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Coupling of the nucleotide $P2Y_4$ receptor to neuronal ion channels

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1 G protein-linked P2Y nucleotide receptors are known commonly to stimulate the phosphoinositide signalling pathway. However, we have previously demonstrated that the cloned $P2Y_2$, $P2Y_6$ and $P2Y_1$ receptors couple to neuronal N-type Ca^{2+} channels and to M-type K^+ channels. Here we investigate the coupling of recombinant, neuronally expressed rat- and human $P2Y_4$ receptors ($rP2Y_4$, $hP2Y_4$) to those channels.

2 Rat sympathetic neurones were nuclear-injected with a $P2Y_4$ cDNA plasmid. A subsequent activation of $rP2Y_4$ or $hP2Y_4$ by UTP (100 μM) in whole-cell (ruptured-patch) mode produced only about 12% inhibition of the N-type Ca^{2+} current ($I_{Ca(N)}$). Surprisingly, in perforated patch mode, UTP produced much more inhibition of $I_{Ca(N)}$ (maximally 51%), with an IC_{50} value of 273 nM. This inhibition was voltage-dependent and was blocked by co-expression of the $\beta\gamma$-binding transducin Gα-subunit. Pertussis toxin (PTX) pretreatment also suppressed $I_{Ca(N)}$ inhibition.

3 UTP inhibited the M-current, recorded in perforated patch mode, by (maximally) 52%, with IC_{50} values of 21 nM for $rP2Y_4$ and 28 nM for $hP2Y_4$. This inhibition was not affected by PTX pretreatment.

4 With $rP2Y_4$, ATP inhibited the M-current (IC_{50} 524 nM, 26 times weaker than UTP), whereas ATP had no agonist activity at $hP2Y_4$. This suggests a difference in agonist binding site between $rP2Y_4$ and $hP2Y_4$.

5 We conclude that, in contrast to other nucleotide receptors studied, the $P2Y_4$ receptor couples much more effectively to M-type K^+ channels than to Ca^{2+} channels. Coupling to the Ca^{2+} channels involves the $\beta\gamma$-subunits of $G_{i/o}$-proteins and requires a diffusible intracellular component that is lost in ruptured-patch recording.

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Abbreviations: GFP, green fluorescent protein; GTP, guanosine 5′-triphosphate; $I_{Ca(N)}$, N-type Ca^{2+} current; IP_3, inositol trisphosphate; I/V, current/voltage; OxoM, oxotremorine M; PTX, Pertussis toxin; $hP2Y_4$, human $P2Y_4$ receptor; $rP2Y_4$, rat $P2Y_4$ receptor SCG, superior cervical sympathetic ganglion; UTP, uridine 5′-triphosphate

Introduction

The P2Y receptors are G-protein-linked nucleotide receptors (North & Barnard, 1997). The family of vertebrate P2Y receptors has nine or so members (depending on the assignment made of species orthologues) cloned and recognized (Barnard & Simon, 2001). At the great majority of these, including the four discussed here, the $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, their agonists stimulate inositol trisphosphate (IP_3) production and mobilization of intracellular Ca^{2+}. P2Y receptor activity has been frequently observed in non-neuronal cells and in recombinant-transfected cell lines (reviewed by Ralevic & Burnstock, 1998), but transduction pathways have not been studied for molecularly identified P2Y receptors in neurones.

An exception to this is that $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors, when expressed in rat superior cervical ganglion (SCG) neurones by nuclear injection of each cDNA, couple with high agonist potency to both N-type Ca^{2+} channels and M-type K^+ channels (Filippov *et al*., 1997; 1998; 1999; 2000). Those studies showed that P2Y receptors inhibit the Ca^{2+} channel current using apparently distinct G-proteins which are either sensitive or insensitive to *Pertussis* toxin (PTX), while they inhibit the M-channel current using only a PTX-insensitive G-protein. Since these ion channels can control neurotransmitter release and excitability, this modulation of ion channels by nucleotides is likely to be of physiological importance in the brain if these receptors are expressed there. Indeed, $P2Y_1$ receptors are expressed in high abundance in many brain regions and their neuronal localization has been demonstrated (Barnard *et al*., 1997; Webb *et al*., 1998a; Moore *et al*., 2000; Moran-Jimenez & Matute, 2000). In contrast, no evidence was found for $P2Y_4$ expression in brain neurones, although it occurs in some other cell types in the brain (Webb *et al*., 1998b), including neonatal cortical astrocytes (Lenz *et al*., 2000). It is therefore of interest to know if it lacks the ability to couple to those neuronal ion channels, a question investigated here in the SCG expression system.

The $P2Y_4$ receptor was cloned first from human placenta (Communi *et al*., 1995) and from genomic human DNA (Nguyen *et al*., 1995); a similar sequence was demonstrated in rat heart (Webb *et al*., 1996; Bogdanov *et al*., 1998) and was

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also cloned and expressed from rat brain (Webb *et al*., 1998b). The rat brain $P2Y_4$ receptor has 84% amino acid sequence identity with human $P2Y_4$ (90% in the combined transmembrane regions and extracellular loops) and 50% homology with the rat $P2Y_2$ receptor. As with the $P2Y_2$ receptor, inositol phosphate accumulation stimulated by $P2Y_4$ was partially sensitive to Pertussis toxin, suggesting a parallel involvement of two distinct G-proteins (Communi *et al*., 1996). We find now that channel interactions of the $P2Y_4$ receptor provide further information on multiple G-protein pathways for this receptor.

Another interesting feature here is that ATP and UTP are equipotent agonists in the Ca^{2+} mobilization response of the rat $P2Y_4$ receptor expressed in transfected cell lines, but at the human $P2Y_4$ receptor, ATP is not an agonist and can even be an antagonist (Webb *et al*., 1998b; Kennedy *et al*., 2000). The mouse $P2Y_4$ receptor has also been described, and in this respect behaves as the rat receptor (Lazarowski *et al*., 2001; Suarez-Huerta *et al*., 2001). We have investigated also whether that agonist specificity applies in the channel coupling of the rat and human $P2Y_4$ receptors.

Methods

cDNA Plasmids

The rat $P2Y_4$ receptor ($rP2Y_4$) cDNA was as described previously (Webb *et al*., 1998b); the human $P2Y_4$ receptor ($hP2Y_4$; Communi *et al*., 1995) was a gift from J.M. Boeynaems and B. Robaye (Brussels). Each was cloned into the pcDNA3.1 vector (Stratagene) and checked by re-sequencing. Plasmids were stored at $-20°C$ for injection in sterile 10 mM Tris /1 mM EDTA, pH 8 (TE solution).

Cell isolation and DNA injection

Isolation and injection procedures were similar to those described previously in detail (Filippov *et al*., 1997; 1998). Briefly, single SCG neurones were isolated from 15–19-day-old rats. Cells were plated on glass coverslips coated with laminin/poly-L-lysine and incubated at 37°C for 4–6 h prior to DNA injection. The plasmid, in sterile TE solution (90–118 ng μl^{-1}), was microinjected into the nucleus. The enhanced green fluorescent (mutant S65T) protein (GFP) cDNA in pcDNA3 (Clontech) was co-injected (10–50 ng μl^{-1}) to identify later the cells with successful expression. Transducin Gα subunit cDNA cloned into pcDNA3 (200 ng μl^{-1}) was also co-injected where stated. These cells were incubated at 37°C for 14–24 h prior to electrophysiological recording.

Electrophysiology and data analysis

Membrane currents were recorded from GFP-labelled neurones at room temperature (20–22°C) in Krebs' solution continuously-flowing at 20–25 ml min^{-1}, using a discontinuous ('switching') amplifier (Axoclamp 2B) sampling voltage at 6–8 kHz. Drugs were applied in this same perfusing solution (bath exchange rate $\leqslant 1$ s); this avoids any change in nucleotide composition due to enzymic action at the surface of the cells. Voltage commands were generated and currents digitized and analysed using 'pClamp 8' software (Axon Instruments, Foster City, U.S.A.).

Currents through voltage-gated Ca^{2+} channels (I_{Ca}), which in these cells were largely N-type ($I_{Ca(N)}$; Filippov *et al*., 1997), were recorded using the whole-cell (disrupted-patch) method or a perforated patch method as described in detail previously (Filippov *et al*., 1999). The bathing solution for $I_{Ca(N)}$ recordings consisted of (mM): tetraethylammonium (TEA) chloride 120, KCl 3, $MgCl_2$ 1.5, $BaCl_2$ 5, HEPES 10, glucose 11.1 and 0.5 μM tetrodotoxin (pH adjusted to 7.35 with NaOH). Patch electrodes (2–3 MΩ) were filled with a solution containing (in mM) CsCl 110, $MgCl_2$ 3, HEPES 40, EGTA 3, Na_2ATP 2, Na_2GTP 0.5 (pH adjusted to 7.4 with CsOH). Amphotericin B, 0.125 mg ml^{-1}, was added to the pipette solution for perforated-patch recordings. Currents were routinely evoked every 20 s with a 50–100 ms depolarizing rectangular test pulse to 0 mV from a holding potential of −90 mV. Current amplitudes were measured isochronally 10 ms from the onset of the rectangular test pulse, i.e., near to the peak of the control current. To eliminate leak currents, Co^{2+} was substituted for Ba^{2+} in the external solution at the end of each experiment to block all Ca^{2+} channel currents and the residual current was digitally subtracted from the corresponding currents in Ba^{2+} solution.

M-type potassium currents, $I_{K(M)}$, were recorded as described in detail previously (Filippov *et al*., 1998) using a perforated-patch method. Patch pipettes (2–3 MΩ) were filled with a solution containing (mM) potassium acetate 90, KCl 20, $MgCl_2$ 3, HEPES 40, BAPTA 0.1, 0.125 mg ml^{-1} amphotericin B (adjusted to pH 7.4 by KOH). The bathing solution contained (mM) NaCl 120, KCl 3, $MgCl_2$ 1.5, $CaCl_2$ 2.5, HEPES 10, glucose 11.1 (adjusted to pH 7.3 with NaOH). Neurones were voltage-clamped at −20 mV or −30 mV and M-currents deactivated with 1 s hyperpolarizing steps at 5 s intervals. Current/voltage (I/V) relationships were obtained using incremental voltage steps of 10 mV between −10 and −100 mV; currents were measured at the end of each hyperpolarizing step. For dose/response curves, currents were measured at −30 mV from a steady-state I/V relationship obtained using a ramp voltage command of 20 s from −20 to −90 mV. Currents measured were leak subtracted. The leak component of current was estimated in both cases by extrapolating a linear fit to the I/V relationship from the negative potential region, where only ohmic currents were observed.

Data are presented as means ± s.e.mean. Statistical significance was verified by Student's test ($P \leqslant 0.05$). Dose–response curves were determined using concentrations added cumulatively, with 1 min exposure times. Curves were fitted (using Origin 5.0 software) to pooled data points using the equation $y = y_{max}x^{nH}/(x^{nH} + K^{nH})$ where y = observed % inhibition, y_{max} = extrapolated maximal % inhibition, x = nucleotide concentration (μM), $K = IC_{50}$ (μM) and n_H = Hill coefficient.

Chemicals

Uridine 5′-triphosphate (UTP) was from Pharmacia Biotech, molecular biology grade; it was 99.5% pure and free from adenine nucleotides. ATP was from Boehringer Mannheim GmbH (Germany) and was freed from nucleotide diphosphates by pre-treatment with creatine kinase/creatine phos-

phate (Filippov *et al*., 2000). Purity was verified on samples by HPLC analysis of nucleotides (Sak *et al*., 2000). Guanosine 5′-triphosphate (GTP), (−)-norepinephrine (noradrenaline) bitartrate, EGTA, BAPTA, amphotericin B were all from Sigma. Oxotremorine-M (OxoM) was from RBI-Sigma, tetrodotoxin from Tocris, Pertussis toxin (PTX) from Porton Products (U.K.), $CoCl_2$ (AnalaR grade) from BDH (U.K.) and $BaCl_2$ and CsCl from Aldrich.

Results

$P2Y_4$ coupling to Ca^{2+} channels

In contrast to $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors, in whole-cell mode activation of the rat- or human $P2Y_4$ receptor by the agonist UTP produced only a marginal inhibition of Ca^{2+} channel current, $I_{Ca(N)}$, up to 12.5±3.2% at 100 μM (Figure 1A,C). Expression of $P2Y_4$ receptors did not prevent modulation of $I_{Ca(N)}$ by endogenous G-protein linked receptors, since noradrenaline (10 μM) inhibited I_{Ca} *via* α_2 receptors by 41.8±4.1% on the same cells.

Surprisingly, in perforated patch mode, UTP (100 μM) significantly inhibited the $I_{Ca(N)}$ current by 45.1±4.3% (Figure 1B,C). As with the three other P2Y receptors studied before (Filippov *et al*., 1997; 1999; 2000), the decrease of current was accompanied by a slowed current onset (Figure 1B), suggesting that the inhibition was voltage-dependent. Voltage-dependence was directly confirmed, since a strong depolarizing pre-pulse restored current onset and decreased inhibition from 47.4±4.5% to 21.5±1.5% (Figure 2).

Pertussis toxin (PTX) pretreatment (0.5 μg ml^{-1}, overnight) dramatically decreased $I_{Ca(N)}$ inhibition in perforated patch mode, to only 9.4±2.4% (Figure 1C). This effect was almost as great as the abolition of α_2-adrenergic inhibition of Ca^{2+} current by PTX in the same cells (Figure 1C).

Together, the voltage-dependence of $P2Y_4$ mediated $I_{Ca(N)}$ inhibition and the effect of PTX suggested the involvement of $\beta\gamma$-subunits of mainly PTX-sensitive G_i/G_o proteins (Caulfield *et al*., 1994; Hille, 1994; Ikeda, 1996; Herlitze *et al*., 1996; Delmas *et al*., 1998a, b). To confirm $\beta\gamma$-subunit involvement, we introduced the transducin Gα subunit known to bind $\beta\gamma$-subunits and confirmed to do so to completion when expressed in SCG neurones (Delmas *et al*., 1999). Co-expression of transducin here almost completely prevented the inhibition of $I_{Ca(N)}$ by UTP (Figure 2). Hence, all of the evidence here demonstrated that $I_{Ca(N)}$ inhibition *via* $P2Y_4$ receptors is mediated by $G\beta\gamma$ subunits.

UTP inhibited $I_{Ca(N)}$ with IC_{50} 272.6±129.6 nM (Figure 1D). Thus, the potency of $P2Y_4$ coupling to N-type Ca^{2+} channels is (for native nucleotide agonists) similar to that of the $P2Y_2$ receptor but of the order of 50 times less than that of $P2Y_1$ or $P2Y_6$ (reviewed by Brown *et al*., 2000).

$P2Y_4$ coupling to M-type K^+ channels

As found with the $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors, activation of the rat- or human $P2Y_4$ receptor by UTP inhibited the M-current (as recorded in perforated patch mode) (Figure 3). In recordings of the M-current (Figure 3A), this inhibition was evident as a decrease of the holding current pre-activated at −20 mV and as a decrease in the amplitude of the deactivation tails after stepping to hyperpolarizing voltages (see Methods). Also, the current measured at the end of the applied hyperpolarizing steps was decreased at all voltages within the M-current activation range (Figure 3B). No voltage dependence of the inhibition occurs. The leak-subtracted current (see Methods) measured at −30 mV was decreased by 52.0±4.1% (Figure 3C). This inhibition was similar to that seen with oxotremorine M (OxoM) acting *via* endogenous M_1 muscarinic receptors. The $P2Y_4$-mediated inhibition was not affected by PTX pretreatment (Figure 3C).

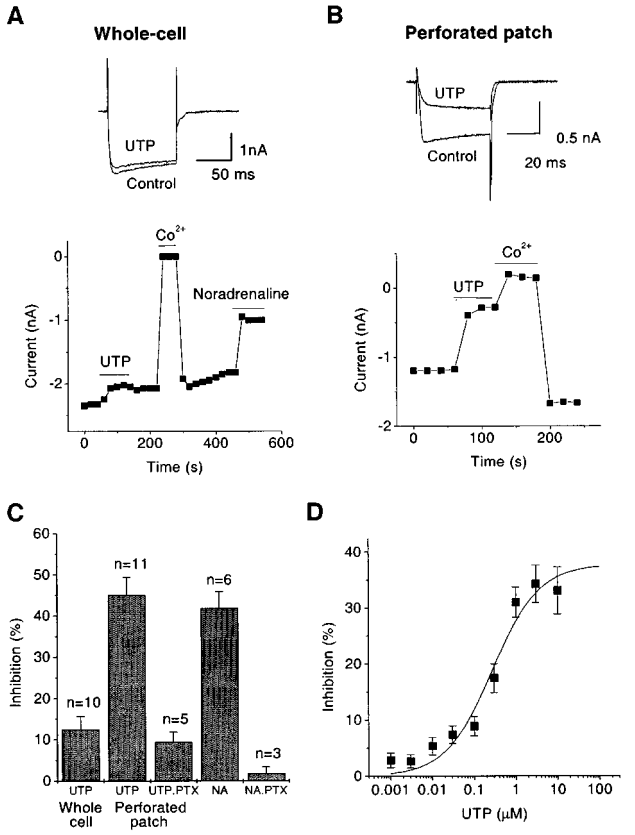

Figure 1 $P2Y_4$ nucleotide receptors expressed in SCG neurones after cDNA injection couple to Ca^{2+} channels in perforated patch mode but do not couple in whole-cell mode. Ca^{2+} channel currents ($I_{Ca(N)}$) were recorded by stepping for 50–100 ms every 20 s from −90 mV to 0 mV and leak-corrected by subtracting currents remaining after substituting 5 mM Co^{2+} for Ba^{2+}. Current amplitude was measured 10 ms from the onset of the test pulse. Records show superimposed leak-subtracted currents in the absence and presence of 100 μM UTP; plots show the time course of changes in current amplitude in whole-cell (ruptured patch) mode (A) and in perforated patch mode (B). Solid bars indicate time of exposure to UTP, Co^{2+} or 10 μM noradrenaline. (C) Bar charts show the mean inhibition of $I_{Ca(N)}$ amplitude by 100 μM UTP and by 10 μM noradrenaline (NA) in neurones pretreated with Pertussis toxin (PTX) and in untreated neurones in whole-cell mode and in perforated patch mode. Bars show s.e.mean; n = number of cells. (D) Concentration-dependence of $I_{Ca(N)}$ inhibition by UTP in perforated patch mode. Points show means±s.e.mean of measurements in three cells, concentrations were added cumulatively, with 1 min exposure times. Curves were fitted to pooled data points using Origin 5.0 software to the Hill equation $y = y_{max}.x^{nH}/(x^{nH} + K^{nH})$ where y = observed % inhibition, y_{max} = extrapolated maximal % inhibition, x = UTP concentration (μM), $K = IC_{50}$ (μM) and n_H = Hill coefficient. Values of Hill constants (mean±s.e.mean) were as follows: $y_{max} = 37.8 \pm 4.3\%$; $K = 272.6 \pm 129.6$ nM; $n_H = 0.8 \pm 0.2$.

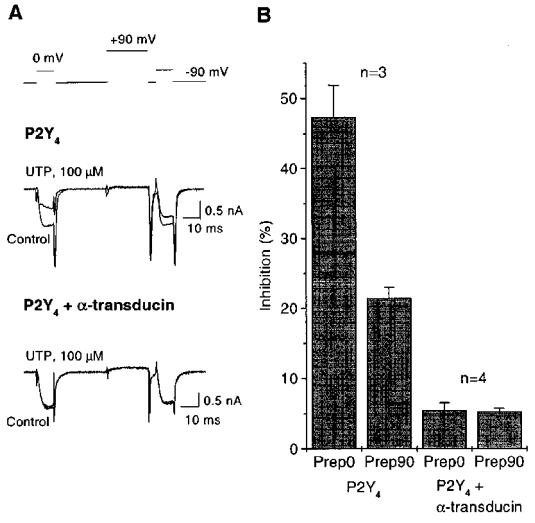

Figure 2 $\beta\gamma$-subunits are responsible for $P2Y_4$ mediated inhibition of Ca^{2+} channel current. (A) records show superimposed $I_{Ca(N)}$ obtained with a double-pulse voltage protocol (top trace) in the absence (control) and presence of 100 μM UTP in cells injected with $P2Y_4$ cDNA alone (upper records) or together with cDNA for transducin (lower records). (B) The bar-charts show the mean per cent current inhibition (measured after 10 ms at 0 mV command potential) produced by UTP before (Prep. 0) and after (Prep. 90) the +90 mV prepulse in cells injected with $P2Y_4$ cDNA alone (two first columns) or together with transducin cDNA (two last columns). Note that the prepulse significantly decreased $P2Y_4$ mediated inhibition of $I_{Ca(N)}$ and eliminated the slowing of the kinetics demonstrating voltage-dependence of the effect in cells expressing $P2Y_4$ alone. However practically no $I_{Ca(N)}$ inhibition and no voltage-dependence can be seen in cells where $\beta\gamma$-subunit buffering transducin was co-expressed with $P2Y_4$ receptor.

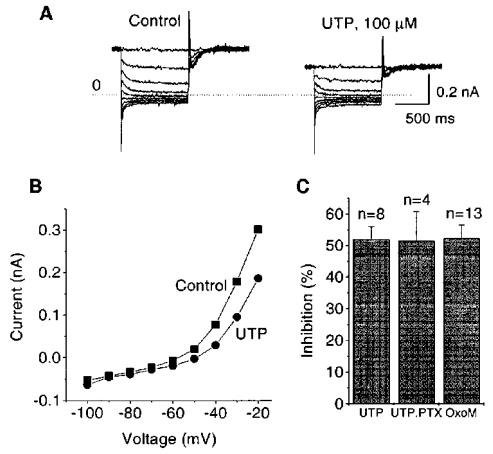

Figure 3 Expressed $P2Y_4$ nucleotide receptors couple to the M-type K^+ channels in SCG neurones. (A) traces show the superimposed membrane currents recorded in perforated-patch mode using a standard voltage protocol for M-type K^+ current (M-current) measurements (see Methods). M-current was pre-activated by holding the membrane potential at -20 mV, then deactivated with a series of 1 s hyperpolarizing steps in increments of 10 mV at 5 s intervals. The dotted line indicates the zero current. Note that UTP decreased an outward current at the holding potential and reduced the amplitude of the deactivation tails during the steps. (B) The graph shows the current amplitude at the end of each 1 s step measured as change from zero current from records in A. Note that UTP reduced the outward rectification of the current–voltage curve positive to -70 mV. (C) Bar charts show mean inhibition of M-current at -30 mV by 100 μM UTP and by 10 μM oxotremorine-M (OxoM) in $P2Y_4$ expressing neurones. The effect of UTP is shown in neurones pretreated with PTX and in PTX-untreated neurones. The effect of OxoM in PTX-untreated neurones is shown for comparison. M-current was leak-subtracted (see Methods). Bars show s.e.mean; n = number of cells. Note that PTX pretreatment did not affect $P2Y_4$ mediated inhibition of M-current.

The inhibition of the M-current by UTP action showed similar concentration dependence for the rat and human $P2Y_4$ receptors (Figure 4A). IC_{50} values were 21.5 ± 4.3 nM (rat receptor) and 27.8 ± 4.7 nM (human receptor). ATP was much less effective than UTP on the rat receptor in M-current inhibition (Figure 4A), with an IC_{50} of 524 ± 273 nM. The greater variability between cells with ATP was unavoidable due to some interference from the response to ATP levels above 1 μM from low but variable levels of endogenous P2X receptors in SCG neurones (Cloues *et al*., 1993). On the human $P2Y_4$ receptor ATP was inactive as an agonist. For example, 1 μM ATP inhibited the M-current by $17.8\pm4.7\%$ for rat $P2Y_4$ but by $3.97\pm2.1\%$ for human $P2Y_4$ (Figure 4B), these values being significantly different (rat) and not significantly different (human) from controls respectively. Although ATP has been reported to be an antagonist (K_B 708 nM) at human $P2Y_4$ receptors expressed by transfection into astrocytoma cells (Communi *et al*., 1996; Kennedy *et al*., 2000), in the neurones here any antagonist activity of ATP on the human receptor was found to be too weak to measure. Thus, the effect of 1 μM UTP in the presence of 1 μM ATP was indistinguishable from that without ATP (data not shown). A K_B value of the order of 10 μM or greater for ATP would not be seen in our cells due to the above-mentioned effect of endogenous P2X receptors.

Comparing the actions of preferred agonists on other P2Y receptors in coupling to the M-type K^+ channel (reviewed by Brown *et al*., 2000), the potency at $P2Y_4$ is about three times less than that for the $P2Y_1$ receptor, but it is comparable to that for the $P2Y_6$ receptor and 75 times greater than that for the $P2Y_2$ receptor.

Discussion

The results demonstrate that the $P2Y_4$ receptor can, under appropriate conditions, couple both to N-type Ca^{2+} channels and to M-type K^+ channels. On the basis of these and our previous results on other P2Y receptors ($P2Y_1$, $P2Y_2$ and $P2Y_6$) we can conclude that the ability to couple to these neuronal ion channels and to inhibit them is a common attribute of nucleotide P2Y receptors. However, there is a significant difference between the channel coupling of the $P2Y_4$ receptor and that of the other P2Y receptors studied: the $P2Y_4$ receptor, alone, couples much more effectively to M-type K^+ channels than to Ca^{2+} channels. M-channels are 13 times more sensitive than Ca^{2+} channels in this case, whereas at other P2Y receptors there was, with a given agonist, either no difference or it was small and in the converse direction (Table 1). Further, the coupling of $P2Y_4$ (but not of the other P2Y receptors) to the Ca^{2+} channels is so vulnerable that it can be observed only in perforated patch recording mode, where intracellular composition is preserved, in contrast to the losses by diffusion occurring in whole-cell mode. This indicates that the $P2Y_4$ receptor action to close

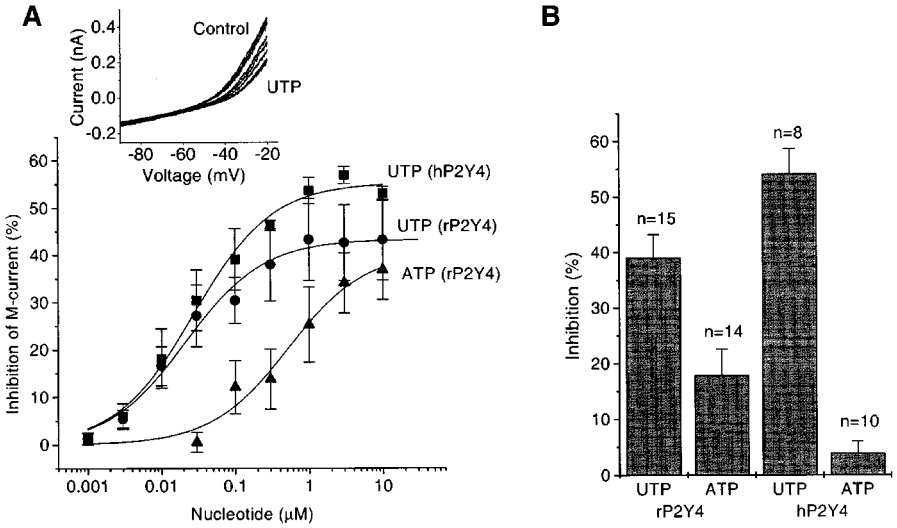

Figure 4 Comparison of UTP- and ATP effects on M-current in neurones expressing $rP2Y_4$- and $hP2Y_4$ receptors. (A) Concentration–dependence of M-current inhibition by UTP in neurones pre-injected with $rP2Y_4$ cDNA or with $hP2Y_4$ cDNA. Also effect of ATP on $rP2Y_4$ is shown. M-current was recorded using a ramp-voltage protocol, leak-subtracted and measured at -30 mV (see Methods). Representative original current records before and after adding increasing concentrations of UTP at $hP2Y_4$ are shown in the inset. Points show means $\pm$ s.e.mean of measurements in 3–5 cells; concentrations were added cumulatively, with 1 min exposure times. Curves were fitted to pooled data points using Origin 5.0 software to the Hill equation (see legend to the Figure 1D). Values of constants (mean $\pm$ s.e.mean) were as follows: UTP ($hP2Y_4$): $y_{max} = 55.3 \pm 1.7\%$; $K = 27.8 \pm 4.7$ nM; $n_H = 0.8 \pm 0.1$; UTP ($rP2Y_4$): $y_{max} = 43.3 \pm 1.6\%$; $K = 21.5 \pm 4.3$ nM; $n_H = 0.8 \pm 0.1$; ATP($rP2Y_4$): $y_{max} = 40.6 \pm 5.9\%$; $K = 523.7 \pm 276.5$ nM; $n_H = 0.8 \pm 0.2$. (B) Bar charts show mean inhibition of M-current at -30 mV by 1 μM UTP and 1 μM ATP in neurones pre-injected with $hP2Y_4$ cDNA, or with $rP2Y_4$ cDNA. Bars show s.e.mean; n = number of cells.

Table 1 Sensitivities of M-type K^+ currents (M-current) and N-type Ca^{2+} currents ($I_{Ca(N)}$) in rat sympathetic neurones to agonists activating expressed P2Y receptors

		*M-current**		$I_{Ca(N)}$		
Receptor	*Agonist*	*[1]IC_{50} (nM)*	*[2]Inh_{max} (%)*	*[1]IC_{50} (nM)*	*[2]Inh_{max} (%)*	*Reference*
$P2Y_1$	ADP	6.9	59	8.2	64	Filippov *et al*., 2000
$P2Y_2$	UTP	1500	61	500	64	Filippov *et al*., 1997; 1998
$P2Y_4$	UTP			$>10^5$	12.5	This study
	UTP	21.5	52	273*	51*	This study
$P2Y_6$	UDP	30	63	5.9	53	Filippov *et al*., 1999

[1]Mean IC_{50} from dose–response curves. [2]Maximum inhibition. *Perforated patch.

the Ca^{2+} channel requires the presence of some intracellular component(s).

It can also be noted that this contrast between the coupling of the other P2Y receptors to the N-type Ca^{2+} channel and the lack of it with the $P2Y_4$ receptor, when all are compared in whole cell patch-clamping, confirms that the channel couplings seen in this neuronal expression system are not some artefact of an overloading of a foreign receptor, since those effects are seen to differ greatly between P2Y subtypes. That difference occurs despite the approximately similar levels of P2Y expression in each case, as judged by the rather similar maximum extents of inhibition of the M-channel or Ca^{2+} channel in all cases (Table 1). Further, both here and with the other P2Y receptors, that maximum is well below 100% and is similar or less than that attainable by activation of the native α_2-adrenergic or M_1 muscarinic receptors in the same cells (Figures 1C, 3C).

In other cases of rat receptor inhibitory coupling to M-channels, only a voltage-independent effect through a PTX-insensitive G-protein is involved (Brown *et al*., 1989; Ikeda *et al*., 1995; Jones *et al*., 1995). This has been identified as the α-subunit of G_q or G_{11} (Jones *et al*., 1995; Haley *et al*., 1998; 2000). This fits the observations on M-channel inhibition by the $P2Y_4$ receptor and by the other PLC-linked P2Y receptors we have previously studied. In contrast, almost all of the $P2Y_4$ modulation of Ca^{2+} channels is sensitive to PTX. This, plus its voltage-dependence and its abolition by transducin Gα co-expression, show the involvement of G protein $\beta\gamma$-subunits, in a direct membrane-delimited action on the Ca^{2+} channel. The parent trimeric G protein is presumed to be one or more of the G_o or G_i subtypes, on the basis of the identifications made in parallel behaviour found in rat SCG neurones with several other receptor types (Delmas *et al*., 1999; Jeong & Ikeda, 2000).

When the $P2Y_4$ receptor was expressed by transfection into astrocytoma cells (Communi *et al*., 1996) it was reported that its activation (*via* UTP) of PLC to form IP_3 comprises a PTX-sensitive ($\sim$60%) and a PTX-insensitive component. For the PTX-insensitive component of the same response (due to native P2Y receptors in rabbit smooth muscle cells,

activatable by either UTP or ATP), evidence was obtained to assign it to PLC-β1 coupling *via* $G\alpha_{q/11}$, and to assign the PTX-sensitive component to PLC-β3 coupling *via* $G\beta\gamma_{i3}$ (Murthy & Makhlouf, 1998). This illustrates how, in our case, the activated $P2Y_4$ receptor could use G-protein $\beta\gamma$ subunits in an IP_3 pathway and in PTX-sensitive closure of a Ca^{2+} channel. It has not been proven that the PLC stimulation actually mediates the latter event, although evidence has been provided to suggest that activation of the $PLC/IP_3/Ca^{2+}$ pathway may be involved in the PTX-insensitive inhibition of M current by UTP *via* native P2Y receptors that is seen in long-term cultures of ganglion cells from newborn rats (Bofill-Cardona *et al*., 2000). These two responses – PTX-insensitive inhibition of $I_{Ca(N)}$ and $I_{K(M)}$ – frequently operate in parallel but may, or may not, use similar downstream signalling pathways (Hille, 1994). However, the initial transduction stages for the $P2Y_4$ receptor must be more complex than has hitherto been described, since they show the first example of a requirement for a diffusible intracellular component as a co-factor of a $\beta\gamma$-subunit effect on Ca^{2+} channels. Such $\beta\gamma$ action would be expected, instead, to be direct and entirely membrane-delimited (reviewed by Hille, 1994; Ikeda, 1996; Kaneko *et al*., 1999). A broad family of diffusible regulators of G-protein signalling (RGS) is now known (Hepler, 1999), but thus far their actions on channel responses are known only as negative regulations. The identity of the positively-acting factor here will be of interest.

We have observed that, at the rat $P2Y_4$ receptor, both UTP and ATP are agonists in inhibiting the M-current whereas, at the human $P2Y_4$ receptor, UTP is an agonist but ATP is not. This unusual specificity difference is not due to the linkage to this ion channel, since it was found earlier with other transductions, i.e. the mobilization of intracellular Ca^{2+} in $P2Y_4$-transfected cells (Webb *et al*., 1998b; Kennedy *et al*., 2000) and *Xenopus* oocyte $P2Y_4$ expression (Bogdanov *et al*., 1998). This indicates a difference within the nucleotide binding site of the rat and human $P2Y_4$ receptors.

With expressed rat $P2Y_4$ receptors UTP was 26 fold more effective than ATP in inhibiting the M-current, but only about 2 fold more effective in mobilization of intracellular Ca^{2+} (Webb *et al*., 1998b; Kennedy *et al*., 2000). The lower ATP potency here cannot be explained by ATP metabolism since we applied fresh nucleotide solutions for each measurement, in continuous and fast superfusion over cells at a low density. Also, UTP was 10 – 30 times more effective in inhibiting the M-current than in its intracellular Ca^{2+} response in rat $P2Y_4$-transfected cells, a confirmation that there was no more nucleotide triphosphate degradation in our experiments than in the latter case. The marked difference in the agonist potency ratio for the two actions does not favour PLC-β activation being the direct precursor of the M-channel closure for this receptor (cf. Bofill-Cardona *et al*., 2000), and further tests of the mechanism of the latter are indicated.

As noted in the Introduction, the $P2Y_4$ receptor has not so far been shown to occur in central neurones, unlike $P2Y_1$ and $P2Y_6$, but is prominent in the brain ventricular system, cardiac and skeletal muscles, some smooth muscles and some other peripheral sites. It is, therefore, not surprising that its coupling to the neurone-specific N-type Ca^{2+} channel *via* G-protein $\beta\gamma$-subunits, imposed on it here, is far less efficient than with those other two P2Y receptors, as cited in Results. Its coupling to the M-type K^+ channel is, exceptionally, far stronger than to the Ca^{2+} channel. The M-type channel is now known to be constituted of KCNQ subunits (Wang *et al*., 1998; Selyanko *et al*., 1999; Schroeder *et al*., 2000). Members of that subunit family are prominent in other channels in, e.g., the cardiac sites of $P2Y_4$ receptors. Since all members of this family can be inhibited by $G_{q/11}$-coupled receptors (Selyanko *et al*., 2000; Schroeder *et al*., 2000), M-channel closure may be a manifestation of the normal non-neuronal role of the $P2Y_4$ receptor; this may explain its ability to couple strongly to the M-channel when introduced in a neurone. This proposal can be tested after co-expressing $P2Y_4$ and each of a series of KCNQ subunits or their combinations in a null host cell line.

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References

BARNARD, E.A. & SIMON, J. (2001). An elusive receptor is finally caught: $P2Y_{12}$, an important drug target in platelets. *Trends Pharmacol. Sci.,* **22,** 388 – 391.

BARNARD, E.A., SIMON, J. & WEBB, T.E. (1997). Nucleotide receptors in the nervous system – an abundant component using diverse transduction mechanisms. *Mol. Neurobiol.,* **15,** 103 – 129.

BOGDANOV, Y.D., WILDMAN, S.S., CLEMENTS, M.P, KING, B.F., & BURNSTOCK, G. (1998). Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. *Br. J. Pharmacol.,* **124,** 428 – 430.

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

BROWN, D.A., FILIPPOV, A.K. & BARNARD, E.A. (2000). Inhibition of potassium and calcium currents in neurones by molecularly-defined P2Y receptors. *J. Autonomic Nervous System,* **81,** 31 – 36.

BROWN, D.A., MARRION, N.V. & SMART, T.G. (1989). On the transduction mechanism for muscarine-induced inhibition of M-current in cultured rat sympathetic neurones. *J. Physiol.,* **413,** 469 – 488.

CAULFIELD, M.P., JONES, S., VALLIS, Y., BUCKLEY, N.J., KIM, G.-D., MILLIGAN, G. & BROWN, D.A. (1994). Muscarinic M-current inhibition via $G\alpha_{q/11}$ and α-adrenoceptor inhibition of Ca^{2+} current via $G\alpha_o$ in rat sympathetic neurones. *J. Physiol.,* **477,** 415 – 422.

CLOUES, R., JONES, S. & BROWN, D.A. (1993). Zn^{2+} potentiates ATP-activated currents in rat sympathetic neurons. *Pflug. Arch.,* **424,** 152 – 158.

COMMUNI, D., MOTTE, S., BOEYNAEMS, J.M. & PIROTTON, S. (1996). Pharmacological characterization of the human $P2Y_4$ receptor. *Eur. J. Pharmacol.,* **317,** 383 – 389.

COMMUNI, D., PIROTTON, S., PARMENTIER, M. & BOEYNAEMS, J.M. (1995). Cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.,* **270,** 30849 – 30852.

DELMAS, P., ABOGADIE, F.C., DAYRELL, M., HALEY, J.E., MILLIGAN, G., CAULFIELD, M.P., BROWN, D.A. & BUCKLEY, N.J. (1998a). G-proteins and G-protein subunits mediating cholinergic inhibition of N-type calcium currents. *Eur. J. Neurosci.,* **10,** 1654 – 1666.

DELMAS, P., ABOGADIE, F.C, MILLIGAN, G., BUCKLEY, N.J. & BROWN, D.A. (1999). $\beta\gamma$ dimers derived from G_o and G_i proteins contribute different components of adrenergic inhibition of Ca^{2+} channels in rat sympathetic neurones. *J. Physiol.,* **518,** 23–36.

DELMAS, P., BROWN, D.A., DAYRELL, M., ABOGADIE, F.C., CAULFIELD, M.P. & BUCKLEY, N.J. (1998b). On the role of endogenous G-protein $\beta\gamma$ subunits in N-type Ca^{2+} current inhibition by neurotransmitters in rat sympathetic neurones. *J. Physiol.,* **495,** 353–366.

FILIPPOV, A.K., BROWN, D.A. & BARNARD, E.A. (2000). The $P2Y_1$ receptor closes the N-type Ca^{2+} channel in neurones, with both adenosine triphosphates and diphosphates as potent agonists. *Br. J. Pharmacol.,* **129,** 1063–1066.

FILIPPOV, A.K., WEBB, T.E., BARNARD, E.A. & BROWN, D.A. (1997). Inhibition by heterologously-expressed $P2Y_2$ nucleotide receptors of N-type calcium currents in rat sympathetic neurones. *Br. J. Pharmacol.,* **121,** 849–851.

FILIPPOV, A.K., WEBB, T.E., BARNARD, E.A. & BROWN, D.A. (1998). $P2Y_2$ nucleotide receptors heterologously-expressed in rat sympathetic neurons inhibit both Ca^{2+} and K^+ currents. *J. Neurosci.,* **18,** 5170–5179.

FILIPPOV, A.K., WEBB, T.E., BARNARD, E.A. & BROWN, D.A. (1999). Dual coupling of heterologously-expressed rat $P2Y_6$ nucleotide receptors to N-type Ca^{2+} and M-type K^+ currents in rat sympathetic neurones. *Br. J.Pharmacol.,* **126,** 1009–1017.

HALEY, J.E., ABOGADIE, F.C., DELMAS, P., DAYRELL, M., VALLIS, Y., MILLIGAN, G., CAULFIELD, M.P., BUCKLEY, N.J. & BROWN, D.A. (1998). The α subunit of G_q contributes to muscarinic inhibition of the M-type potassium current in sympathetic neurons. *J. Neurosci.,* **18,** 4521–4531.

HALEY, J.E., DELMAS, P., OFFERMANNS, S., ABOGADIE, F.C., SIMON, M.I., BUCKLEY, N.J. & BROWN, D.A. (2000). Muscarinic inhibition of calcium current and M current in Galpha q deficient mice. *J. Neurosci.,* **20,** 3973–3979.

HEPLER, J.R (1999). Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol. Sci.,* **20,** 376–382.

HERLITZE, S., GARCIA, D.E., MACKIE, K., HILLE, B., SCHEUER, T. & CATTERALL, W.A. (1996). Modulation of Ca^{2+} channels by G-protein $\beta\gamma$ subunits. *Nature,* **105,** 113–119.

HILLE, B. (1994). Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci.,* **17,** 531–536.

IKEDA, S.R. (1996). Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature,* **380,** 255–258.

IKEDA, S.R., LOVINGER, D.M., MCCOOL, B.A. & LEWIS, D.L. (1995). Heterologous expression of metabotropic glutamate receptors in adult rat sympathetic neurons: subtype-specific coupling to ion channels. *Neuron,* **14,** 1029–1038.

JEONG, S.W. & IKEDA, S.R. (2000). Effect of G protein heterotrimer composition on coupling of neurotransmitter receptors to N-type Ca^{2+} channel modulation in sympathetic neurons. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 907–912.

JONES, S., BROWN, D.A., MILLIGAN, G., WILLER, E., BUCKLEY, N.J. & CAULFIELD, M.P. (1995). Bradykinin excites rat sympathetic neurons by inhibition of M current through a mechanism involving B_2 receptors and $G\alpha_{q/11}$. *Neuron,* **14,** 399–405.

KANEKO, S., AKAIKE, A. & SATOH, M. (1999). Receptor-mediated modulation of voltage-dependent Ca^{2+} channels via heterotrimeric G-proteins in neurons. *Jpn. J. Pharmacol.,* **81,** 324–331.

KENNEDY, C., QI, A.D., HEROLD, C.L., HARDEN, T.K. & NICHOLAS, R.A. (2000). ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. *Mol. Pharmacol.,* **57,** 926–931.

LAZAROWSKI, E.R., ROCHELLE, L.G., O'NEAL, W.K., RIBEIRO, C.M., GRUBB, B.R., ZHANG, V., HARDEN, T.K. & BOUCHER, R.C. (2001). Cloning and functional characterization of two murine uridine nucleotide receptors reveal a potential target for correcting ion transport deficiency in cystic fibrosis gallbladder. *J. Pharmacol. Exp. Ther.,* **297,** 43–49.

LENZ, G., GOTTFRIED, C., LUO, Z., AVRUCH, J., RODNIGHT, R., NIE, W.J., KANG, Y. & NEARY, J.T. (2000). P_{2Y} purinoceptor subtypes recruit different Mek activators in astrocytes. *Br. J. Pharmacol.,* **129,** 927–936.

MORAN-JIMENEZ, M.J. & MATUTE, C. (2000). Immunohistochemical localization of the $P2Y_1$ purinergic receptor in neurons and glial cells of the central nervous system. *Mol. Brain Res.,* **78,** 50–58.

MOORE, D., CHAMBERS, J., WALDVOGEL, H., FAULL, R. & EMSON, P. (2000). Regional and cellular distribution of the $P2Y_1$ purinergic receptor in the human brain: striking neuronal localisation. *J. Comp. Neurol.,* **421,** 374–384.

MURTHY, K.S. & MAKHLOUF, G.M. (1998). Coexpression of ligand-gated P_{2X} and G protein-coupled P_{2Y} receptors in smooth muscle. Preferential activation of P_{2Y} receptors coupled to phospholipase C (PLC)-β1 via $G\alpha_{q/11}$ and to PLC-β3 via $G\beta\gamma_{i3}$. *J. Biol. Chem.,* **273,** 4695–4704.

NGUYEN, T., ERB, L., WEISMAN, G.A., MARCHESE, A., HENG, H.H., GARRAD, R.C., GEORGE, S.R., TURNER, J.T. & O'DOWD, B.F. (1995). Cloning, expression, and chromosomal localization of the human uridine nucleotide receptor gene. *J. Biol. Chem.,* **270,** 30845–30848.

NORTH, R.A. & BARNARD, E.A. (1997). Nucleotide receptors. *Current Opin. Neurobiol.,* **7,** 346–357.

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

SAK, K., BARNARD, E.A. & JARV, J. (2000). Dual effect of nucleotides on P2Y receptors. *IUBMB Life,* **50,** 99–103.

SCHROEDER, B.C., HECHENBERGER, M., WEINREICH, F., KUBISCH, C. & JENTSCH, T.J. (2000). KCNQ5, a novel potassium channel broadly expressed in brain, mediates M-type currents. *J. Biol. Chem.,* **275,** 24089–24095.

SELYANKO, A.A., HADLEY, J.K., WOOD, I.C., ABOGADIE, F.C., DELMAS, P., BUCKLEY, N.J., LONDON, B. & BROWN, D.A. (1999). Two types of K(+) channel subunit, Erg1 and KCNQ2/3, contribute to the M-like current in a mammalian neuronal cell. *J. Neurosci.,* **9,** 7742–7756.

SELYANKO, A.A., HADLEY, J.K., WOOD, I.C., ABOGADIE, F.C., JENTSCH, T.J. & BROWN, D.A. (2000). Inhibition of KCNQ1-4 channels expressed in mammalian cells via M_1 muscarinic acetylcholine receptors. *J.Physiol.,* **522,** 349–355.

SUAREZ-HUERTA, N., POUILLON, V., BOEYNAEMS, J. & ROBAYE, B. (2001). Molecular cloning and characterization of the mouse $P2Y_4$ nucleotide receptor. *Eur. J. Pharmacol.,* **416,** 197–202.

WANG, H.S., PAN, Z., SHI, W., BROWN, B.S., WYMORE, R.S., COHEN, I.S., DIXON, J.E. & MCKINNON, D. (1998). KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science,* **282,** 1890–1893.

WEBB, T.E., BOLUYT, M.O. & BARNARD, E.A. (1996). Molecular biology of P2Y purinoceptors: Expression in rat heart. *J. Autonom. Pharmacol.,* **16,** 303–307.

WEBB, T.E., SIMON, J. & BARNARD, E.A. (1998a). Regional distribution of [^{35}S] 2′-deoxy 5′-O-(1-thio) ATP binding sites and the $P2Y_1$ mRNA within the chick brain. *Neuroscience,* **84,** 825–837.

WEBB, T.E., HENDERSON, D.J., ROBERTS, J.A. & BARNARD, E.A. (1998b). Molecular cloning and characterization of the rat $P2Y_4$ receptor. *J. Neurochem.,* **71,** 1348–1357.

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