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# $P2X_4$, $P2Y_1$ and $P2Y_2$ receptors on rat alveolar macrophages

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**1** ATP receptors present on rat alveolar macrophages (NR8383 cells) were identified by recordings of membrane current, measurements of intracellular calcium, RT–PCR and immunocytochemistry.

**2** In whole-cell recordings with a sodium-based internal solution, ATP evoked an inward current at −60 mV. This reversed at 0 mV. The $EC_{50}$ for ATP was 18 $\mu$M in normal external solution (calcium 2 mM, magnesium 1 mM). The currents evoked by 2′,3-*O*-(4-benzoyl)benzoyl-ATP were about five-fold smaller than those observed with ATP. ADP, UTP and $\alpha\beta$-methylene-ATP ($\alpha\beta$meATP) (up to 100 $\mu$M) had no effect. ATP-evoked currents were potentiated up to ten-fold by ivermectin and were unaffected by suramin (30–100 $\mu$M), pyridoxal-phosphate-6-azophenyl-(2,4-sulphonic acid) (30–100 $\mu$M), and brilliant blue G (1 $\mu$M).

**3** In whole-cell recordings with a potassium-based internal solution and low EGTA (0.01 mM), ATP evoked an inward current at −60 mV that was followed by larger outward current. ADP and UTP (1–100 $\mu$M) evoked only outward currents; these reversed polarity at the potassium equilibrium potential and were blocked by apamin (10 nM). Outward currents were also blocked by the phospholipase C inhibitor U73122 (1 $\mu$M), and they were not seen with higher intracellular EGTA (10 mM). Suramin (30 $\mu$M) blocked the outward currents evoked by ATP and UTP, but not that evoked by ADP. PPADS (10 $\mu$M) blocked the ADP-evoked outward current without altering the ATP or UTP currents.

**4** RT–PCR showed transcripts for P2X subunits 1, 4 and 7 (not 2, 3, 5, 6) and P2Y receptors 1, 2, 4 and 12 (not 6). Immunocytochemistry showed strong $P2X_4$ receptor expression partly associated with the membrane, weak $P2X_7$ staining that was not associated with the cell membrane, and no $P2X_1$ receptor immunoreactivity.

**5** We conclude that rat alveolar macrophages express (probably homomeric) $P2X_4$ receptors, but find no evidence for other functional P2X subtypes. The P2Y receptors are most likely $P2Y_1$ and $P2Y_2$ and these couple through phospholipase C to an increase in intracellular calcium and the opening of SK type potassium channels.

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**Keywords:** P2X; purinergic receptor; P2Y; phospholipase C; SK channel; macrophages

**Abbreviations:** BzATP, 2′3-*O*-(4-benzoyl)benzoyl-ATP; $\alpha\beta$meATP, $\alpha\beta$-methylene-ATP; PPADS, pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid; LPS, lipopolysaccharide; U73122, 1-[6-[[17 $\beta$-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1-*H*-pyrrole-2,5-dione

## Introduction

The alveolar macrophage is the first line of defence against inhaled pathogens, and they appear to be involved in the production and maintenance of airway inflammation in asthma and other inflammatory lung diseases (Holgate, 2002; Barnes, 2003). They produce and release inflammatory mediators such as interleukin-1$\beta$ (IL-1$\beta$), TNF-$\alpha$ and macrophage inflammatory protein 1$\alpha$ (MIP-1$\alpha$), which can contribute in the short term to bronchoconstriction and hyper-reactivity, and in the longer term to the development of pulmonary fibrosis (Kelly *et al*., 2003). One of the principal stimuli to the release of these inflammatory cytokines from lipopolysaccharide-(LPS) primed macrophages in other tissues is ATP acting at P2 receptors (Di Virgilio *et al*., 2001). ATP causes a rise in intracellular calcium concentration ($[Ca]_i$) in macrophages; part of this action ($\mu$M in concentrations, mimicked by ADP, UTP and UDP) is independent of extracellular calcium, whereas part of it (mM in concentrations of ATP, not mimicked by other nucleotides) requires calcium entry from outside (Sung *et al*., 1985; Greenberg *et al*., 1988). Thus, both P2Y and P2X receptors have been implicated in these effects.

The preponderance of studies have since focused on the part played by the $P2X_7$ (formerly P2Z) receptor (Surprenant *et al*., 1996). Activation of this receptor results in processing and release of IL-1$\beta$ in macrophages (Perregaux & Gabel, 1994; Ferrari *et al*., 1997; Perregaux *et al*., 2000) by the shedding of membrane micovesicles (Mackenzie *et al*., 2001), and LPS-primed peritoneal macrophages from mice with a $P2X_7$ gene disruption do not release IL-1$\beta$ in response to ATP (Solle *et al*., 2001). The main aim of this work was to study the actions of ATP on lung macrophages and to identify the P2 receptor types involved.

The NR8383 cell line was originally cloned from resident macrophages lavaged from the lungs of Sprague–Dawley rats (Helmke *et al*., 1987). The cells synthesize and release IL-1$\beta$

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and TNF-α in response to activation of CD8α (Lin *et al*., 2000). TNF-α and MIP-1α are also released by agonists at leukotriene ($LTD_4$) receptors (Menard & Bissonnette, 2000). The cells respond to ATP with an increase in intracellular calcium concentration ($[Ca]_i$) (Zhang *et al*., 1997), but otherwise there is little known about the expression of P2 receptors by these cells, or the consequences of their activation. As an initial step in the characterization of the receptors we have studied the actions of some purines and pyrimidines on membrane currents using patch-clamp recording, we have measured the changes in $[Ca]_i$ that they cause, and we have sought the presence of P2 receptor transcripts by RT–PCR.

## Methods

### *Immunocytochemistry and RT–PCR*

The cells used were NR8383, a rat alveolar macrophage cell line (ATCC, Rockville, MD, U.S.A.). For immunostaining, cells were fixed with Zamboni's fixative (30 min), blocked with 5% goat serum in buffered saline with 2% Triton X-100 (30 min) and incubated with primary antibody for 2–3 h at room temperature. Cells were rinsed and FITC-labelled secondary antibody applied for 1 h, rinsed and mounted on slides for viewing under confocal microscope. Antibodies used were: rabbit polyclonal anti-$P2X_1$ ($10\,\mu g\,ml^{-1}$); anti-$P2X_4$ ($0.6\,\mu g\,ml^{-1}$), anti-$P2X_7$ ($0.6-2\,\mu g\,ml^{-1}$) from Alomone Labs (Israel).

RNA was extracted from $1 \times 10^6$ cells using an RNeasy kit (Qiagen West Sussex UK.), including DNase I treatment according to the manufacturer's instructions. First-strand cDNA was prepared from 5 μg total RNA in a 20 μl reaction using an oligo(dT) primer and Superscript II First Strand Synthesis System for RT–PCR (Invitrogen Paisley, UK). Reverse transcriptions were also performed in the absence of reverse transcriptase as a control for contaminating genomic DNA. PCR reactions were performed with mRNA-specific primer pairs for P2X and P2Y as listed below. The reaction contained 1 μl cDNA, 1 μl forward primer (10 pmol), 1 μl of reverse primer (10 pmol), 25 μl 2 × Reddymix PCR master mix (ABgene: contains 1.25 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP) and 22 μl $H_2O$. Amplification conditions were 60 s at 94°C, 30 s at 55°C, 45 s at 72°C for 35 cycles and finally 5 min at 72°C. Products were resolved on a 1.5% agarose gel by electrophoresis. The primers used (forward, reverse and product size (bp)) were as follows: $P2X_1$: CAGAAAGGAAAGCCCAAGGTATTC, TGACGACGGTTTGTCCCATTC, 508; $P2X_2$ GTGACTACCTCAAGCATTG, CTGTTGGGAAGGCTCAGGGAC, 785; $P2X_3$: GGACATAAAGAGGTGCCGCTTC, AACACTGGGTTGGTTGACGCAG, 534; $P2X_4$: GGAACATCCTCCCCAACATCAC,TTCATCTCCCCCGAAAGACC, 553; $P2X_5$: TGTCACGCTGGGGAGTCTGTTGTAG, TTGCTATTCTGCTTCCTGCCAC, 869; $P2X_6$: AGAGTAGTGCTGTGCCCAGGAAAC, CCTCAAAGTCCCCTCCAGTCATAG, 228; $P2X_7$: AATGAGTCCCTGTTCCCTGGCTAC, CAGTTCCAAGAAGTCCGTCTGG, 468; $P2Y_1$: TCCTCTTCATTCCGATGTGCC, TCTTCTTCTTGAGCCTGCCCAG, 391; $P2Y_2$: GGGACGAACTGGGTTACAAATGTC, GGTGTGGCAACTGAGGTCAAGTG, 785; $P2Y_4$: CAACCAATGCCAATGGAACTACC, ACTTGTCCCCCGTGAAGAGATAG, 414; $P2Y_6$: TGCTTGGGTGGTATGTGGAGTC, TGTTGTGTGAAGTAGAAGAGGATAGGG, 489; $P2Y_{12}$: TCAGCCAACACCACCTCCATTC, CCAGACCAAACTCCGACTTCAAG, 544; β-actin: GGCTCTCTTCCAGCCTTCCTTCTTG, CACAGAGTACTTGCGCTCAGGAGG, 241.

### *Electrophysiology and data analysis*

Whole-cell patch-clamp recordings were made at room temperature using an EPC9 amplifier and Pulse software (HEKA, Germany); agonists were applied by fast-flow using the RSC system (Bio-Logic Science Instruments, France). Patch pipettes were filled with high EGTA solution (mM): NaCl or KCl 145, HEPES 10, glucose 10, EGTA 10; or low EGTA solution (mM): NaCl or KCl 145, HEPES 10, glucose 10, EGTA 0.01, $CaCl_2$ 0.1. The external solution was NaCl 145, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10; solutions were maintained at pH 7.3 and osmolarity of 300–310 $mosmol\,l^{-1}$.

Results are expressed as mean ± s.e.m. for number of cells examined and curves were fit from pooled data using Kaleidagraph (Synergy Software, Reading, PA, U.S.A.). Agonist concentration–response curves for inward currents and changes in $[Ca]_i$ were fit by $I/I_{max} = 100\,([A]^n/[A]^n + EC_{50}{}^n)$, where $I$ is the peak current evoked by concentration [A] as a percent of maximum current. ATP, 2′,3-*O*-(4-benzoyl)benzoyl-ATP (BzATP), α,β methylene ATP (αβmeATP), ivermectin and Coomassie brilliant blue G, were obtained from Sigma Dorset UK; suramin was from Bayer (Germany), pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) from Sigma, apamin from Sigma and U73122 was from Calbiochem Nottingham UK.

### *Fluo-4 measurements of intracellular calcium*

NR8383 cells were incubated for 30 min at 37°C with Fluo-4 AM (3 μM; Molecular Probes, Eugene, OR, U.S.A.) in the following buffer (mM): NaCl 136, KCl 1.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 5, $CaCl_2$ 2, glucose 6, HEPES 20 and EGTA 5. Cells were washed three times, resuspended in the same buffer without EGTA after which $3 \times 10^5$ $cells\,ml^{-1}$ were placed in 1 ml volumes into cuvettes for fluorescence measurements (488 nm) using the Cairn Integra fluorometer (Cairn Research, Kent, U.K.). Concentration–response curves were obtained from cumulative (UTP) and non-cumulative (UTP and ADP) additions of agonist; no differences in UTP dose–response curves were obtained using either type of agonist application and so results have been pooled.

## Results

### *P2X receptor-mediated currents*

We first used a high EGTA NaCl-based internal solution (see Methods) in order to isolate potential P2X-mediated currents because the high EGTA would be expected to abrogate intracellular calcium rises due to activation of P2Y receptors and the internal Na blocks all potassium currents (see below). ATP (30 μM) evoked relatively sustained inward currents at the holding potential of −60 mV in all cells examined ($n = 56$; Figure 1a); the current reversed at 0 mV and showed slight

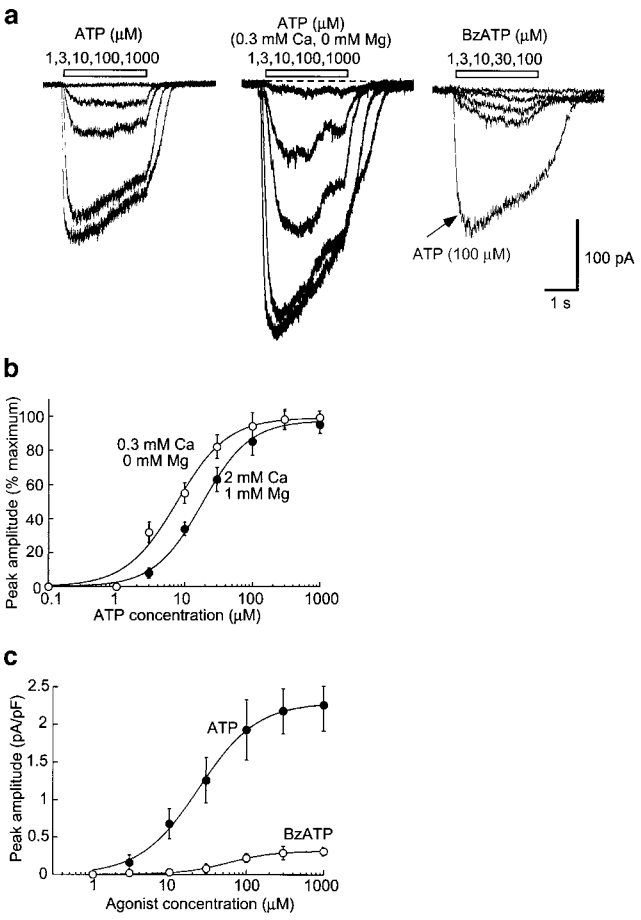



**Figure 1** ATP-induced currents. (a) Currents evoked by ATP (left), ATP in solution with reduced divalent ion concentration (middle) and BzATP (right). (b) Concentration–response curves for ATP to cause inward current, both in normal solution and solution containing reduced divalent ion concentration. Current normalized to maximum current in each case: $n=4$–8 cells for each point. (c) Relative effectiveness of ATP and BzATP to evoke inward current. Currents normalized to cell capacitance.

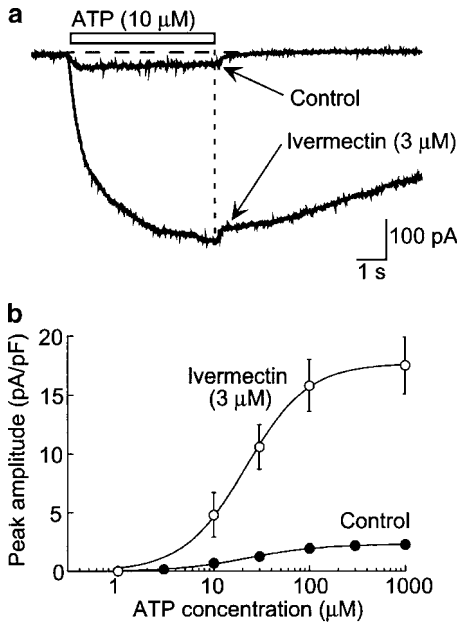



**Figure 2** Ivermectin potentiates ATP-evoked current. (a) Representative traces of current before and after adding ivermectin (3 $\mu$M). (b) Averaged data show that ivermectin increased the ATP-evoked current about 10-fold but did not alter the $EC_{50}$ ($n=5$).

inward rectification (see Figure 4b), consistent with typical P2X-mediated cationic currents (North, 2002). ATP concentration–response curves were constructed in normal external solution and also in a low calcium/magnesium-free external solution, which is known to potentiate $P2X_7$ receptor-mediated currents by several fold (North, 2002); $EC_{50}$ values were $18\pm2$ and $7\pm1.5\,\mu$M ($n=10$), respectively (Figure 1). Maximum current responses in low divalent external solution were 1.3–2-fold greater than in normal external solution (Figure 1a). A similar small increase in maximum ATP-mediated currents in low divalent solutions was observed from recordings made in HEK293 cells transfected with rat $P2X_4$ receptors (data not shown). BzATP, which is several fold more potent than ATP at $P2X_7$ receptors, was a weak partial agonist, evoking currents of about 15% that were evoked by ATP (Figure 1a, c); this relationship was not altered in low divalent external solutions ($n=3$). ADP, UTP, GTP and $\alpha\beta$me-ATP (all at 30–100 $\mu$M, $n=4$–10) were without effect when recordings were made with this high EGTA NaCl internal solution.

The rat $P2X_4$ receptor is not inhibited by high concentrations of either suramin or PPADS, while the rat $P2X_7$ receptor is fully inhibited by a concentration of BBG (0.3–1 $\mu$M) that has little action at $P2X_4$ receptors (Buell *et al*., 1996; Jiang *et al*., 2001). Therefore, we examined these compounds on the ATP-evoked currents in NR8383 cells. Neither suramin (30, 100 $\mu$M) nor PPADS (30, 100 $\mu$M), nor BBG (1 $\mu$M) inhibited ATP- or BzATP-mediated currents ($n=3$–6). Ivermectin, a channel modulator that has been shown to enhance specifically $P2X_4$-mediated currents in heterologous expression systems (Khakh *et al*., 1999), potentiated ATP-mediated currents in NR8383 cells by up to 20-fold without significantly altering the $EC_{50}$ value (Figure 2).

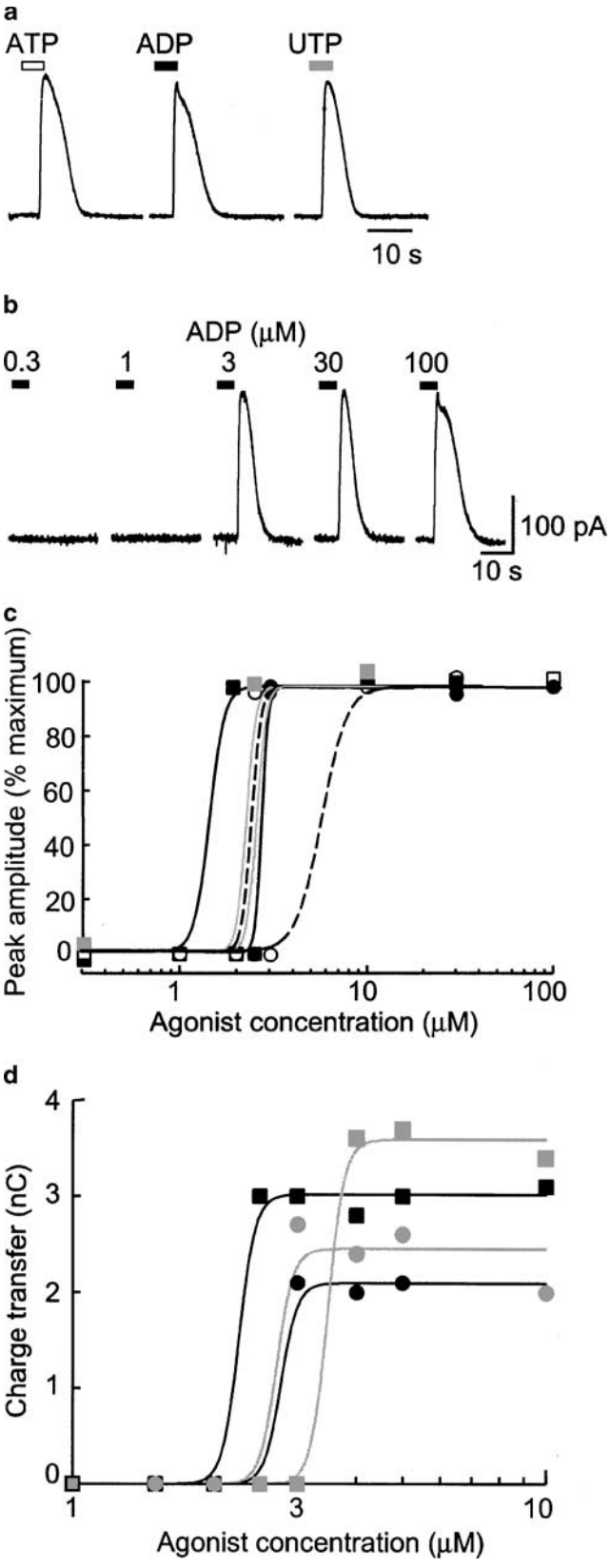
These currents elicited by ATP were not obviously different when the EGTA concentration of the internal solution was 0.01 mM, or when a potassium-based internal solution was used containing 10 mM EGTA (in the latter condition, delayed rectifier potassium currents were obvious when the cells were depolarized).

### *P2Y receptor-mediated currents*

We next used a potassium-based internal solution with low EGTA (0.01 mM). At −60 mV ATP evoked an inward current, and this was similar in all respects to that described above. However, within a few seconds of beginning the application ($5\pm0.4$ s, $n=10$) this reversed to a large outward current ($n>30$; Figures 3–6). ADP and UTP did not elicit any inward current (see above) but were as effective as ATP to elicit the outward current (Figure 3a). The time to onset of the outward current induced by ADP or UTP at −60 mV was $3.8\pm0.1$ s ($n=20$, e.g. Figure 4a). It was difficult to examine the concentration-dependence of the outward current because, in any given cell, a very small increase in concentration (typically from 1 to 3 $\mu$M) was sufficient to activate fully the current (Figure 3b–d).

The currents evoked by ADP and ATP showed a linear current–voltage relationship with reversal potential at −105 mV in normal external solution (Figure 4a, b); the delayed component of the ATP-evoked current showed similar properties, even though the initial inward component (at −60 mV) reversed at 0 mV (Figure 4b). The reversal potential of the UTP- and ADP-evoked currents in external solutions containing 2, 20 and 80 mM potassium changed as expected for a potassium-selective current (Figure 4c).

The phospholipase C inhibitor U73122 (1 μM) abolished agonist-evoked outward currents without affecting the ATP-induced inward current ($n = 6$; Figure 5a), while the DMSO vehicle was without effect on either inward or outward current ($n = 2$). This concentration of U73122 was effective within 2 min of introduction into the superfusion solution and reached maximum effect within 4 min; no reversal of blockade was observed over a 10-min washout period ($n = 4$). Agonist-evoked outward currents were also abolished by the SK2/SK3-channel blocker apamin (100 nM, $n = 4$) (Figure 5b) but were



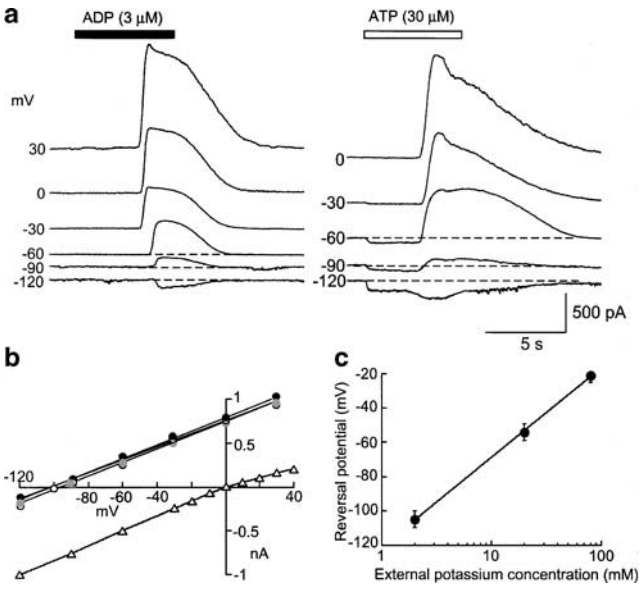




**Figure 4** P2 agonists open potassium-selective channels. (a) Examples of currents recorded in response to ADP (left) and ATP (right) at holding potentials from −120 to 30 mV. Note that ATP but not ADP evokes an inward current prior to the outward current. (b) Averaged current voltage plots from experiments such as those shown in (a) circles are ATP, UTP and ADP respectively with potassium-based internal solution (normalized to 1 at 30 mV). Open triangles are ATP-evoked currents with sodium-based internal solution (normalized to 1 at −120 mV). Standard error bars are smaller than symbol size. (c) Reversal potential ($E_{rev}$) for outward current (pooled using ATP, UTP and ADP) as a function of extracellular potassium concentration ($[K]_o$). The fitted line is $E_{rev} = 58 \log\{[K]_o/147\}$.

**Figure 3** P2 agonists evoke outward currents. (a) Outward currents recorded at −60 mV in one cell in response to ATP, ADP and UTP (each 3 μM). Note that this concentration of ATP does not evoke any inward current. (b) Currents in response to increasing concentrations of ADP, as indicated. (c) Concentration–response curves plotted for individual cells from six experiments such as that as illustrated in (b) (ADP two cells: black circles, squares, line; UTP two cells: grey circles, squares, line; ATP two cells: open circles and squares, broken line). (d) Concentration–response curves plotted for individual cells from four experiments, using several concentrations in the 1–10 μM range. In this case, the time integral of the current is plotted. ADP two cells: black circles, squares, line; UTP: grey circles, squares, line.

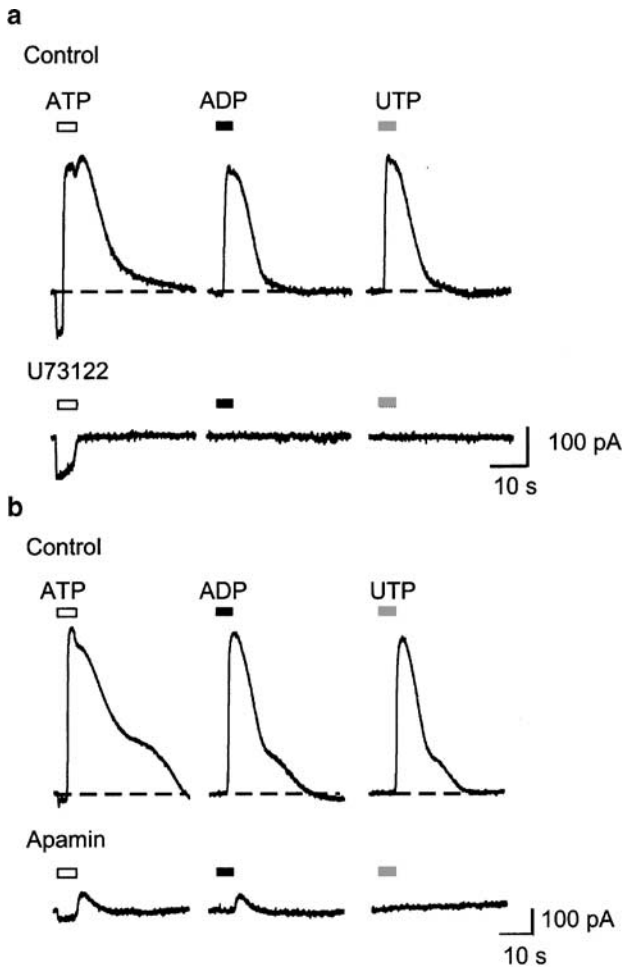



**Figure 5** P2 agonists open calcium-activated, SK potassium channels. (a) Currents in one cell in response to ATP, ADP and UTP (each at 30 $\mu$M). The outward currents were completely blocked by the phospholipase C inhibitor U73122 (1 $\mu$M). (b) A similar experiment in another cell in which apamin (100 nM) blocked the outward currents. Note that the inward current evoked by ATP was not changed by U73122 or apamin.

unaltered by tetraethylammonium (1–10 mM). The inhibition by apamin was irreversible during a 20-min washout period.

Suramin (30 $\mu$M) abolished the currents elicited by ATP and UTP but had no effect on the ADP-evoked outward current (Figure 6b). Conversely, PPADS (10 $\mu$M) abolished currents evoked by ADP (3, 10, 30 $\mu$M) and had no significant effect on ATP or UTP-evoked currents (3–30 $\mu$M; Figure 5a). Neither ADP nor UTP evoked any currents when the high EGTA concentration (10 mM) was used with the potassium chloride internal solution ($n=5$).

### *Fluo-4 measurements of intracellular calcium*

ADP and UTP consistently evoked concentration-dependent increases in intracellular calcium $[Ca]_i$. The threshold for activation by ADP was 10–30 nM with an $EC_{50}$ of $0.8\pm0.5$ $\mu$M ($n=4$); for UTP the threshold was approximately 300 nM and the $EC_{50}$ was $4\pm0.9$ $\mu$M ($n=4$) (Figure 7). PPADS (10 $\mu$M) inhibited ADP-mediated responses but had no significant effect on UTP-evoked responses (Figure 7d). Suramin (100 $\mu$M) did not alter ADP responses but strongly inhibited

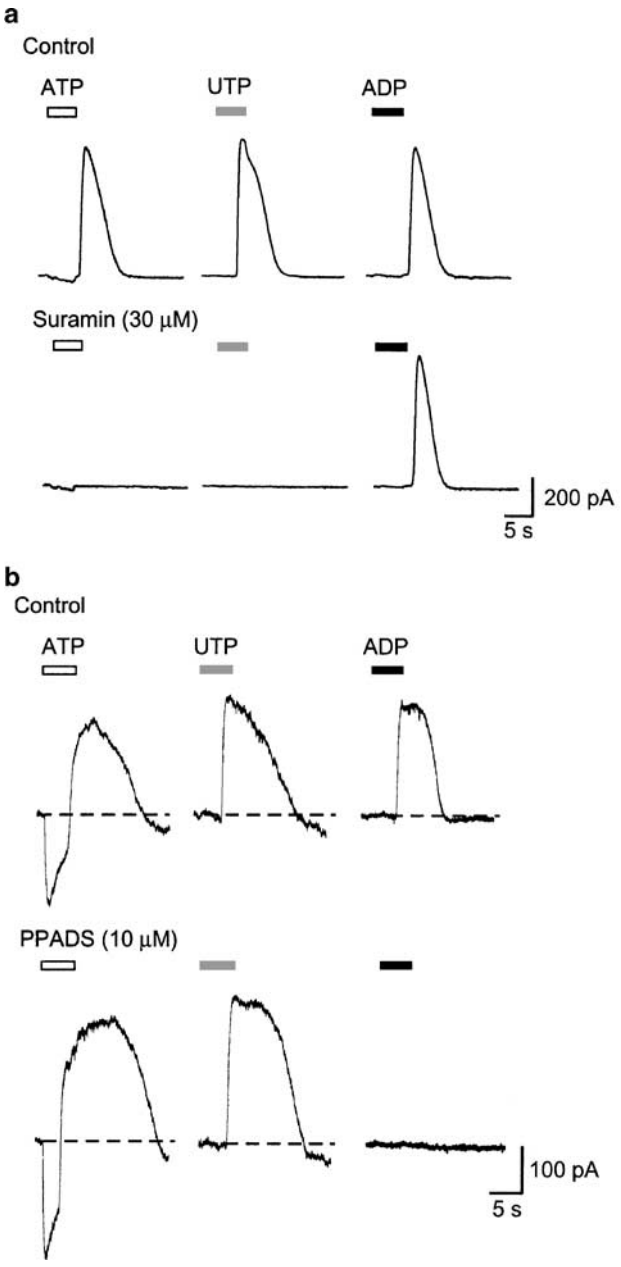



**Figure 6** Antagonists selectively block the outward current evoked by ADP, and by ATP or UTP. (a) Currents recorded from one cell in response to 5 s applications of ATP, UTP and ADP (each 3 $\mu$M), first in control solution and then in suramin (3 $\mu$M). (b) Currents recorded from one cell in response to 5 s applications of ATP, UTP and ADP (each 30 $\mu$M), first in control solution and then in PPADS (10 $\mu$M). Note that ATP-evoked inward currents are unaffected by PPADS.

UTP-evoked responses (Figure 7d). Preincubation with U73122 at the concentration (1 $\mu$M) that abolished ADP- and UTP-evoked potassium currents reduced calcium responses by about 40%, although a higher concentration (10 $\mu$M) inhibited responses by >85% (Figure 7).

### *mRNA expression and immunocytochemistry*

Figure 8 shows that NR8383 cells express mRNA transcripts for $P2X_1$, $P2X_4$, $P2X_7$ as well as for $P2Y_1$, $P2Y_2$, $P2Y_4$, and

$P2Y_{12}$, but not for $P2X_2$, $P2X_3$, $P2X_5$ or $P2X_6$ or $P2Y_6$ (Figure 8a). Immunocytochemistry with $P2X_1$-, $P2X_4$- and $P2X_7$-specific antibodies showed no staining for $P2X_1$ subunits, strong immunoreactivity for $P2X_4$ subunits (Figure 8b), and weaker staining for $P2X_7$ subunits (Figure 8b). $P2X_4$ immunoreactivty was present both in the plasma membrane and underlying cytoplasm, whereas the $P2X_7$ immunoreactivity was weak and punctate throughout the cytoplasm with no clear labelling of the cell membrane apparent.

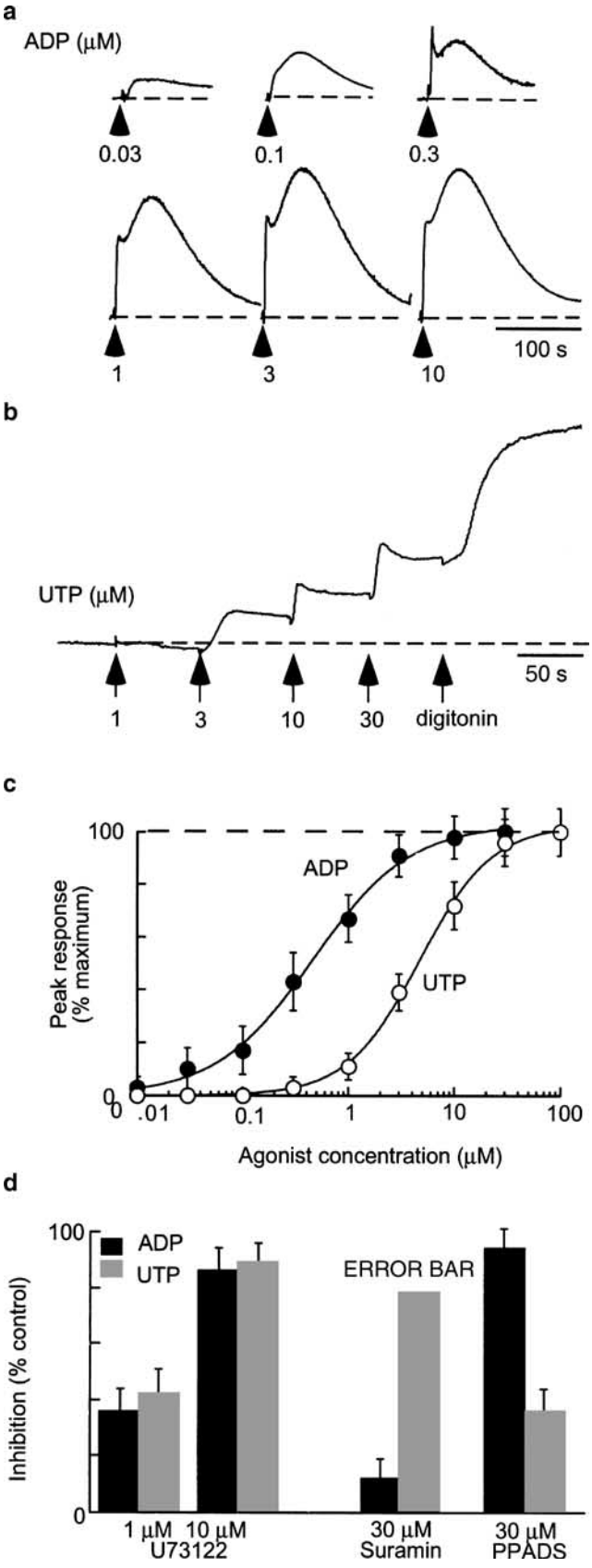



**Figure 7** Rises in $[Ca]_i$ evoked by ADP and UTP. (a) Representative fluorescence traces recorded from Fluo-4 loaded cells in response to ADP (concentrations as indicated). Responses are from separate experiments to avoid desensitization that was observed with ADP; each is normalized to the signal observed with digotonin. (b) Fluorescence traces during cumulative additions of UTP; note minimal desensitization. (c) Summary of experiments such as those shown in (a) and (b) (ADP $n=4$, UTP $n=3$). (d) Inhibition of $[Ca]_i$ signals evoked by ADP (filled bars, 3 $\mu$M) and UTP (grey bars, 3 $\mu$M) by U73122, suramin and PPADS ($n=5$ for each histogram).

## Discussion

The main conclusion of this work is that the NR8383 cells express both P2X and P2Y receptors. It is convenient to discuss the evidence for this in turn.

### *P2X receptors*

The rapid-onset inward current elicited by ATP but not UTP or ADP is indicative of P2X receptor involvement. The effective concentrations of ATP ($EC_{50}$ about 15 $\mu$M), the time course of the current, and the reversal potential close to 0 mV are all fully consistent with P2X receptors. Several further distinguishing features of the current allow us to conclude that it results from activation of $P2X_4$ receptors (see North, 2002). These are the lack of effect of $\alpha\beta$meATP, the potentiation by ivermectin and the lack of any blockade by suramin or PPADS (Buell *et al*., 1996; North, 2002). Three features, in particular, distinguish it from currents at $P2X_7$ receptors: these are the relatively low effective concentrations of ATP (Figure 1), the finding that BzATP is much less potent than ATP (Figure 1c), and the weak potentiation by reducing the concentration of divalent cations (Figure 1b). We have observed similar small effects of these changes in divalent ion concentrations in HEK293 cells transfected with rat $P2X_4$ subunit (data not shown), whereas the currents at rat $P2X_7$ receptors are typically increased more than five-fold (Surprenant *et al*., 1996). Three features exclude the presence of a significant component through $P2X_1$ receptors: these are the weak desensitization of the currents, the lack of action of $\alpha\beta$meATP and the absence of any block by suramin and PPADS. Together with the results from the RT–PCR and immunocytochemistry, these findings allow us to conclude that the current induced by ATP on NR8383 cells results from the activation of $P2X_4$ receptors.

Membrane currents induced by ATP in macrophages and related cells have recently been reviewed (North, 2002). In some cases, the current described has properties that closely resemble those of the cloned $P2X_7$ receptor, such as the requirement for mM concentrations of ATP, marked potentiation by low divalent ion concentrations, and the observation that BzATP is more potent than ATP. These include J774 cells

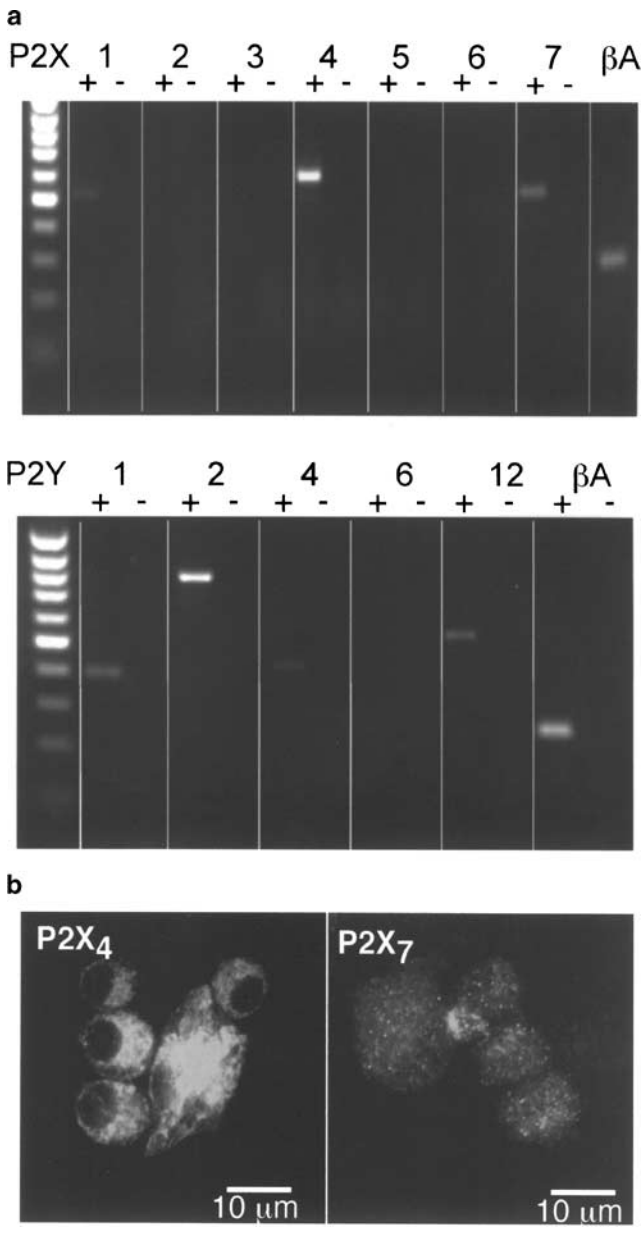



**Figure 8** Detection of P2 receptor mRNAs and protein. (a) RT–PCR shows amplification of appropriately sized products corresponding to $P2X_1$, $P2X_4$ and $P2X_7$ subunits (top panel), and $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{12}$ (bottom panel). No $P2X_2$, $P2X_3$, $P2X_5$, $P2X_6$ or $P2Y_6$ was detected. Vertical lines separate lanes for clarity. (b) Confocal microscope images of cells exhibiting immunoreactivity for $P2X_4$ (left) and $P2X_7$ (right) subunits.

(Buisman *et al*., 1988; Surprenant *et al*., 1996), NTW8 microglia (Chessell *et al*., 1997) and human monocyte-derived macrophages (Rassendren *et al*., 1997). However, there are other studies for which the agreement is much less good (monocyte-derived macrophages; Eschke *et al*., 2002; brain amoeboid microglia: Haas *et al*., 1996). In particular, rat microglia after 7–10 days in culture express two distinct components to the inward current activated by ATP and BzATP, the properties of which correspond very closely to those expected for $P2X_4$ and $P2X_7$ receptors (Visentin *et al*., 1999). Cells other than macrophages can exhibit responses to ATP that are very similar to those reported here for NR8383 cells. These include submandibular gland cells (Buell *et al*., 1996) and osteoclasts (Naemsch *et al*., 1999).

### *P2Y receptors*

The outward current component observed in response to ATP is clearly a potassium-selective current (Figures 4 and 5). The block by apamin identifies it as a member of the SK family of potassium channels (Bond *et al*., 1999). The block by U73122 indicates that it is activated as a result of calcium released by inositol 1,4,5-trisphosphate generated by the action of phospholipase C on phosphatidyl inositol 4,5-bisphosphate. The finding that the outward current was not observed with a high (10 mM) EGTA concentration inside the cell is consistent with this interpretation. The delay in the onset of the current and its explosive 'all-or-nothing' nature as a function of the agonist concentration might also be expected from an intracellular transduction mechanism providing considerable intracellular signal amplification.

Our results with Fluo-4 loaded cells show that ADP and UTP at concentrations that were effective to increase the potassium conductance also caused a substantial increase in $[Ca]_i$. We have not attempted to quantify the $[Ca]_i$ in these experiments, or to probe in any detail its source within the cell. The main point is that neither ADP nor UTP activated any inward current similar to that caused by ATP, but both caused substantial rises in $[Ca]_i$ that were largely ($\approx 85\%$) blocked by inhibition of phospholipase C with U73122 (Figure 7d). It is therefore likely that the P2 receptors activated by ADP and UTP in these experiments lead directly to stimulation of phospholipase C.

Potassium currents activated by P2Y receptor stimulation have been described in many cells and occur either by direct coupling to the channel (i.e. G protein $\beta\gamma$ subunit) or through an increase in $[Ca]_i$. The former case has been most widely described in neurons and cardiac muscle (e.g. Friel & Bean, 1990; Ikeuchi & Nishizaki, 1996; Matsuura & Ehara, 1996). The latter case has been best characterized in hepatocytes (Yamashita *et al*., 1996) and intestinal smooth muscle, where it brings about an ATP-mediated inhibitory postsynaptic potential (Koh *et al*., 1997). In all these tissues, an apamin-sensitive potassium conductance was involved. Microglia also exhibit an outward current in response to ATP (Langosch *et al*., 1994; Norenberg *et al*., 1994), but probably the cells having the response closest in its properties to NR8383 cells are rat osteoclasts (which of course have many of the properties and cell markers of macrophages). Weidema *et al*. (1997; 2001) describe that the inward current evoked by ATP is followed by calcium-activated potassium current. Only the potassium current was activated by UTP; the potassium current, but not the preceding inward current, was also blocked by suramin.

A main focus of the present work was the identification of the underlying P2Y receptor and this can be approached by comparing the currents with the known properties of rat P2Y receptors in heterologous expression systems (reviewed by von Kugelgen & Wetter, 2000; Sak & Webb, 2002). It is clear from the experiment illustrated in Figure 6 that PPADS (10 $\mu$M) and suramin (30 $\mu$M) distinguish two P2Y receptors. The current activated by ADP that was selectively blocked by PPADS, and the increase in $[Ca]_i$ with similar properties (Figure 7), probably results from activation of the $P2Y_1$ receptor. PPADS

(30 $\mu$M) has previously been shown to block the rat $P2Y_1$ receptor (Schachter *et al*., 1997). PPADS did not affect the current activated by ATP and UTP, which therefore involves a different P2Y receptor. The rat $P2Y_4$ receptor is activated by both ATP and UTP but is not blocked by suramin (100 $\mu$M) (Bogdanov *et al*., 1998) on the basis of our current measurements, we can therefore exclude its involvement. On the other hand, the rat $P2Y_2$ receptor is activated equally well by ATP and UTP and this is sensitive to blockade by suramin (Chen *et al*., 1996). Involvement of the $P2Y_6$ receptor, which is more selectively activated by UDP, is unlikely given that we failed to amplify its mRNA. The $P2Y_{12}$ (formerly P2T) receptor (Hollopeter *et al*., 2001), which is also expressed by NR8383 cells at the mRNA level, is most commonly linked to inhibition of adenyl cyclase ($G_{io}$ coupled) and thus perhaps less likely to underlie the increase in $[Ca]_i$ and potassium current.

The most parsimonious explanation of the present results is therefore that NR8383 cells express both $P2Y_1$ and $P2Y_2$ receptors. Such coexpression has previously been reported for *inter alia* rat glioma (C6) cells (Jin *et al*., 2001; Sabala *et al*., 2001), rat hepatocytes (Dixon *et al*., 2000) and human keratinocytes (Burrell *et al*., 2003). However, in rat hepatocytes, the ADP-mediated calcium influx, which was attributed to activation of $P2Y_1$ receptors, was suramin-sensitive (Dixon *et al*., 2000) in contrast to the suramin-insensitive ADP response in the present study on NR8383 cells. Thus, it remains possible that a distinct P2Y receptor, or perhaps a heteromeric metabotropic purine receptor, underlies the ADP-mediate response in either NR8383 cells or rat hepatocytes. In any event, given that the downstream signalling pathways are similar, at our present level of understanding, the advantage of such coexpression would be to generalize the sensitivity to a wider range of purine and pyrimidine nucleotides.

The significance of this to immune cells such as the lung macrophage is not yet clear. It has been widely held that macrophages release inflammatory cytokines such as IL-1$\beta$ as a result of activation of $P2X_7$ receptors. However, our failure in the present experiments to detect any functional $P2X_7$ receptors makes it important to determine whether NR8383 release IL-1$\beta$ in response to any purine nucleotide and, if they do, to investigate the underlying mechanism. It is possible that $P2X_7$ receptors are upregulated under conditions of inflammation in these lung macrophages. This might occur in response to the activation of P2Y receptors. The P2 receptors might even interact in a more direct way, such that P2Y activation results in phosphorylation of P2X receptors, as has been suggested for a parallel pathway in the case of the $P2X_3$ receptors on sensory neurons (see North, 2002). Future studies might distinguish these and other possibilities.

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