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SPECIAL REPORT

Inhibition by heterologously-expressed $P2Y_2$ nucleotide receptors of N-type calcium currents in rat sympathetic neurones

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The $P2Y_2$ nucleotide receptor has previously been shown to stimulate phosphoinositide breakdown. We now show that, when $P2Y_2$ receptors are heterologously expressed by cRNA injection into dissociated rat sympathetic neurones, activation of these receptors by uridine 5′-triphosphate (UTP) or adenosine 5′-triphosphate (ATP) inhibits the N-type voltage-gated calcium current by ~65%, with an IC_{50} of 0.5 μM. Thus, the same molecular species of nucleotide receptor can link to two different effector pathways.

Keywords: Nucleotide receptors; P_2 receptors; uridine triphosphate; adenosine triphosphate; sympathetic neurones; calcium currents

Introduction The $P2Y_2$ receptor is an adenosine 5′-triphosphate (ATP)/uridine 5′-triphosphate (UTP)-sensitive G protein-linked nucleotide receptor ('P_{2U} receptor') which normally stimulates the phosphoinositide signalling pathway (Parr *et al*., 1994; Chen *et al*., 1996). It was originally cloned from the NG108-15 neuroblastoma × glioma cell line (Lustig *et al*., 1993). However, when we applied UTP to these cells, we also noted that it inhibited voltage-activated Ca^{2+} currents (Filippov & Brown, 1996). This might have resulted from stimulation of another receptor or it might mean that the same receptor activates two different signalling pathways.

To test this, we have used the approach introduced by Ikeda *et al*. (1995), to assess whether the molecularly-defined rat $P2Y_2$ receptor could inhibit neuronal N-type Ca^{2+} currents when expressed in sympathetic neurones.

Methods Neurones were dissociated from superior cervical ganglia isolated from 15–19 day old rats (killed by CO_2 asphyxiation) and plated on poly-D-lysine-coated glass cover slips. Complementary RNA (cRNA) for the rat $P2Y_2$ receptor (Chen *et al*., 1996) was prepared, capped and poly-adenylated as described by Webb *et al*. (1993). Dissociated neurones were micro-injected with 1.25 or 0.5 μg μl^{-1} $P2Y_2$ cRNA plus 1.25 μg μl^{-1} cRNA for green fluorescent protein (GFP); controls were injected with GFP cRNA alone. After culturing for 14–24 h at 37°C, whole-cell Ca^{2+} currents were recorded from fluorescent cells as described by Caulfield *et al*. (1994), by use of patch-electrodes of 2–3 MΩ resistance filled with (mM): CsCl 110, $MgCl_2$ 3, HEPES 40, EGTA 3, Na_2ATP 2, Na_2GTP 0.5 (adjusted to pH 7.4 with CsOH). Cells were superfused at 10–12 ml min^{-1} with (mM): tetraethylammonium chloride 120, KCl 3, $MgCl_2$ 1.5, $BaCl_2$ 5 (or $CaCl_2$), HEPES 10, D-glucose 11 and tetrodotoxin 0.5 μM (buffered to pH 7.35 with NaOH; temperature 20–24°C). Ba^{2+} was used as charge carrier unless otherwise stated. Currents were recorded by discontinuous voltage-clamp (Axoclamp 2A, 6–8 kHz at 50% duty cycle) and leak-corrected by subtracting currents remaining after substituting 5 mM Co^{2+} for Ca^{2+} or Ba^{2+}. Data are given as mean ± s.e.mean (significance at $P<0.05$ by Student's *t* test).

Results Ca^{2+} currents were substantially (66.7 ± 9.2%; $n=6$) and irreversibly inhibited by 200–300 nM ω-conotoxin GVIA (CTX; 8 min exposure) but insignificantly (5.8 ± 4.1%; $n=5$) by 2 μM nifedipine. Hence, the currents were predominantly N-type, with negligible contamination by L-type currents; the nature of the residual (CTX/dihydropyridine-insensitive) current is not known (see Pan *et al*., 1996, and references therein).

In cells pre-injected with 1.25 μg μl^{-1} $P2Y_2$ cRNA, UTP (100 μM) inhibited currents by 64.9 ± 1.9% ($n=17$) with Ba^{2+} as charge carrier (Figure 1Ab) and by 68.7 ± 3.3% ($n=12$) with Ca^{2+} as a charge carrier. At 0.5 μg μl^{-1} $P2Y_2$ cRNA, inhibition of Ba^{2+} currents was 50.3 ± 2.1% ($n=5$). No significant inhibition was recorded in cells injected with GFP cRNA alone (Figure 1Aa). UTP inhibited the residual current in CTX solution by 23.9 ± 4.6% ($n=6$), corresponding to ~8% of total current before addition of CTX. Thus, UTP inhibited the CTX-sensitive component of I_{Ca} by ~85%.

The IC_{50} for UTP was 0.50 ± 0.03 μM at 1.25 μg μl^{-1} cRNA and 0.90 ± 0.05 μM at 0.5 μg μl^{-1} cRNA (Figure 1B). Corresponding values for ATP, inosine triphosphate (ITP) and adenosine 5′-diphosphate (ADP) at 1.25 μg μl^{-1} cRNA (3–4 cells for each) were (μM): ATP, 0.50 ± 0.03; ITP, 3.1 ± 0.20; ADP, 25.3 ± 11.1. At 10 μM agonist, the order of diminishing inhibition was UTP ≥ ATP ≥ ITP > ADP > UDP ≈ GTP (guanosine 5′-triphosphate) (Figure 1C).

Discussion The principal point emerging from these experiments is that the rat $P2Y_2$ receptor can activate two quite different signalling pathways with comparable facility. Thus, the IC_{50} values for inhibition of N-type Ca^{2+} currents by UTP and ATP (~0.5 μM) were near to their EC_{50} for phosphoinositide signalling (0.2 μM; Chen *et al*., 1996); and the relative activities of the different nucleotides tested accords broadly with their ability to stimulate Ca^{2+} release (Lustig *et al*., 1993; Chen *et al*., 1996) or inositol phosphate production (Nicholas *et al*., 1996) in other $P2Y_2$-transfected cells.

It is unlikely that Ca^{2+} current inhibition is a consequence of intracellular Ca^{2+} release (leading to Ca^{2+} channel inactivation) since: (i) phospholipase C (PLC)-linked receptor activation produces very little (<20 nM) rise in intracellular Ca^{2+} in these cells and any such rise is prevented by 3 mM EGTA (S.J. Marsh, unpublished observations); (ii) Ca^{2+}-induced inactivation would simply reduce current amplitude, not slow its activation as in Figure 1Ab; (iii) the analogous inhibition of I_{Ca} by the endogenous P_{2Y} receptor in NG108-15 cells was not prevented by 20 mM intracellular BAPTA (Filippov & Brown, 1996); and (iv) preliminary experiments (A.K. Filippov, unpublished data) indicate that inhibition is substantially (~60%) prevented by

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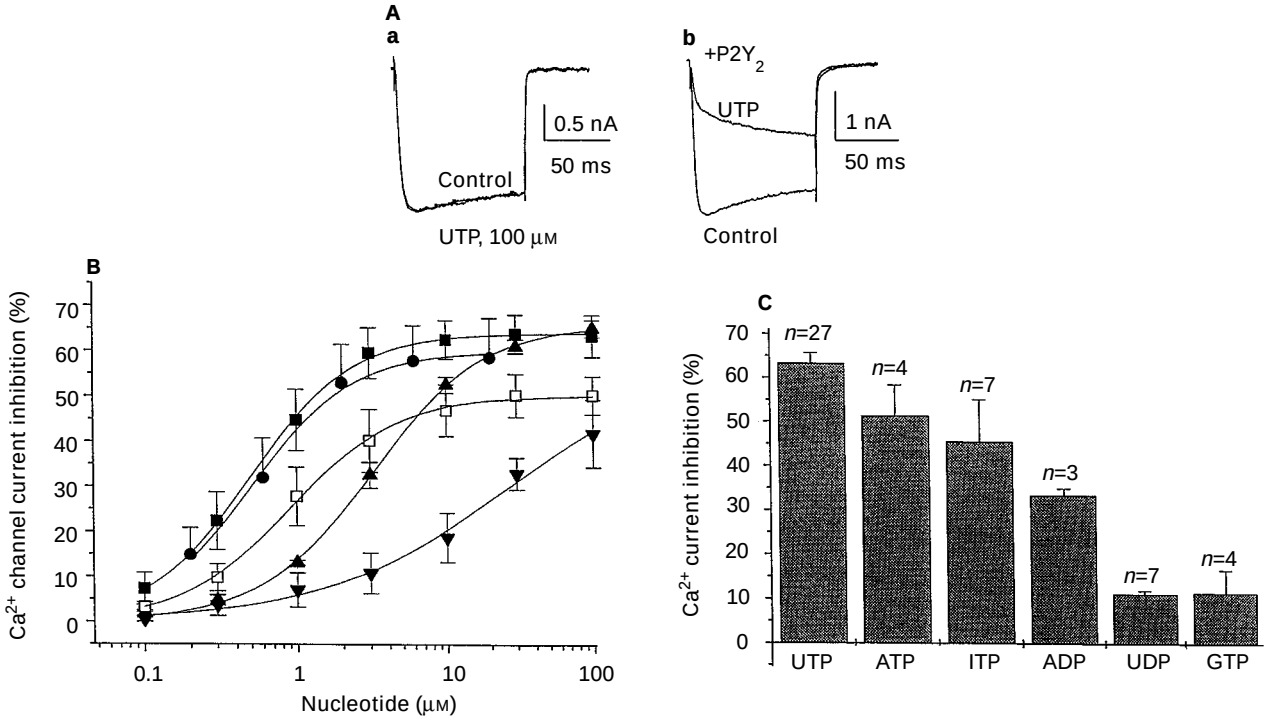

Figure 1 (A) Effects of UTP on Ca^{2+} channel currents (recorded with Ba^{2+} as charge carrier; see Methods) in two cells pre-injected with (a) $1.25\,\mu g\,\mu l^{-1}$ GFP cRNA and (b) $1.25\,\mu g\,\mu l^{-1}$ GFP cRNA plus $1.25\,\mu g\,\mu l^{-1}$ $P2Y_2$ receptor cRNA. Currents were recorded by stepping for 100 ms every 20 s from -90 mV to 0 mV. Records show superimposed leak-subtracted currents in the absence and presence of 100 μM UTP. (B) Concentration-dependence of Ca^{2+} channel current inhibition by UTP (■), ATP (●), ITP (▲) and ADP (▼) in cells pre-injected with $1.25\,\mu g\,\mu l^{-1}$ $P2Y_2$ cRNA. Current amplitude was measured 10 ms after the onset of the test pulse. Open symbols (□) show effects of UTP on cells pre-injected with $0.5\,\mu g\,\mu l^{-1}$ cRNA. Points show means and vertical lines s.e.mean of measurements in 3–4 cells for each nucleotide. Concentrations were added cumulatively, with 1 min exposure times; desensitization was negligible since the maximum inhibition produced at 100 μM UTP after cumulative addition (63.4±4.7%) was indistinguishable from that following a single application (see text). Curves were fitted to pooled data points by use of Origin 4.0 software to the equation $y = y_{max}x^{n_H}/(x^{n_H} + K^{n_H})$ where y = observed % inhibition, y_{max} = extrapolated maximal % inhibition, x = nucleotide concentration (μM), $K = IC_{50}$ (μM) and n_H = Hill coefficient. Values of constants (mean±s.e.mean) were as follows: UTP: $y_{max} = 64.0 \pm 0.75\%$; $K = 0.50 \pm 0.03\,\mu$M; $n_H = 1.29 \pm 0.07$; ATP: $y_{max} = 59.9 \pm 1.09\%$; $K = 0.50 \pm 0.03\,\mu$M; $n_H = 1.30 \pm 0.10$; ITP: $y_{max} = 65.9 \pm 1.24\%$; $K = 3.12 \pm 0.20\,\mu$M; $n_H = 1.16 \pm 0.07$; ADP: $y_{max} = 59.0 \pm 7.6\%$; $K = 25.3 \pm 11.1\,\mu$M; $n_H = 0.68 \pm 0.08$; UTP after $0.5\,\mu g\,\mu l^{-1}$ cRNA: $y_{max} = 50.2 \pm 0.61\%$; $K = 0.90 \pm 0.05\,\mu$M; $n_H = 1.21 \pm 0.06$. (C) Ca^{2+} channel current inhibition produced by different nucleotides (10 μM) in cells pre-injected with $1.25\,\mu g\,\mu l^{-1}$ $P2Y_2$ cRNA. Columns show mean±s.e.mean; n = number of cells.

pretreatment with Pertussis toxin (PTX), implying the involvement of a different (PTX-sensitive) G-protein to that responsible for phosphoinositide stimulation (cf. Lin, 1994). Indeed, the slowed current kinetics induced by UTP mirrors the effect of several other transmitters on these cells and implies that part, at least, of the inhibition results from a direct action of the activated G protein on channel gating (see Hille, 1994).

The observations suggest a novel function for $P2Y_2$ receptors. Thus, in sympathetic neurones, inhibition of Ca^{2+} currents reflects one mechanism whereby transmitters can reduce their own release from neuronal processes (see Boehm & Huck, 1996). Since mRNA for the $P2Y_2$ receptor is present in the brain (Lustig *et al*., 1993; Webb & Barnard, unpublished data), a comparable effect on Ca^{2+} currents in central neurones might provide a potential mechanism for auto-inhibition of ATP release from purinergic nerve endings.

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