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P2Y receptor regulation of cultured rat cerebral cortical cells: calcium responses and mRNA expression in neurons and glia

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1 We have investigated increases in cytosolic Ca^{2+} in response to nucleotides in mixed rat cerebrocortical cultures (neurons and glia in similar numbers) and in essentially neuron-free glial cultures.

2 In both cultures, the agonist-response profile was 2-methylthioADP(2MeSADP) > 2-methylthioATP(2MeSATP) > ADP > ATP > adenosine 5′-*O*-(3-thiotriphosphate), consistent with a $P2Y_1$ receptor. The maximal responses to 2MeSADP, 2MeSATP and ADP were identical, but that to ATP was higher.

3 Suramin, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid, reactive blue 2 (RB2), and adenosine biphosphate (A3P5P) were antagonists with apparent pA_2 values of 5.5 for suramin, 6.4 for RB2, and 4.7 for A3P5P.

4 Single cell imaging divided the cells from the mixed neuronal – glial cultures into two populations: responsive (neurons) and unresponsive (glial cells) to high $[K^+]$. The response of cells to nucleotides was almost exclusively limited to those not responsive to high K^+.

5 In the presence of extracellular Mn^{2+}, the response of the mixed cultures to 30 mM K^+ and 20 μM Bay K 8644 was attenuated. However, when 2MeSADP was added there was no reduction in response in cultures previously loaded with Mn^{2+}. This further indicated that the 2MeSADP response was not in the neurons.

6 Reverse transcriptase – polymerase chain reaction studies detected transcripts for $P2Y_1$, $P2Y_4$ and $P2Y_6$ in RNA preparations from embryonic rat cortex, and from both mixed and glial cultures. $P2Y_2$ transcripts were not detected in the embryonic cortex.

7 Based on this and previous work, it is proposed that the principal P2Y influences in the brain are on cytosolic Ca^{2+} in glial cells and presynaptic sites on neurons.
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Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); A3P5P, adenosine-3′-phosphate-5′-phosphate; FLIPR, fluorimetric imaging plate reader; GFAP, glial fibrillary acidic protein; HPLC, high-pressure liquid chromatography; 2-MeSADP, 2-methylthioADP; 2-MeSATP, 2-methylthioATP; RTPCR, reverse transcriptase-polymerase chain reaction; TTX, tetrodotoxin

Introduction

ATP is known to be a major neurotransmitter in the peripheral nervous system, modulating physiological events by action at both presynaptic and postsynaptic sites (Burnstock, 1972; 1976; Shinozuka *et al*., 1988; Kurz *et al*., 1993; Allgaier *et al*., 1994; von Kugelgen *et al*., 1995; Kumagai & Saino, 2001; Vartian *et al*., 2001). UTP has also been shown to regulate neurotransmitter release from peripheral nerve terminals (Norenberg *et al*., 2000; Boehm, 1998). These nucleotides act directly on P2 receptors, which are divided into two subfamilies. The P2X receptors have intrinsic ion channels and are essentially receptors for ATP, while the G protein-coupled P2Y receptors are responsive to a wider range of endogenous nucleotides (notably ATP, ADP, UTP, UDP). The mammalian P2Y receptor family comprises eight cloned receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$ (Fredholm *et al*., 1997; Boarder & Hourani, 1998; Communi *et al*., 1999; 2001; Hollopeter *et al*., 2001; Zhang *et al*., 2001, 2002; Abbrachio *et al*., 2003). Each has a distinct profile of activation by different agonists. In the absence of a range of selective antagonists, the pharmacological characterisation of the P2 receptors has largely depended on the rank order of agonist potencies.

The role of nucleotides in signalling between cells in the brain is not well established. There is evidence that ATP may act as a fast neurotransmitter in the brain by acting at central P2X receptors (e.g. Edwards *et al*., 1992). Excitatory postsynaptic responses at central synapses have also been ascribed to activation of P2Y receptors (Shen & North, 1993; Nieber *et al*., 1997). The most substantial body of evidence for

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functional neuronal P2Y receptors in the brain concerns a presynaptic role. Initially this related to presynaptic P2Y receptor regulation of release of the amines noradrenaline, 5-hydroxytryptamine and dopamine (von Kugelgen *et al*., 1994; Koch *et al*., 1995; 1997; Krugel *et al*., 2001a, b). However, we and others have recently presented evidence that directly implicates P2Y receptors in the presynaptic regulation of glutamate release (Bennett & Boarder, 2000; Inoue & Koizumi, 2001). These investigations suggest a widespread influence of P2Y receptors on brain function.

In this report we have taken a different approach, namely looking for evidence of regulation by nucleotides of cytosolic Ca^{2+} levels in primary cell preparations from neonatal rat brain. It has previously been shown that ATP can elicit increases in cytosolic Ca^{2+} in rat cortical cell preparations (King *et al*., 1996; Centemeri *et al*., 1997; Lalo & Kostyuk, 1998). Here, we investigate this response with respect to the pharmacological profile and the identity of the cells containing P2Y receptors that generate the rise in Ca^{2+}, and we also examine the expression of P2Y receptors at the mRNA level.

Methods

Materials

DMEM, Hank's balanced salt solution (HBSS) and B27 were from Gibco; Neutral protease (DispaseII) was from Boehringer, Mannheim, Germany; DNAse and papain were from Sigma; poly-D-lysine-coated fluorimetric imaging plate reader (FLIPR) plates and 96-well culture plates were from Becton Dickenson; poly-D-lysine was from Sigma; Fura-2/AM Fluo3-AM was from Molecular Probes; anti-β-tubulin and anti-glial fibriallary acidic protein (GFAP) antibodies were from DAKO and fluorescent secondary antibodies were from Molecular Probes; all nucleotides were from Sigma apart from 2-methylthioATP (2MeSATP) and 2-methylthioADP (2MeSADP), which were from RBI; suramin was from RBI; pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), reactive blue 2 (RB2) and adenosine biphosphate (A3P5P) were from Sigma; Gibco supplied all reverse transcriptase – polymerase chain reaction (RT – PCR) reagents; ethidium bromide was from Sigma; P2 receptor primers were synthesised by Sigma-Genosys. All other reagents were from Sigma.

Cell preparation

Embryonic rat brains were obtained 14 days after mating, cortices dissected free of membranes, chopped and digested with 0.01% papain, 0.1% neutral protease, 0.01% DNAse and 12.4 mM $MgSO_4$ in a HEPES-buffered HBSS. After three rounds of incubation (37°C for 30 min) and trituration, the cells were centrifuged at $1500 \times g$ for 5 min, incubated with 0.01% DNAse in DMEM (with 3 g l^{-1} glucose), and resuspended in DMEM with 10% bovine serum. Cells were plated at 60×10^3 cells per well in 96-well plates (poly-D-lysine coated) for Ca^{2+} studies and RT – PCR analysis, and at 70×10^3 cells per 22 mm coverslip (poly-D-lysine coated) for single cell imaging and for immunohistochemistry. After incubation overnight, the medium was replaced with DMEM with 3 g l^{-1} glucose and the neuronal survival supplement B27 (Sigma). Cells were used at 7 – 9 days of culture. These mixed cell cultures, comprising similar numbers of neuronal and glial cells (see below), were in some cases compared to glial cultures, prepared in the same way but cultured in the presence of 10% heat-inactivated bovine serum in place of the neuronal survival supplement B27. At 7 days these cultures were almost completely free of neurons, as judged by immunocytochemistry and lack of sensitivity to high K^+ (see below).

Cytosolic Ca^{2+} measurement in 96-well plates

Cytosolic Ca^{2+} was measured simultaneously in Fluo-3-labelled cells in 96-well plates using a FLIPR. Cells were cultured for 8 days in 96-well FLIPR plates, then prior to study were washed twice with 200 μl FLIPR buffer (HBSS, 2 mM $CaCl_2$, 2.5 mM probenecid and 10 mM HEPES, pH 7.4) per well. Cells were loaded by addition of 75 μl of 2 μM Fluo-3/AM in the presence of 0.001% pluronic acid for 45 min at 37°C, followed by a 15 min hydrolysis period during which antagonists were added (25 μl at 4 × final concentration) as indicated. At the end of this period, the medium was aspirated and replaced with 100 μl FLIPR buffer with 100 nM tetrodotoxin (TTX) and antagonists as appropriate. Wells were then excited at 505 nm with emission recorded at 530 nm, and changes in intracellular Ca^{2+} levels recorded as changes in arbitrary fluorescence units, captured up to three times every second. Basal levels were recorded, typically for 5 s, before agonists were added in 100 μl as indicated. The purity of nucleotide agonists was checked by high-pressure liquid chromatography and found to be 95% or greater, and since data were plotted as peak heights measured within seconds of agonist addition, it is unlikely that agonist interconversion significantly affected the outcome of these experiments. At 3 min after addition of nucleotide agonists, carbachol (30 μM final concentration) was added. Peak heights for the nucleotide responses were obtained using the statistics function of the FLIPR software, and data plotted using Graphpad Prism. All points are expressed as a percentage of the control agonist concentration – response curve maximum in that particular plate.

Calculation of antagonist potencies

Families of concentration – response curves for various agonists were constructed, using the FLIPR methodology, in the presence of increasing concentrations of antagonists, and the results computed to generate estimates of antagonist potencies. In almost every case, the maxima of the concentration – effect curves were depressed at the higher concentrations of antagonists, probably as a result of the rapid, transient nature of the response peak. Despite this, we computed an estimate of apparent pA_2 values using a rearrangement of the Schild equation:

Apparent $pA_2 = -\log_{10}$ ([B]/(CR-1)), where [B] is the concentration of antagonist tested and CR is the concentration ratio of curve mid-point in the presence and absence of antagonist.

Cytosolic Ca^{2+} measurement in single cells

Cells on coverslips were loaded for 45 min with 5 μM Fura-2/AM in HEPES-buffered HBSS with 0.001% pluronic acid and

250 μM sulphinpyrazone. Cells were then washed, incubated for a further 10 min, and mounted into a perfusion chamber on a microscope stage and visualised using a $\times 40$ objective. Cells were excited at 340/380 nm, emission recorded at 510 nm and the results displayed ratiometrically using Axon Imaging Workbench software. At the end of each experiment, cells were exposed to 10 μM ionomycin followed by 5 mM $MnCl_2$ to determine background fluorescence.

RT–PCR

Cells in 96-well plates were extracted from all wells with Trizol, into a final pooled volume of 1.2 ml. For direct extraction of brain tissue, cortices were chopped and extracted into 1 ml Trizol per 100 mg tissue using a polytron homogeniser. Following chloroform extraction, the RNA was precipitated with isopropyl alcohol. cDNA was synthesised from 5 μg RNA in a 40 μl reaction volume using 400 U of Superscript II and PCR carried out as described in Harper *et al.* (1998).

Immunohistochemistry

Immunohistochemistry utilised simultaneous visualisation of immunoreactivity specific for neurons (anti-β-tubulin) and glia (GFAP) with a general nuclear (chromatin) counterstaining with DAPI to visualise all cells. Cells on coverslips were fixed with 4% paraformaldehyde and exposed to mouse anti-β-tubulin III (DAKO) at 1 : 500 followed by alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) at 1 : 200, and rabbit anti-cow GFAP (DAKO) at 1 : 1000 followed by alexa 594-conjugated goat anti-rabbit IgG (Molecular Probes) at 1 : 200. Cells were then stained with 10 μg ml^{-1} DAPI. Coverslips were mounted and viewed under the fluorescent microscope.

Results

Immunocytochemical characterisation of mixed cerebrocortical cells in culture

Cerebrocortical cells cultured with B7 supplement for 7 days were simultaneously stained with anti-β-tubulin with a secondary antibody coupled to a green fluorescent dye (neuronal marker), anti-GFAP with a secondary antibody coupled to a red fluorescent dye (glial marker) and a nonselective blue nuclear counterstain. The differential staining showed that there were approximately equal numbers of glia and neurons, with essentially no other cell types present. By 7 days in culture both cell types were stellate in morphology, forming long processes that made contact with other cells.

Immunocytochemical characterisation of glial cells in culture

Cerebrocortical cells were cultured for 7 days in the presence of 10% heat-inactivated bovine serum in place of the neuronal survival supplement B27. Simultaneous staining with anti-β-tubulin with a secondary antibody coupled to a green fluorescent dye (neuronal marker), anti-GFAP with a secondary antibody coupled to a red fluorescent dye (glial marker), and a nonselective blue nuclear counterstain (DAPI) revealed that after 7 days the culture consisted of almost exclusively glial cells.

Cytosolic Ca^{2+} measurement in 96-well plates: agonist profiles

When Fluo-3-loaded cerebrocortical cells were recorded in FLIPR experiments in the absence of TTX, each well of the 96-well plate showed spontaneous calcium spiking behaviour. This was not seen in cells that had been cultured for less than 5 days (not shown). The frequency of the calcium oscillations varied, but was typically about 50 s peak to peak. Spontaneous oscillations were abolished in the presence of either 100 nM TTX or 1 mM Mn^{2+}. To provide a stable baseline, 100 nM TTX was included in all subsequent experiments.

Figure 1 shows typical traces from FLIPR wells stimulated with high [K^+], ATP, ADP or 2MeSATP. In each case, this was followed by exposure to carbachol to provide a reference response. The results show that each of these nucleotide agonists generated a typical peak-and-plateau response. Also shown in Figure 1 are the responses in the absence of extracellular Ca^{2+} (Ca^{2+} was omitted from the buffer and 100 μM EGTA was added). While this completely eliminated the KCl-stimulated transients, it left a residual peak response to nucleotides, but no sustained phase. This is consistent with a classical Ca^{2+} response to G protein-coupled receptors, in which the initial phase of the transient is largely the result of mobilisation of Ca^{2+} from intracellular stores, and the sustained phase is entry from the extracellular compartment.

To characterise the system, responses to some non-nucleotide agonists were tested (data not shown). Glycine and the GABA agonist RS1467 gave no response at 100 μM, while glutamate gave a large response at 100 μM, which was approximately three-fold greater in magnitude than that elicited by 100 μM ATP. A maximally effective concentration of carbachol (30 μM) gave a response that was typically less than half that generated by 100 μM ATP.

The peak heights of responses to a variety of nucleotides, plotted as concentration – effect curves, were used to generate data for mixed cerebrocortical cultures (Figure 2a; Table 1). 2MeSADP was highly potent, with an EC_{50} of 6.3 nM in the mixed cultures. The potency order was 2MeSADP > 2MeSATP > ADP $\geq$ ATP > ATPγS. UDP, AMP, adenosine, α,β-methylene-ATP and β,γ-methylene-ATP all failed to give a response at up to 300 μM. UTP (300 μM) gave a very small response ($\sim$5% of response to ATP) in three out of seven experiments, and no response in the remainder.

In a further series of experiments, concentration – response curves were constructed for glial cultures. We first established that these cultures do not generate a significant Ca^{2+} transient to 30 mM K^+ (data not shown), unlike neuron-containing mixed cell cultures (see Figure 1). The glial cell cultures displayed very similar agonist response profiles (Figure 2b; Table 1) to the mixed cultures: 2MeSADP > 2MeSATP > ADP > ATP > ATPγS.

For both these cultures, the pEC_{50} values, maximum response and slopes are shown in Table 1. In each case the slopes are not appreciably different from 1. ATP had a similar potency to ADP and generated a higher maximum ($P<0.05$) in these studies (Figure 2 and Table 1).

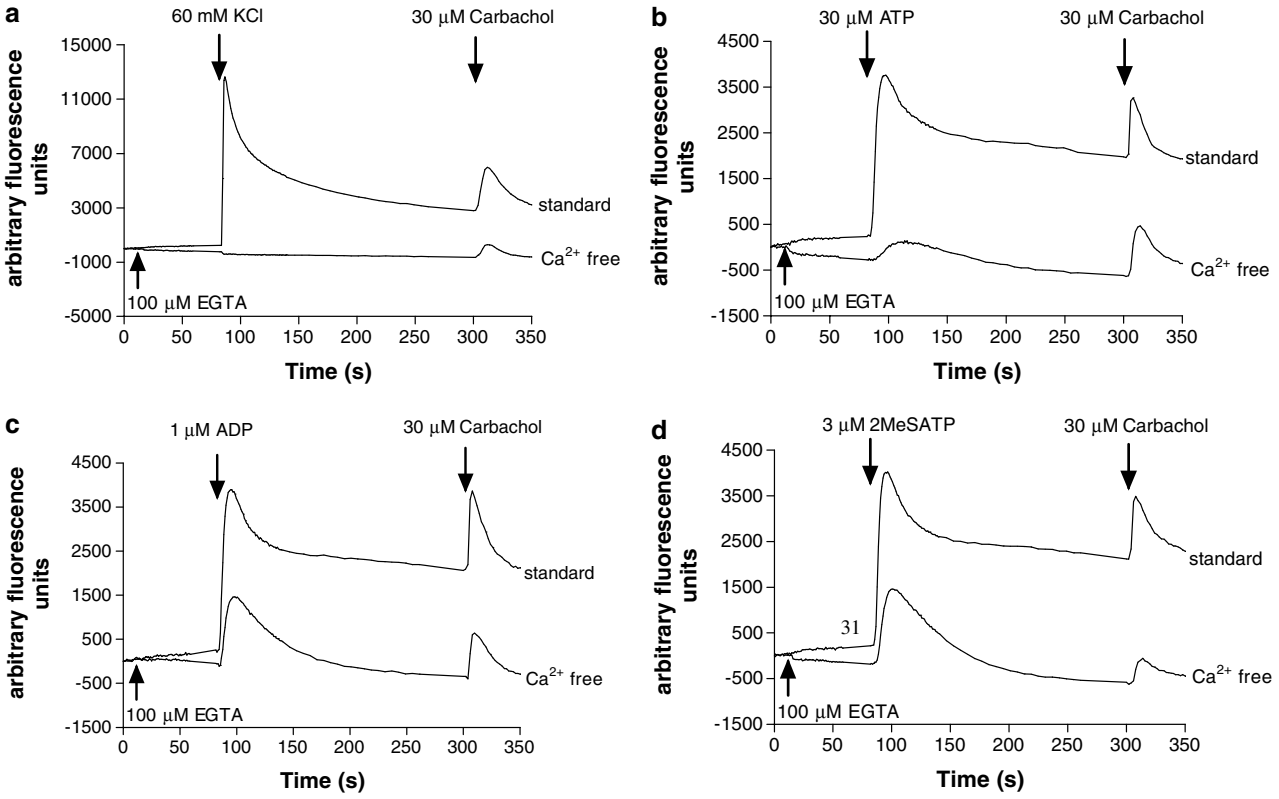

Figure 1 Cytosolic Ca^{2+} responses of mixed cerebrocortical cultures to addition of nucleotides, carbachol or high K^+. Cultures in 96-well plates were loaded with Fluo3-AM and stimulated in the presence of TTX using the FLIPR. For the traces labelled 'Ca^{2+}-free' recordings were with Ca^{2+}-free buffer with 100 μM EGTA added at the time indicated. Agonists and high K^+ were added to both standard (2 mM Ca^{2+}) and Ca^{2+}-free wells at the times indicated. Concentrations of agonists and K^+ indicated are the final concentrations. Each trace is a single experiment, typical of several experiments undertaken with three separate cell preparations.

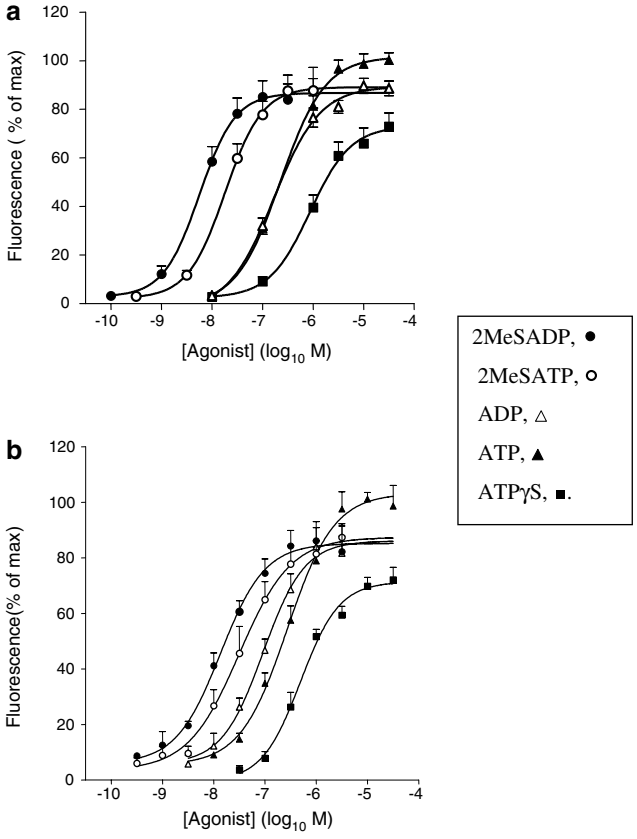

Figure 2 Concentration – effect curves to purine agonists. Cultures of mixed (a) or glial (b) cells were loaded with Fluo3-AM in 96-well plates and agonists were added in the presence of TTX using the FLIPR. Peak fluorescence was calculated and plotted as percentage of mean maximum response for that plate. Data points are mean ± s.e.m. from six to 14 separate experiments. Curves were fitted to a logistic function.

Mn^{2+} quench experiments

In the mixed cultures, the presence of the L-type Ca^{2+} channel enhancer Bay K 8644 (20 μM) enhanced the size of the Ca^{2+} transient generated in response to 30 mM K^+ (data not shown). In the presence of high [K^+] and Bay K 8644, the addition of extracellular Mn^{2+} leads to Mn^{2+} entry through open L-type channels and the quenching of Ca^{2+} Fluo-3 signals (Figure 3a). Cells were then stimulated at 20 s with 20 μM Bay K 8644 and 30 mM K^+ with or without the simultaneous addition of 1 mM Mn^{2+} (Figure 3b). The presence of Mn^{2+} effectively quenched the response to combined L-type channel enhancer and high K^+. However, when nucleotides were subsequently added, the size of the Ca^{2+} response was unaffected by the prior Mn^{2+} entry. This is illustrated in Figure 3b for addition of the $P2Y_1$ agonist 2MeSADP (10 μM). We conclude from this that the response to 2MeSADP occurred on different cells from those stimulated to permit Mn^{2+} entry by L-channel opening.

Table 1 Agonist concentration–effect curve parameters in mixed cerebrocortical and glial cell cultures

	2MeSADP	*2MeSATP*	*ADP*	*ATP*	*ATPγS*
Mixed cells					
pEC_{50}	8.20±0.08	7.67±0.06	6.76±0.07	6.70±0.09	6.16±0.07
Max.	86.79±6.77	89.17±5.08	89.45±3.99	101.70±4.59	73.12±6.17
Slope	1.17±0.13	1.23±0.05	1.07±0.12	1.12±0.10	1.20±0.07
Number of curves	8	13	12	14	13
Glial cells					
pEC_{50}	7.87±0.17	7.49±0.18	7.08±0.09	6.58±0.09	6.21±0.15
Max.	85.24±5.47	87.89±3.38	86.14±7.83	105.29±3.84	72.53±3.67
Slope	1.16±0.20	1.10±0.08	1.16±0.11	1.03±0.18	1.08±0.16
Number of curves 6	6	6	6	6	6

Ca^{2+} transients were measured in Fluo-3-AM-loaded cells in the presence of 100 nM TTX. Mixed cerebrocortical cultures (upper) or glial (lower) cultures were used. A logistic function was fitted to each curve (using GraphPad) to generate the negative logarithm of the concentration of agonist giving half the maximal response (pEC_{50}), the top of the concentration curve, and the slope of the curve. The results are expressed as the mean±s.e.m., and the number of experiments for each agonist are also shown.

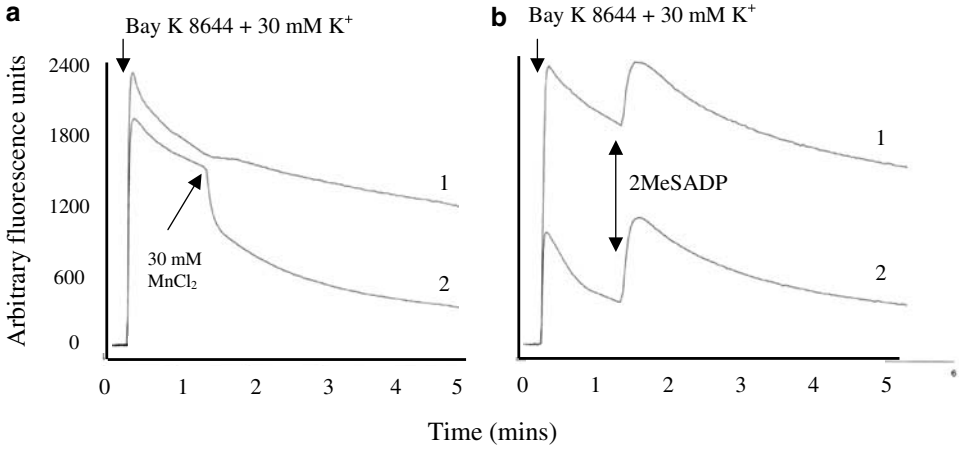

Figure 3 Effects of Mn^{2+} loading on K^+- and 2MeSADP-stimulated Ca^{2+} responses. (Left panel a) Trace 1: addition of Bay K 8644 (20 μM) and K^+ (30 mM) and addition of vehicle control 1 min later; trace 2: addition of Bay K 8644 and K^+ followed by $MnCl_2$ (1 mM) after 1 min. (Right panel b) Trace 1: stimulation with 30 mM K^+ and 20 μM Bay K 8644 followed by stimulation with 10 μM 2MeSADP; trace 2, same experiment but in the presence of 1 mM $MnCl_2$. Traces are from individual representative experiments.

Cytosolic Ca^{2+} measurement in 96-well plates: antagonist profiles

Concentration – effect curves were constructed for a variety of nucleotide agonists, in the presence of increasing concentrations of the P2 antagonists suramin, PPADS, RB2, and A3P5P (Figures 4 and 5 and Table 2). Suramin caused a progressive rightward displacement of concentration – effect curves to ATP, ADP, 2MeSADP (Figure 4a – c), ATPγS and 2MeSATP (not shown). At the highest concentration used (100 μM) there was also a reduction in the maxima, except with ADP (Figure 4a – c). The apparent pA_2 values for suramin were approximately 5.5 for all the agonists (Table 2). PPADS, in the range 0.1 – 10 μM, depressed the maxima of the concentration – effect curves to ATP, ADP, 2MeSADP (Figure 4d – f), ATPγS and 2MeSATP (not shown). The extent of this insurmountable antagonism, in the presence of little movement of the curves to the right, prevented estimation of pA_2 values for PPADS. RB2, in the range 0.3 – 30 μM, progressively displaced the concentration – effect curves to the right. At concentrations of 3 μM and above RB2 also caused a reduction in the maxima of these curves. Despite this, apparent pA_2 values were calculated to be about 6.4 *versus* all agonists, with no significant difference for the different agonists (Table 2).

A3P5P, in the range 10 – 100 μM, also displaced curves to the right, but with a smaller reduction in maxima than seen with the other antagonists. pA_2 values were estimated to be approximately 4.7 *versus* all agonists (Table 2).

Single cell imaging studies of cytosolic Ca^{2+}

Ratiometric imaging of Fura-2-loaded cells was used to provide further information concerning the cell type responding to nucleotides with elevated cytosolic Ca^{2+} levels. Cells were assigned to either neuronal-type (K^+ responsive) or glial-type (not K^+ responsive), and the numbers of each which showed an increase in cytosolic Ca^{2+} on application of various nucleotides were recorded (Table 3). The vast majority of nucleotide responses were seen in glial cells. Of the cells identified as neurons, only 9% responded to ATP, 11% to ADP and 15% to 2MeSATP. By contrast, of the cells identified as glial 59% responded to ATP, 92% to ADP and

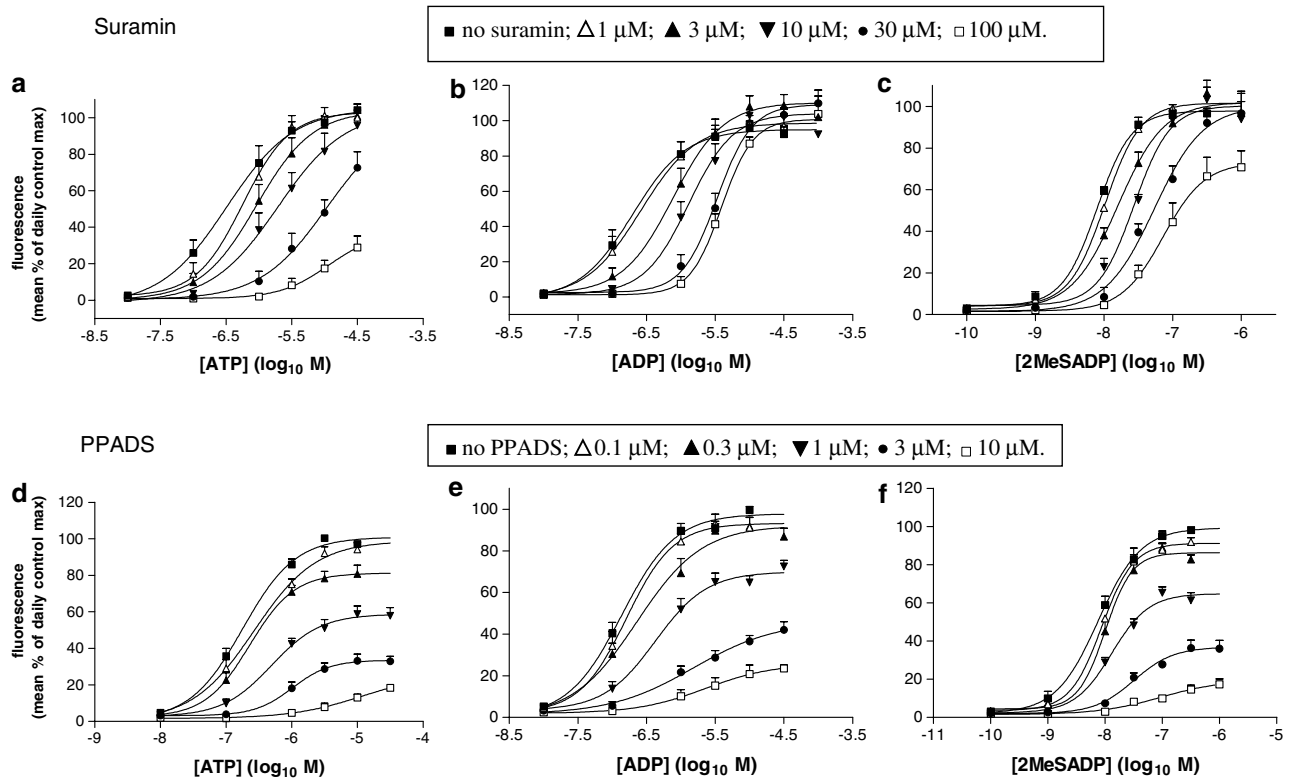

Figure 4 Agonist concentration – response curves in the presence of increasing concentrations of suramin or PPADS. Cells were stimulated with ATP (panels a and d), ADP (panels b and e) or 2MeSADP (panels c and f) at the concentrations indicated in the presence of suramin (panels a – c) or PPADS (panels d – f) at the concentrations indicated. Antagonists were added 15 min prior to addition of agonists. Data are mean ± s.e.m. from four to five separate experiments.

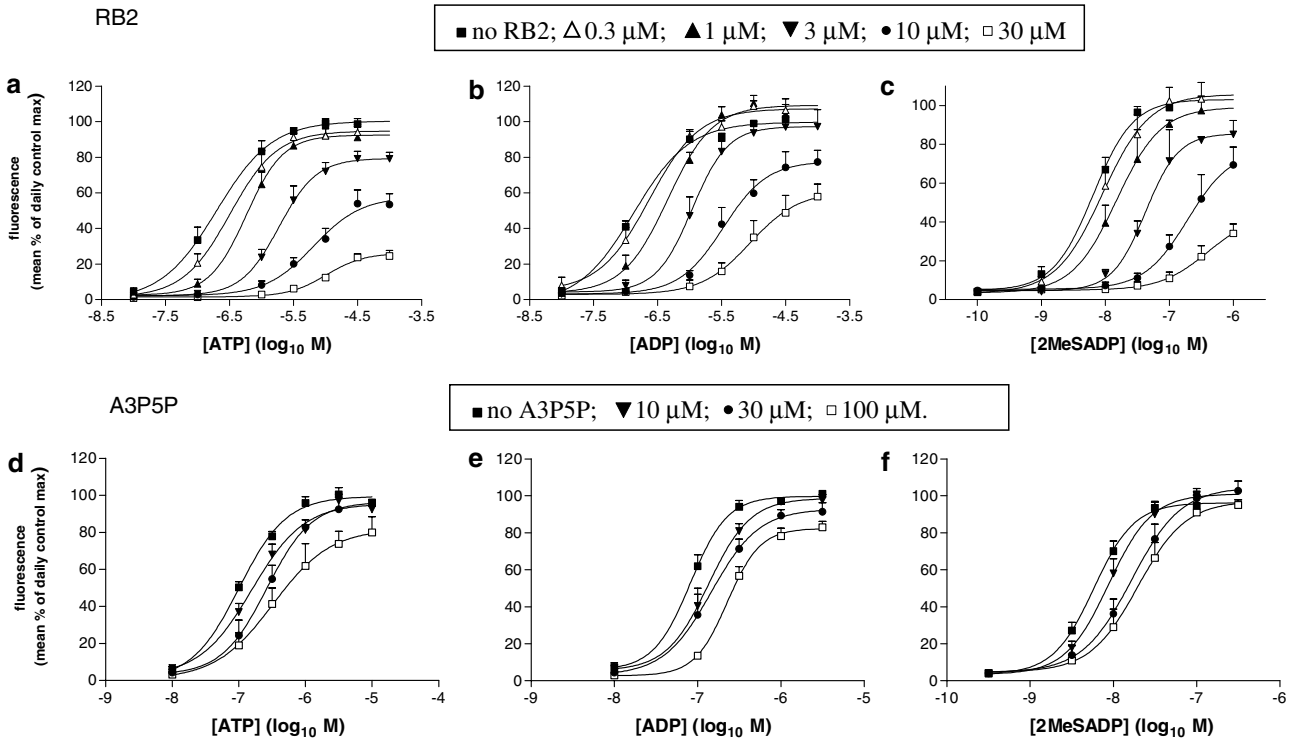

Figure 5 Agonist concentration – response curves in the presence of increasing concentrations of RB2 or A3P5P. Cells were stimulated with ATP (panels a and d), ADP (panels b and e) or 2MeSADP (panels c and f) at the concentrations indicated in the presence of RB2 (panels a – c) or A3P5P (panels d – f) at the concentrations indicated. Antagonists were added 15 min prior to addition of agonists. Data are mean ± s.e.m. from four to five separate experiments.

Table 2 Apparent pA_2 values for P2 receptor antagonists in cerebrocortical cells

	ATP	*ADP*	*ATPγS*	*2MeSATP*	*2MeSADP*
Suramin	5.59±0.11, $n=6$	5.51±0.14, $n=5$	5.46±0.17, $n=5$	5.76±0.09, $n=5$	5.45±0.09, $n=4$
RB2	6.44±0.13, $n=5$	6.44±0.12, $n=5$	6.38±0.18, $n=5$	6.31±0.12, $n=5$	6.40±0.14, $n=4$
ABP	4.65±0.18, $n=4$	4.65±0.11, $n=4$	4.89±0.19, $n=4$	4.79±0.20, $n=4$	4.72±0.05, $n=5$

Using mixed cultures of cerebrocortical cells in the presence of 100 nM TTX, concentration–effect curves to the agonists were constructed in the presence of different concentrations of the antagonists shown. Antagonists were added 15 min prior to the agonists. Data are expessed as mean±s.e.m. from the number of experiments indicated.

Table 3 Proportions of neuronal and glial cells responsive to nucleotides

	Neurons		*Glia*	
	Fraction	*(Percentage)*	*Fraction*	*(Percentage)*
ATP	12/132	(9%)	22/37	(59%)
ADP	6/51	(11%)	12/13	(92%)
2MeSATP	4/26	(15%)	11/11	(100%)
α,β-meATP	6/35	(17%)	0/12	(0%)
UTP	0/13	(0%)	0/4	(0%)

Epifluorescent responses to nucleotides of individual Fura-2-loaded cells were measured. Fields of cells were subsequently stimulated with 50 mM KCl and identified as neuronal if they responded with an increase in Ca^{2+} and as glial if they did not. The data are shown as the fraction, and percentage, of cells that responded to a particular nucleotide.

100% to 2MeSATP. Of neurons, 17% responded to α,β-methylene ATP, but no glial cells responded to this P2X agonist. None of the cells tested responded to UTP.

RT–PCR studies

The sequences for genes encoding for rat $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ are available to enable the design of primers for PCR work. Figure 6 shows the results of analysis of embryonic rat cortex, mixed cerebrocortical cells and glial cells, the latter each at 7 days in culture. In embryonic cortex no $P2Y_2$ transcripts were detectable, in mixed neuron–glia cultures all four were clearly detected, while in glial cells $P2Y_1$, $P2Y_2$ and $P2Y_6$ were invariably detected, but the signal for $P2Y_4$ was weak, variable, and in some cases undetectable.

Discussion

The cerebrocortical cells in this study developed processes over a period of 7–9 days in culture, at which time they displayed spontaneous spiking behaviour, as previously reported in neuronal cultures that have formed synaptic connections (Ogura *et al*., 1987; Muramoto *et al*., 1993). This may be because of synchronised neuronal depolarisations, propagation of which is dependent on release of glutamate (Koizumi & Inoue, 1997), generating an increase in cytosolic Ca^{2+} concentrations in both glia and neurons. In the present study, we observed that either TTX or extracellular Mn^{2+} abolished spontaneous spiking, indicating a role for Na^+ channels and Ca^{2+} entry.

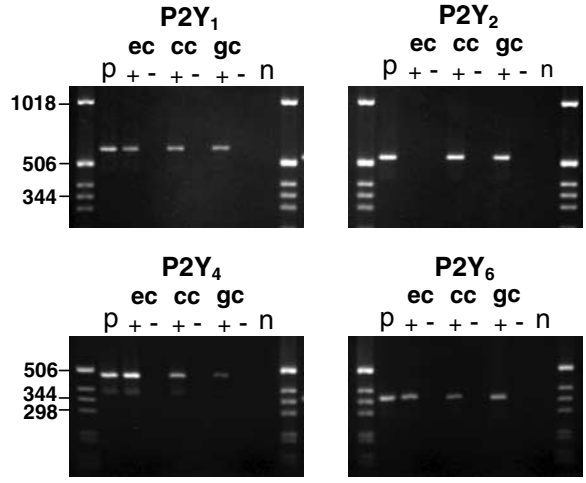

Figure 6 RT–PCR of embryonic cortex, mixed cerebrocortical cultures, and glial cultures. Embryonic rat brain cortical tissue (ec), mixed cerebrocortical cell culture (cc) and glial cell culture (gc) RNA extracts were subjected to RT–PCR with P2Y receptor-specific primers. In each case, the first lane (p) is the plasmid-template positive control, each extract is shown with a no-reverse transcriptase negative control (−) run with each primer pair, and the last lane in each case is the no template negative control for each primer pair. The results are representative of three separate experiments on different cell cultures.

The RT–PCR results reported here show transcripts for $P2Y_1$ receptors present at easily detectable levels in the mixed cerebrocortical and glial cultures. These transcripts are also detectable in uncultured embryonic cortex, suggesting that the Ca^{2+} responses we describe in cultured cells may represent events in the immature rat brain. The $P2Y_2$ receptor transcript is not detectable in the uncultured developing cortex, but appears on culture of cells. Messenger RNA encoding $P2Y_4$ and $P2Y_6$ receptors are also seen in our extracts of immature and developing brain, and cell cultures. Prior reports have shown functional responses to pyrimidine nucleotides in astrocyte cultures (e.g. Neary *et al*., 1991; Bruner & Murphy, 1993; Centemeri *et al*., 1997; King *et al*., 1996a, b). However, the lack of response to pyrimidine nucleotides seen here implies that, under our culture conditions, the presence of $P2Y_2$, $P2Y_4$ and $P2Y_6$ transcripts does not imply the presence of functional receptors. In other studies, it has been noted that the presence of mRNA encoding for a particular receptor does not invariably result in the expression of functional receptor; this presumably reflects a failure to accumulate sufficient functional protein appropriately inserted in the plasmalemma. We have no data concerning expression of transcripts for rat $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors.

The RT–PCR studies demonstrated expression of P2Y transcripts in both mixed (neuron and glia) and glial cultures. The P2Y responses reported here were also present in both types of culture. Two pieces of evidence from the mixed cultures suggest that this response is mediated by glial cells and not neurons. First, selective loading of neurons with Mn^{2+} quenched the Ca^{2+} signal in these cells, but the subsequent calcium transient in response to 2MeSADP was unaffected. Loading with Mn^{2+} is dependent on opening of Ca^{2+} channels by depolarisation, and we have shown that this is largely restricted to the mixed cultures (i.e. it occurs in neurons and not glial cells). We show here that Mn^{2+} quenching of the Fluo-3-dependent signal occurs rapidly and effectively, analogous to the Fura-2 quenching by Mn^{2+} widely used as a surrogate for stimulated Ca^{2+} influx (Merritt *et al*., 1989). Selective Mn^{2+} quenching of Ca^{2+} signals in neurons leaving nucleotide responses unaffected provides strong support for the notion that the nucleotide responses are restricted to glial cells. Secondly, single cell studies revealed that most of the cells that give a Ca^{2+} response to K^+ depolarisation do not respond to nucleotides. This shows that cells responding to nucleotides are mainly those lacking the functional response expected of neurons (we have shown that the response to K^+ does not occur in the glial cultures). Again, this indicates that the Ca^{2+} response to nucleotides is largely restricted to the glial cells.

When nucleotides were added to these mixed cultures (in the presence of TTX) there was a peak-and-plateau response consistent with that expected from action at a seven-transmembrane receptor, G protein coupled to activation of phospholipase C. Subsequent mobilisation of Ca^{2+} from intracellular stores, followed by entry from the extracellular compartment, explains the partial dependency of the response on extracellular Ca^{2+} reported here.

In this study, we use both agonist and antagonist profiles to provide a pharmacological characterisation of the receptors responding to nucleotides. 2MeSADP was the most potent agonist, with an EC_{50} of around 10 nM. Of the cloned P2Y receptors, 2MeSADP is a potent agonist for $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors (Palmer *et al*., 1998; Communi *et al*., 2001; Zhang *et al*., 2001; 2002), with EC_{50} values of 10 nM or less when these receptors are heterologously expressed. However, it is unlikely that the response to 2MeSADP is mediated by $P2Y_{12}$ or $P2Y_{13}$ receptors, since these receptors reportedly do not couple to Ca^{2+} signalling pathways (except in those expression systems specifically engineered to create such a response). In addition, the antagonist experiments discussed below are inconsistent with a response at $P2Y_{12}$ receptors. The effects of 2MeSADP and ADP in the experiments described here are consistent with action at $P2Y_1$ receptors. Furthermore, 2MeSATP was a full agonist, although with lower potency than 2MeSADP. It has been suggested that this nucleotide does not act at $P2Y_1$ receptors (Leon *et al*., 1997; Hechler *et al*., 1998). However, Dixon (2000) presented evidence that 2MeSATP does act at the $P2Y_1$ receptor expressed by rat hepatocytes. In addition, Palmer *et al*. (1998) have shown that 2MeSATP is a low-efficacy agonist at human $P2Y_1$ receptors, which can sustain a full response when there is sufficient receptor reserve. Both these studies used conditions that minimise agonist interconversions. In the experiments described here the responses started immediately when agonists were added, and the data reported are from peaks occurring a few seconds after addition of the agonists. Under these conditions agonist interconversion is likely to be limited. However, some degree of conversion of 2MeSATP to 2MeSADP at the cell surface may contribute to the results shown here. On balance, however, it seems most likely that responses to either 2MeSATP or 2MeSADP are because of direct nucleotide action on $P2Y_1$ receptors.

ATP has also been described as a low-efficacy agonist at $P2Y_1$ receptors (Palmer *et al*., 1998). In Figure 2 and Table 1, ATP has a similar EC_{50} to ADP and gives a significantly larger maximum response. It is possible that an additional receptor for ATP is expressed. This is unlikely to be $P2Y_2$ as UTP is ineffective, or $P2Y_{11}$ since this receptor is not antagonised by PPADS (Communi *et al*., 1999). The data presented in Figure 1 show that the response to ATP is more dependent on extracellular Ca^{2+} than the responses to other nucleotides, which may indicate contribution from P2X receptors, but the lack of response to α,β-methlylene ATP clearly militates against involvement of $P2X_1$ or $P2X_3$ receptors. Thus, these results indicate that the response to ATP is likely to be mediated by $P2Y_1$ and P2X (possibly $P2X_2$) receptors.

Antagonist data presented in this study are largely consistent with the majority of the responses being mediated by $P2Y_1$ receptors (Boyer *et al*., 1996; Charlton *et al*., 1996a, b; Schachter *et al*., 1996; Hansmann *et al*., 1997). The tendency of higher concentrations of antagonist to depress the maximum of the agonist concentration – response curves is likely to be a consequence of the experimental design, generating a pseudo-irreversible antagonism. Antagonists were added 15 min prior to agonist addition. If the dissociation rates of antagonists such as suramin were slow compared to the association rates of agonists, then a depression of the maximum response at higher concentrations of antagonist is likely even if, under equilibrium conditions, the antagonist would be seen to be competitive and reversible. The data presented in Figures 4 and 5, and Table 1, are consistent with a response mediated via $P2Y_1$ receptors. One inconsistency is that the apparent pA_2 for A3P5P of 4.6–4.9 is lower than reported values of 5.7 for turkey $P2Y_1$, 5.8 for bovine $P2Y_1$ and 6.0 for human platelet $P2Y_1$ (Boyer *et al*., 1996; Fagura *et al*., 1998). Park *et al*. (1998) reported that a related antagonist was rapidly broken down by ectonucleotidases. In the present study, the antagonist was exposed to the cells for 15 min prior to adding agonist, by which time the concentration of antagonist may have been reduced. Of the known P2Y receptors, A3P5P is thought to be selective for the $P2Y_1$ receptor (Boyer *et al*., 1996), so even with this apparently low potency, the profile is consistent with the response being largely at $P2Y_1$ receptors.

Importantly, the antagonist results exclude the involvement of $P2Y_{12}$ receptors. Even at high concentrations A3P5P did not inhibit the ADP-induced inhibition of adenylyl cyclase in platelets (Jin *et al*., 1998) or PC12 cells (Unterberger *et al*., 2002); both these responses are at native $P2Y_{12}$ receptors. There is currently insufficient pharmacological data to similarly evaluate $P2Y_{13}$ receptors.

In conclusion, we have shown that in rat-cultured brain cells the dominant calcium mobilisation response to nucleotides has the characteristics expected of $P2Y_1$ receptors and is largely located on glial cells. Neurons in these cultures apparently lack P2Y receptors coupled to cytosolic Ca^{2+} responses. There is evidence of an additional response to ATP, possibly via activation of a population of P2X receptors.

There is a body of evidence that provides a clear indication of a widespread role for P2Y receptors in rat cerebral neurons – a presynaptic role on nerve terminals. Accordingly, several reports have shown that release of biogenic amines (noradrenaline, dopamine and 5-hydroxytryptamine) and glutamate is regulated by presynaptic P2Y receptors (von Kugelgen *et al*., 1994; Koch *et al*., 1995; 1997; Zhang *et al*., 1995; Bennett & Boarder, 2000; Inoue & Koizumi, 2001). From the current studies there emerges evidence for a second role for P2Y receptors in rat brain: glial cells are apparently influenced by nucleotides via a phospholipase C-coupled $P2Y_1$ receptors. It would be intriguing to determine, given these two contrasting roles, the net influence that nucleotides impart on cerebral function.

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