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Pharmacological characterisation of pyrimidinoceptor responses in NG108-15 cells

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Abstract

In the present study, the P2Y receptor(s) mediating the effects of the pyrimidines UTP and UDP on phospholipase C activation in the mouse neuroblastoma × rat glioma hybrid cell line NG108-15 was investigated. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis detected transcripts for the $P2Y_6$ and $P2Y_2$ receptors, but not for $P2Y_1$ and $P2Y_4$. UTP and UDP were equipotent agonists and their effects were partially additive. Suramin, reactive blue 2 and pyridoxal phosphate-6-azophenyl-2′,4′disulfonic acid (PPADS) antagonised the phospholipase C response to both UTP and UDP. High micromolar concentrations of adenosine, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS-21680), 2′,3′-*O*-isopropylideneadenosine (iPAdo) and adenosine 3′:5′-cyclic monophosphate (3′,5′-cAMP) were able to antagonise the effect of UTP on phospholipase C but not that of UDP. The additivity of the UTP and UDP responses, novel P2 receptor antagonist profile and the distinguishing action of adenosine may indicate the expression of a pyrimidine selective P2Y receptor in addition to the $P2Y_6$ type in these cells. © 2001 Published by Elsevier Science B.V.

Keywords: NG108-15 cell line; Pyrimidinoceptor; UTP-preferring receptor; Inositol phospholipid; Adenosine

1. Introduction

The role of G protein-coupled P2Y receptors in the nervous system is well established (Ralevic and Burnstock, 1998; Barnard et al., 1997). We have previously demonstrated that the NG108-15 mouse neuroblastoma × rat glioma cell line provides a model system for investigation of pyrimidinoceptors coupled to the activation of phospholipase C as only uracil nucleotides were found to be active (Sak et al., 1998, 1999). Furthermore, the pharmacological profile determined for this cell line revealed an equipotency of UTP and UDP (UTP ≥ UDP ≫ UMP > ATP = ADP). This profile was found to be a common feature of a number of neuroblastoma cell lines (Sak et al., 1998, 1999).

Such a pharmacological profile would exclude the functional expression of adenine nucleotides selective $P2Y_1$ (Webb et al., 1993) and $P2Y_{11}$ receptors (Communi et al., 1999) and the $P2Y_2$ receptor at which UTP and ATP have equal activity (Lustig et al., 1993). Furthermore, the rat and mouse $P2Y_4$ receptors have the same agonist profile as $P2Y_2$ (Webb et al., 1998; Bogdanov et al., 1998; Suarez-Huerta et al., 2000) and thus a contribution of this receptor to the phospholipase C responses detected may also be excluded. Indeed, the $P2Y_6$ receptor is the single recombinant P2Y receptor subtype activated preferentially by uracil nucleotides (Communi et al., 1996). However, this receptor has a clear selectivity for UDP over UTP, with EC_{50} values of 6 nM and 0.47 μM, respectively (Li et al., 1998). Thus, the equipotency of UTP and UDP in a range of mouse neuroblastomas and the NG108-15 cell line does not appear to be compatible with the expression of one or more of the recombinant P2Y receptors.

In the present study, we have investigated the expression of P2Y receptor transcripts in the NG108-15 cell line and performed experiments to characterise those functionally expressed in these cells.

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2. Materials and methods

2.1. Chemicals

UTP, UDP, ATP, ADP, AMP (5′-AMP) and adenosine were obtained from Boehringer Mannheim. Adenosine-5′-carboxylic acid (AdoC), 2′,3′-*O*-isopropylideneadenosine (iPAdo), 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS-21680), adenosine 2′-monophosphate (2′-AMP), adenosine 3′-monophosphate (3′-AMP), adenosine 2′:3′-cyclic monophosphate (2′,3′-cAMP), 3′,5′-cAMP, UMP, uridine, reactive blue 2, theophylline and forskolin were the products of Sigma. Pyridoxal phosphate-6-azophenyl-2′,4′disulfonic acid (PPADS) and suramin were purchased from RBI. 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]-ethyl)phenol (ZM 241385) was the product of Tocris. Trichloroacetic acid and ammonium formate were obtained from Acros Organics. Formic acid was purchased from Riedel-de Haën. Diethyl ether was from Den Norske Eter-Fabrikk. Biotaq was purchased from Bioline U.K., Moloney-murine leukaemia virus reverse transcriptase, RNase-free DNase I and Trizol were all obtained from Gibco BRL. Oligonucleotides were synthesised by Genosys. All other molecular biology reagents were obtained from Sigma.

All nucleotides were analysed and purified by High-Performance Liquid Chromatography (HPLC; Gilson) on an anion exchange column Mono Q (Amersham Pharmacia Biotech) monitoring the absorbance at 258 nm for adenine nucleotides and 270 nm for uracil nucleotides. Linear gradients from 0 to 0.8 M NaCl in 50 mM phosphate buffer (pH = 7.0) and from 0.1 to 1.2 M ammonium carbonate in water at the flow rate 1 ml/min were used. The latter gradient was used for preparative purification of nucleotides.

2.2. Cell culture

NG108-15 cell line was from European Collection of Cell Cultures (ECACC). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco), supplemented with 10% (v/v) of fetal calf serum, 8 μg/ml of tylosine, 0.1 mM hypoxanthine, 1 μM aminopterin and 16 μM thymidine. Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in 75-cm^2 tissue culture flasks.

2.3. Reverse transcriptase-polymerase chain reaction

Cells were seeded at 1×10^6 cells per 9-cm dishes and were assayed 3 days later. RNA was prepared ($n = 3$) using Trizol, according to the manufacturer's instructions, and was treated with RNase-free DNase I for 15 min at 37°C and then re-extracted with Trizol. First strand cDNA was prepared from 5 μg RNA using an oligo(dT) 18 primer and Moloney-murine leukaemia virus reverse transcriptase in a 40-μl reaction volume, according to the manufacturer's instructions. Reverse transcriptions were also performed in the absence of the enzyme as a control for contaminating DNA. PCR reactions for the four rodent/murine P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$) were performed with primer pairs designed to amplify partial cDNAs from each species sequence:

$P2Y_1$F 5′-TGGTGGCCATCTCCCCTATTCTCTT-3′;
$P2Y_1$R 5′-ATCTCGTGCCTTCACAAACT-3′;
$P2Y_2$F 5′-TTCCACGTCACCCGCACCCTCTATTACT-3′;
$P2Y_2$R 5′-CGATTCCCCAACTCACACATACAAA-TGATTG-3′;
$P2Y_4$F 5′-CTTCTCTGCCTGGGTGTTTGGTTG-GTAGTA-3′;
$P2Y_4$R 5′-TCCCCCGTGAAGAGATAGAG-CACTGGA-3′;
$P2Y_6$F 5′-GCCAGTTATGGAGCGGGACAATGG-3′;
$P2Y_6$R 5′-AGGAACAGGATGCTGCCGTGTAG-GTTG-3′.

PCR reactions were performed using 7.5% (v/v) of each first strand cDNA reaction and 15 pmol of each subtype-specific forward and reverse primer, using 2.5 units of Biotaq with the buffer supplied, 200 μM of each deoxy-nucleotide triphosphate and 1.5 mM $MgCl_2$. Amplification conditions were 60 s at 94°C, 30 s at 55°C, 45 s at 72°C for 35 cycles and finally 10 min at 72°C. Positive control amplifications were performed in parallel using plasmid DNA (2.5 ng) encoding each receptor subtype. Amplicons (10 μl) were resolved on a 2% (w/v) agarose gel by electrophoresis and sequenced directly using an automated DNA sequencer to confirm their identity.

2.4. Inositol phosphates assay

For measurements of total inositol phosphates NG108-15 cells were seeded in a 24-well culture plate at density of $\sim 5 \times 10^4$ cells/well. The cells were assayed after 3 days in culture when still subconfluent. Inositol lipids were radiolabelled by overnight incubation of cells with *myo*-[2-^{3}H]inositol (2 μCi/ ml, Amersham) in 200 μl of inositol-free and serum-free DMEM. No changes in medium were made subsequent to the addition of [^{3}H]inositol. The assay was initiated by addition of ligand solution in 50 mM LiCl and the assay mixture was incubated for 10 min at 37°C. Antagonists were added approximately 1 min before agonist solution. The assay was terminated by aspiration of the media and addition of 500 μl of ice-cold 5% trichloroacetic acid. The acid-containing supernatant was extracted three times with 500 μl of diethyl ether and inositol phosphates were isolated by using Dowex AG1-X8 gel (BioRad, 100–200 mesh, formate form). The columns (bed volume 0.8 ml) were washed with water (2 × 4 ml) and 50

mM ammonium formate (8 ml) and inositol phosphates were eluted with 1 M ammonium formate in 0.1 M formic acid (2×3 ml). The amount of ^{3}H-labelled inositol phosphates formed was determined by adding 12 ml of scintillation cocktail (OptiPhase HiSafe III, Wallac) to each eluates and counting the radioactivity. The basal value for inositol phosphate formation was 2460 ± 135 cpm.

2.5. Data analysis

Results were calculated as means $\pm$ S.E.M. from the experiments performed in triplicate and were representative of three independent experiments. The dose–response curves were processed by a non-linear regression analysis program (Prism™ version 2.00). The maximal response of UDP (initiated by 10 μM solution) was equated to 100% and the experimental data were transformed to percentile scale. Statistically significant effects were determined by Two-way Analysis of Variance (two-way ANOVA) and antagonistic constants K_i were calculated by the Gaddum equation (Lazareno and Birdsall, 1993).

3. Results

3.1. P2Y transcript expression in NG108-15 cells

cDNA de rived from NG108-15 cell RNA ($n = 3$) was subjected to 35 cycles of PCR amplification using oligonucleotide primers specific for the four cloned rodent/murine P2Y subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$). An abundant amplicon was detected for the $P2Y_6$ transcript with a $P2Y_2$ receptor amplification product present at lower levels (Fig. 1). There was no detectable amplification of either the $P2Y_1$ or $P2Y_4$ receptor transcripts. PCR products with the predicted sizes for each of the P2Y receptor subtypes were detected when plasmid DNA was used as a template. The specificity of the amplified products was confirmed by sequence analysis.

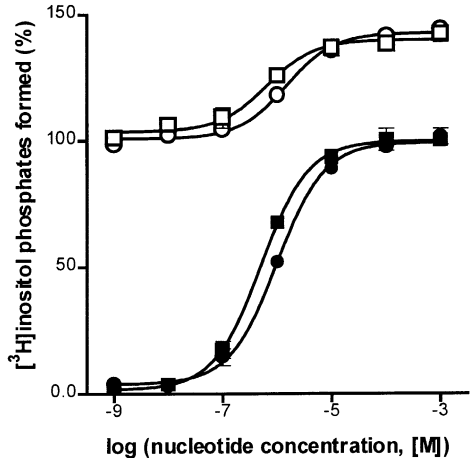

Fig. 2. Additivity of agonist induced inositol phosphates formation: UTP (■), UDP (●), activity of UTP in the presence of 10 μM UDP (□) and UDP in the presence of 10 μM UTP (○). The results are expressed as the percent of the maximal response of UDP.

3.2. Co-application of UTP and UDP give an additive response in NG108-15 cells

The P2Y receptors coupled to the activation of phospholipase C in NG108-15 cell line are activated by UTP and UDP, with ATP and ADP being considerably less effective (Sak et al., 1998, 1999). As reported previously, UTP and UDP were both able to evoke the accumulation of inositol phosphates at high nanomolar concentrations and displayed similar potency, with EC_{50} values of 0.50 ± 0.05 and 0.98 ± 0.11 μM, respectively (Fig. 2).

In an attempt to ascertain if UTP and UDP act at the same or different receptors the effect of co-application of these agonists was assessed. The concentration–effect relationship for UTP was determined in the presence of a concentration of UDP that gave a maximal response when applied alone (10 μM). The accumulation of inositol phosphates was raised under these conditions with an apparent EC_{50} for UTP of 0.65 ± 0.23 μM (Fig. 2). The

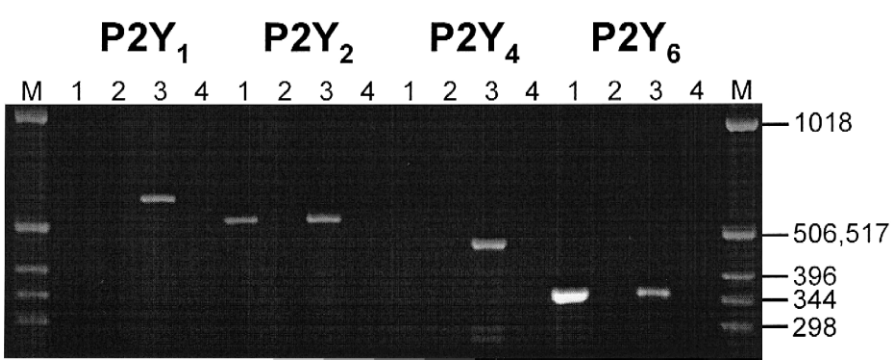

Fig. 1. RT-PCR analysis of P2Y receptor transcripts present in NG108-15 cells. Agarose gel electrophoresis of PCR products, see Section 2 for further details. (M) Size markers: 1 kbp ladder, appropriate sizes are indicated. For each receptor sequence amplification, lanes 1 and 2 incorporate cDNA syntheses where reverse transcriptase was present or absent, respectively, lane 3 is a PCR reaction using the appropriate plasmid construct, lane 4 is a no added template control. The figure is representative of three independent experiments.

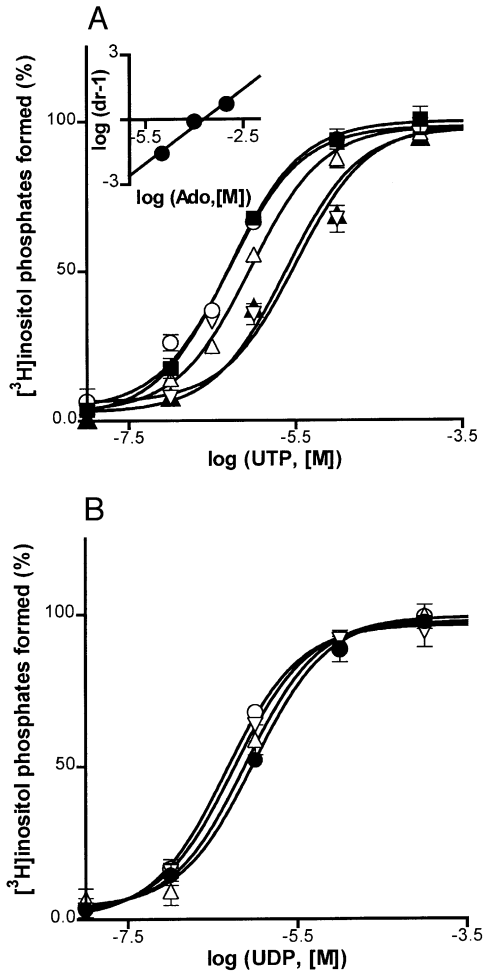

Fig. 3. (A) Effects of 10 μM (○, $P = 0.7051$), 100 μM (△, $P = 0.0121$) and 1 mM (▽, $P < 0.0001$) adenosine on the dose-dependent stimulation of inositol phosphates synthesis by UTP (■), and the ineffectiveness of 180 μM ZM 241385 to suppress the inhibitory action of 1 mM adenosine (▲). The inset shows the Schild plot of the data. (B) Effects of 10 μM (▽, $P = 0.2883$), 100 μM (○, $P = 0.5887$) and 1 mM (△, $P = 0.3254$) adenosine on the dose-dependent stimulation of inositol phosphates synthesis by UDP (●). The results are expressed as the percent of the maximal response of UDP, P compared with the nucleotide alone induced PLC response.

reciprocal experiment was also performed (i.e. the concentration dependent effect of UDP in the presence of 10 μM UTP was assessed). In this case, the determined EC_{50} value for the effect of UDP was 1.49 ± 0.29 μM in the presence 10 μM UTP (Fig. 2). In each case, co-application of these agonists was additive up to approximately 140% of the level produced by 10 μM UDP alone. The same level of accumulation of inositol phosphates (~140%) was observed when the cells were challenged simultaneously with either agonist in the presence of 10 mM NaF.

3.3. Differential effect of adenosine on the phospholipase C response to UTP and UDP application

The effect of adenosine and some of its structural analogues on the UTP and UDP stimulated activation of phospholipase C was determined. None of the ligands tested were able to modulate phospholipase C activity in their own right up to a concentration of 1 mM (data not shown). However, adenosine was able to shift the UTP dose–response curve to the right and behaved as a competitive antagonist. A Schild analysis revealed a slope of 1.14 ± 0.19 and the K_i for adenosine was 185 μM (Fig. 3A). Of the other compounds tested (Table 1) only iPAdo, 3′,5′-cAMP and adenosine A_{2A} receptor agonist CGS-21680 were also able to antagonise the action of UTP. However, the adenosine A_2 receptor antagonist ZM 241385 at a concentration of 180 μM neither suppressed nor abolished the inhibitory action of adenosine on the phospholipase C response to UTP (Fig. 3A). Furthermore, the general adenosine receptor antagonist theophylline was also without effect, the EC_{50} for UTP in the presence of 1 mM adenosine was 3.16 ± 0.48 μM and was 2.95 ± 0.38 μM when determined in the presence of 1 mM adenosine and 1 mM theophylline. To establish if the inhibitory actions of adenosine were mediated via adenylate cyclase the concentration dependent effect of UTP on phospholipase C activation was determined in the presence of 10 μM forskolin. This treatment was found to be without effect, the EC_{50} for UTP in the absence and presence of forskolin was 0.50 ± 0.05 and 0.60 ± 0.08 μM.

CGS-21680, iPAdo and 3′,5′-cAMP were unable to antagonise the phospholipase C response evoked by UDP (data not shown). Adenosine also failed to act as an antagonist but slightly potentiated the UDP response although this effect was not statistically significant (Fig. 3B).

3.4. Effect of P2 receptor antagonists on the synthesis of inositol phosphates evoked by UTP and UDP application

The non-phosphate antagonists of P2 receptors, suramin, PPADS and reactive blue 2 were all tested for their ability to antagonise the effect of UTP and UDP on phospholipase C in NG108-15 cells, in an attempt to further characterise

Table 1
Effect of adenosine and its derivatives on the synthesis of inositol phosphates initiated by UTP in NG108-15 cells

Compound	pK_i
Adenosine	3.73 ± 0.07[a]
CGS-21680	3.24 ± 0.03[a]
iPAdo	4.26 ± 0.08[a]
AdoC	N.E.[b]
AMP (5′-AMP)	N.E.
2′-AMP	N.E.
3′-AMP	N.E.
2′,3′-cAMP	N.E.
3′,5′-cAMP	4.32 ± 0.14[a]

The pK_i values were calculated by the Gaddum equation (Lazareno and Birdsall, 1993).
[a] Significant effect ($P < 0.05$).
[b] N.E. = no significant effect at 300 μM.

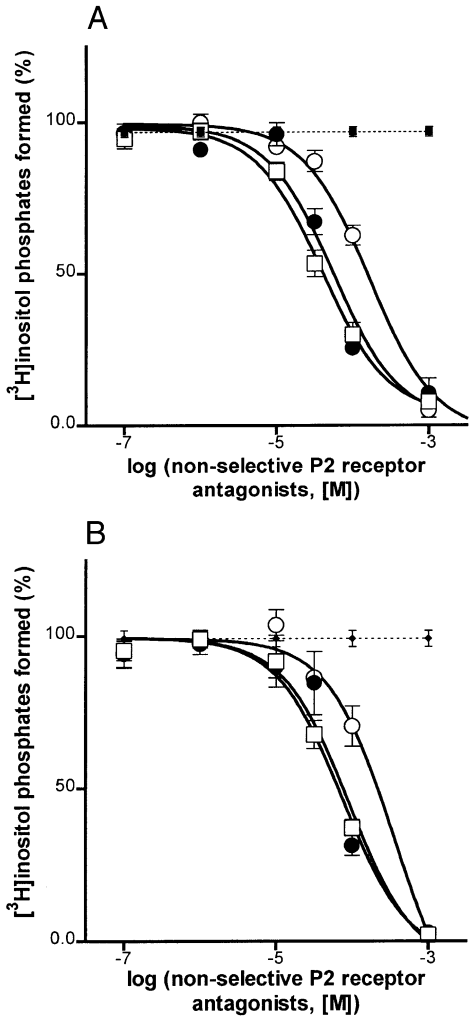

Fig. 4. (A) Effects of the P2 receptor antagonists suramin (□), reactive blue 2 (●) and PPADS (○) on 1 μM UTP (■) induced hydrolysis of inositol phospholipids. The results are expressed as the percent of the response to 1 μM UTP. (B) Effects of the P2 receptor antagonists suramin (□), reactive blue 2 (●) and PPADS (○) on 1 μM UDP (♦) induced hydrolysis of inositol phospholipids. The results are expressed as the percent of the response to 1 μM UDP.

the pyrimidinoceptor(s) expressed in this cell line. All three compounds were able to antagonise the response to 1 μM UTP (Fig. 4A). Suramin and reactive blue 2 had a similar inhibitory activity, with IC_{50} values of 39.7 ± 6.4 and 55.9 ± 14.6 μM, respectively, while PPADS had lower activity with an IC_{50} value of 166.7 ± 33.0 μM. These antagonists had the same rank order of activity at phopholipase C activation mediated by 1 μM UDP (IC_{50} values (in μM): suramin 74.6 ± 14.7, reactive blue 2 91.0 ± 27.4, PPADS 363.6 ± 135.3) (Fig. 4B).

4. Discussion

Previously, mRNA for the $P2Y_6$ receptor, as well as the $P2Y_1$, $P2Y_2$, but not the $P2Y_4$ has been detected by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis in NG108-15 cells (Webb and Barnard, 1999) used for studies into $P2Y_2$ receptor modulation of ion channel activity (Filippov et al., 1994). Analysis by RT-PCR of the cells used in the current study, using conditions which allow the detection of the murine as well as rodent P2Y receptor sequences, revealed an abundant expression of the $P2Y_6$ receptor mRNA (UDP > UTP) and detected the $P2Y_2$ receptor transcript (UTP = ATP) at considerably lower levels. The inactivity of ATP at these cells excludes the functional expression of the $P2Y_2$ receptor. Thus, the only characterised P2Y receptor subtype expressed in this clone of the NG108-15 cell line is the $P2Y_6$ receptor. The equipotency of the UTP and UDP evoked inositol phosphates responses in NG108-15 cells are not compatible with the pharmacological profile of this receptor subtype. UTP is full agonist at this receptor, but with a potency of ~70 times less than UDP for the generation of second messengers (Li et al., 1998). However, in a separate study where the ability of the recombinant $P2Y_6$ receptor to couple to ion channels was investigated, UTP was found to be ~4 times less potent than UDP (Filippov et al., 1999). In the present study, purified UTP and UDP were applied to the cells, thus a considerable breakdown of UTP to UDP would be required to explain the apparent shift in potency. However, we found that at 10 μM concentration, only a small proportion of the UTP (up to 20%) was degraded in the reaction mixture during assay time (data not shown). This led us to consider the possibility that these two uracil nucleotides activate more than one P2Y receptor subtype in these cells. The additive nature of the UTP and UDP phospholipase C responses suggests that this is the case. That application of UTP gives an enhanced phospholipase C response in the presence of UDP and UDP application gives a similar enhancement in the presence of UTP indicates that UDP and UTP act primarily at different receptor sites. Furthermore, the effect of NaF, a G protein activator, in combination with either UTP or UDP gave the same degree of additivity indicating that a maximal level of stimulation had been observed. In addition, a similar level of stimulation has been observed previously upon the co-application of UTP and bradykinin in this cell line (Lin, 1994).

The differential ability of adenosine to modulate UTP and UDP responses in the NG108-15 cell line provides further evidence for the presence of two different receptors for these uracil nucleotides for a number of reasons. Adenosine behaved as low affinity antagonist for the UTP mediated accumulation of inositol phosphates in these cells but was found to be without inhibitory action at the UDP response. Although slight potentiation of UDP curve was observed in the presence of adenosine this effect was not significant. Both adenosine A_{2A} and A_{2B} receptor subtypes have been reported to be functionally expressed by NG108-15 cells and are coupled to the stimulation of adenylate cyclase (Mundell and Kelly, 1998). However, the inhibitory effect of adenosine at the UTP response does

not appear to be adenosine receptor mediated for the following reasons. Firstly, derivatives of adenosine which have no activity at adenosine A_2 receptors for example, 2′,3′-*O*-isopropylideneadenosine and adenosine 3′:5′-cyclic monophosphate (Ijzerman personal communication) had clear antagonistic activity on the UTP response. Secondly, neither theophylline, the general adenosine receptor antagonist, nor the adenosine A_{2A} receptor antagonist ZM 241385 (used in this study at a concentration at which it would also act at the adenosine A_{2B} receptor, Poucher et al., 1995), were able to modulate the inhibitory action of adenosine. Moreover, recently it has been indicated that NG108-15 cells express mainly adenosine A_{2A} receptor (Ohkubo et al., 2000). Thirdly, the specific and very potent agonist for adenosine A_{2A} receptor CGS-21680 (Jarvis et al., 1989) had a smaller inhibitory effect on the UTP response than did adenosine, the reverse might be expected if this effect was mediated by the adenosine A_{2A} receptor. Finally, an involvement of adenylate cyclase per se could be excluded as forskolin was found to have no effect on UTP stimulation of phospholipase C.

P2 receptor antagonists were used in an attempt to discriminate between the actions of UDP and UTP. However, the rank order of activity for the inhibition of inositol phosphates accumulation upon either UTP or UDP application was suramin ≥ reactive blue 2 > PPADS. This antagonist activity order is not compatible with data obtained for the known P2Y receptor subtypes (Jacobson et al., 1999). Specifically, these antagonists have been reported to have a rank order of activity: reactive blue 2 > PPADS ≫ suramin at the $P2Y_6$ receptor (Robaye et al., 1997). It should be noted that suramin has a number of non-receptor mediated effects including inhibition of ectonucleotidase (Hourani and Chown, 1989). However, these data are again suggestive of expression of a further P2Y receptor other than $P2Y_6$ in these cells. This study joins other examples of atypical pyrimidine-preferring receptors found in the literature (Wilson et al., 1999; Yang et al., 1996; Laubinger and Reiser, 1998; Ko et al., 1997; Bourke et al., 1999).

It is interesting to compare the pharmacological activity profile measured in the present study with the data published previously on NG108-15 cells. Basing on the ligand potency order such studies can be divided into two categories. Firstly, similarly to our observations (Sak et al., 1998, 1999) the P2Y receptors expressed have been characterised by the rank order of potency: UTP ≥ UDP ≫ ATP ~ ADP (Lin, 1994; Chueh et al., 1995). In parallel to these studies, there is clear evidence for the functional expression of $P2Y_2$ receptors with the agonist activity order UTP = ATP > ATPγS ≫ UDP ~ ADP (Lustig et al., 1993; Filippov et al., 1994; Matsuoka et al., 1995; Czubayko and Reiser, 1996; Lin et al., 1993). This may indicate that there are two variants of NG108-15 cell line or this could be a consequence of differences in culturing conditions.

In summary, we have evidence for the expression of a novel UTP-preferring pyrimidinoceptor subtype in NG108-15 cells at which adenosine acts as a low affinity antagonist. Its expression in a neuronal cell line suggests a potential physiological role in nervous system. The determination of its molecular structure and functional properties will be the subject of future studies.

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