Genius PCR machine using the following protocol: denaturation at 94°C for 5 min; repeated cycles of denaturation at 94°C, followed by annealing at 57°C and extension at 72°C, each for 30 s; final extension at 72°C for 10 min. A total of 35 cycles were used in most cases, except for tyrosine hydroxylase (TH) and β-actin amplification, for which 30 cycles were used. The amplification products were examined by electrophoresis on a 1% agarose gel and visualized using ethidium bromide under UV light. Positive controls used were TH for confirmation of the sympathetic nature of the tissue, and β-actin was used to confirm that there was no genomic DNA contamination (Calvert & Evans, 2004). The primers for β-actin were designed such that they include an intron from the genomic sequence. Amplification products from genomic DNA would contain the intron and therefore have a larger size than expected. Amplification of mRNA would be the correct size due to removal of the intron by splicing. Given the nature of the SCG sample, the possibility that some of the P2Y receptor subunits could have been amplified from non-neuronal cells cannot be discounted.

Data analysis

Statistical tests were performed using an unpaired *t*-test, with a *P*-value of <0.05 considered as significant. All data are expressed as a percentage of the mean agonist responses measured before and after the test solution, except in the case of the antagonists, in which case it is the percentage of the initial agonist response. Data are expressed as the mean $\pm$ standard error of the mean, unless otherwise stated. Data on neurons and glia were collected from at least three animals.

Concentration–response data was collected from individual neurons at three or more concentrations of agonist. Data were normalized to the maximal response. A maximal concentration for all agonists used was 1 mM. Data were fitted in Origin 6 using the Hill equation: $y=(V_{max}x^n)/(EC_{50}^n+x^n)$, where V_{max} is the maximum response (100%), n the Hill coefficient, and EC_{50} the concentration required to evoke a half-maximal response. Where saturation of the concentration response curve was not seen at 1 mM, an estimate of the maximal response using origin was obtained and used to estimate the EC_{50} value. This is likely to be an underestimate and is quoted as '$EC_{50}>$ value' in the text to indicate this.

Results

$P2Y_{1,2,4,6\&11}$ receptors couple to phospholipase C, generation of IP_3 and a rise in intracellular calcium (Dubyak, 2003). In this study, the complement of $G\alpha_q$-coupled P2Y receptors was determined in cultured adult SCG using a combination of molecular and functional studies. RT–PCR studies using P2Y subtype-selective primers amplified products corresponding to $P2Y_{1,2\&6}$ receptors but not the $P2Y_4$ receptor from the SCG (Figure 1) and DRG (data not shown). The sequence for the mouse $P2Y_{11}$ receptor has yet to be cloned, and primers for $hP2Y_{11}$ were ineffective. There was no difference in the P2Y receptors identified by RT–PCR between intact ganglia and those that had been placed in tissue culture. This indicates that the culture procedure does not modify the expression of P2Y receptors in these ganglia and that these cultured cells provide a good model to study calcium imaging in peripheral ganglia. In calcium imaging studies, we determined the response of SCG neurons and glial cells to the P2 receptor agonists ATP, ADP, UTP and UDP and the antagonists suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and pyridoxalphosphate-6-azophenyl-2′,5′-disulphonate (iso-PPADS) in order to identify functional P2Y receptors in the SCG.

SCG neuron P2 receptors

The agonists ATP, ADP, UTP and UDP (all 100 μM) evoked rises in intracellular calcium in SCG neurons (Table 2). The peak responses corresponded to $\sim$50–75% of the calcium rise evoked by depolarization to 60 mM KCl (mean amplitude 1.4 ± 0.03, $n=466$). In all cases, the peak rise in intracellular calcium (occurring at 10–17 s) was not sustained during agonist application and generally decayed by 30–50% during the 40 s application (Figures 2–4). However, there was a

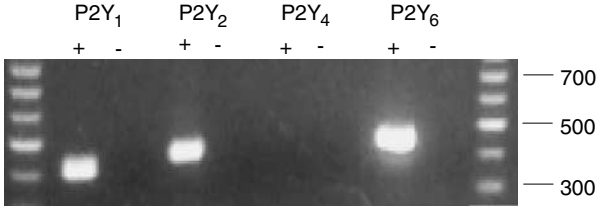

Figure 1 RT–PCR amplification products from SCG using subunit-selective P2Y receptor primers. Products were run on a 1% agarose gel and visualized using ethidium bromide. The gel shows amplification of message for $P2Y_{1,2\&6}$ receptors but not for $P2Y_4$ receptors. + indicates the presence of reverse transcriptase and − indicates that no bands were amplified in the absence of reverse transcriptase showing there is no DNA contamination of the samples.

Table 2 Summary of results for SCG neurons and glia

	Percentage of cells responding	*Number of cells responding*	*Amplitude (% increase over basal)*	*EC_{50}* (μM)	*pEC_{50}*	*Ca^{2+} free (% of control)*	*Suramin (% of control)*	*PPADS (% of control)*	*Iso-PPADS (% of control)*
Neurons									
ADP	82.6	328/397	29±2	~22	~4.7±0.1	97.6±8.7	28.2±6.7	18.6±10.1	7.3±2.4
UTP	68.1	94/138	24±4	~85	~4.2±0.1	98.5±9.7	19.7±14	52.5±10.4	34.1±34.1
UDP	17.7	32/181	19±11	>90	~5.9±0.1	134±25	0± 0	0±0	0±0
Glia									
ADP	48.4	62/128	64±11	~28	~4.5±0	80.2±26.5	22.9±8.1	9.3±9.3	2.2±1.5
UTP	60	30/50	67±16	~25	~4.6±0	94.6±17.2	23.5±14	41.7±13.8	62.2±37.2
UDP	17.1	14/82	82±15	>172	~6.5±2.4	79.2±19.9	4.8±4.8	0±0	0±0

The table shows cells responding, the percent increase over basal, the EC_{50} for each agonist, the calcium response in nominally free extracellular calcium and the effects of the antagonists suramin, PPADS and iso-PPADS (expressed as % of the response to 100 μM agonist).

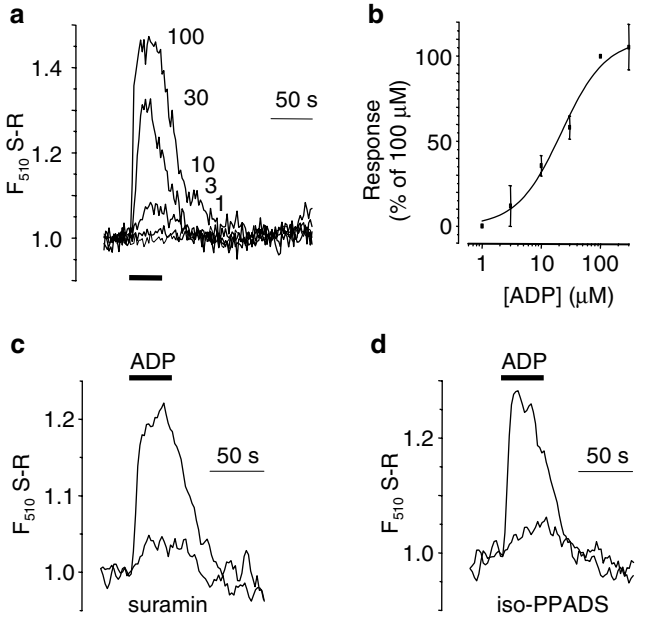

Figure 2 Characteristics of the ADP response in SCG neurons. (a) Example traces from an individual neuron in which multiple concentrations of ADP (μM) were tested. (b) Concentration–response curve for ADP. Mean EC_{50} ~22 μM. (c) The response to ADP (100 μM) was reduced to 28.2±6.7% ($n=23$) of control in the presence of suramin (100 μM). (d) Iso-PPADS (30 μM) caused an inhibition to 7.3±2.4% ($n=23$) of control of the response to ADP (100 μM).

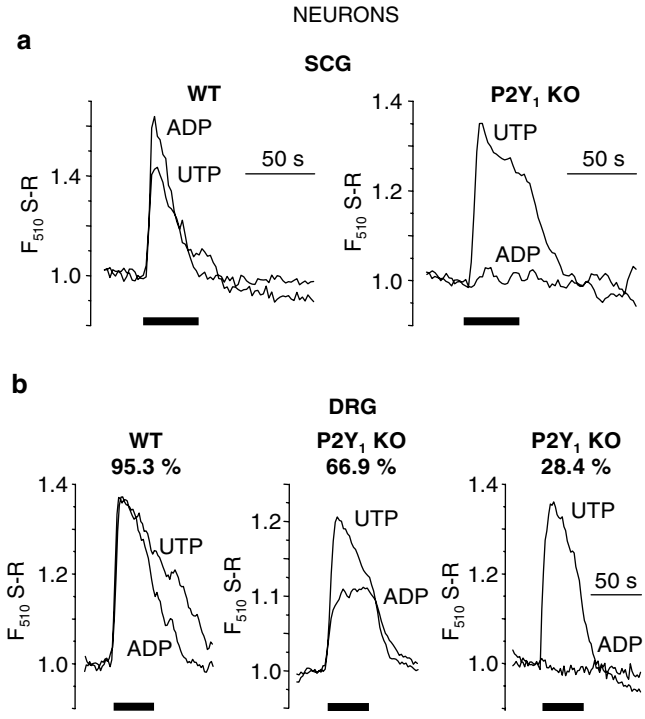

Figure 3 ADP evoked calcium signalling in neurons from WT and $P2Y_1$ receptor-deficient mice. (a) ADP and UTP (both 100 μM) evoked rises in intracellular calcium from SCG neurons. The response to ADP was abolished in neurons from $P2Y_1$ receptor-deficient mice; however, the response to UTP was unaffected. (b) ADP and UTP (both 100 μM) evoked responses in DRG neurons. In $P2Y_1$ receptor-deficient mice, the response was reduced by ~75% in 67% of neurons and in the remainder ADP responses were abolished; however, UTP-evoked responses were unaffected in the $P2Y_1$ receptor-deficient mice.

marked difference in their characteristics, depending on the agonist, including the proportion of cells responding (see below), suggesting that there is heterogeneity in expression of the receptors within SCG neurons.

ATP (100 μM) was the most effective agonist (mean increase in calcium self-ratio of 1.54±0.03) evoking responses in ~80% of neurons. Previous studies have shown that ATP acts through P2X receptors in SCG neurons to mediate a rise in intracellular calcium and that these responses are abolished by removal of extracellular calcium (Calvert & Evans, 2004). In this study, removing calcium from the extracellular medium had no effect on the responses to ADP, UTP or UDP but abolished the response to ATP (all agonists 100 μM) (Table 2). These results indicate that ADP, UTP and UDP mediate their actions through the release of calcium from intracellular stores by the activation of $G\alpha_q$-coupled P2Y receptors. These results are consistent with the lack of effect of these agonists at P2X receptors and the reduction by >80% of the calcium rise following depletion of calcium stores with cyclopiazonic acid (Calvert & Evans, 2004) (data not shown). ATP acts solely through P2X receptors in SCG neurons and shows that the ATP-sensitive mouse $P2Y_2$ and $P2Y_4$ receptors (Dubyak, 2003) (Table 1) are not involved in mediating P2Y receptor-mediated calcium increases in SCG neurons.

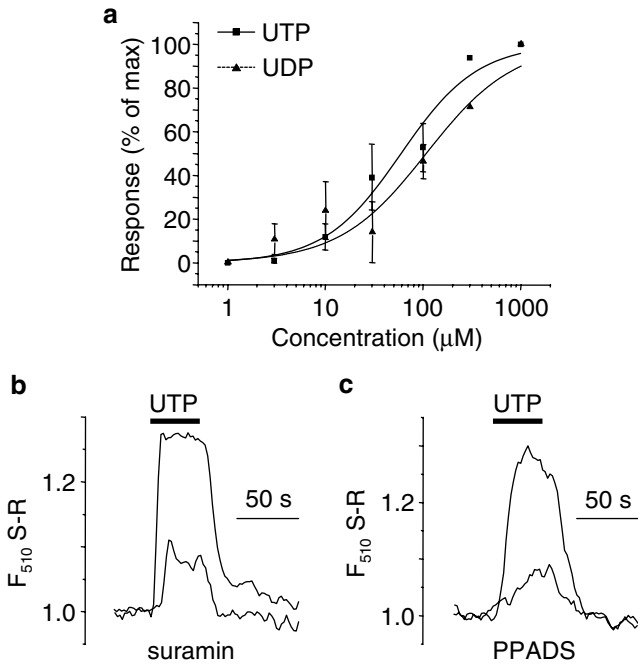

Figure 4 Characteristics of the UTP and UDP responses in SCG neurons. (a) Average concentration–response curves for UTP and UDP, plotted as % of the maximal agonist concentration tested (1 mM). Mean EC_{50} ~85 and >90 μM, respectively. (b) Suramin (100 μM) caused a reduction of the UTP (100 μM) response to 19.7±14% ($n=8$) of control. (c) PPADS (30 μM) caused an inhibition of the UTP control response (100 μM).

Characterization of the ADP-sensitive $P2Y_1$ receptor in SCG neurons

ADP (100 μM) evoked responses in 82.6% of neurons with a mean peak increase in fluorescence of ~29%. The effects of ADP were concentration dependent with an EC_{50} of ~20 μM (Figure 2). The purinergic antagonists suramin (100 μM), PPADS and iso-PPADS (both 30 μM) reduced the ADP (100 μM)-evoked rise in intracellular calcium (Figure 2, Table 2). The $P2Y_1$ receptor antagonist MRS2179 (Boyer *et al*., 1998) also reduced the responses to ADP (100 μM) to 44.9±7.0 and 16.6±4.5% of control values for MRS2179 at 1 and 10 μM, respectively ($n=22$).

To determine the contribution of $P2Y_1$ receptors to the ADP-evoked responses, we compared SCG neurons from wild-type (WT) and $P2Y_1$ receptor-deficient mice (Figure 3a). ADP-responsive neurons were essentially abolished in SCG neurons from $P2Y_1$ KO mice. In contrast, there was no effect on the response to UTP between WT and $P2Y_1$ KO neurons (WT: 64% responded, 89/138, amplitude of 1.48±0.06; KO: 61% responded, 138/226, amplitude of 1.35±0.05). The residual response to ADP in ~1% of neurons (3/226) most likely reflects some interconversion of ADP to ATP and activation of endogenous P2X receptors. These results indicate that the $P2Y_1$ receptor underlies the ADP-sensitive rise in intracellular calcium in SCG neurons.

$P2Y_1$ receptors do not account for all ADP-mediated calcium rises in sensory neurons

$P2Y_1$ receptors are expressed in DRG (Bao *et al*., 2003), thought to be involved in sensation (Nakamura & Strittmatter, 1996), and may be upregulated in DRG following injury models (Xiao *et al*., 2002). We therefore took advantage of the $P2Y_1$ receptor-deficient mouse to determine whether ADP-evoked rises in intracellular calcium in DRG neurons, like those for the SCG, were mediated by $P2Y_1$ receptors (Figure 3b). ADP (100 μM) evoked responses in ~95% of DRG neurons (121/127) with a mean increase in calcium self-ratio of 1.55±0.13 ($n=121$). For $P2Y_1$ KO mice, the percentage of cells responding to ADP was reduced to 66.9% (93/139), and the amplitude of these responses was also significantly reduced by ~75% (to a self-ratio of 1.13±0.02, $n=93$, range 1.01–1.97) (ADP evoked responses in WT DRG glial cells, but had no effect on calcium levels in DRG glial cells from KO mice, $n=21$). UTP (100 μM)-evoked responses were essentially the same for WT (76% responded, 136/179) and KO mouse (77%, 108/139) DRG neurons (1.35±0.04 and 1.4±0.05 peak increase in self-ratio for WT and KO respectively, $n=136$, 108). These results demonstrate that $P2Y_1$ receptors contribute substantially to the ADP-evoked responses in DRG, but in contrast to the SCG are not solely responsible for the ADP-evoked response in all neurons.

Characterization of the UTP-sensitive response in SCG neurons

UTP (100 μM) evoked responses in ~68% of neurons with a mean peak increase in calcium of 24% (the amplitude of UTP responses from these MF1 mice is lower than that of UTP from the C57/Bl6 mice used in the ADP and $P2Y_1$ receptor-deficient studies detailed above and most likely reflects differences based on the background strain of the mouse). UTP had an EC_{50} of ~85 μM (Figure 4a, Table 2). The UTP (100 μM) response was reduced by the purinergic antagonists suramin (100 μM), PPADS (30 μM) and iso-PPADS (30 μM) (Figure 4b, Table 2). These pharmacological properties, in particular the lack of ATP-sensitive P2Y receptors in SCG neurons, do not fit with the currently identified profiles of murine P2Y receptors.

Characterization of the UDP response of SCG neurons

In 18.8% of neurons tested, 100 μM UDP evoked peak increases in calcium of ~19% over basal (Table 2). These neurons were also responsive to UTP (100 μM) (13/15 UDP-sensitive neurons were also sensitive to UTP). The UDP effects were concentration dependent with an $EC_{50}>90$ μM and were abolished by suramin (100 μM), PPADS (30 μM) and iso-PPADS (30 μM) (Table 2). These results are consistent with the properties of murine $P2Y_6$ receptors.

Glial P2Y receptors

Glial cells were also present in the SCG cultures and we have characterized the properties of P2Y receptors expressed in these cells. ATP, ADP, UTP and UDP (all 100 μM) evoked increases in intracellular calcium. The UDP-, ADP- and UTP-evoked responses were unaffected by removal of the extracellular calcium, suggesting that calcium influx from the extracellular space does not contribute to P2Y receptor-mediated calcium signalling under these conditions (Table 2). However, in contrast to SCG neurons, the response to ATP in glial cells was not abolished by removal of extracellular

calcium but reduced by 26.1±14% ($n=7$) (Figure 5a). These results demonstrate that in SCG glial cells ATP can mediate a rise in intracellular calcium through P2Y receptors (most likely the UTP-sensitive $P2Y_2$ receptor, see below). The calcium-sensitive component of the ATP response in glial cells indicates that these may express P2X receptors. Previous reports on glial cells have indicated the presence of P2X receptors (James & Butt, 2001), and Muller glial cells (Neal *et al*., 1998) showed the presence of an α,β-meATP-sensitive response suggesting the contribution of P2X receptors. We have shown that α,β-meATP evoked responses in a minority of glial cells that were absent in tissues taken from $P2X_1$ receptor-deficient mice (JA Calvert and RJ Evans, unpublished observations).

ADP-sensitive P2Y$_1$ receptors

ADP (100 μM) evoked calcium responses (peak self-ratio 1.64±0.11, $n=57$) in ~50% of SCG glial cells (Table 2). These responses were concentration dependent with an EC_{50} of ~29 μM. Suramin (100 μM), PPADS (30 μM) and iso-PPADS (30 μM) reduced the response to ADP (100 μM) (similar block to that seen for the neurons) (Figure 5b, Table 2). The $P2Y_1$ receptor antagonist MRS2179 reduced the responses to ADP (100 μM) to 43.0±5.7 and 21.2±4.0% of control values for MRS2179 at 1 and 10 μM, respectively ($n=22$). In SCG glia from $P2Y_1$ receptor-deficient mice, there was an ~90% decrease in the number of cells responding to ADP (100 μM). The residual responses had a peak self-ratio of 1.72±0.2 ($n=4$ of 94 cells) (Figure 6). There was no difference in the response to UTP (100 μM) between WT and $P2Y_1$ receptor-deficient glial cells (data not shown). These results indicate that the $P2Y_1$ receptor underlies the ADP-evoked rise in intracellular calcium in the vast majority of SCG glial cells.

UTP-sensitive responses

UTP (100 μM) evoked responses in 60% of glial cells (30/50) with a mean peak increase of ~67% (Table 2). UTP had an EC_{50} of ~25 μM. Suramin (100 μM), PPADS (30 μM) and iso-PPADS (30 μM) reduced the response to UTP (100 μM) (Figure 5b, Table 2). These properties are suggestive of the existence of functional $P2Y_2$ receptors in the SCG glial cells.

UDP receptors

UDP evoked responses in 17.1% of glia (14/82) with a mean peak self-ratio of 1.82±0.15. UDP evoked concentration-dependent responses with an EC_{50} of >170 μM (Table 2). Suramin (100 μM), PPADS (30 μM) and iso-PPADs (30 μM) abolished responses to UDP (100 μM) (Table 2). Taken together with the RT–PCR data, these results suggest the presence of $P2Y_6$ receptors in the glial cells.

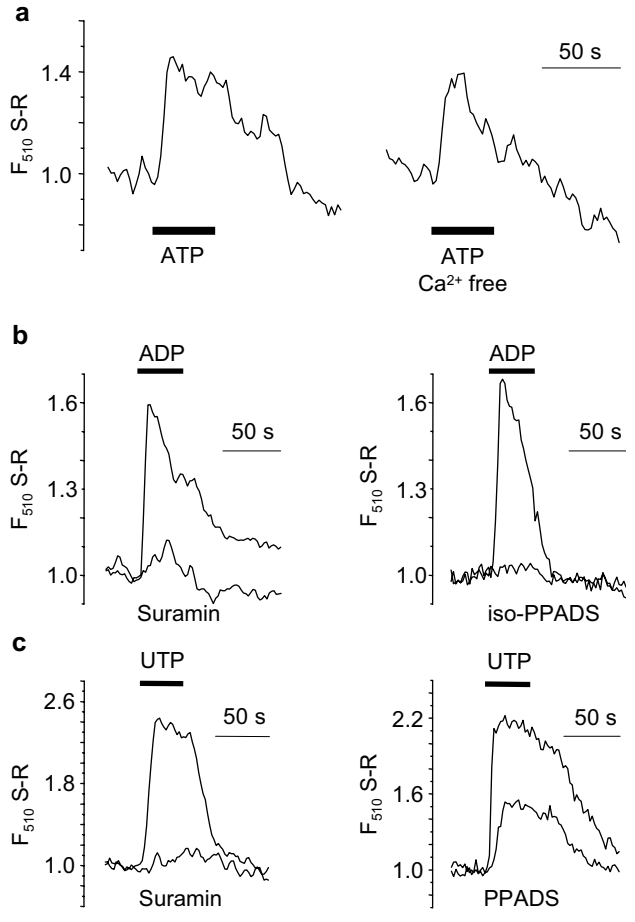

Figure 5 Glial P2Y receptor-mediated increases in intracellular calcium. (a) Example traces of the response to ATP (100 μM) in the presence and absence of extracellular Ca^{2+}. (b and c) Responses to ADP (b, 100 μM) and UTP (c, 100 μM) were inhibited by the P2 antagonists suramin (100 μM) and iso-PPADS (30 μM).

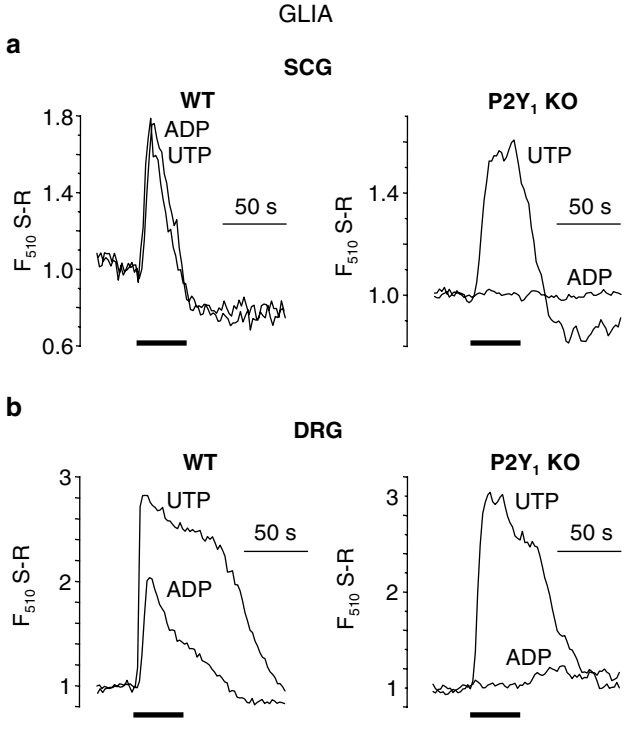

Figure 6 ADP-evoked responses are abolished in glia from $P2Y_1$ receptor-deficient mice. (a) SCG derived glia from WT mice respond to ADP and UTP (100 μM), and ADP but not UTP responses are abolished in glia from $P2Y_1$ receptor-deficient mice. (b) DRG-derived glia from WT mice respond to ADP and UTP (100 μM), and ADP but not UTP responses are abolished in glia from $P2Y_1$ receptor-deficient mice.

Discussion

P2Y receptors can be divided based on their signalling mechanisms into those that couple predominantly to an increase in intracellular calcium ($P2Y_{1,2,4,6\&11}$) and those that inhibit adenylyl cyclase activity ($P2Y_{12\&13}$). Calcium signalling plays an important role in neuronal and glial cell function and nucleotides acting at P2Y receptors have been implicated in nervous system signalling including neuronal–glial cell communication (Hansson & Ronnback, 2003). Our RT–PCR analysis indicated the expression of $P2Y_{1,2\&6}$ receptors in mouse SCG, which is consistent with Northern analysis of rat SCG (Moskvina *et al*., 2003). In addition, we have identified functional P2Y receptors corresponding to $P2Y_{1,2\&6}$ receptors linked to rises in intracellular calcium in neurons and glial cells from the SCG. These P2Y receptor-mediated responses show a heterogeneous distribution within the SCG indicating some degree of specialization of function of both neuronal and glial cells.

ADP in the nervous system is generally considered to be generated following nucleotidase-mediated breakdown of ATP released from neurons (Moskvina *et al*., 2003) by mechanical stimulation (Neary *et al*., 2003) or tissue damage. ADP-mediated calcium rises were detected in SCG neurons and glial cells and these were essentially abolished in the SCG from $P2Y_1$ receptor-deficient mice. This is the first time that the $P2Y_1$ receptor-deficient mouse has been used to study neurons and glial cells and confirms previous studies on astrocytes that pharmacologically indicated the presence of ADP-sensitive $P2Y_1$ receptors (Fam *et al*., 2000; James & Butt, 2001). In glial cells, $P2Y_1$ receptor stimulation may also lead to downstream activation of ERK kinases (Neary *et al*., 2003) and similar signalling pathways may also exist in SCG neurons. The presence of $P2Y_1$ receptors on both SCG neuronal and glial cells may therefore provide a mechanism for communication or synchronizing the behaviour of these SCG in response to local changes in ATP/ADP (Fields & Stevens, 2000).

The $P2Y_1$ receptor however is not universally responsible for ADP-evoked increases in calcium in neurons as shown by ADP-sensitive calcium rises in a significant proportion of DRG neurons from $P2Y_1$ receptor-deficient mice (ADP-mediated rises in calcium however were abolished in DRG glial cells from $P2Y_1$ receptor KO mice). These results suggest that there is an additional ADP-sensitive P2Y receptor in the DRG. One possibility is that it could result from the expression of ADP-sensitive $P2Y_{12}$ or $P2Y_{13}$ receptors (these were detected in the DRG by RT–PCR, unpublished observations) that could either be coupled to PLC (in the rat SCG, heterologously expressed P2Y receptors are capable of coupling through multiple signalling pathways; Boehm, 2003) or possibly form a dimer with $G\alpha_q$-coupled P2Y receptors.

At $P2Y_2$ receptors, ATP and UTP are agonists (Dubyak, 2003). These nucleotides are also agonists at $P2Y_{4\&11}$ receptors (Dubyak, 2003); however, these have not been detected in neuronal tissues (Ralevic & Burnstock, 1998; Communi *et al*., 2001). In the present study, RT–PCR analysis indicated the expression of $P2Y_2$ receptors in the SCG and this is consistent with the UTP- and ATP-mediated, store-dependent, suramin- and PPADS-sensitive, rises in intracellular calcium in SCG glial cells. Similar $P2Y_2$ receptor-dependent signalling has been described in optic nerve (James & Butt, 2002) and spinal cord (Fam *et al*., 2003) glial cells.

The UTP-sensitive response in the SCG neurons cannot be accounted for by the properties of current molecularly identified/characterized P2Y receptors (Table 1). UTP-sensitive P2Y receptors show ATP sensitivity (Dubyak, 2003) (Table 1). In the SCG, ATP responses in neurons are mediated by P2X receptors and are dependent on calcium influx (Calvert & Evans, 2004; this study) ruling out a contribution of P2Y receptors that are sensitive to both UTP and ATP. Interconversion of agonists has complicated the analysis of agonist sensitivity in some preparations. However, it is unlikely that the UTP response in neurons results from breakdown of UTP to UDP and activation of $P2Y_6$ receptors, as $\sim$70% of neurons responded to UTP (100 μM) but only $\sim$20% responded to UDP (100 μM). In the rat SCG, there is evidence for distinct ATP and UTP receptors (Boehm *et al*., 1995); however, in contrast to the present study, the UTP response was suramin insensitive (Boehm *et al*., 1995). The molecular identification for the UTP-sensitive P2Y receptor in mouse SCG neurons remains to be determined.

The UDP-sensitive response in $\sim$20% of SCG neurons and glia most likely corresponds to the $P2Y_6$ receptor (Dubyak, 2003) that was detected in the SCG in the present RT–PCR studies. Similar UDP-sensitive responses have been described previously for rat SCG neurons (von Kugelgen *et al*., 1997); however, in contrast, these were suramin and PPADS insensitive (Bofill-Cardona *et al*., 2000), suggesting that there may be species differences in antagonist sensitivity as have been documented for molecularly defined $P2X_4$ receptors (Garcia-Guzman *et al*., 1997).

In this study, we have characterized P2Y receptor-mediated calcium rises in cultured adult SCG neurons and glia. Our data suggest that there are $P2Y_1$ and $P2Y_6$ receptors in neurons and glia, $P2Y_2$ receptors in glial cells and a novel UTP-sensitive receptor in SCG neurons. There is heterogeneity in responses suggesting some degree of specialization/coding within the neurons and glial cells. The existence of multiple P2Y receptor subtypes in neurons and glial cells in the SCG raises the possibility that nucleotides may be effective molecules in mediating communication between neurons and glial cells (Auld & Robitaille, 2003).

This work was supported by the MRC, the Wellcome Trust and INSERM, Association de Recherche et de Development en Medecine et Sante Publique, EFS-Alsace, Fondation de France (no. 2002005149).

References

ABBRACCHIO, M.P., BOEYNAEMS, J.M., BARNARD, E.A., BOYER, J.L., KENNEDY, C., MIRAS-PORTUGAL, M.T., KING, B.F., GACHET, C., JACOBSON, K.A., WEISMAN, G.A. & BURNSTOCK, G. (2003). Characterization of the UDP-glucose receptor (re-named here the P2Y(14) receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol. Sci.,* **24,** 52–55.

AULD, D.S. & ROBITAILLE, R. (2003). Glial cells and neurotransmission: an inclusive view of synaptic function. *Neuron,* **40,** 389–400.

BAO, L., JIN, S.X., ZHANG, C., WANG, L.H., XU, Z.Z., ZHANG, F.X., WANG, L.C., NING, F.S., CAI, H.J., GUAN, J.S., XIAO, H.S., XU, Z.Q., HE, C., HOKFELT, T., ZHOU, Z. & ZHANG, X. (2003). Activation of delta opioid receptors induces receptor insertion and neuropeptide secretion. *Neuron,* **37,** 121–133.

BOEHM, S. (2003). P2Ys go neuronal: modulation of Ca^{2+} and K^+ channels by recombinant receptors. *Br. J. Pharmacol.,* **138,** 1–3.

BOEHM, S., HUCK, S. & ILLES, P. (1995). UTP- and ATP-triggered transmitter release from rat sympathetic neurones *via* separate receptors. *Br. J. Pharmacol.,* **116,** 2341–2343.

BOFILL-CARDONA, E., VARTIAN, N., NANOFF, C., FREISSMUTH, M. & BOEHM, S. (2000). Two different signalling mechanisms involved in the excitation of rat sympathetic neurons by uridine nucleotides. *Mol. Pharmacol.,* **57,** 1165–1172.

BOYER, J.L., MOHANRAM, A., CAMAIONI, E., JACOBSON, K.A. & HARDEN, T.K. (1998). Competitive and selective antagonism of $P2Y_1$ receptors by N6-methyl 2'-deoxyadenosine 3',5'-bisphosphate. *Br. J. Pharmacol.,* **124,** 1–3.

CALVERT, J.A. & EVANS, R.J. (2004). Heterogeneity of P2X receptors in sympathetic neurons: contribution of neuronal $P2X_1$ receptors revealed using knockout mice. *Mol. Pharmacol.,* **65,** 139–148.

COMMUNI, D., SUAREZ GONZALEZ, N., DETHEUX, M., BREZILLON, S., LANNOY, V., PARMENTIER, M. & BOEYNAEMS, J.M. (2001). Identification of a novel human ADP receptor coupled to Gi. *J. Biol. Chem.,* **276,** 41479–41485.

CONNOLLY, G.P., HARRISON, P.J. & STONE, T.W. (1993). Action of purine and pyrimidine nucleotides on the rat superior cervical ganglion. *Br. J. Pharmacol.,* **110,** 1297–1304.

DUBYAK, G.R. (2003). Knock-out mice reveal tissue-specific roles of P2Y receptor subtypes in different epithelia. *Mol. Pharmacol.,* **63,** 773–776.

EVANS, R.J. & KENNEDY, C. (1994). Characterization of P2-purinoceptors in the smooth muscle of the rat tail artery: a comparison between contractile and electrophysiological responses. *Br. J. Pharmacol.,* **113,** 853–860.

FAM, S.R., GALLAGHER, C.J., KALIA, L.V. & SALTER, M.W. (2003). Differential frequency dependence of $P2Y_1$- and $P2Y_2$-mediated Ca^{2+} signaling in astrocytes. *J. Neurosci.,* **23,** 4437–4444.

FAM, S.R., GALLAGHER, C.J. & SALTER, M.W. (2000). $P2Y_1$ purinoceptor-mediated Ca^{2+} signaling and Ca^{2+} wave propagation in dorsal spinal cord astrocytes. *J. Neurosci.,* **20,** 2800–2808.

FIELDS, R.D. & STEVENS, B. (2000). ATP: an extracellular signaling molecule between neurons and glia. *Trends Neurosci.,* **23,** 625–633.

GARCIA-GUZMAN, M., SOTO, F., GOMEZ-HERNANDEZ, J.M., LUND, P.-E. & STUHMER, W. (1997). Characterization of recombinant human $P2X_4$ receptor reveals pharmacological differences to the rat homologue. *Mol. Pharmacol.,* **51,** 109–118.

HANSSON, E. & RONNBACK, L. (2003). Glial neuronal signaling in the central nervous system. *FASEB J.,* **17,** 341–348.

HE, W., MIAO, F.J-P., LIN, D.C-H., SCHWANDNER, R.T., WANG, Z., GAO, J., CHEN, J.-L., TIAN, H. & LING, L. (2004). Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature,* **429,** 188–193.

INBE, H., WATANABE, S., MIYAWAKI, M., TANABE, E. & ENCINAS, J.A. (2004). Identification and characterization of a cell-surface receptor, $P2Y_{15}$, for AMP and adenosine. *J. Biol. Chem.,* **279,** 19790–19799.

JAMES, G. & BUTT, A.M. (2001). P2X and P2Y purinoreceptors mediate ATP-evoked calcium signalling in optic nerve glia *in situ*. *Cell Calcium,* **30,** 251–259.

JAMES, G. & BUTT, A.M. (2002). P2Y and P2X purinoceptor mediated Ca(2+) signalling in glial cell pathology in the central nervous system. *Eur. J. Pharmacol.,* **447,** 247–260.

LEON, C., HECHLER, B., FREUND, M., ECKLY, A., VIAL, C., OHLMANN, P., DIERICH, A., LEMEUR, M., CAZENAVE, J.-P. & GACHET, C. (1999). Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. *J. Clin. Invest.,* **104,** 1731–1737.

MOSKVINA, E., UNTERBERGER, U. & BOEHM, S. (2003). Activity-dependent autocrine–paracrine activation of neuronal P2Y receptors. *J. Neurosci.,* **23,** 7479–7488.

NAKAMURA, F. & STRITTMATTER, S.M. (1996). $P2Y_1$ purinergic receptors in sensory neurons: contribution to touch-induced impulse generation. *Proc. Natl. Acad. Sci. USA,* **93,** 10465–10470.

NEAL, M.J., CUNNINGHAM, J.R. & DENT, Z. (1998). Modulation of extracellular GABA levels in the retina by activation of glial P2X-purinoceptors. *Br. J. Pharmacol.,* **124,** 317–322.

NEARY, J.T., KANG, Y., WILLOUGHBY, K.A. & ELLIS, E.F. (2003). Activation of extracellular signal-regulated kinase by stretch-induced injury in astrocytes involves extracellular ATP and P2 purinergic receptors. *J. Neurosci.,* **23,** 2348–2356.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

VON KUGELGEN, I., NORENBERG, W., ILLES, P., SCHOBERT, A. & STARKE, K. (1997). Differences in the mode of stimulation of cultured rat sympathetic nerurons between ATP and UDP. *Neuroscience,* **78,** 935–941.

XIAO, H.S., HUANG, Q.H., ZHANG, F.X., BAO, L., LU, Y.J., GUO, C., YANG, L., HUANG, W.J., FU, G., XU, S.H., CHENG, X.P., YAN, Q., ZHU, Z.D., ZHANG, X., CHEN, Z. & HAN, Z.G. (2002). Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proc. Natl. Acad. Sci. USA,* **99,** 8360–8365.

(Received May 26, 2004
Revised July 14, 2004
Accepted July 16, 2004)