DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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# SOME ASPECTS OF LIGAND SPECIFICITY OF P2Y RECEPTORS

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# SOME ASPECTS OF LIGAND SPECIFICITY OF P2Y RECEPTORS

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PRESS

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# CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

**I** Sak, K., Kreegipuu, A., Järv, J. (2000) P2Y-receptor-ligand database. *Trends Biochem. Sci.*, **25**, 35.

**II** Sak, K. (2000) Are $P2Y_1$ purinoceptors expressed in turkey erythrocytes? *Neurosci. Lett.*, **293**, 78–80.

**III** Sak, K. & Järv, J. (2000) Adenosine triphosphate is full antagonist at human $P2Y_1$ purinoceptors. *Neurosci. Lett.*, **284**, 179–181.

**IV** Sak, K., Raidaru, G., Webb, T. E., Järv, J. (2000) Phosphate-substituted ATP analogs are antagonists at human $P2Y_1$ purinoceptors. *Arch. Biochem. Biophys.*, **381**, 171–172.

**V** Sak, K., Uri, A., Enkvist, E., Raidaru, G., Subbi, J., Kelve, M., Järv, J. (2000) Adenosine-derived non-phosphate antagonists for $P2Y_1$ purinoceptors. *Biochem. Biophys. Res. Commun.*, **272**, 327–331.

**VI** Sak, K., Webb, T. E., Samuel, K., Kelve, M., Järv, J. (1999) Only pyrimidinoceptors are functionally expressed in mouse neuroblastoma cell lines. *Mol. Cell Biol. Res. Commun.*, **1**, 203–209.

**VII** Sak, K., Samuel, K., Kelve, M., Webb, T. E. (2000) Pharmacological evidence for a novel pyrimidinoceptor in NG108-15 cells. Submitted.

**VIII** Sak, K., Kelve, M., Uri, A., Järv, J. (1998) Pyrimidinoceptor potentiation by ATP in NG108-15 cells. *FEBS Lett.*, **439**, 107–109.

# ABBREVIATIONS

Names and abbreviations of the most often used P2Y receptor ligands.

| No. | Abbreviation | Name |
|---|---|---|
| 1 | ATP | Adenosine 5'-triphosphate |
| 2 | 2MeSATP | 2-methylthioATP |
| 3 | 2ClATP | 2-chloroATP |
| 4 | BzBzATP | 3'-O-(4-benzoyl)benzoylATP |
| 5 | dATP | 2'-deoxyATP |
| 6 | ATPαS | Adenosine 5'-O-(1-thiotriphosphate) |
| 7 | ATPγS | Adenosine 5'-O-(3-thiotriphosphate) |
| 8 | Ap[$CH_2$]pp | αβMethyleneATP |
| 9 | App[$CH_2$]p | βγMethyleneATP |
| 10 | App[NH]p | βγImidoATP |
| 11 | dATPαS | 2'-deoxyadenosine 5'-O-(1-thio)triphosphate |
| 12 | ADP | Adenosine 5'-diphosphate |
| 13 | 2MeSADP | 2-methylthioADP |
| 14 | ADPβS | Adenosine 5'-O-(2-thiodiphosphate) |
| 15 | Ap[$CH_2$]p | αβMethyleneADP |
| 16 | A3P5P | Adenosine 3',5'-bisphosphate |
| 17 | Ap4A | $P^1$,$P^4$-diadenosine tetraphosphate |
| 18 | UTP | Uridine 5'-triphosphate |
| 19 | 5BrUTP | 5-bromoUTP |
| 20 | UTPγS | Uridine 5'-O-(3-thiotriphosphate) |
| 21 | UDP | Uridine 5'-diphosphate |
| 22 | 5BrUDP | 5-bromoUDP |

Other abbreviations used in this dissertation.

| | |
|---|---|
| 3',5'-cAMP | Adenosine 3':5'-cyclic monophosphate |
| AC | Adenylate cyclase |
| Ado | Adenosine |
| AdoC | Adenosine-5'-carboxylic acid |
| AMP | Adenosine 5'-monophosphate |
| AMPαS | Adenosine 5'-O-(thiomonophosphate) |
| Asp | Aspartic acid |
| CGS-21680 | 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine |
| CHO | Chinese hamster ovary |
| DMEM | Dulbecco's Modified Eagle's Medium |
| EDTA | Ethylenediaminetetraacetic acid |
| Fura 2 | 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid |

| | |
|---|---|
| Gly | Glycine |
| HEK | Human embryonic kidney |
| HPLC | High Performance Liquid Chromatography |
| Indo 1 | 4-(6-Carboxy-2-indolyl)-4'-methyl-2,2'-(ethylenedioxy)dianiline-N,N,N',N'-tetraacetic acid |
| InP | Inositol phosphates |
| iPAdo | 2',3'-O-isopropylideneadenosine |
| PEI | Polyethyleneimine |
| PPADS | Pyridoxal phosphate-6-azophenyl-2',4'disulfonic acid |
| Pro | Proline |
| Quin 2 | 2-([2-bis-(Carboxymethyl)amino-5-methylphenoxy]methyl)-6-methoxy-8-bis(carboxymethyl)aminoquinoline |
| RT-PCR | Reverse Transcriptase-Polymerase Chain Reaction |
| Sar | Sarcosine |
| TCA | Trichloroacetic acid |
| TLC | Thin Layer Chromatography |
| UV | Ultraviolet |
| ZM 241385 | 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol |

# 1. INTRODUCTION

Drury and Szent-Györgyi published the first report about the potent action of extracellular adenine compounds in mammalian heart in 1929 (Drury & Szent-Györgyi, 1929). This finding stimulated the studies of the role of extracellular purine nucleotides and nucleosides in mammals and in 1972 Burnstock proposed that ATP could be a neurotransmitter involved in nerve-mediated responses (Burnstock, 1972). Purinergic receptors were first mentioned by the same author in 1978, when he distinguished between two major types of these receptors: P1-purinoceptors, at which adenosine is the principal natural ligand; and P2-purinoceptors, which are selective for nucleotides (Burnstock, 1978). Later the P2 receptors were divided into P2X receptors (ligand-gated cation channels) and P2Y receptors (G protein-coupled receptors) (Abbracchio & Burnstock, 1994). Today we know that extracellular nucleotides are important signalling molecules and mediate several physiologically essential processes (Ralevic & Burnstock, 1998). The present dissertation is focused on some aspects of ligand specificity of the P2Y type nucleotide receptors.

# 2. P2Y RECEPTORS

The first P2Y receptors were cloned in 1993 (Webb *et al.*, 1993; Lustig *et al.*, 1993) and these publications initiated an extensive work in this field. The "dynamics" of these studies is illustrated in Fig. 1 as time-dependence of the number of publications devoted to interaction of purinoceptors with synthetic and natural ligands. These activity data were collected within this study and were made available in Internet as "P2Y Receptor Ligand Database" (*http://bioorg.chem.ut.ee/p2y/*) (**Paper I**). The following survey of literature is based on the analysis of this database.

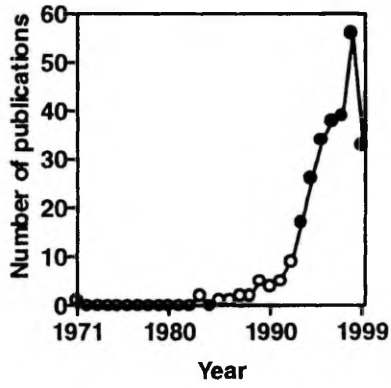


**Figure 1.** Time-dependence of the number of publications devoted to the interaction of nucleotide receptors with synthetic and natural ligands. Publications before (○) and after (●) cloning of the P2Y receptors.

## 2.1. Structure and biochemical responses of P2Y receptors

P2Y receptors have seven well-conserved transmembrane hydrophobic domains and a short extracellular N-terminus, while the intracellular C-terminus is more variable (Abbracchio & Burnstock, 1998; Barnard *et al.*, 1997). The P2Y receptors are predominantly coupled with phospholipid turnover and intracellular $Ca^{2+}$ mobilisation phenomena via $G_q$ proteins (Chen ZP *et al.*, 1995). In parallel, coupling of some P2Y receptors to adenylate cyclase has been described (Communi *et al.*, 1997b; Communi *et al.*, 1999; Van der Weyden *et al.*, 2000).

## 2.2. Assay of P2Y receptors

P2Y receptors have been mainly assayed by three methods. First, breakdown of inositol phospholipids can be followed either by measuring of inositol 1,4,5-triphosphate or by determination of the total amount of inositol phosphates formed (Shears, 1997; Berridge, 1986). Second, intracellular $Ca^{2+}$ mobilisation can be followed by fluorimetric analysis, using Ca-indicators like Fura 2, Quin 2 and Indo 1 (Takahashi *et al.*, 1999). Third, modulation of ion-channel activity (inhibition or activation of the N-type $Ca^{2+}$ and M-type $K^+$ currents) can be recorded by the whole-cell patch-clamp method (Filippov *et al.*, 1994; Barnard *et al.*, 1997; Filippov *et al.*, 2000). If the receptors are coupled to adenylate cyclase, changes in cyclic AMP concentration can be determined (Communi *et al.*, 1997b; Communi *et al.*, 1999).

Although the radioligand binding is a widespread technique for receptor studies, the application of this method for nucleotide receptors was found to be complicated by the presence of numerous nucleotide-binding proteins of non-receptor origin on cell surface (Schachter & Harden, 1997a).

The three main assay methods mentioned above differ somewhat in experimental conditions used. If the electrophysiological recordings are normally made at room temperature (20–24°C), in other procedures the cells are incubated at 37°C. Other differences concern duration of experiments. If intracellular $Ca^{2+}$ mobilisation experiments and electrophysiological recordings can be made during seconds, measurement of total inositol phosphates requires minutes. These differences may be essential if ligands are metabolised by ecto-nucleotidases (Zimmermann & Braun, 1999) or nucleoside diphosphokinases (Harden *et al.*, 1997) on the target cells. Sometimes these effects may be reduced by application of triphosphate or diphosphate regenerating systems (Harden *et al.*, 1997).

Proceeding from the "P2Y Receptor Ligand Database" statistical comparison of different assay procedures was made. This analysis revealed that there was no systematic influence of the assay procedure on the results (Fig. 2a–d). In spite of this the results of different publications are rather scattered. Therefore special attention should be paid on reproducibility of experimental conditions and ligand purity.

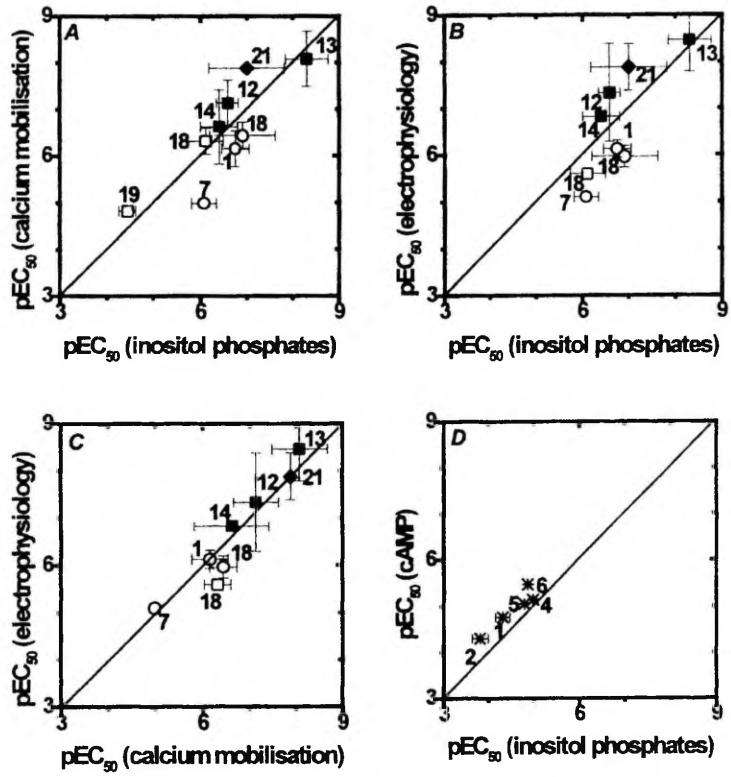


**Figure 2.** Comparison of ligand activities at P2Y receptor transfected cells measured by different assay procedures. Numbers in the figure correspond to the numeration of ligands listed in Abbreviations (■ — $P2Y_1$, ○ — $P2Y_2$, □ — $P2Y_4$, ◆ — $P2Y_6$, ✱ — $P2Y_{11}$).

## 2.3. Time-line of P2Y receptor cloning

To date considerable number of P2Y receptor subtypes has been cloned and designated as $P2Y_1$ to $P2Y_n$. The subscript allocation in this terminology was based on the chronology of cloning (Boeynaems *et al.*, 2000) and the time-line of these works is illustrated in Fig. 3.

The $P2Y_1$ receptor was first cloned from chick brain (Webb *et al.*, 1993). Thereafter homologues of this subtype were cloned from turkey brain (Filtz *et al.*, 1994), mouse insulinoma cells (Tokuyama *et al.*, 1995), rat insulinoma cells (Tokuyama *et al.*, 1995), rat ileal myocytes (Pacaud *et al.*, 1996), bovine endothelium (Henderson *et al.*, 1995), bovine brain (Deng *et al.*, 1998), human erythroleukemia cells (Ayyanathan *et al.*, 1996), human prostate and ovary (Janssens *et al.*, 1996), human placenta (Leon *et al.*, 1996), and human brain (Schachter *et al.*, 1996). The identity of these receptors was high within mammalian homologues (95–100%) and lower if the mammalian and avian homologues were compared (83–86%).

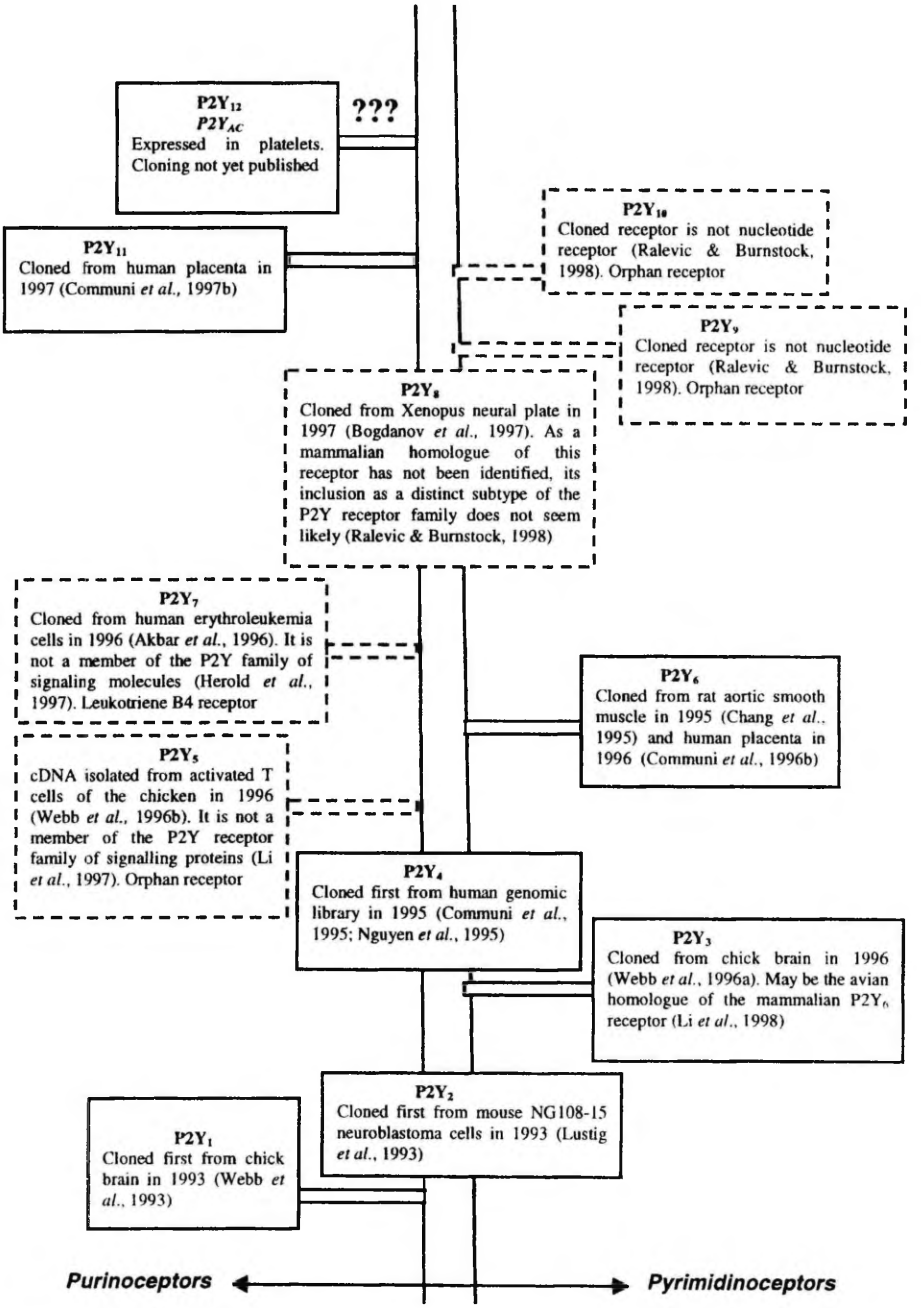


**Figure 3.** Evolution of P2Y receptor studies. Functional subtypes are presented by using straight lines, orphan receptors by dotted lines.

The similar sequence variations can be observed within all P2Y subtypes cloned from different species. For example, the sequence analysis revealed 96–100% amino acid identity within the rodent $P2Y_2$ receptors as well as within the human $P2Y_2$ receptor clones, while the amino acid homology between rodent and human $P2Y_2$ receptors was only 86–89%. Further, comparison of sequences of rat and human $P2Y_6$ receptors revealed 88% identity in amino acids. Similarly, the amino acid sequence analysis revealed that rat and human $P2Y_4$ receptors shared 83–84% amino acid identity. Summary of these sequence comparison data is presented in Table 1. It can be seen that identity level above 80% is considered as sufficient for receptors of the same subtype. At the same time rather significant variations in amino acid sequence can be observed if different subtypes are compared.

In several cases no functional activity was associated with cloned receptors. For example, $P2Y_5$ subtype cloned in 1996 (Webb *et al.*, 1996b) was later shown as "orphan receptor" (Boeynaems *et al.*, 2000). Similarly, $P2Y_7$ receptor was not fitted into the family of signalling P2Y receptors (Herold *et al.*, 1997). Moreover, this receptor was later identified as leukotriene $B_4$ receptor (Boeynaems *et al.*, 2000). The $P2Y_8$, $P2Y_9$ and $P2Y_{10}$ receptors were also considered as orphan receptors (Bogdanov *et al.*, 1997; Boeynaems *et al.*, 2000).

The last P2Y receptor cloned so far, $P2Y_{11}$, was identified in human placenta in 1997 (Communi *et al.*, 1997b). At least two further subtypes of P2Y receptors have yet to be identified on molecular level (Webb & Barnard, 1999). The first of these is most extensively characterised on platelets ($P2Y_{AC}$). The second P2Y receptor with hitherto unknown structure is activated by diadenosine polyphosphates (Miras Portugal *et al.*, 1996).

In summary, there are five functionally active P2Y receptors cloned so far ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$). On the other hand, within each of these subtypes the structural similarity of the receptor proteins may still differ up to 20%. These differences in amino acid sequences may influence affinity of ligands and thus change the ligand activity order even within the same subtype. This possibility, in turn, may additionally complicate the classification of P2Y receptors by ligand specificity patterns.

**Table 1.** Percentage of amino acid sequence identities of cloned P2Y receptor subtypes. Data are from articles used for compilation of the "P2Y Receptor Ligand Database".

| % | $P2Y_1$ | $P2Y_2$ | $P2Y_3$* | $P2Y_4$ | $P2Y_6$ | $P2Y_{11}$ |
|---|---|---|---|---|---|---|
| **$P2Y_1$** | **≥ 83** | 34–38 | 39 | 30–35 | 35–38 | 33 |
| **$P2Y_2$** | | **≥ 86** | 41 | 47–54 | 37–44 | 28 |
| **$P2Y_3$*** | | | **≥ 98** | 40 | *65* | ~30 |
| **$P2Y_4$** | | | | **≥ 83** | 36–40 | 30 |
| **$P2Y_6$** | | | | | **≥ 88** | ~30 |
| **$P2Y_{11}$** | | | | | | **100** |

* May be the avian homologue of mammalian $P2Y_6$ receptor

## 2.4. Functionally active P2Y receptor subtypes

Proceeding from ligand selectivity, the functionally active receptors can be characterised as purinoceptors, pyrimidinoceptors and receptors with mixed ligand selectivity. These receptors differentiate also between nucleoside tri-, di- and monophosphates, although the latter nucleotides are not effective ligands at any of these subtypes. Therefore only nucleoside di- and triphosphates and their derivatives have been used for characterisation of ligand specificity of these receptors. However, scattering and sometimes even contradictory results have hampered perfection of this procedure. For example, ATP and its derivatives have been concurrently described as full agonists, partial agonists or even as full antagonists at the human $P2Y_1$ subtype, even if the same receptor clone was used in experiments. Moreover, for some other compounds it has been even hard to decide whether they are active or not.

Most clearly these problems can be observed while analysing the "P2Y Receptor Ligand Database" (*http://bioorg.chem.ut.ee/p2y/*). This database was used to calculate the mean $EC_{50}$ values for main nucleotides and nucleotide derivatives, most often used for study of P2Y receptors. The results of this procedure are listed in Table 2 and used to summarise the present understandings about ligand activities at functional P2Y receptor subtypes. Some queries raised by this analysis were the objectives of the present dissertation.

### 2.4.1. $P2Y_1$ receptor

This receptor is specific for adenine nucleotides (Table 2). It is certain that ADP and its derivatives act as full agonists at $P2Y_1$ receptor (Harden *et al.*, 1998). Modification at the adenine base can be made and so 2MeSADP is more potent agonist than ADP itself (Table 2). Some alterations at the phosphate moiety of ADP are also tolerated at this subtype. For example, substitution of the double-bonded oxygen atoms by sulphur yielded agonistic ligands, while ADP analogue with methylene group instead of the oxygen atom intervening phosphorus atoms was not active (Table 2).

ATP and its analogues have been described as full agonists at the chick (Webb *et al.*, 1993), turkey (Filtz *et al.*, 1994; Schachter *et al.*, 1996), rat (Dixon *et al.*, 2000; Filippov *et al.*, 2000) and bovine (Henderson *et al.*, 1995; Deng *et al.*, 1998) $P2Y_1$ receptors. However, the apprehensions about activity type of ATP at human $P2Y_1$ receptor have passed significant changes. So, in early nineties ATP has been described as full agonist (O'Grady *et al.*, 1996; Schachter *et al.*, 1996; Schachter & Harden, 1997a). However, no agonistic activity of ATP was found in other studies of calcium mobilisation (Leon *et al.*, 1997; Vigne *et al.*, 1999). Moreover, it was shown that purified ATP, 2ClATP, 2MeSATP, BzBzATP and ATPαS behaved as antagonists at this receptor.

These results were supported by experiments where ATP regeneration system (creatine phosphate and creatine phosphokinase) was added into the assay system (Hechler *et al.*, 1998a).

On the other hand, Palmer and co-workers (1998) suggested that the type of activity of ATP could be determined by different level of the receptor expression in different transfected cells (Palmer *et al.*, 1998). All these contradictory results have hampered understanding of the role of these receptors in human and definition of structural requirements for antagonists of this $P2Y_1$ receptor. Therefore elucidation of the type of ATP effect at this receptor was one of the objectives of the present work.

**Table 2.** Summary of activity data for nucleotides and their analogues at P2Y receptors. The constants listed ($pEC_{50}$ ± standard deviation in 95% of confidence level) were calculated from "P2Y Receptor Ligand Database" as mean values of published data for transfected systems. Number of separate determination used for calculation is given in parenthesis.

| No. | Compound | $P2Y_1$ | $P2Y_2$ | $P2Y_4$ | $P2Y_6$ | $P2Y_{11}$ |
|---|---|---|---|---|---|---|
| 1 | ATP | ? | 6.4±0.4 (13) | ? | N.A. | 4.5±0.3 (3) |
| 2 | 2MeSATP | ? | N.A. | ? | N.A. | 4.0±0.3 (3) |
| 3 | 2ClATP | ? | 5.6 (1) | | | |
| 4 | BzBzATP | ? | N.A. | | | 5.1±0.1 (2) |
| 5 | dATP | | | | | 4.9±0.2 (2) |
| 6 | ATPαS | ? | N.A. | | | |
| 7 | ATPγS | ? | 5.7±0.6 (5) | ? | | 5.2±0.4 (2) |
| 8 | Ap[$CH_2$]pp | N.A. | N.A. | N.A. | | |
| 9 | App[$CH_2$]p | N.A. | N.A. | | | |
| 10 | App[NH]p | 4.8±0.2 (2) | ? | | | |
| 11 | dATPαS | ? | 4.6 (1) | N.A. | N.A. | |
| 12 | ADP | 6.9±0.5 (16) | N.A. | ? | N.A. | N.A. |
| 13 | 2MeSADP | 8.3±0.5 (17) | N.A. | | N.A. | N.A. |
| 14 | ADPβS | 6.5±0.5 (7) | N.A. | | N.A. | 4.2±0.4 (2) |
| 15 | Ap[$CH_2$]p | N.A. | | | | |
| 17 | Ap4A | ? | 6.4±0.3 (3) | ? | N.A. | N.A. |
| 18 | UTP | N.A. | 6.4±0.6 (16) | 6.1±0.4 (12) | N.A. | N.A. |
| 19 | 5BrUTP | | 5.7 (1) | 4.6±0.3 (3) | N.A. | |
| 20 | UTPγS | | 6.6 (1) | 5.8 (1) | N.A. | |
| 21 | UDP | N.A. | N.A. | N.A. | 7.4±0.8 (7) | N.A. |
| 22 | 5BrUDP | | | N.A. | 7.6±1.0 (2) | |

? — activity type unclear

N.A. — not active or very weak agonistic activity at 100 μM concentration

Another problem has been associated with the usage of turkey erythrocytes as model system for study of nucleotide receptors since middle of 1980s. The receptor expressed in these cells has been believed to belong to the $P2Y_1$ subtype (Boyer *et al.*, 1996b) and many structure activity relationships were proposed for this subtype on the basis of these data (Filtz *et al.*, 1994; Berrie *et al.*, 1989; Boyer *et al.*, 1998; Boyer *et al.*, 1995; Boyer *et al.*, 1996a; Boyer *et al.*, 1996c; Burnstock *et al.*, 1994; Camaioni *et al.*, 1998; Fischer *et al.*, 1993; Nandanan *et al.*, 1999; Nandanan *et al.*, 2000; Kim *et al.*, 2000). However, a more complete analysis of this aspect revealed that several ligands behave rather differently at nucleotide receptors from turkey erythrocytes and other sources (**Paper II**). For example, the agonistic constants for ADP were approximately two magnitudes lower in erythrocytes if compared with the same values for $P2Y_1$ receptors derived from chick, turkey, rat or human brain. At the same time ADPβS revealed similar activity in these systems. Moreover, the full and competitive antagonists for $P2Y_1$ receptor (adenosine 3',5'-bisphosphate and its sulphate-containing analogue) behaved as partial agonists in turkey erythrocytes. These differences demonstrate that the P2Y receptors expressed in turkey erythrocytes were not the same as the receptors derived from brain of different species. Therefore the structure-activity relationships for the former system cannot directly transferred to genuine $P2Y_1$ subtype.

### 2.4.2. $P2Y_2$ receptor

Both ATP and UTP activated the $P2Y_2$ receptors (Table 2) and no activity of ADP and UDP was found, if possibility of contamination of these compounds by appropriate triphosphates was eliminated (Harden *et al.*, 1998; Nicholas *et al.*, 1996b). In addition to ATP and UTP diadenosine tetraphosphate (Ap4A) (Lazarowski *et al.*, 1995) and adenosine and uridine γ-thiotriphosphates (ATPγS and UTPγS) (Lazarowski *et al.*, 1995; Lazarowski *et al.*, 1996) were described as $P2Y_2$ agonists. At the same time other substitutions in ATP molecule were not tolerated by this receptor.

### 2.4.3. $P2Y_4$ receptor

The human clone of this receptor was first described as UTP and UDP specific subtype (Communi *et al.*, 1995; Nguyen *et al.*, 1995; Communi *et al.*, 1996a). However, under the conditions where purity and stability of agonists were under control, UDP was found to be inactive at this subtype (Nicholas *et al.*, 1996a; Nicholas *et al.*, 1996b). UTPγS was rather similar to UTP and 5BrUTP was less potent (Table 2). Furthermore, it was found that ATP acted as antagonist at the human receptor (Kennedy *et al.*, 2000). However, studies with rodent $P2Y_4$ receptors revealed that ATP (Bogdanov *et al.*, 1998; Webb *et al.*, 1998; Suarez-

Huerta *et al.*, 2000) and Ap4A (Bogdanov *et al.*, 1998; Kennedy *et al.*, 2000) were agonists at this receptor.

### 2.4.4. $P2Y_6$ receptor

This receptor was activated by UDP and its derivatives (Table 2), whereas very weak activity of 2MeSATP and ADP was described (Communi *et al.*, 1996a; Nicholas *et al.*, 1996b; Harden *et al.*, 1998). UTP was inactive if its degradation into UDP was excluded (Nicholas *et al.*, 1996b; Communi *et al.*, 1997a). 5BrUDP was almost equipotent with UDP (Harden *et al.*, 1998; Nicholas *et al.*, 1996b).

Considering structural similarity, it has been suggested that $P2Y_3$ receptor might be the avian homologue of mammalian $P2Y_6$ receptor (Li *et al.*, 1998). These both receptors were activated by UDP and 5BrUDP, but adenine nucleotides were more potent at the avian $P2Y_3$ receptors (Li *et al.*, 1998; Weisman *et al.*, 1998).

### 2.4.5. $P2Y_{11}$ receptor

This receptor is selective against ATP and its derivatives (Table 2) that makes it different from the $P2Y_1$ subtype. ATPγS, BzBzATP and dATP were shown to be more potent than ATP and 2MeSATP was less potent (Table 2). Furthermore, two thiophosphates ADPβS and AMPαS were identified as partial agonists at $P2Y_{11}$ receptor (Communi *et al.*, 1999; Van der Weyden *et al.*, 2000).

In summary, distribution of nucleotide receptor ligands according to their affinity was analysed proceeding from data in Table 2. The results of this analysis are illustrated in Fig. 4. It can be seen that affinities of most of the agonists remain between 0.1 and 10 μM. The highest agonist affinity can be observed in the case of $P2Y_1$ subtype, where the $pEC_{50}$ values 8.3 can be found for 2MeSADP. Thus, no exceptionally potent agonists can be found among natural nucleotides and their derivatives, obtained through modification of both nucleotide base and phosphate groups. Therefore search for potent compounds, which also may provide some interest as potential drugs, should be directed to more radical alteration of ligand structure. Within this dissertation such attempt has been made by search of agonistic and antagonistic effects of peptide-nucleoside conjugates, synthesised in our laboratory by Dr. Asko Uri.

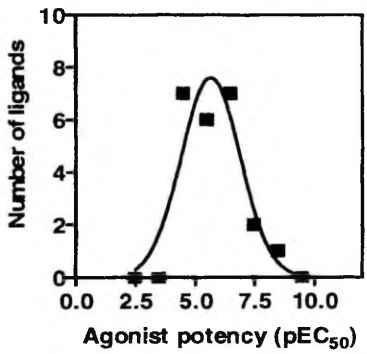


**Figure 4.** Distribution of nucleotide receptor ligands according to their affinity for the receptor. Data were taken from Table 2.

## 2.5. P2Y receptor antagonists

Although the need for potent and subtype selective P2Y receptor antagonists is evident, the list of such compounds is very short. In 1996 it was shown that adenosine 2',5'- and 3',5'-bisphosphates behaved as competitive antagonists and weak partial agonists in turkey erythrocytes (Boyer *et al.*, 1996a). The same compounds were full antagonists for $P2Y_1$ receptor derived from human brain and expressed into 1321N1 human astrocytoma cells (Boyer *et al.*, 1996a). Further modifications in these molecules were done with the purpose to increase potency of these antagonists. However, as all these compounds were tested in turkey erythrocytes, their effect at genuine $P2Y_1$ receptors is still not known explicitly.

It was demonstrated that the modifications which increased the potency and selectivity of bisphosphates included $N^6$-methyl, 2-chloro, 2'-deoxy and carbocyclic analogue of ribose (Camaioni *et al.*, 1998; Boyer *et al.*, 1998; Nandanan *et al.*, 1999; Nandanan *et al.*, 2000; Kim *et al.*, 2000). On the other hand, all these phosphate-containing compounds were relatively unstable as enzymes present on cell surface may catalyse their decomposition through hydrolysis and various phosphate transfer reactions (Harden *et al.*, 1997).

In addition, several polyaromatic and polysulfonated compounds, including suramin, reactive blue 2 and PPADS, have been found to be able to inhibit the responses of several P2Y receptors (Brown *et al.*, 1997; Charlton *et al.*, 1996a; Charlton *et al.*, 1996b; Chen BC *et al.*, 1996). However, the application of these inhibitors is limited by irreversibility of their antagonism as well as by relatively low potency. Moreover, all the latter inhibitors are not subtype-selective among P2X and P2Y receptors and even not specific against the class of the P2 receptors (Lambrecht *et al.*, 1999; Jacobson *et al.*, 1998). Therefore

the development of stable and subtype selective antagonists for $P2Y_1$ receptors is still a "hot" topic. This problem was also dealt with in this dissertation, as evaluation of activity of several non-phosphate nucleotide analogues was made at the human $P2Y_1$ receptor.

## 2.6. Objectives of the present study

The main objectives of the present study arise from unsolved problems, mentioned in the literature overview, and can be formulated as follows:

1. Revision of ligand activity order of the $P2Y_1$ receptor subtype of human origin, with special emphasis on determination of the activity type of ATP and its analogues.
2. Investigation into the effect of adenosine-derived non-phosphate nucleotide analogues at the human $P2Y_1$ subtype. These compounds were synthesised in our laboratory as possible purinoceptor agonists or antagonists.
3. Studies of functional P2Y receptors in cell lines of neuronal origin with the purpose to investigate their selectivity against adenine and uracil nucleotides.

# 3. EXPERIMENTAL PROCEDURES

**Cultured cells** were used for assay of nucleotide receptors and the exploited cell lines are listed in Table 3. The cells were grown at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM; Gibco), supplemented with 10% (v/v) of foetal calf serum and tylosine (8 μg/ml). Specific ingredients used with $hP2Y_1$-1321N1 and NG108-15 cells are mentioned in **Papers V** and **VIII**, respectively. Most cell culture work was done at the National Institute of Chemical Physics and Biophysics, Tallinn, Estonia with appreciated assistance by Külli Samuel. The experiments with rat brain capillary endothelial cells B10 were carried out in the Department of Physiology and Medical Biophysics, Uppsala University, Sweden.

**Table 3.** Cell lines used in this project.

| Cell lines | | Origin |
|---|---|---|
| Human embryonic kidney cells | **HEK293** | From Dr. T.E. Webb (Department of Biological Sciences, De Montfort University, UK) |
| Rat brain capillary endothelial cells | **B10** | From Dr. K.E.O. Åkerman (Department of Physiology and Medical Biophysics, Uppsala University, Sweden) |
| Chinese hamster ovary cells | **CHO-K1** | From cell culture collection of Institute of Molecular and Cell Biology, Tartu University, Estonia |
| Rat glioma and mouse neuroblastoma hybrid cells | **NG108-15** | From European Collection of Cell Cultures |
| Mouse neuroblastoma cells | **NB4 1A3** | From European Collection of Cell Cultures |
| Mouse neuroblastoma cells | **N1E 115** | From European Collection of Cell Cultures |
| Mouse neuroblastoma cells | **Neuro 2a** | From European Collection of Cell Cultures |
| Human neuroblastoma cells | **SH-SY5Y** | From European Collection of Cell Cultures |
| Human neuroblastoma cells | **SK-N-SH** | From European Collection of Cell Cultures |
| 1321N1 human astrocytoma cells stably expressing the human $P2Y_1$ receptor (Ayyanathan *et al.*, 1996) | **$hP2Y_1$-1321N1** | From Dr. S. P. Kunapuli (Department of Physiology, Temple University Medical School, Philadelphia, PA) on the request of Prof. E. A. Barnard (Department of Pharmacology, University of Cambridge, UK) |

**Ligands** used in this study can be divided into two categories.

First, nucleotides and their derivatives were purchased from Boehringer Mannheim, Sigma, RBI, Tocris and Amersham. HPLC analysis revealed that purity of these products was in most cases less than 98%. Therefore these ligands were additionally purified on an anion exchange column Mono Q (Amersham Pharmacia Biotech) before their use. The chromatographic pro-

cedures were performed by Gerda Raidaru in our Institute. In addition, adenosine $A_{2A}$ receptor specific agonist CGS-21680 and antagonist ZM 241385, non-selective P2 receptor inhibitors reactive blue 2, PPADS, suramin and triphosphate regeneration system (creatine phosphate and creatine phosphokinase) were used in this study.

Second, peptide conjugates of adenosine and adenosine-5'-carboxylic acid were designed and synthesised by Dr. A. Uri in our Institute and were generously given in for assay of their activity at nucleotide receptors.

**Kinetics of ATP degradation** in the assay mixture was studied in Dulbecco's Modified Eagle's Medium (DMEM) in the presence of the $hP2Y_1$-1321N1 cells. In these experiments [2-$^3$H]-ATP (Amersham) was used and the products of degradation were determined by radioactivity after their separation on TLC plates (PEI/$UV_{254}$; Macherey-Nagel, Germany). For elution the solution of 4 M LiCl in 1 M acetic acid (2:8, v/v) was used as proposed in literature (Randerath & Randerath, 1967).

**Assay of inositol phosphates** was performed by the standard method (Shears, 1997). The time and conditions of loading of the cells with *myo*-[2-$^3$H]-inositol (2 μCi/ml; Amersham) and the density of cells in the culture plates were optimised. Also, the assay conditions where the incubation time could be changed without loss of exactness of the results were elaborated. Shortly, NG108-15 and N1E 115 cells were seeded in a 24-well culture plates at density of ~5 × $10^4$ cells/well and all other cell lines tested (HEK293, B10, CHO-K1, NB4 1A3, Neuro 2a, SH-SY5Y, SK-N-SH and $hP2Y_1$-1321N1) were plated at a density of ~1 × $10^5$ cells/well. The cells were used for assay after 3 days in culture when still subconfluent. Inositol lipids were labelled with *myo*-[2-$^3$H]-inositol in 200 μl of inositol-free and serum-free DMEM. The medium was not changed after [$^3$H]-inositol addition. The assay was initiated by addition of ligand and the mixture was incubated at 37°C. Antagonists were added 1 min before agonist. The assay was terminated by aspirating the media and adding 500 μl of ice-cold 5% TCA. The TCA-containing supernatant was extracted three times with diethyl ether (500 μl) and inositol phosphates were isolated on Dowex AG1–X8 columns (BioRad, 100–200 mesh, formate form, bed volume 0.8 ml). Results of assay were calculated as means±S.E.M. from three independent experiments.

**Data analysis** was made by a non-linear regression analysis program GraphPad Prism™ (GraphPad Software Inc., San Diego, CA). Antagonistic constants $K_i$ were calculated by the Schild or Gaddum equations (Kenakin, 1997; Lazareno & Birdsall, 1993) and statistically significant effects were determined by Student's t-test (Wardlaw, 2000).

# 4. RESULTS AND DISCUSSION

## 4.1. Effect of ATP and its analogues at human $P2Y_1$ receptor

This part of the dissertation was motivated by contradictory data about the type of activity of ATP and its analogues at human $P2Y_1$ receptor (see part 2.4.1 above) and results of these studies (**Papers III** and **IV**) can be summarised as follows.

Initially we observed that the human $P2Y_1$ receptor, stably expressed in 1321N1 human astrocytoma cells, was activated by ATP. But this response was weak and its maximal value was below the effect of regular agonists (ADP, 2MeSADP). Moreover, the effect depended on the incubation time used for assay of inositol phosphates. At longer incubation the dose response curve was shifted to the lower ATP concentrations and the amplitude of the response increased. These data led to the hypothesis that the substance evoking the agonistic effect formed during the experiment and was presumably ADP. Therefore kinetics of ATP degradation in the assay medium was investigated and the time course of this process was compared with the dynamics of the receptor response observed in the presence of ATP.

Indeed, it was found that ATP concentration decreased relatively rapidly in the incubation medium containing the standard amount of the cells. During the time interval under discussion the main degradation product was ADP. Moreover, the agonistic activity measured in the presence of ATP was abolished in the presence of the triphosphate regeneration system in the reaction mixture (creatine phosphate / creatine phosphokinase). All these experiments supported the hypothesis that ATP itself was not the agonist at the human $P2Y_1$ receptor. Subsequently ATP was tested as antagonist at the human $P2Y_1$ receptor. It was found that in the presence of ATP the receptor response of 2MeSADP was effectively inhibited. Even though degradation of ATP could not be avoided in these experiments and only estimate of the antagonistic constant for ATP was obtained, this conclusion was important to establish the fact that the transfer from diphosphate to triphosphate changed agonist (ADP) to antagonist (ATP).

Proceeding from this fact it was interesting to study the activity of ATP analogues with modified structure of the phosphate moiety. These derivatives, where methylene or imido groups substituted oxygen atoms between phosphorus atoms, have been described to be more stable than ATP (Yount, 1975; Cascalheira & Sebastiao, 1992; Cunha *et al.*, 1987; Yount *et al.*, 1971; Schuurmans Stekhoven *et al.*, 1983). Therefore it was expected that their antagonistic properties should be less shaded by agonistic properties of diphosphates formed during the assay. This suggestion was approved by the following experiments.

These experiments revealed that methylene-derivatives of ATP, App[$CH_2$]p and Ap[$CH_2$]pp, did not activate the synthesis of inositol phosphates and were full competitive antagonists at the human $P2Y_1$ receptor if purified ligands were used. This latter aspect is stressed because in the commercial sample of

App[NH]p significant amount (up to 10%) of the appropriate diphosphate (App[$NH_2$], adenylyl-5'-yl phosphoramidate) was found by HPLC analysis. Most probably for this reason App[NH]p has been described as agonist at $P2Y_1$ receptor in literature (see Table 2).

In summary, the following ligand activity order could be elaborated for the human $P2Y_1$ receptor, 2MeSADP > ADP > ADPβS >> AMP, while ATP and its phosphate-modified analogues App[$CH_2$]p, Ap[$CH_2$]pp and App[NH]p are antagonists. Thus the presence of triphosphate moiety seems to be a crucial structural factor for antagonistic behaviour of ligand at this receptor. It is interesting to add that adenosine bisphosphate, still the most important competitive antagonist at the same receptor, has similarly to ATP four negative charges in phosphate moieties. Proceeding from this fact non-phosphate nucleotide analogues were synthesised and their interaction with the human $P2Y_1$ receptor was studied in the next part of this dissertation.

## 4.2. Effect of adenosine-derived non-phosphate nucleotide analogues at human $P2Y_1$ receptor

Two different series of compounds were synthesised in our laboratory and their effects were tested at the human $P2Y_1$ receptor in this study (**Paper V**). These compounds can be defined as non-phosphate ATP analogues, containing adenosine-5'-carboxylic acid (I) or adenosine (II) moiety coupled with negatively charged peptide fragment $Asp_n$ (Fig. 5).

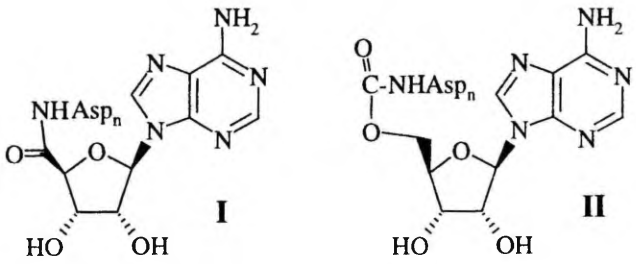


**Figure 5.** Structure of adenosine-5'-carboxylic acid derivatives AdoCAsp$_n$ (I) and adenosine peptide conjugates AdoOC(O)Asp$_n$ (II).

The compounds of the first type included besides the $Asp_n$ fragment also glycine, sarcosine and proline residues to modify the structure of these compounds (Table 4).

Assay of activity of these compounds revealed the following. Firstly, the conjugates of adenosine-5'-carboxylic acid with negatively charged peptide fragments (AdoCAsp$_n$ and AdoCXAsp$_n$, X=Gly, Sar, or Pro; n=1–4) had neither agonistic nor antagonistic effect at the human $P2Y_1$ receptor. Secondly, non of

the adenosine-peptide conjugates ($AdoOC(O)Asp_n$, n=1–4) had agonistic effect, but all these compounds at 1 mM concentration were able to inhibit the breakdown of inositol phospholipids initiated by 2MeSADP in $hP2Y_1$-1321N1 cells. The antagonistic effect was clearly dependent on the length of the peptide fragment in the rank order of potency $AdoOC(O)Asp_2$> $AdoOC(O)Asp_3$> $AdoOC(O)Asp_1$> $AdoOC(O)Asp_4$. These effects were characterised by the ratio of $EC_{50}$ values of 2MeSADP dose effect curves measured in the presence and absence of 1 mM antagonists (Table 4). The effect of the most potent antagonist $AdoOC(O)Asp_2$ was studied at various concentrations and characterised by antagonistic constant $pA_2$ 5.4±0.1.

**Table 4.** Adenosine-derived non-phosphate nucleotide analogues tested at human $P2Y_1$ receptor. dr characterises the ratio of $EC_{50}$ values of 2MeSADP concentration effect curves measured in the presence and absence of 1 mM $AdoOC(O)Asp_n$.

| Derivatives of adenosine-5'-carboxylic acid | | Derivatives of adenosine | |
|---|---|---|---|
| $AdoCAsp_n$ (n=1–4) | No agonistic or | $AdoOC(O)Asp_n$ | |
| $AdoCGlyAsp_n$ (n=1–4) | antagonistic activity | n=1 | dr=6.1 |
| $AdoCSarkAsp_n$ (n=1–4) | | n=2 | dr=34.6 |
| $AdoCProAsp_n$ (n=1–4) | | n=3 | dr=13.9 |
| | | n=4 | dr=4.8 |

The latter data revealed an interesting structure-activity relationship for this reaction series. Namely, the antagonistic effect (dr) revealed clear maximum at n=2 (Fig. 6a). In the figure 6b the affinities of AMP, ADP and ATP at human $P2Y_1$ receptor were plotted. Surprisingly the same type of plot was obtained with maximum at n=2. In the latter case, however, the type of ligand activity was also dependent on the number of phosphate groups as ADP behaved as full agonist and ATP as full antagonist at this receptor.

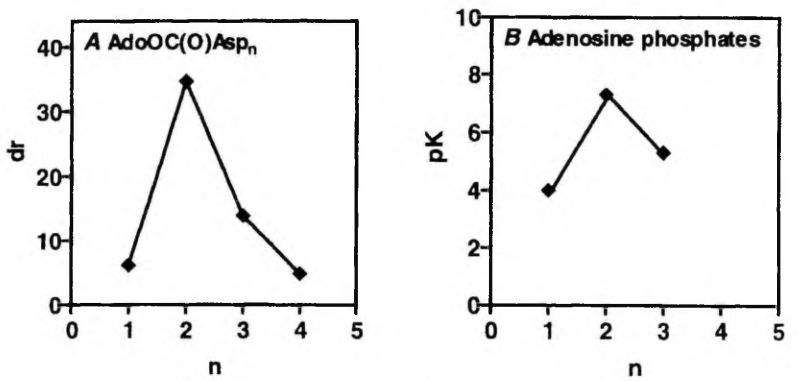


**Figure 6. A.** Dependence of antagonistic activity of $AdoOC(O)Asp_n$ on length of peptide fragment. **B.** Dependence of pK value of adenosine phosphates on number of phosphate residues.

In summary, the conjugates AdoOC(O)$Asp_n$ are promising leads for the development of potent antagonists of nucleotide receptors. The investigation of interaction of these compounds with other subtypes within the P2Y receptor family will be the subject of future studies.

## 4.3. Assay of P2Y receptors in native cell lines

Proceeding from "P2Y Receptor Ligand Database" statistical comparison of agonist effects in transfected and native cells was made. This analysis revealed that nucleotides and their analogues have systematically higher activity in the transfected cells (Fig.7), while the rank order of agonist potency is similar in the transfected and native cells. Although indications of the same trend have been described in the case of some concrete receptor systems before (Fagura *et al.*, 1998), no clear explanation of this phenomenon was suggested. Therefore, we also paid some attention on P2Y receptors in native cell lines (**Papers VI**, **VII** and **VIII**).

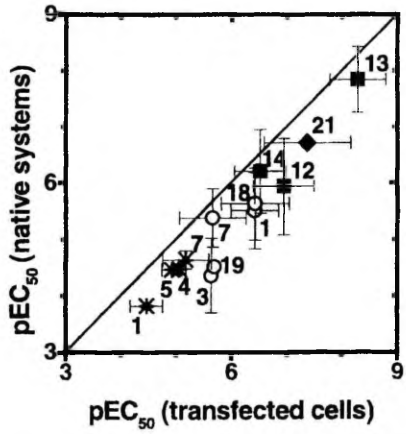


**Figure 7.** Comparison of P2Y receptor ligand activities measured in transfected cells and native systems. Numbers in the figure correspond to the numeration of ligands listed in Abbreviations (■ — $P2Y_1$, ○ — $P2Y_2$, □ — $P2Y_4$, ◆ — $P2Y_6$, ✷ — $P2Y_{11}$).

The ratio of the effects measured in the presence of ATP, ADP, UTP and UDP (100 μM) and without these external ligands (basal activity) was calculated for different cell lines (Table 5). It can be seen that the effect of nucleotides on turnover of inositol phospholipids can be rather different. So, the nucleotide-initiated synthesis of inositol phosphates in human embryonic kidney cells HEK293, chinese hamster ovary cells CHO-K1, and rat brain capillary endothelial cells B10 was too slow to be used for reliable assay of these receptors, although the functional expression of nucleotide receptors in all these

cells has been reported (Schachter *et al.*, 1997b; Iredale & Hill, 1993; Megson *et al.*, 1995; Feolde *et al.*, 1995; Vigne *et al.*, 1998; Hechler *et al.*, 1998b). On the other hand, the effect of nucleotides was considerably high in the rat glioma and mouse neuroblastoma hybrid line NG108-15 and in the mouse neuroblastoma line NB4 1A3. As both UTP and UDP gave responses in the rodent neuroblastoma lines (N1E 115, Neuro 2a, NB4 1A3 and NG108-15) these cells seem to be suitable model systems for investigation into properties of pyrimidinoceptors (**Paper VI**). At the same time, nucleotides were not able to activate phospholipase C in human neuroblastoma cell lines SH-SY5Y and SK-N-SH, although this signalling pathway exists in these cells and is used by muscarinic receptor.

**Table 5.** Effect of nucleotides (100 μM) on rate of formation of inositol phosphates in native cell lines.

| Cell line | Source | Increase in InP concentration* | | | |
|---|---|---|---|---|---|
| | | ATP | ADP | UTP | UDP |
| **hP2Y$_1$-1321N1** | Human astrocytoma | 1** | **3.6** | 1 | 1 |
| **HEK293** | Human kidney | **1.4** | **1.5** | **1.5** | 1 |
| **B10** | Rat brain | 1 | 1 | 1 | 1 |
| **CHO-K1** | Hamster ovary | **1.3** | 1 | **1.3** | 1 |
| **NG108-15** | Mouse neuroblastoma, rat glioma | **2.1** | **1.7** | **5.4** | **6.3** |
| **NB4 1A3** | Mouse neuroblastoma | 1 | 1 | **9.0** | **9.0** |
| **N1E 115** | Mouse neuroblastoma | 1 | 1 | **1.8** | **1.8** |
| **Neuro 2a** | Mouse neuroblastoma | 1 | 1 | **2.4** | **2.2** |
| **SH-SY5Y** | Human neuroblastoma | 1 | 1 | 1 | 1 |
| **SK-N-SH** | Human neuroblastoma | 1 | 1 | 1 | 1 |

* Calculated by dividing the amount of inositol phosphates formed by stimulating the cells with 100 μM nucleotide with the respective amount in basal conditions
** Measured in the presence of creatine phosphate and creatine phosphokinase system

## 4.4. Functional P2Y receptors in cell lines of mouse neuroblastoma origin

The effect of UTP and UDP in mouse neuroblastoma lines N1E 115, Neuro 2a, NB4 1A3 and NG108-15 was studied more thoroughly. These ligands were almost equipotent in these cells, whereas ATP and ADP gave a small response only in NG108-15 cells (**Paper VI**). Such ligand specificity profile is not compatible with properties of any recombinant mammalian P2Y receptors, as follows from data collected in Table 2. Shortly, all subtypes that are activated by adenine nucleotides can be excluded ($P2Y_1$, $P2Y_2$, rodent $P2Y_4$, and $P2Y_{11}$) and the only receptor specific for uracil nucleotides ($P2Y_6$) has clear selectivity

for UDP over UTP. Therefore, existence of a novel receptor subtype can be expected to explain the ligand specificity pattern observed in mouse neuroblastoma cell lines and two possibilities should be considered. Firstly, these cells express a novel pyrimidinoceptor that is equipotent against UTP and UDP. Secondly, the response of UDP is mediated by $P2Y_6$ receptor and UTP acts on a novel UTP-specific subtype. These possibilities were studied with NB4 1A3 and NG108-15 cells (**Paper VII**).

It was found that co-application of UTP and UDP in both NG108-15 and NB4 1A3 cell lines yielded higher response than each of these ligands alone. This increase was approximately 140% in both cases and was in a good agreement with results reported by Lin about elevation of response in NG108-15 cells at co-application of UTP and bradykinin, an agonist of phospholipase C-coupled bradykinin receptor (Lin, 1994). These results point to the fact that UTP and UDP act on different receptors in rodent neuroblastoma cells. The same conclusion was reached by RT-PCR analysis of NG108-15 cells, carried out by Dr. T. E. Webb at the Department of Biological Sciences, De Montfort University, UK. In co-operation with our group she demonstrated an abundant expression of the $P2Y_6$ receptor mRNA in NG108-15 cells, while the $P2Y_2$ receptor transcript was at considerably lower levels (private communication). Thus, the UDP-specific receptor could be the $P2Y_6$ subtype and UTP-specific receptor could be a novel subtype in the P2Y receptor family.

The presence of two receptor subtypes in NG108-15 and NB4 1A3 cells was also confirmed by different influence of adenosine and its derivatives (iPAdo, CGS-21680) on UTP and UDP effects in these cells: if the response evoked by UTP was inhibited, there was no effect on UDP response. This somewhat surprising phenomenon can be explained by the putative UTP receptor "cross-talk" with adenosine $A_2$ receptors, also expressed in NG108-15 cells (Mundell & Kelly, 1998). However, this possibility can be excluded by the following data, as shown in **Paper VII**.

Firstly, the adenosine $A_{2A}$ receptor antagonist ZM 241385 had no effect on the inhibition of UTP response by adenosine. As specified by Ohkubo and co-workers, NG108-15 cells express mainly adenosine $A_{2A}$ receptor (Ohkubo *et al.*, 2000). Moreover, the ligand ZM 241385 was used at high concentration where it inhibits also adenosine $A_{2B}$ receptor responses (Poucher *et al.*, 1995).

Secondly, specific $A_{2A}$ receptor agonist CGS-21680 had a smaller inhibitory effect on UTP mediated inositol phosphates formation than did adenosine, although its affinity for this adenosine receptor is higher.

Thirdly, adenosine derivatives iPAdo and 3',5'-cAMP, which have no activity on adenosine $A_2$ receptors (personal communication with Dr. Ijzerman, Department of Medicinal Chemistry, Leiden University, The Netherlands) had clear antagonistic effect on UTP response.

In summary, these data confirm the possibility that a novel UTP-specific P2Y receptor is expressed in NG108-15 and NB4 1A3 cells, and that adenosine

acts as an antagonist at this receptor. Further we are planning to determine the molecular structure of this putative pyrimidinoceptor.

## 4.5. Potentiation of pyrimidinoceptors by ATP in NG108-15 cells

As adenosine inhibited the putative pyrimidinoceptor in NG108-15 and NB4 1A3 cells, the effect of AMP, ADP and ATP on both UTP and UDP responses in the same cells were studied. It has been found that neither AMP nor ADP changed the UTP and UDP responses. However, in the presence of ATP significant dose-dependent potentiation of UTP and UDP responses was observed in NG108-15 cells. For example, 1 mM ATP shifted the dose response curve of UTP up to three powers of magnitude (**Paper VIII**). At the same time ATP analogues (ATPγS, App[$CH_2$]p, BzBzATP and dATPαS) had no influence on neither UTP nor UDP responses. This fact as well as abolishment of the potentiation effect by EDTA leads to hypothesis that extracellular phosphorylation of pyrimidinoceptors could be involved in this phenomenon. This potentiation mechanism may be physiologically significant in the regulation of cellular processes by uracil nucleotides. Further it was shown (**Paper VI**) that this receptor potentiation phenomenon was not present in the mouse neuroblastoma cell line NB4 1A3, also responding to UTP and UDP. Therefore, it is possible that the extracellular kinases involved in the potentiation phenomenon belong to rat glioma rather than mouse neuroblastoma component of the hybrid cell line parentage of the NG108-15 cells.

In summary, our results indicate that activity parameters measured for P2Y receptor ligands may reflect more factors than the receptor- ligand and the receptor- G-protein interactions. These factors may involve interaction of receptors with endogenous adenosine and ATP that in addition to ligand metabolism by ectoenzymes may be different in various biological systems and cause scattering of results, despite the continuous improvement of receptor assay methods during the two decades of P2Y receptor study.

# 5. CONCLUSIONS

1. The "P2Y Receptor Ligand Database" was compiled within this dissertation for overview of the main trends in P2Y receptor research. The statistical analysis of activity parameters published for P2Y receptor ligands led to the following conclusions:
   a) Results of different assay procedures (inositol phosphates formation, intracellular calcium mobilisation, cyclic AMP accumulation and electrophysiological recording) are comparable, but most of ligands have higher affinity in the P2Y receptor transfected cells if compared with native cells.
   b) Turkey erythrocytes, although widely used as model system for $P2Y_1$ receptor studies, do not express the same subtype derived from different species and originally designated as $P2Y_1$ receptor.
2. ATP and its structural analogues App[$CH_2$]p, Ap[$CH_2$]pp and App[NH]p are full antagonists at the human $P2Y_1$ receptor and the agonistic activity often described in the literature belongs to the respective diphosphates formed during assay.
3. Adenosine-peptide conjugates (AdoOC(O)$Asp_n$, n=1-4) behave as antagonists for human $P2Y_1$ receptor with antagonistic constant of ~4 μM for AdoOC(O)$Asp_2$, the most effective antagonist in this series.
4. Rate of formation of inositol phosphates is considerably fast in neuronal origin cells and the rodent neuroblastoma cell lines (N1E 115, Neuro 2a, NB4 1A3 and NG108-15) reveal clear selectivity for uracil nucleotides, providing a good model system for investigation into the cellular processes regulated by pyrimidinoceptors.
5. NB4 1A3 and NG108-15 cells express a novel UTP-preferring P2Y receptor subtype, at which adenosine behaves as an antagonist.
6. ATP is able to potentiate pyrimidinoceptors in NG108-15 cells, probably via extracellular phosphorylation of these proteins. The enzymes involved in this potentiation phenomenon derive probably from rat glioma rather than mouse neuroblastoma parentage of NG108-15 hybrid cells.

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# MÕNINGAID ASPEKTE P2Y RETSEPTORITE LIGANDSPETSIIFILISUSEST

## Kokkuvõte

P2Y retseptorid on nukleotiidide poolt aktiveeritavad ja G-valguga seotud rakumembraani retseptorid. Käesoleval ajal teatakse viit P2Y retseptori alatüüpi, mille aktivatsioon viib füsioloogilise vastuseni raku sees. Nukleotiidi retseptorite erinevate alatüüpide aminohappelise järjestuse sarnasus on 30–55% ning ühe alatüübi isendi homoloogide järjestused võivad erineda kuni 20%. Erinevate looduslike ja sünteetiliste ainete toimet neile retseptoritele on uuritud alates 1970. aastate lõpust ning see töö intensiivistus 1993. aastal pärast P2Y retseptorite esmakordset kloonimist. Käesoleva dissertatsiooni raames koostati kirjanduses publitseeritud ligandide aktiivsusandmetest "P2Y Retseptorite Ligandide Andmebaas" ning viidi läbi aktiivsusparameetrite statistiline analüüs. Selle tulemusena näidati, et ligandide aktiivsuse määramiseks kasutatud erinevad mõõtmismeetodid annavad võrreldavad tulemused, kuigi konstantide hajuvus on suur. Samas leiti, et ligandide afiinsused transfekteeritud rakuliinides on süstemaatiliselt suuremad, võrreldes looduslikes rakuliinides mõõdetutega. Lisaks neile P2Y retseptorite uurimist kirjeldavatele üldistele järeldustele leiti andmebaasi analüüsi käigus mitmeid vastuolusid ligandide aktiivsustüübi määratlemisel.

Käesolevas dissertatsioonis uuriti lähemalt $P2Y_1$ retseptori ligandspetsiifilisust, tehti kindlaks, et ATP ja tema analoogid on inimese $P2Y_1$ retseptori antagonistid ja vastav nukleotiidi retseptori alatüüp on ADP-spetsiifiline. Samuti leiti, et sarnaselt ATP-ga on adenosiini ja peptiidi konjugaatidel ($AdoOC(O)Asp_n$, n=1–4) neile retseptoritele inhibeeriv toime. Nimetatud konjugaadid on esimesed mittefosfaatsed nukleotiidi analoogid, millel on P2Y retseptoritele antagonistlik aktiivsus. Teiseks uuriti nukleotiidi retseptorite funktsionaalset ekspressiooni mitmetes looduslikes rakuliinides ning näidati, et hiire neuroblastoomi rakuliinides ekspresseeruvad uratsüül-nukleotiidide spetsiifilised P2Y retseptori alatüübid, millest UTP-spetsiifiline retseptor on uus alatüüp P2Y retseptorite perekonnas. Käesoleva dissertatsiooni tulemusena näidati, et P2Y retseptorite jaoks mõõdetud aktiivsuskonstandid võivad olla mõjutatud mitmete faktorite poolt peale senitunnustatud ligand–retseptor ja retseptor–G-valk interaktsioonide. Kuna ligandide degradatsioon ektoensüümide poolt, retseptorite interaktsioon endogeense adenosiini ja ATP-ga on eri rakuliinides erinevad, võib see olla põhjuseks, miks aktiivsusparameetrid sageli hajuvad, vaatamata mõõtmise metoodikate pidevale täiustamisele.

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# PUBLICATIONS

# I

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P2Y-receptor-ligand database. *Trends Biochem. Sci.*, **25**, 35.

# P2Y-receptor-ligand database

Since the mediatory role of extracellular nucleotides was proposed in the early 1970s[1], nine nucleotide receptors belonging to the P2Y-receptor family have been cloned and expressed[2]. This family of receptors are G-protein-coupled receptors, which are able to evoke intracellular responses (e.g. increase in cytosolic calcium concentration, accumulation of inositol phosphates) upon stimulation with extracellular nucleotides. Five of these receptors have been found to cause an increase in physiological responses through activation of the classical secondary messenger pathways[3]. These downstream signalling effects have been used to analyse the ligand-binding properties of these receptors. We have collected most of this information into a computer-operated database and have made this available for the purinoceptor research community at http://bioorg.chem.ut.ee/p2y/.

This database can be searched online and it includes information about the biological system used in experiments, the putative receptor subtype(s) involved, the activity of the ligands, the assay methods and the references to the original papers. The present version involves 1976 records for 267 distinct compounds. Although the database is focused on G-protein-coupled P2Y receptors, also some data for P2X receptors (ligand-gated ion channels activated by extracellular nucleotides) were included. No critical analysis of the data was made before their listing. Thus, the data collected provide also information about the development of the assay methods as well as about our understandings of the purity requirements for the ligands tested.

Any comments and references, but also any new data to add to the database should be addressed to: p2y-data@bioorg.chem.ut.ee.

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# II

Sak, K. (2000)
Are $P2Y_1$ purinoceptors expressed in turkey erythrocytes?
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# Are $P2Y_1$ purinoceptors expressed in turkey erythrocytes?

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**Abstract**

Since the beginning of purinoceptor research turkey erythrocytes have been widely used as the model systems for studying the pharmacology of $P2Y_1$ nucleotide receptors. In this report the statistical analysis of the activity parameters of several purinoceptor agonists and antagonists in the turkey erythrocytes and $P2Y_1$ receptor transfected cells is presented. As a results of this analysis several differences in the ligand activity orders measured in these biological systems were found. These data indicate that the receptors expressed in turkey erythrocytes and $P2Y_1$ transfected cells are probably not the same. Whether it has to do with co-expression of several purinoceptor subtypes in turkey erythrocytes or novel P2Y receptors needs the further investigation. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords*: Turkey erythrocyte; $P2Y_1$ purinoceptor; Pharmacological activity order

Turkey erythrocytes have been widely used to study the G-protein and receptor regulated phospholipase C since middle of the 1980s [1,4,5,22,23]. This signal transduction pathway turned out to be mediated by the purinergic receptors as the synthesis of inositol phosphates was initiated by nucleotides [1,4,5], being clearly selective to adenine nucleotides [2,4,5,7,13]. The same physiological pathway was selectively activated by adenine nucleotides also in the case of $P2Y_1$ receptors cloned first from the chick brain in 1993 [32] and later from the turkey brain in 1994 [20] and expressed in the null cell lines. Although some nucleotides had about ten-fold differences in activities in $P2Y_1$ receptor transfected cells [20,30] and turkey erythrocytes [1,5,7,13,20] the receptors expressed in erythrocytes were believed to belong to the $P2Y_1$ subtype judged by the pharmacological selectivity and second messenger system preferences [9]. Moreover, the transcript of the turkey brain $P2Y_1$ receptor was also present in adult turkey blood [9]. Considering these experimental data the turkey erythrocytes were chosen for a natural model system to study the $P2Y_1$ receptors [6,8,10,14,21,27].

However, during the extensive progress in P2Y receptor research in the last years the existence of novel purinergic receptors having selectivity to adenine nucleotides and being able to initiate the synthesis of inositol phosphates was demonstrated [17,18]. Also, the transcripts of various purinergic receptors were found in turkey blood and some novel P2Y receptors have been cloned from the turkey blood cDNA library with pharmacological and biochemical properties clearly different from the $P2Y_1$ receptors [3,11]. These findings pointed to some weaknesses in the initial definition of the purinoceptor subtype in turkey erythrocytes. Moreover, in the case of erythrocytes which is a natural biological system the expression of some other undetected and unknown subtypes cannot be excluded. Therefore, the question arises if the turkey erythrocytes express (only) $P2Y_1$ receptors and if the use of these cells as the model system for studying this subtype is justified.

In the present report the statistical analysis of activity parameters describing the ability of adenine nucleotides to evoke the second messenger signalling responses in the intact turkey erythrocytes, turkey erythrocyte membranes and $P2Y_1$ receptor transfected systems is presented. This analysis was possible by using the P2Y Receptor Ligand Database (P2Yr-LDB) available on the Internet at http://bioorg.chem.ut.ee/p2y/ where the parameters describing the activities of various natural and synthetic compounds measured in the different biological systems have been compiled [29]. The mean values of the constants ($pEC_{50}$) with the standard deviations were calculated and used for the pharmacological comparison of the receptors in turkey erythrocytes and $P2Y_1$ transfected systems.

The comparison of ligand activities in initiating the breakdown of inositol phospholipids pointed to some essential differences (Fig. 1). Firstly, the data for adenosine

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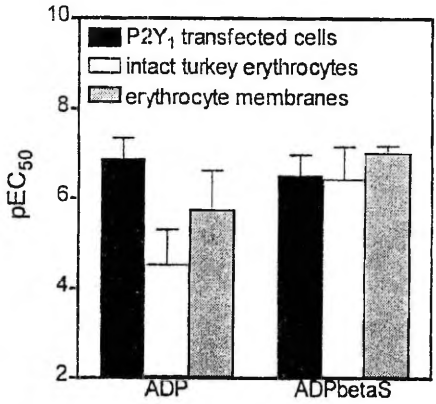


Fig. 1. Comparison of the mean $pEC_{50}$ values for activation of phospholipase C by ADP and ADPβS measured in the $P2Y_1$ receptor transfected systems, intact turkey erythrocytes and turkey erythrocyte membranes. The data were derived from the P2Yr-LDB [29] and standard deviations were calculated with 95% of confidence level.

diphosphate (ADP) and its thio-substituted analogue ADPβS known as full agonists of $P2Y_1$ receptors were selected for the analysis. The constants ($pEC_{50}$) for ADP measured in the intact turkey erythrocytes (4.56 ± 0.76) were approximately two magnitudes lower than those measured in the $P2Y_1$ transfected systems (6.88 ± 0.47). It was also interesting to find out that the activity of ADP in the intact erythrocytes was lower than in the membranes (5.76 ± 0.87). At the same time, ADP thio-substituted analogue ADPβS had similar activity in all these systems (Fig. 1).

Secondly, in the last years it has been demonstrated that adenosine triphosphate and its structural analogues (e.g. 2MeSATP, 2ClATP and BzATP) have antagonistic activity in the $P2Y_1$ transfected systems [25,26,28,31]. In the case of turkey erythrocytes these compounds have only been shown to be agonists [29] and the novel synthetic compounds are still tested against 2MeSATP [6,8,14].

Thirdly, adenosine 3′,5′-bisphosphate (A3P5P) and its sulfate-containing analogue (A3P5PS) were found to behave as partial agonists and competitive antagonists of the purinoceptors in turkey erythrocyte membranes [8,9,14]. Both of these compounds were strictly competitive antagonists in $P2Y_1$ receptor transfected systems and exhibited no agonistic activity [8,9,19,24].

Finally, also the non-selective P2 receptor antagonists behaved differently in turkey erythrocytes and $P2Y_1$ receptor transfected cells. Although the inhibitory constants were similar, suramin was a non-competitive antagonist in turkey erythrocyte membranes [12] but a competitive antagonist in $P2Y_1$ receptor transfected systems [15,16]. On the contrary, PPADS was characterised as a competitive antagonist in turkey erythrocyte membranes [12] but was a non-competitive antagonist in $P2Y_1$ transfected cells [15].

The differences in the behaviour of purinoceptor ligands in turkey erythrocytes and $P2Y_1$ receptor transfected cells indicate that these compounds probably do not act on the same receptors. Whether it has to do with co-expression of several purinoceptor subtypes in turkey erythrocytes or novel P2Y receptors remains unclear on the basis of existing data.

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# III

Sak, K. & Järv, J. (2000)
Adenosine triphosphate is full antagonist at human $P2Y_1$ purinoceptors.
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# Adenosine triphosphate is full antagonist at human $P2Y_1$ purinoceptors

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**Abstract**

Both agonistic and antagonistic effects have been reported for ATP at $P2Y_1$ purinoceptors at micromolar ligand concentrations. These conflicting data hamper specification of the true pharmacological profile as well as structural requirements for antagonistic ligands of this receptor. In this report the type of ATP activity at human $P2Y_1$ receptors in $hP2Y_1$–1321N1 cells was revisited. In parallel, kinetics of degradation of ATP in the assay mixture was analysed. It was found that transformation of this ligand to ADP was responsible for initiation of synthesis of inositol phosphates, observed in the presence of ATP in $hP2Y_1$–1321N1 cells. This agonistic effect was abolished in the presence of the triphosphate regeneration system (CP/CPK). On the other hand, if the agonistic effect caused by degradation product of ATP was taken into consideration, this ligand behaved as a full antagonist at $P2Y_1$ receptors and was characterized by the apparent inhibitory constant 5 μM. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords*: ATP; Antagonist; $P2Y_1$ purinoceptor; Degradation; Inositol phosphates assay

Besides other physiological functions nucleotides participate in regulation of various cell processes via G-protein coupled P2Y receptors [8]. Among these receptors the $P2Y_1$ subtype reveals clear specificity against adenine nucleotides [1]. These purinoceptors have been intensively investigated during last years, including determination of their primary structure [12] and characterisation of their pharmacological profiles [6]. While adenosine diphosphate (ADP) and analogous diphosphates behave as agonists at $P2Y_1$ subtype [3–5,11], understanding about the type of the effect of adenosine triphosphate (ATP) at these receptors has passed significant changes.

Until late nineties ATP has been recognised as an agonist, evoking breakdown of inositol phospholipids via $hP2Y_1$ receptors [11]. Its agonistic activity at this subtype has also been mentioned in several $Ca^{2+}$-mobilisation experiments. More data of this kind can be found in the ' P2Y Receptor Ligand Database' on the Internet [10].

Later the same ligand has been described as a partial agonist with some antagonistic effect in $Ca^{2+}$-mobilisation experiments [2,5]. Starting from these studies the possibility of ATP degradation during the assay procedure has been discussed, but obviously not excluded, although the contact time of ATP with cells was shorter in $Ca^{2+}$-mobilisation experiments if compared with the assay of inositol phosphates.

Finally, experiments were made in the presence of creatine phosphate and creatine phosphokinase (CP/CPK) to regenerate ATP during the assay procedure [4]. In these studies ATP behaved as a typical antagonist, pointing to the possibility that the agonistic effect can be caused by ADP, formed through degradation of the triphosphate during the assay.

However, this chemically sound reasoning of the agonistic effect observed in the presence of ATP has further been confused by suggestion that the capacity of this ligand to activate $Ca^{2+}$-mobilisation response in $hP2Y_1$–1321N1 cells might be related to the degree of the receptor reserve. It has been found that in the case of high receptor expression ATP behaved as agonist and if the receptor reserve was lowered by receptor desensitisation, antagonistic properties of this ligand appeared [7]. However, as the triphosphate regenerating system was not used in this study, the possibility of rapid ATP breakdown at cell surface was not excluded, although the bulk concentration of ADP in the superfused assay medium was low. On the other hand, pre-incubation of cells with ADP or its analogues for receptor desensitisation might also decrease activity of cell-surface phosphatases,

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reducing in parallel the rate of ATP decomposition in experiments with the down-regulated receptor. Certainly, all these possibilities have additionally complicated our understanding of the role of ATP at $P2Y_1$ receptors. Therefore the influence of ATP on this receptor subtype was revisited in this report by measuring breakdown of inositol phosphates at different assay times and by running separate experiments in the presence of the CP/CPK system to regenerate ATP in situ.

The concentration of ATP decreased relatively rapidly in the assay medium containing the standard amount of the cells (Fig. 1). At 2 μM initial concentration of the ligand the half-life of this process was about 12 min. The chemical background of this process must be rather complex, as the spontaneous degradation was evidently assisted by various enzymatic activities. The same analysis demonstrated that ADP was relatively stable if compared with ATP, as only a small fraction of adenosine monophosphate (AMP) was formed during the time interval used for the analysis. At the same time AMP had negligibly low effect at the $hP2Y_1$ receptors [11]. This means that the transformation of ATP into ADP could be treated as the main route of triphosphate decomposition, affecting synthesis of inositol phosphates during the assay. The same experiment was also performed in the presence of the triphosphate regenerating CP/CPK system. In this case no significant decrease in ATP concentration was observed during 20 min that was long enough for the assay (Fig. 1).

The breakdown of inositol phospholipids in $hP2Y_1$-1321N1 cells was assayed in the presence of various concentrations of ATP and 2MeSADP, which were purified by HPLC before their use. The dose-response curves were monitored at incubation times from 3 to 20 min. As the latter ligand is a generally recognised full agonist at $P2Y_1$ receptor subtype [3,5,11], the maximal response was calculated from the dose-response curves for 2MeSADP at different incubation times. Further these values of the maximal response were used to calculate the relative effects, observed in the presence of ATP under the same assay conditions and at the same different incubation times. The dependencies of the relative effect upon ATP concentration are shown in Fig. 2.

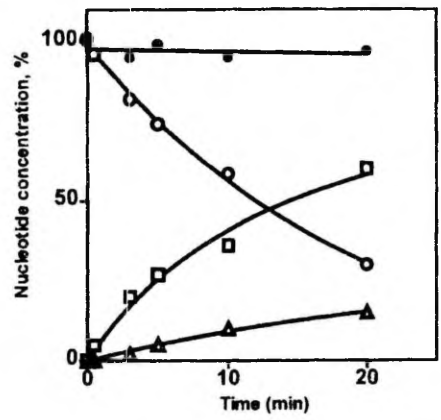


Fig. 1. Degradation of 2 μM ATP in assay of $hP2Y_1$ receptors in 1321N1 cells in absence (ATP-○, ADP-□, AMP-△) and in the presence (ATP-●) of CP/CPK. Degradation was studied in Dulbecco's Modified Eagle Medium (DMEM), containing the standard amount of $hP2Y_1$-1321N1 cells. Nucleotides were separated on TLC plates (PEI/$UV_{254}$), eluent 4 M LiCl/1 M $CH_3COOH$ (2:8, v/v). [2-$^3$H]-ATP was used and nucleotides were assayed by counting radioactivity.

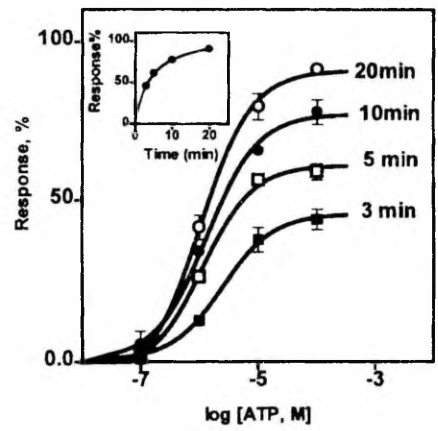


Fig. 2. Dose-response plots for formation of inositol phosphates in $hP2Y_1$-1321N1 cells, observed in the presence of ATP at different incubation times. The maximal effect (100%), used for calculation of the plotted relative effects, was separately measured with 2MeSADP at each incubation time shown in figure. The inset shows the time-dependence of the relative effect, observed in the presence of 100 μM ATP. The assay of inositol phosphates was performed as described before [9].

In the absence of the triphosphate regenerating system clear dose-dependent responses were observed with ATP, while the maximal effect of this ligand was in most cases lower than observed with 2MeSADP (Fig. 2, inset). Previously this behaviour of ATP was noticed and related to its partial agonistic activity [2]. However, the present study also revealed that the effect of ATP, calculated relatively to the maximal effect of 2MeSADP, was clearly time-dependent (Fig. 2). Therefore, at sufficiently long incubation time the maximal responses elicited in the presence of ATP and by 2MeSADP became equal. This time-dependence of the relative effect of ATP pointed to the fact that the substance, actually responsible for formation of inositol phosphates in $hP2Y_1$-1321N1 cells, was not ATP itself, but was formed during the assay.

In further experiments with ATP the assay was performed

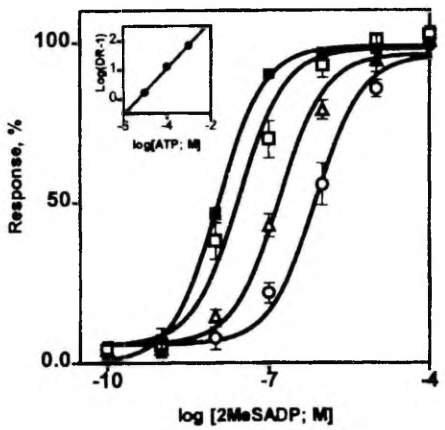


Fig. 3. Inhibition of 2MeSADP response at hP2Y$_1$-1321N1 cells (■) by ATP at initial concentrations 10 μM (□), 100 μM (△), and 1 mM (○). The assay time was 10 min, other conditions were as described before [9]. The response caused by ATP degradation product was subtracted. The inset shows the Schild plot of the data.

in the presence of the triphosphate regenerating system. In this case no increase in breakdown of inositol phospholipids was observed after addition of ATP, even if the incubation time up to 10 min was used. Thus, the results suggested that the response observed in the presence of ATP occurred in parallel with formation of ADP and thus should be caused by the latter ligand. At the same time ATP itself had no significant agonistic activity at the human P2Y$_1$ receptors.

In further experiments antagonistic effect of ATP on 2MeSADP induced response was studied at P2Y$_1$ receptors. As the CP/CPK system cannot be applied to avoid ATP degradation in these experiments, the effect of ADP, formed from ATP during the assay time, was measured separately and subtracted from the overall response. It was found that the dose-response curves for 2MeSADP were clearly shifted in the presence of ATP, pointing to its antagonistic activity at these receptors. The inhibitory effect of ATP was characterised by the $pK_i$ value 5.3 ± 0.1, calculated from the Schild plot (Fig. 3). This value is in a reasonably good agreement with earlier reports, where antagonistic properties of ATP were assessed [4]. However, for explicit quantification of the appropriate inhibitory constant the decrease of ATP and the increase in ADP concentrations during the assay, as well as potency of the latter agonist, should be taken into consideration. In turn, these calculations call for a complex kinetic study of ATP degradation at various ligand concentrations.

In summary, the present analysis demonstrated that ATP can be considered as a full antagonist at P2Y$_1$ receptor subtype and can be indeed treated as a lead compound for design of chemically and biologically stable P2Y$_1$ antagonists.

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# IV

Sak, K., Raidaru, G., Webb, T. E., Järv, J. (2000)
Phosphate-substituted ATP analogs are antagonists at human $P2Y_1$ purinoceptors.
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# RESEARCH REPORT

## Phosphate-Substituted ATP Analogs Are Antagonists at Human $P2Y_1$ Purinoceptors[1]

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***Key Words:*** **ATP phosphate-substituted analog; antagonist; $P2Y_1$ receptor; inositol phosphate assay.**

Among the G-protein-coupled nucleotide receptors the $P2Y_1$ subtype can be distinguished by its specificity for adenine nucleotides (1). ADP is a potent agonist at this receptor and its activity tolerates several chemical modifications at the diphosphate group as well as at the adenosine moiety (2–4). In contrast, ATP has been found to be an antagonist at this subtype (3, 5), although its inhibitory effect may be (and commonly is) overshadowed by the agonistic activity of ADP, rapidly formed through ATP degradation under assay conditions (3, 5, 6). To avoid this accompanying effect, it is necessary to test more chemically stable ATP derivatives, obtained through substitution of the phosphorus-bridging oxygen atoms by methylene or imido groups in the triphosphate moiety. In this study, the activity of these ATP derivatives at the transfected human $P2Y_1$ receptors was studied by measuring their effect on the breakdown of inositol phospholipids in $hP2Y_1$-1321N1 cells.

ATP analogs with a methylene group between the first and the second phosphorus atoms ($\alpha\beta$methyleneATP or Ap[$CH_2$]pp) and the second and the third phosphorus atoms ($\beta\gamma$methyleneATP or App[$CH_2$]pp) were obtained from Sigma. The purity of these ligands was above 99% as determined by HPLC analysis (7) and no appropriate diphosphates were detected in the samples used. It was found that these ATP analogs were stable under the assay conditions used and were unable to initiate the $P2Y_1$ receptor response in $hP2Y_1$-1321N1 cells at ligand concentrations of up to 1 mM. At the same time both these derivatives inhibited the receptor response, elicited by 2MeSADP (Figs. 1 and 2). The Schild plot for the inhibitory effect for App[$CH_2$]p had a slope of 1.0 ± 0.1 and a $K_i$ of 66 ± 13 $\mu$M, while that for Ap[$CH_2$]pp had a slope 1.1 ± 0.2 and a $K_i$ of 191 ± 60 $\mu$M. Thus, both of these derivatives were competitive antagonists at the $P2Y_1$ receptors. However, the positioning of the methylene group had a clear influence on the potency of these ATP derivatives.

The response, elicited by 2MeSADP in $hP2Y_1$-1321N1 cells, was also inhibited by $\beta\gamma$imidoATP (App[NH]p), the only commercially available imido-derivative of ATP (Fig. 3). Some activation of the inositol phosphate formation was observed in the presence of App[NH]p at micromolar concentrations. However, as the extent of this effect was clearly increased if longer assay incubation times were used, this agonist response was related to the degradation products of App[NH]p and is analogous to the agonistic activity observed in the presence of ATP and discussed in detail in our previous report (5). Although the chemical stability of App[NH]p is known to be greater than that of ATP (8, 9), a significant amount (up to 10%) of the appropriate diphosphate App[$NH_2$] was found even in the commercial sample (Sigma, A2647) of this ATP analog. Therefore, the antagonistic effect of App[NH]p was quantified after subtraction of the small agonist response observed and a $K_i$ of 3.8 ± 0.9 $\mu$M was calculated from the Schild plot (Fig. 3).

The latter $K_i$ value was similar to that obtained for ATP (5.0 ± 1.2 $\mu$M), measured under the same assay conditions (5). This is not surprising as the spatial structures of these ligands are rather similar, as the angles between the P–O–P and P–N–P bonds remain close, 130 and 127°, respectively (8). On the other hand, introduction of the methylene group into the triphosphate moiety yielded the P–C–P angle of 117° (8), obviously leading to a more significant disturbance of the ligand conformation.

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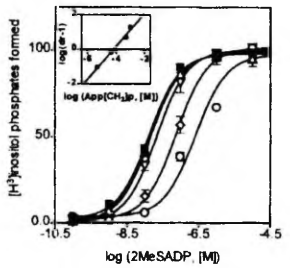


**FIG. 1.** Antagonistic effect of βγmethyleneATP (App[$CH_2$]p, △, 5 μM; ▽, 50 μM; ◇, 500 μM; ○, 1 mM) on formation of inositol phosphates, initiated by 2MeSADP (■) in hP2$Y_1$-1321N1 cells. The assay time was 10 min, other conditions were as described previously (7, 11). The inset shows the Schild plot of the data.

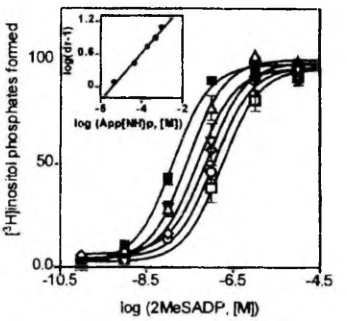


**FIG. 3.** Antagonistic effect of βγimidoATP (App[NH]p, △, 5 μM; ▽, 50 μM; ◇, 200 μM; ○, 500 μM; □, 1 mM) on the formation of inositol phosphates, initiated by 2MeSADP (■) in hP2$Y_1$-1321N1 cells. The assay time was 10 min, other conditions were as described previously (7, 11). The inset shows the Schild plot of the data.

In summary, substitution of the phosphorus-bridging oxygen atoms of ATP by methylene or imido groups was tolerated by the P2$Y_1$ receptor subtype and all these ATP derivatives were indeed antagonists at this receptor. These data are indicative of the importance of the triphosphate motif for antagonistic activity of ligands at this human receptor, as ADP and its derivatives (e.g., ADPβS, 2MeSADP) were full agonists in hP2$Y_1$-1321N1 cells (2–4). However, the methylene-substituted ADP analog Ap[$CH_2$]p (αβmethyleneADP) has been found to be unable to elicit agonistic response in these cells (4). Thus, P2$Y_1$ receptor agonists seem to be more sensitive to chemical modification of the phosphate moiety if compared with antagonists. It should also be noted that substitutions in other regions of the molecule appear to be able to override the presence of the triphosphate motif. For example at the P2$Y_1$ receptor expressed endogenously in rat hepatocytes 2MeSATP has properties similar to those of 2MeSADP (10). Thus, the P2$Y_1$ receptor seems to possess different selectivity criteria for agonists and antagonists, even if the nucleoside part of these ligands retains the same structure.

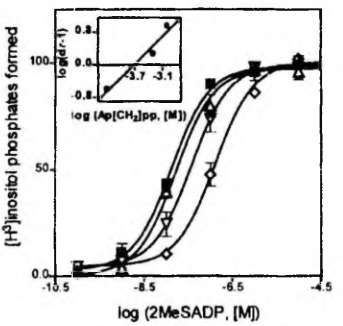


**FIG. 2.** Antagonistic effect of αβmethyleneATP (Ap[$CH_2$]pp, △, 50 μM; ▽, 500 μM; ◇, 1 mM) on the formation of inositol phosphates, initiated by 2MeSADP (■) in hP2$Y_1$-1321N1 cells. The assay time was 10 min, other conditions were as described previously (7, 11). The inset shows the Schild plot of the data.

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V

Sak, K., Uri, A., Enkvist, E., Raidaru, G., Subbi, J., Kelve, M., Järv, J. (2000) Adenosine-derived non-phosphate antagonists for $P2Y_1$ purinoceptors. *Biochem. Biophys. Res. Commun.*, **272**, 327–331.

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# Adenosine-Derived Non-Phosphate Antagonists for $P2Y_1$ Purinoceptors

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**Novel type antagonists for $P2Y_1$ adenine nucleotide receptors were synthesized by coupling of adenosine 5′-OH group with oligo-aspartate chain via a carbonyl linker. All these conjugates (AdoOC(O)$Asp_n$, $n$ = 1–4) inhibited the 2MeSADP-stimulated synthesis of inositol phosphates in 1321N1 human astrocytoma cells stably expressing human $P2Y_1$ receptors. This inhibitory effect followed the rank order AdoOC(O)$Asp_2$> AdoOC(O)$Asp_3$> AdoOC(O)$Asp_1$> AdoOC(O)$Asp_4$ with antagonistic constant $pA_2$ = 5.4 for AdoOC(O)$Asp_2$. Potency of this non-phosphate inhibitor was comparable with the previously known adenosine 3′,5′- and 2′,5′-bisphosphates. Chemical and biological stabilities of these novel adenosine derived antagonists of the nucleotide receptor provide perspectives of their pharmacological implication.** © 2000 Academic Press

*Key Words:* **non-phosphate nucleotide analogue; adenosine–peptide conjugate; antagonist; $P2Y_1$ purinoceptor.**

Purinoceptors of $P2Y_1$ subtype belong to the family of G-protein-coupled nucleotide receptors and reveal clear specificity for adenine nucleotides (1). These receptors are activated by adenosine 5′-diphosphate (ADP), its thiophosphate analogues and 2-substituted derivatives (1). Adenosine triphosphate (ATP) and its derivatives (2MeSATP, 2C1ATP and BzATP) behave as antagonists at this subtype (2, 3). Besides these triphosphates adenosine 3′,5′- and 2′,5′-bisphosphates and their derivatives have recently been demonstrated to be antagonists at $P2Y_1$ receptors (4, 5). However, all these phosphate-containing compounds are relatively unstable while in contact with cells, as numerous phosphatases and other enzymes catalyze their decomposition through hydrolysis and various phosphate transfer reactions (6). Therefore much attention has been paid to design of non-phosphate antagonists of nucleotide receptors. Indeed, it has been found that several polyaromatic and polysulfonated compounds like suramin, Reactive Blue 2, PPADS and others inhibit $P2Y_1$ receptors (2, 7, 8). However, these inhibitors are not selective towards nucleotide receptors and have been suggested to interact with other components of the cellular signal transduction system (7, 8).

Therefore an attempt was made to design specific non-phosphate purinoceptor ligands by coupling adenosine-5′-carboxylic acid (AdoC) with negatively charged peptide fragments (Fig. 1, **I**). In 1994 these compounds with 3 and 4 aspartic acid residues ($AdoCAsp_3$ and $AdoCAsp_4$) were synthesized and tested on C6 rat glioma cells (9) and some tissue preparations (10). Although weak effects were observed in some of these experiments, the results of different assays were conflicting and their interpretation was hampered by the fact that these assay systems contained several P2 receptors of not defined types. Therefore the same ligands were further tested within the present project using human $P2Y_1$ receptors stably expressed in 1321N1 human astrocytoma cells. None of these AdoC derivatives revealed agonistic or antagonistic activity in this assay system.

Recently Pehk and Uri demonstrated on the basis of NMR analysis that the transformation of the 5′ carbon atom of adenosine nucleotides into carboxylic group of AdoC derivatives noticeably altered the 3-D structure of the adenosine moiety (11). Therefore another series of adenosine derivatives AdoOC(O)$Asp_n$ was synthesized by coupling adenosine with aspartic acid residues *via* a carbonyl linker (Fig. 1, **II**). In these compounds the sugar puckering mode as well as the positioning of

Abbreviations used: 2MeSATP, 2-methylthio-ATP; 2C1ATP, 2-chloro-ATP; BzATP, 2′- and 3′-O-(4-benzoylbenzoyl)-ATP; AdoC, adenosine-5′-carboxylic acid; 2MeSADP, 2-methylthio-ADP; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance.

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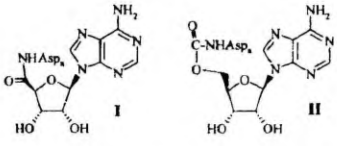


**FIG. 1.** Structure of adenosine-5′-carboxylic acid derivatives $AdoCAsp_n$ (I) and adenosine derivatives $AdoOC(O)Asp_n$ (II).

the adenine base resemble more closely those in native adenine nucleotides. In the present study these novel adenosine conjugates were tested and identified as non-phosphate antagonists at $P2Y_1$ receptors.

## MATERIALS AND METHODS

*Synthesis of $AdoOC(O)Asp_n$.* Synthesis of adenosine–aspartate conjugates $AdoOC(O)Asp_n$ followed the method previously described for preparation of $AdoOC(O)Asp_4$ (11). Shortly, oligo-L-aspartic acid chain was assembled on a Wang-type resin by using the conventional Fmoc peptide chemistry. N-terminal Fmoc group was removed by treatment with 20% piperidine in dimethylformamide. The carbonyl linker and adenosine group were attached to the aspartate chain by using 5′-(4-nitrophenylcarbonate) of 2′,3′-*O*-isopropylidene adenosine. The protection groups (*t*-Bu for Asp and isopropylidene for adenosine) were removed and the compounds were cleaved from the resin by treatment with 90% trifluoroacetic acid. The conjugates were purified by HPLC (C18 reversed phase column, water-acetonitrile gradient, 0.1% trifluoroacetic acid) and freeze-dried to obtain white solids. Purity of the products was checked by HPLC (Mono Q HR 5/5 ion exchange column, Pharmacia Biotech). Linear gradient of ammonium bicarbonate was applied as mobile phase (solution A: 0.04 M ammonium bicarbonate in 10% methanol; solution B: 1.2 M ammonium bicarbonate in water; 0–14 min, 0–70% B). The structures of $AdoOC(O)Asp_n$ were verified with mass spectrometry (gridless time-of-flight MALDI mass spectrometer built at the National Institute of Chemical Physics and Biophysics, Tallinn, Estonia) and $^1H$ and $^{13}C$ NMR (Bruker AC 200) techniques.

*$AdoOC(O)Asp_1$.* $^1H$ NMR: (DMSO-$d_6$) $\delta$ 2.54 (dd, 1H, 16.4 and 8.2Hz, 1H $\beta$ Asp), 2.72 (dd, 1H, 16.4 and 5.6Hz, 1H $\beta$ Asp), 4.0–4.4 (m, 5H, 2H-5′, H-3′, H-4′, H-$\alpha$ Asp $\delta$ = 4.33), 4.64 (t, 1H, 5.6 and 5.2Hz, H-2′), 5.95 (d, 1H, 5.6Hz, H-1′), 7.67 (d, 1H, 8.4Hz, NH), 8.35 (s, 3H, H-2, $NH_2$-6), 8.53 (1H, H-8). $^{13}C$ NMR: (DMSO-$d_6$) $\delta$ 35.95 (C-$\beta$ Asp), 50.40 (C-$\beta$ Asp), 64.37 (C-5′), 70.36 (C-3′), 73.09 (C-2′), 82.35 (C-4′), 87.25 (C-1′), 118.84, 140.53, 149.02, 149.42, 153.55, 155.49(5 adenine carbons and OCONH), 171.34 (CO Asp), 172.27 (CO Asp). MS: 427 (M + H, calculated M = 426). UV spectroscopy: $\lambda_{max}$ (water, pH 7.0) = 259 nm. HPLC (Mono Q): $R_t$ = 7.20 min, purity 97%.

*$AdoOC(O)Asp_2$.* MS: 542 (M + H), calculated M = 541). UV spectroscopy: $\lambda_{max}$ (water, pH 7.0) = 259 nm. HPLC (Mono Q): $R_t$ = 9.05 min, purity 97%.

*$AdoOC(O)Asp_3$.* MS: 657 (M + H), calculated M = 656). UV spectroscopy: $\lambda_{max}$ (water, pH 7.0) = 259 nm. HPLC (Mono Q): $R_t$ = 10.68 min, purity 91%.

*$AdoOC(O)Asp_4$.* $^1H$ and $^{13}C$ NMR spectra were in accordance with spectra published before (11). MS: 772 (M + H), calculated M = 771). UV spectroscopy: $\lambda_{max}$ (water, pH 7.0) = 259 nm. HPLC (Mono Q): $R_t$ = 12.05 min, purity 98%.

Synthesis and characterization of derivatives of adenosine-5′-carboxylic acid $AdoCAsp_n$ (n = 1, 2, and 4) has been described elsewhere (9, 11).

The compounds were well soluble (>10 mM) and stable in phosphate buffer (pH 7.4) and remained intact in the presence of cells during the receptor assay as detected by HPLC.

*Nucleotides and their purification.* 2MeSADP and adenosine 3′,5′-bisphosphate were obtained from Sigma. Purity of 2MeSADP was checked by HPLC (Gilson) on a Mono Q column by using UV detector (258 nm). Linear gradient from 0 to 1 M NaCl in 40 mM phosphate buffer (pH 7.0) at flow rate 1 ml/min was used for elution.

*Assay of inositol phosphates.* 1321N1 human astrocytoma cells stably expressing the human $P2Y_1$ receptor (kindly donated by Dr. S. P. Kunapuli, Department of Physiology, Temple University Medical School, Philadelphia, PA) were used. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% (v/v) of fetal calf serum, tylosine (8 $\mu$g/ml) and geneticin (400 $\mu$g/ml) (Gibco). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ in 75-$cm^2$ culture dishes. For the assay the cells were seeded on 24-well culture plates at a density of $\sim 1 \times 10^5$ cells/well and grown to subconfluence during 3 days. [$^3$H]-inositol (Amersham) was added to the cells in 200 $\mu$l of inositol-free DMEM at 2 $\mu$Ci/ml 18 h before the assay. No changes in medium were made subsequent to [$^3$H]-inositol addition. The assay was initiated by addition of ligand solution in 50 mM LiCl and 100 mM phosphate buffer (pH 7.4) and the assay mixture was incubated for 10 min at 37°C. Antagonists were added approximately 1 min before 2MeSADP solution. For termination of the assay the medium was aspirated and ice-cold 5% trichloroacetic acid (0.5 ml) was added. The acidic supernatant was extracted three times with 0.5 ml of diethyl ether and inositol phosphates were isolated on Dowex AG1-X8 columns (BioRad; 100–200 mesh, formate form, bed volume 0.8 ml). These columns 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). Scintillation cocktail (OptiPhase HiSafe III; Wallac) was added and radioactivity of the samples was counted to determine the amount of [$^3$H]-inositol phosphates formed. The basal radioactivity of samples remained between 600 and 700 cpm/sample and the maximal radioactivity was 3200–3400 cpm/sample.

The results were calculated as means ± S.E.M. from three independent experiments and the dose response curves were processed by a non-linear regression analysis program Prism (Version 2.00).

## RESULTS

### *Novel Adenosine Derivatives*

Adenosine-peptide conjugates $AdoOC(O)Asp_n$ (n = 1–4) were prepared according to a solid-phase synthetic method (11) and structures of these compounds were verified by using MALDI TOF MS and $^1H$ and $^{13}C$ NMR techniques. Comparison of $^1H$ and $^{13}C$ NMR spectra of $AdoOC(O)Asp$ with NMR spectra of AMP and other adenine nucleotides revealed similarity of patterns of chemical shifts of the adenosine part of these different types of compounds. So, 2′-H shift $\delta$ = 4.64 ppm for $AdoOC(O)Asp$ was close to the value for AMP (12, 13), revealing predominantly *anti*-positioning of the adenine ring and sugar moiety in these compounds. The difference in $^{13}C$ chemical shifts of C2′ and C3′ atoms was 2.73 ppm for $AdoOC(O)Asp$, also pointing to the *anti* conformation of this ligand (14). Finally, close values of the proton-proton $J_{1'2'}$ coupling constants for $AdoOC(O)Asp$ (5.6 Hz) and adenosine phosphates (13) indicated similar sugar puckering modes in these compounds. Thus, introduction of a carbonyl group be-

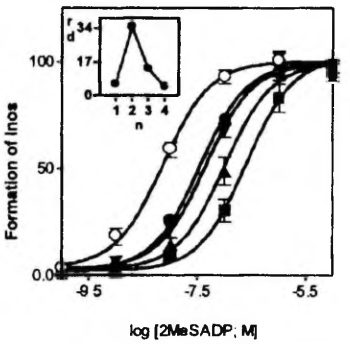


**FIG. 2.** The effect of AdoOC(O)Asp$_n$ (▼, n = 1; ■, n = 2; ▲, n = 3; and ●, n = 4; 1 mM) on the synthesis of inositol phosphates evoked by 2MeSADP (○) in hP2Y$_1$-1321N1 cells. The inset shows the dependence of the antagonistic activity on the number of aspartic acid residues (n).

tween the adenosine O5′ atom and the N-terminal of oligo-aspartates in AdoOC(O)Asp$_n$ (II) did not cause considerable change in the 3-D structure of the adenosine moiety if compared with natural adenosine phosphates. However, the adenosine-oligoaspartate conjugates were chemically and biologically more stable if compared with nucleotides.

### *Activation of Breakdown of Inositol Phospholipids in hP2Y$_1$-1321N1 Cells*

None of the compounds AdoCAsp$_n$ (I, n = 1, 2, and 4) and AdoOC(O)Asp$_n$ (II, n = 1–4) activated synthesis of inositol phosphates in hP2Y$_1$-1321N1 cells. At the same time this response was initiated by 2MeSADP and the EC$_{50}$ value 7.3 ± 1.1 nM was calculated for this ligand from the dose response curve (Fig. 2). The latter value was in a good agreement with other data for the same ligand, collected in the *P2Y Receptor Ligand Database* (http://bioorg.chem.ut.ee/p2y/) (15).

### *Inhibition of Breakdown of Inositol Phospholipids in hP2Y$_1$-1321N1 Cells*

The breakdown of inositol phospholipids initiated by 2MeSADP in hP2Y$_1$-1321N1 cells was inhibited by AdoOC(O)Asp$_n$ (Fig. 2). At the same time the derivatives of adenosine carboxylic acid AdoCAsp$_n$ had no effect on 2MeSADP response under the same conditions.

The inhibitory effect of AdoOC(O)Asp$_n$ was characterized by the shift of 2MeSADP dose-response curve and was clearly dependent on the length of the peptide fragment in rank order AdoOC(O)Asp$_2$> AdoOC(O)Asp$_3$> AdoOC(O)Asp$_1$> AdoOC(O)Asp$_4$ (Fig. 2). The effect of the most effective antagonist AdoOC(O)Asp$_2$ was measured at various ligand concentrations and the inhibitory constant pA$_2$ = 5.4 ± 0.1 was calculated from the Schild plot (Fig. 3).

For reference the inhibitory effect of a selective P2Y$_1$ receptor antagonist adenosine 3′,5′-bisphosphate (4, 5) was measured under the same assay conditions and the pA$_2$ value 5.5 ± 0.2 was obtained. This value was in a good agreement with other data reported for P2Y$_1$ receptors of the same cell line (16) or in turkey erythrocyte membranes (4, 16).

## DISCUSSION

Both ligand series, AdoCAsp$_n$ (I) and AdoOC(O)Asp$_n$ (II), were designed proceeding from the structure of native nucleotides by replacing their phosphate residues with aspartates. We expected that this replacement could simulate the negatively charged part of nucleotides while interacting with the receptor site. The present results demonstrated that this consideration was indeed valid, if a proper linker group between the sugar moiety and the peptide fragment was used. So, the derivatives of adenosine carboxylic acid (I) were not active at P2Y$_1$ receptors while the conjugates containing intact adenosine (II) were antagonists.

The chemical nature of the linker group may affect the structure of the conjugates in different ways. Firstly, transformation of the sp$^3$ carbon atom at 5′ position of adenosine into the sp$^2$ configuration in adenosine carboxylic acid may substantially change

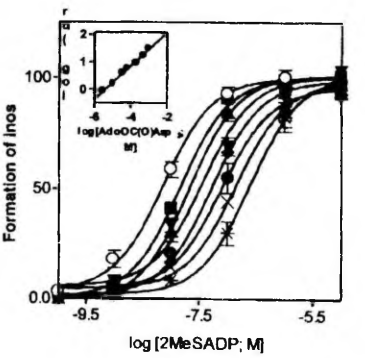


**FIG. 3.** Antagonistic effect of AdoOC(O)Asp$_2$ (■, 3 μM; ▲, 10 μM; ▼, 30 μM; ◆, 70 μM; ●, 200 μM; ×, 450 μM; and *, 1 mM) on the synthesis of inositol phosphates initiated by 2MeSADP (○) in hP2Y$_1$-1321N1 cells. The antagonistic constant pA$_2$ of 5.4 ± 0.1 and the slope 0.61 ± 0.02 were found from the Schild plot presented in the inset.

the mutual orientation of the peptide and the nucleoside moiety. Moreover, this transformation affects also flexibility of this connection.

Secondly, the two extra atoms intervening the peptide and adenosine moieties change the distance between adenine and the charged atoms of aspartates. This, in turn, may alter the accessibility of these negatively charged and probably metal-bound aspartate residues for interaction with the receptor protein.

Thirdly, an intramolecular hydrogen bond between the N3 atom of adenine base and NH hydrogen of the first amide group of oligo-aspartate seems to lock adenine in *syn* conformation in AdoCAsp$_n$ (11, 17, 18). However, the X-ray diffraction method has revealed *anti* conformation of adenine in nucleotides bound in active sites of enzymes (19). Moreover, the same *anti* configuration has been used in molecular modeling studies of the human P2Y$_1$ receptor (19).

The dependence of antagonistic activity of AdoOC(O)Asp$_n$ upon the number of aspartic acid residues had a clear maximum at n = 2 (Fig. 2). This feature somewhat resembled the behavior of native adenine nucleotides with different number of phosphate residues, if their affinity at P2Y$_1$ receptors was considered. In the latter case, however, the type of pharmacological effect was also dependent on the number of phosphate groups. So, AMP (pEC$_{50}$ ~4) behaved as very weak agonist (20), ATP (pEC$_{50}$ = 6.7 ± 0.1) as full agonist (2) and ATP (pA$_2$ = 5.3 ± 0.1) as full antagonist at these receptors (21).

The adenine group was introduced into ligands AdoOC(O)Asp$_n$ to meet specificity requirements for the adenine nucleotide receptors, particularly the P2Y$_1$ subtype. The present results for this subtype agree well with this suggestion. Although a thorough analysis of subtype selectivity of these novel ligands is in progress, the preliminary experiments have revealed that these compounds were not effective at pyrimidinoceptors of NG108-15 cells (our unpublished data).

In summary, the novel adenosine derivatives **II** provide a promising lead for further development of specific non-phosphate antagonists for nucleotide receptors. A real advantage of these adenosine-peptide conjugates is that the knowledge gained from the structure-activity studies on adenine nucleotides (5) can also be adapted for the novel type of ligands. On the other hand, before the possibilities of pharmacological implication of these novel non-phosphate antagonists can be defined more clearly, the influence of the peptide fragment on potency and selectivity of these adenosine–peptide conjugates should be systematically studied. For this purpose the conventional methods developed for design of peptide-type hormones can be applied.

## ACKNOWLEDGMENTS

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# Only Pyrimidinoceptors Are Functionally Expressed in Mouse Neuroblastoma Cell Lines

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**The ability of UTP, UDP, ATP, and ADP to influence inositol phospholipid hydrolysis in neuroblastoma origin cell lines was assessed. The mouse neuroblastoma lines N1E 115, Neuro 2a, and NB4 1A3 and the rat glioma/mouse neuroblastoma hybrid line NG108-15 gave robust responses to both UTP and UDP, which were essentially equipotent. Thus a range of cell lines of mouse neuroblastoma origin express a pyrimidine-selective P2Y receptor. The NG108-15 cells were the only cell type tested at which ATP and ADP displayed activity with $EC_{50}$ values of greater than 100 μM, compared with values of 0.58 and 1.25 μM for UTP and UDP, respectively. In contrast to the cell lines derived from mouse neuroblastoma, the human neuroblastoma lines SH-SY5Y and SK-N-SH did not respond to any nucleotides, although both responded well to carbachol.** © 1999 Academic Press

The concept of purinergic signalling in the nervous system is well established (1). The existence of a pyrimidine nucleotide-activated receptor was first proposed in 1989 (2) and there is evidence that such receptors also have a signalling role in the nervous system. For example, UTP has inhibitory effects on evoked transmitter release from brain tissue (3) while UTP and UDP trigger neurotransmitter release from sympathetic nerve terminals (4, 5).

Nucleotides act via P2 receptors of which there are two distinct types, the ionotrophic P2X receptors ($P2X_{1-7}$) and the metabotropic P2Y receptors ($P2Y_1$-$P2Y_{11}$) (6). None of the P2X receptors respond to pyrimidine nucleotides while three subtypes of P2Y receptor do: the $P2Y_2$ receptor, at which ATP and UTP are equipotent and diphosphate nucleotides are inactive (7), the $P2Y_4$ receptor where the same agonist profile is found in the case of the rat receptor (8), and the $P2Y_6$ receptor which is activated preferentially by UDP and UTP (9).

Neuroblastoma derived cell lines provide a model system for the study of the signal transduction pathways for a variety of neurotransmitter receptors including those for nucleotides. Indeed, the $P2Y_2$ receptor was originally cloned from the NG108-15 line (10). Further studies have indicated that other P2 receptors are expressed in this neuroblastoma x glioma hybrid. The P2Z receptor, now known as $P2X_7$, is functionally expressed by these cells (11–13) and RT-PCR data has demonstrated the presence of transcripts for the $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors while the $P2Y_4$ receptor transcript was absent (14). Other evidence for the presence of metabotropic receptors other than the $P2Y_2$ subtype comes from studies of nucleotide inhibition of channel activity in these cells. For example, the M-type $K^+$ current is equally inhibited by UTP and ATP (suggesting a $P2Y_2$ receptor), while the N-type $Ca^{2+}$ current is inhibited by nucleotides with a rank order of potency UDP > UTP > ATP and the L-type $Ca^{2+}$ current is inhibited by nucleotides with a rank order of potency of UDP > UTP = ATP (15, 16). However, the receptor species involved in these responses remains unclear in some cases, partially due to the complications of contamination of the applied nucleotides with the respective triphosphate or diphosphate, nucleotide breakdown and the action of ectoenzymes during the course of the assay (17).

In this study we have investigated the ability of nucleotides to generate a PLC response in a number of human and mouse derived neuroblastoma origin cell lines. The human lines did not respond to any nucleotides while the cell lines derived from mouse neuroblastoma gave the robust responses both to UTP and

Abbreviations used: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; UTP, uridine 5′-triphosphate; UDP, uridine 5′-diphosphate; Carb, carbachol; InP, inositol phosphates; PLC, phospholipase C-β; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid; HPLC, high performance liquid chromatography.

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UDP. The agonist profile indicate the functional expression of pyrimidine selective P2Y receptor subtype(s) in the mouse neuroblastoma lines.

## MATERIALS AND METHODS

### *Chemicals*

UTP, UDP, ATP and ADP (Boehringer Mannheim) were analyzed and purified by 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. Carbachol was obtained from Sigma Aldrich.

### *Cell Culture*

SH-SY5Y, SK-N-SH, N1E 115, Neuro 2a, NB4 1A3, and NG108-15 cell lines were from ECACC. All the cells were grown in Dulbecco's Modified Eagle's Medium (Gibco Ltd), supplemented with 10% (v/v) of fetal calf serum and tylosine (8 $\mu$g/ml). The medium for NG108-15 cells was further supplemented with 0.1 mM hypoxanthine, 1 $\mu$M aminopterin and 16 $\mu$M 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.

### *Inositol Phosphates Assay*

For measurements of total InP N1E 115 and NG108-15 cells were seeded in a 24-well culture plate at density of $\sim 5 \times 10^4$ cells/well and all other cell lines tested were plated at a density of $\sim 10^5$ cells/well. The cells were assayed after 3 days in culture when still subconfluent. Inositol lipids were radiolabeled by overnight incubation of cells with *myo*-[2-$^3$H]inositol (2 $\mu$Ci/ml, Amersham) in 200 $\mu$l of inositol-free and serum-free DMEM. No changes in medium were made subsequent to the addition of [$^3$H]inositol. Cells were incubated with the agonists, which were applied in a total volume of 50 $\mu$l in 50 mM LiCl, for 10 min at 37°C. The assay was terminated by aspirating the media and adding 500 $\mu$l of ice-cold 5% TCA. The TCA-containing supernatant was extracted three times with 500 $\mu$l of diethyl ether and inositol phosphates were isolated by using Dowex AG1-X8 gel (BioRad, 100-200 mesh, formiate form). The columns (bed volume 0.8 ml) were washed with water (2 × 4 ml) and 50 mM ammonium formiate (8 ml) and inositol phosphates were eluated with 1 M ammonium formiate in 0.1 M formic acid (2 × 4 ml). The amount of [$^3$H]InP formed was determined by adding 12 ml of scintillation cocktail (OptiPhase HiSafe III, Wallac) to each eluates and counting the radioactivity. The results were calculated as means ± S.E.M. from three independent experiments each performed in triplicate and the dose-response curves were processed by a non-linear regression analysis program Prism (Version 2.00).

## RESULTS

Six cell lines of neuroblastoma origin: the human SH-SY5Y and SK-N-SH lines, the mouse N1E 115, Neuro 2a, and NB4 1A3 lines and the rat glioma/mouse neuroblastoma hybrid NG108-15 were challenged with ATP, ADP, UTP, and UDP (1 and 100 $\mu$M) and the increase in InP synthesis was determined (Fig. 1A–1F). No increase in InP accumulation was detected upon addition of any of these four nucleotides to the SH-SY5Y and SK-N-SH cell lines (Fig. 1A, 1B). However, the transduction mechanism for this signalling pathway was present in both these cell types as stimulation of these cells with carbachol (100 $\mu$M), an agonist of the PLC-coupled muscarinic receptor subtype(s) expressed in these cell lines (18–21), gave rise to a 2.1-fold increase of InP accumulation for both SH-SY5Y and SK-N-SH cell lines (Fig. 1A, 1B) with $EC_{50}$ values of 13.60 ± 0.31 $\mu$M and 17.40 ± 0.40 $\mu$M, respectively (data not shown). These $EC_{50}$ values are in good accordance with the data presented by Mei *et al.* (18) and Thompson *et al.* (20). Thus, neither of these human neuroblastoma cell lines express P2Y receptors coupled to inositol phospholipid hydrolysis.

The pyrimidine nucleotides were able to activate the inositol phospholipid breakdown on the mouse neuroblastoma cell lines (Fig. 1C, 1D, 1E). The increase in InP concentration following stimulation of the cells with 100 $\mu$M UTP and UDP varied from 1.8 to 9-fold between the different mouse neuroblastoma cell lines tested (Table 1). No activity of adenine nucleotides was detected at 100 $\mu$M concentration in any of these three cell lines (Fig. 1).

In the case of mouse neuroblastoma/rat glioma hybrid cell line NG108-15, UTP, and UDP were again found to be active but some activity of adenine nucleotides was also seen. The increase in InP accumulation following stimulation of these cells by 100 $\mu$M UTP and UDP was ~ 2.5-fold greater than that evoked by 100 $\mu$M ATP and ADP (Fig. 1F, Table 1).

To further characterise the pharmacological selectivity of the P2Y receptors on the murine origin cell lines, the concentration dependence of nucleotide stimulated InP accumulation was determined (Fig. 2 and Table 1). Although UTP and UDP were found to be active in all the mouse neuroblastoma derived cell lines characterized in the present study (N1E 115, Neuro 2a, NB4 1A3 and NG108-15) there were some differences in the $EC_{50}$ values derived from the concentration–response relationships for them (Fig. 2 and Table 1). A relationship

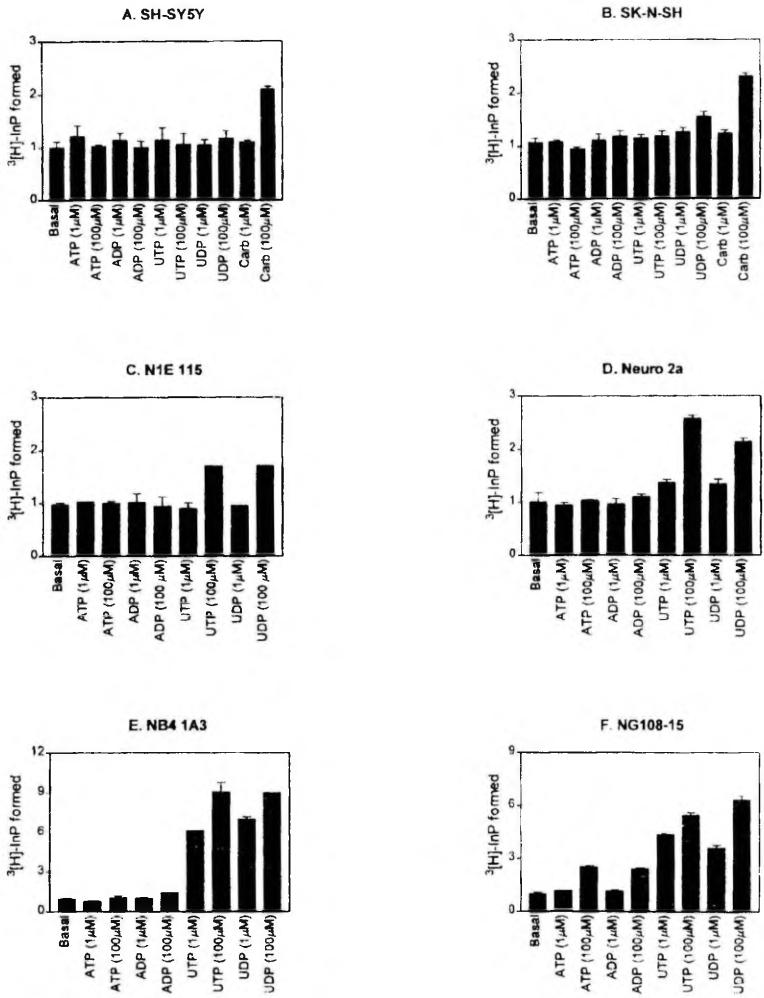


**FIG. 1.** Inositol phosphates accumulation in neuroblastoma origin cell lines in response to nucleotides. Basal accumulation of [$^3$H]InP formation was 900-1100 cpm/well and it is equated to one. Results are expressed as the means ± SEM of three independent experiments each carried out in triplicate.

between the potency of nucleotides and the magnitude of the InP accumulation was observed; a lower $EC_{50}$ value for an agonist correlated with a greater level of InP synthesis (Table 1).

Additionally, we have studied the effect of simultaneous addition of 100 $\mu$M ATP and UTP and 100 $\mu$M ATP and UDP on the InP formation in mouse neuroblastoma NB4 1A3 cell line (Fig. 3). The basal level of response remained constant as 100 $\mu$M ATP itself was without activity at these cells (Fig. 1). The $EC_{50}$ values for UTP and UDP were not significantly altered by the addition of 100 $\mu$M ATP, being 0.57 ±

TABLE 1

$EC_{50}$ Values of Nucleotides for Mouse Neuroblastoma-Derived Cell Lines

| | UTP | | UDP | | ATP | ADP |
|---|---|---|---|---|---|---|
| Cell lines | $EC_{50}$ ($\mu$M) | Increase in InP conc.[a] | $EC_{50}$ ($\mu$M) | Increase in InP conc.[a] | $EC_{50}$ ($\mu$M) | $EC_{50}$ ($\mu$M) |
| N1E 115 | 11.14 ± 3.08 | 1.8 | 10.49 ± 3.96 | 1.8 | NE[b] | NE |
| Neuro 2a | 8.03 ± 2.31 | 2.4 | 13.15 ± 4.10 | 2.2 | NE | NE |
| NB4 1A3 | 0.67 ± 0.13 | 9.0 | 0.51 ± 0.11 | 9.0 | NE | NE |
| NG108-15 | 0.58 ± 0.08 | 5.4 | 1.25 ± 0.19 | 6.3 | 113.1 ± 25.1 | 105.8 ± 17.3 |

[a] Increase in total InP concentration was calculated by dividing the mean cpm-s obtained by stimulating the cells with 100 $\mu$M nucleotide with the mean cpm-s of basal value.

[b] NE, no effect at 100 $\mu$M. $EC_{50}$ values were calculated from a set of the dose–response curves of the type shown in Fig. 2. All values are expressed as the mean ± standard error of three independent experiments each carried out in triplicate.

0.10 $\mu$M and 0.70 ± 0.16 $\mu$M, respectively (Fig. 3). Furthermore, addition of 100 $\mu$M ATP had no influence on the maximal response to uracil nucleotides in this cell line. Thus ATP does not potentiate the PLC response to uracil nucleotides in the NB4 1A3 cell line.

## DISCUSSION

The first observation worthy of comment is the lack of InP accumulation in the human neuroblastoma cell lines in response to nucleotide application. As both cell lines responded to carbachol this would appear to in-

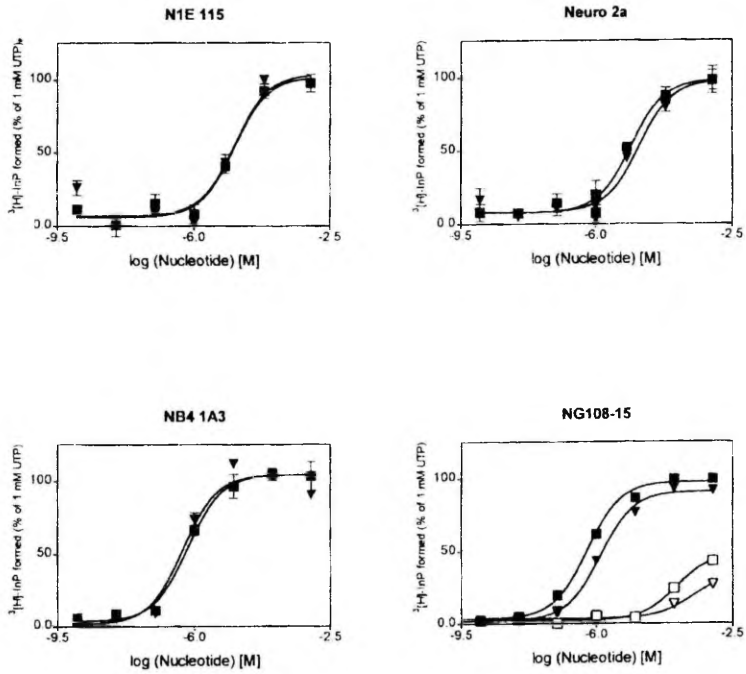


FIG 2. Concentration dependent stimulation of [$^3$H]InP formation induced by nucleotides in mouse neuroblastoma derived cell lines (■ - UTP, ▼ - UDP, □ - ATP, ▽ - ADP). See Methods section for further detail. Results are expressed as the means ± SEM of three independent experiments each carried out in triplicate.

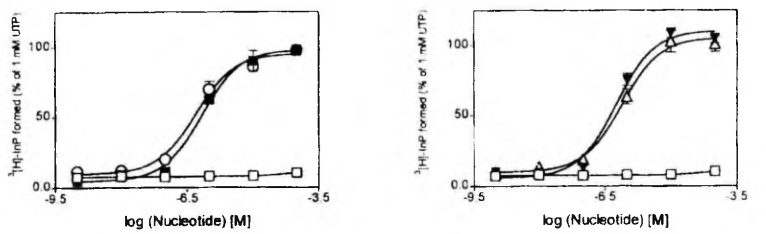


**FIG 3.** Effect of 100 $\mu$M ATP on the response to UTP and NB4 1A3 cells (■ - UTP, ▼ - UDP, □ - ATP, ○ - UTP in the presence of 100 $\mu$M ATP, △ - UDP in the presence of 100 $\mu$M ATP). Results are expressed as the means ± SEM of three independent experiments each carried out in triplicate.

dicate the absence of functionally expressed PLC-coupled P2Y receptors. This is in marked contrast to the results obtained with the mouse derived neuroblastoma cell lines where robust responses were obtained to both UTP and UDP.

With regard to the identity of the receptor(s) expressed in the mouse neuroblastomas, the agonist profile is most like that of the $P2Y_6$ receptor, as this is the only receptor subtype that has been cloned so far which is selective for pyrimidines. Indeed, mRNA for this receptor has been detected in the mouse neuroblastoma/rat glioma NG108-15 cell line by RT-PCR, as have mRNA for $P2Y_1$ and $P2Y_2$ (14). However, we can exclude the functional expression of the latter subtypes as $P2Y_1$ is activated only by adenine nucleotides (6) and $P2Y_2$ is activated by both ATP and UTP at micromolar concentrations (7). Therefore, the activity of only uracil nucleotides leads us to suggest that the receptor participating in these signal transduction pathway is the $P2Y_6$ subtype. However, we cannot exclude the possibility that a novel pyrimidine receptor subtype is expressed in the mouse neuroblastoma cell lines.

The $EC_{50}$ values for UTP and UDP are in accordance with the results published previously for the cell lines Neuro 2a and NG108-15 (22, 23). In the Neuro 2a line UTP and UDP were more than 300-fold more potent than ATP (22), while in NG108-15 cells UTP and UDP were 300- and 100- fold more potent than ATP in stimulating InP formation (24). However, we have not detected a clear $P2Y_2$-like response in any of the cell lines tested as has been previously found in the NG108-15 and N1E 115 cells, in which ATP and UTP stimulated both InP accumulation and $Ca^{2+}$ mobilization (10, 25).

The recombinant rat $P2Y_6$ receptor has been studied in a number of heterologous expression systems, using different assay methodologies. Li *et al.* studied the activity of pure nucleotides in the 1321N1 cell line stably expressing the receptor and measured a half-maximal concentration for UDP of 6 nM by using both the inositol phosphates measurements and intracellular calcium mobilisation assay (9). A nanomolar $IC_{50}$ value for UDP was also determined by Filippov *et al.*, measuring the inhibition of both N-type $Ca^{2+}$ and M-type $K^+$ currents in $P2Y_6$ receptor mRNA injected rat sympathetic neurones (5.9 nM and 30 nM, respectively) (15). However, the potency of UTP was more than 50-fold lower in the case of rat $P2Y_6$-1321N1 cells (9) and 3-fold lower in $P2Y_6$ injected rat sympathetic neurones (15). An endogenous pyrimidine receptor of cultured sympathetic neurones has been characterized, at which UTP and UDP are equipotent ($EC_{50}$ ~ 1 $\mu$M) for the inhibition of the M-type $K^+$ current (26). In all of these cases ATP was inactive while ADP acted as a full agonist with reduced potency being 1000- and 30-fold less active than UDP (9, 26). The equipotency of UTP with UDP and the lack of activity of ADP in the mouse neuroblastoma lines tested here may be indicative of a novel receptor subtype. However, it could also be a consequence of differences in receptor reserves in the different expression systems. This could also explain why the $EC_{50}$ values for UTP and UDP vary in the different neuroblastoma origin cell lines studied in the present paper and to some extent the lack of activity of ADP.

Previously, we have studied the effect of ATP on the response to UTP and UDP in the NG108-15 cell line. There we found that ATP at a concentration of both 100 $\mu$M and 1 mM can potentiate the actions of UTP and UDP in the NG108-15 cell line (23). In this study we assessed whether the same phenomenon occurred with the NB4 1A3 line. We found that it did not which leads us to speculate that the ectoenzymes involved in the potentiation in the case of the NG108-15 cell line derive from its rat glioma rather than the mouse neuroblastoma parentage.

The absence of purine nucleotide stimulated InP accumulation in the three neuroblastoma cell lines suggests that these three clones can be used as model systems to investigate the cellular processes regulated by pyrimidine activated P2Y receptors. Indeed, the expression of such a receptor in a neuroblastoma cell line *per se* may be indicative of a significant role for pyrimidines in the nervous system.

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# VII

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Pharmacological evidence for a novel pyrimidinoceptor in NG108-15 cells.
Submitted.

# Pharmacological Evidence for a Novel Pyrimidinoceptor in NG108-15 Cells

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## Abstract

Two distinct pyrimidinergic receptors are coupled to the activation of phospholipase C in the mouse neuroblastoma × rat glioma hybrid cell line NG108-15. High micromolar concentrations of adenosine and some of its structural analogues are able to antagonize the effect of UTP on phospholipase C but not that of UDP. Based on the obtained pharmacological profile (UTP ≥ UDP >> ATP = ADP) formation of inositol phosphates was mediated by the $P2Y_6$ receptor and a novel UTP-preferring subtype. Suramin, reactive blue 2 and PPADS antagonized the action of UTP at this novel receptor.

*Key words*: NG108-15 cell line; pyrimidinoceptor; UTP-preferring receptor; hydrolysis of inositol phospholipids; adenosine

*Abbreviations*: AdoC, adenosine-5'-carboxylic acid; iPAdo, 2',3'-O-isopropylideneadenosine; CGS-21680, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine; A2'P, adenosine 2'-monophosphate; A3'P, adenosine 3'-monophosphate; A2'3'cP, adenosine 2':3'-cyclic monophosphate; A3'5'cP, adenosine 3':5'-cyclic monophosphate; ZM 241385, (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol)

# 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 x rat glioma cell line provides a model system for the investigation of pyrimidinergic receptors coupled to the activation of phospholipase C as only uracil nucleotides were found to be active (Sak *et al.*, 1998; Sak *et al.*, 1999). This is a common feature of a number of neuroblastoma cell lines (Sak *et al.*, 1999). Considering the recombinant mammalian P2Y receptors (Ralevic and Burnstock, 1998) we have proposed that this receptor was of the $P2Y_6$ type (Sak *et al.*, 1999). Thus, the NG108-15 cell line can be used to investigate the ability of other signaling molecules to modulate the transduction mechanisms of this P2Y receptor subtype.

Previously, augmentation of the PLC response of nucleotides acting at the $P2Y_2$ receptor by the adenosine A1 receptor expressed in CHO cells has been reported (Megson *et al.*, 1995), as have synergistic increases in MAP kinase activity (Dickenson *et al.*, 1998). However, activation of the adenosine A2 receptors in NG108-15 cells leads to an inhibition of inositol phosphate production and calcium mobilization evoked by bradykinin (Campbell *et al.*, 1990). Here we present evidence that adenosine and some of its derivatives are able to reduce the level of inositol phosphates released in response to challenge with UTP but not UDP and that this effect is not mediated by an adenosine receptor. Furthermore, we present other data consistent with the expression of two pyrimidinergic receptors in the NG108-15 cell line, the $P2Y_6$ receptor and a novel pyrimidinoceptor.

# 2. Materials and methods

## 2.1. Chemicals

UTP, UDP, ATP, ADP, AMP (A5'P) and adenosine were obtained from Boehringer Mannheim. AdoC, iPAdo, CGS-21680, A2'P, A3'P, A2'3'cP, A3'5'cP, UMP, uridine and reactive blue 2 were the products of Sigma. PPADS and suramin were purchased from RBI. ZM 241385 was the product of Tocris. Trichloroacetic acid (TCA) and ammonium formate were obtained from Acros Organics. Formic acid was purchased from Riedel-de Haën. Diethyl ether was from A/S Den Norske EterFabrikk.

All nucleotides were analysed and purified by 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 ECACC. The cells were grown in Dulbecco's Modified Eagle's Medium DMEM (Gibco Ltd), 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. Inositol phosphates assay

For measurements of total inositol phosphates NG108-15 cells were seeded in a 24-well culture plate at density of ~ $5 \times 10^4$ cells/ well. The cells were assayed after 3 days in culture when still subconfluent. Inositol lipids were radiolabeled 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 aspirating the media and adding 500 μl of ice-cold 5% TCA. The TCA-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]InP formed was determined by adding 12 ml of scintillation cocktail (OptiPhase HiSafe III, Wallac) to each eluates and counting the radioactivity.

### 2.4. Data analysis

Results were calculated as means ± 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 percental scale. Statistically significant effects were determined by Student's *t*-test and antagonistic constants Ki were calculated by the Gaddum equation (Lazareno and Birdsall, 1993).

# 3. Results

## 3.1. Differential effect of adenosine on the PLC response to UTP and UDP application

The P2Y receptor coupled to the activation of phospholipase C in NG108-15 cell line is activated by UTP and UDP, with ATP and ADP being considerably less effective (Sak *et al.*, 1998; Sak *et al.*, 1999). As reported previously, UDP and UTP were both able to evoke the accumulation of inositol phosphates at high nanomolar concentrations and displayed similar potency, with $EC_{50}$ values of 0.98 $\pm$ 0.11 μM and 0.50 $\pm$ 0.05 μM respectively (Fig. 1A and 1B).

The effect of adenosine and its structural analogues on the UTP concentration-effect relationship was determined. None of the ligands tested were able to modulate PLC 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 $\pm$ 0.19 and the $K_i$ for adenosine was 185 μM (Fig.1A). Of the other compounds tested (Table 1) only iPAdo, A3'5'cP and $A_{2A}$ adenosine receptor agonist CGS-21680 were also able to antagonize the action of UTP. However, the $A_{2A}$ adenosine receptor antagonist ZM 241385 neither suppressed nor abolished the inhibitory action of adenosine on the PLC response to UTP (Fig. 1A).

Surprisingly, adenosine was unable to antagonize the PLC response evoked by UDP (Fig. 1B). Although adenosine slightly potentiated the UDP response this effect was not statistically significant.

## 3.2. UTP and UDP act at distinct P2Y receptors in the NG108-15 cell line

The opposing effect of adenosine on the PLC response to UTP and UDP led us to hypothesis that there were potentially two pyrimidine receptors functionally expressed on the NG108-15 cells under investigation. In an attempt to ascertain if UTP and UDP act at the same receptor 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 inositol phosphates accumulation was raised under these conditions with an apparent $EC_{50}$ for UTP of 0.65$\pm$ 0.23 μM compared with 0.50$\pm$0.05 μM when applied alone (Fig. 2A). Co-application of these agonists was additive up to approximately 140% of the control level, and a similar level of inositol phosphates accumulation was observed when the cells were challenged simultaneously with 10 μM UDP and 10 mM NaF (Fig. 2B).

### 3.3. Effect of non-selective P2 receptor antagonists on the synthesis of inositol phosphates evoked by UTP

To further characterize the UTP receptor, the non-selective and non-phosphate antagonists of P2 receptors, suramin, PPADS and reactive blue 2 were all tested for their ability to antagonize the effect of UTP on PLC in NG108-15 cells. All three were able to inhibit the response to 1 μM UTP (Fig. 3). Suramin and reactive blue 2 had a similar inhibitory activity, with $IC_{50}$ values of 39.7 ± 6.4 μM and 55.9 ± 14.6 μM respectively while PPADS had lower activity with an $IC_{50}$ value of 166.7 ± 33.0 μM.

## 4. Discussion

The ability of adenosine to modulate pyrimidinoceptor signaling in the NG108-15 cell line was investigated. Adenosine behaved as low affinity antagonist for the UTP mediated accumulation of inositol phospholipids 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. The inhibitory effect at the UTP response does not appear to be achieved via interaction with adenosine receptors, although both the $A_{2A}$ and $A_{2B}$ receptor subtypes have been reported to be functionally expressed in NG108-15 cells and are coupled to the stimulation of adenylate cyclase (Mundell and Kelly, 1998), on the basis of the following observations. Firstly, derivatives of adenosine which have no activity at $A_2$ receptors for example, 2',3'-O-isopropylideneadenosine and adenosine 3':5'-cyclic monophosphate (*Dr. Ijzerman personal communication*) had clear antagonistic activity on the UTP response. Secondly, the $A_{2A}$ receptor antagonist ZM 241385, used in this study at a concentration at which it would act also at the $A_{2B}$ receptor (Poucher *et al.*, 1995), had no effect on the inhibitory action of adenosine. Moreover, recently it has been indicated that NG108-15 cells express mainly $A_{2A}$ receptor (Ohkubo *et al.*, 2000). Thirdly, the $A_{2A}$ receptor specific agonist CGS-21680 (Jarvis *et al.*, 1989) had a smaller inhibitory effect on the UTP response than did adenosine. In light of these observations, adenosine could be used as a lead compound for the design of specific antagonists for this UTP-preferring receptor.

The differential effect of adenosine on the UTP and UDP PLC responses led us to consider the possibility that these two uracil nucleotides preferentially activate different P2Y receptor subtypes in these cells. The additive nature of the UTP and UDP PLC responses further indicates that this is the case, a similar level of stimulation has been observed previously upon the co-application of UTP and bradykinin in this cell line (Lin, 1994). The pharmacological profile we have determined for these cells (UTP≥ UDP>> UMP> ATP= ADP) (Sak *et*

*al.*, 1998; Sak *et al.*, 1999), excludes 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 which is equally activated by UTP and ATP (Lustig *et al.*, 1993). Although the human $P2Y_4$ receptor has been demonstrated to be activated by UTP and antagonized competitively by ATP (Kennedy *et al.*, 2000) the rat $P2Y_4$ receptor has the same agonist profile as $P2Y_2$ (UTP=ATP) (Webb *et al.*, 1998; Bogdanov *et al.*, 1998) and therefore can also be excluded. The $P2Y_6$ receptor is the single P2Y receptor subtype activated preferentially by uracil nucleotides with the selectivity for UDP over UTP (Communi *et al.*, 1996; Li *et al.*, 1998), which may account for the UDP response detected in this study. It should be noted that the NG108-15 cell line is a hybrid of the mouse neuroblastoma C1300 and the rat glioma C6 and therefore the UTP- preferring receptor could be of rat or mouse origin. Previously, mRNA for the $P2Y_6$ receptor, as well as the $P2Y_1$, $P2Y_2$, but not the $P2Y_4$ has been detected by RT-PCR analysis in NG108-15 cells (Webb and Barnard, 1999) used for studies into P2Y 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 transcripts, revealed an abundant expression of the $P2Y_6$ receptor mRNA and detected the $P2Y_2$ receptor transcript at considerably lower levels (data not shown). Thus the UDP-preferring receptor is the $P2Y_6$ subtype and the UTP-sensitive receptor is likely to be a novel nucleotide receptor subtype rather than the murine $P2Y_4$ receptor, which has recently been shown to have the same agonist profile as that of the rodent receptor (Suarez-Huerta *et al.*, 2000).

Further evidence for the expression of a novel receptor comes from use of P2 receptor antagonists. The rank order of potency for the inhibition of inositol phosphates accumulation upon UTP application was suramin $\geq$ reactive blue 2 > PPADS. This antagonist activity order is not compatible with data obtained for the known P2Y receptor subtypes (Jacobson *et al.*, 1999). Thus this study joins other examples of UTP-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; Sak *et al.*, 1999) the P2Y receptors expressed have been characterized by the rank order of potency: UTP $\geq$ 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 > ATP$\gamma$S >> 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 clear evidence for the expression of a novel UTP-preferring pyrimidinoceptor subtype in NG108-15 cells at which adenosine acts as a low activity antagonist. As this receptor is widely expressed in neuronal cell lines its potential physiological role in nervous system necessitates its further study and determination of its molecular structure.

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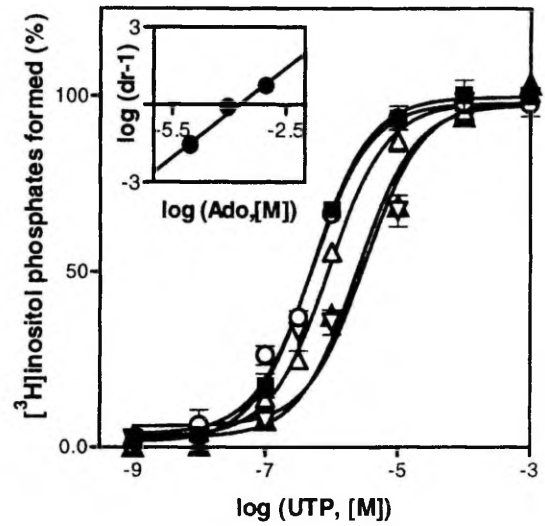
A

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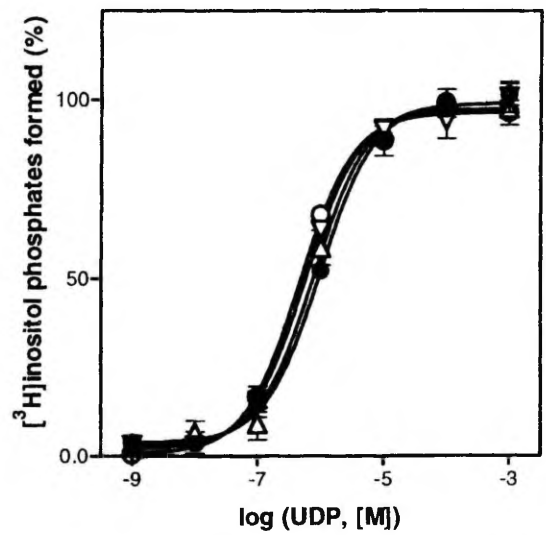
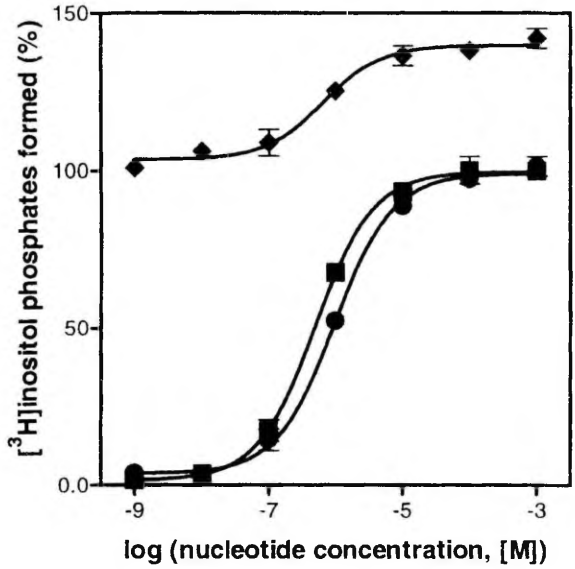



**Figure 1A.** Effect of 10 μM (○), 100 μM (△) and 1 mM (▽) adenosine on the dose dependent stimulation of inositol phosphates synthesis by UTP (■), and the ineffectiveness of 180 μM ZM 241385 to suppress the inhibitory action of 1 mM adenosine (▲). The inset shows the Schild plot of the data. **1B.** Effect of 10 μM (○), 100 μM (△) and 1 mM (▽) adenosine on the dose dependent stimulation of inositol phosphates synthesis by UDP (●).

A

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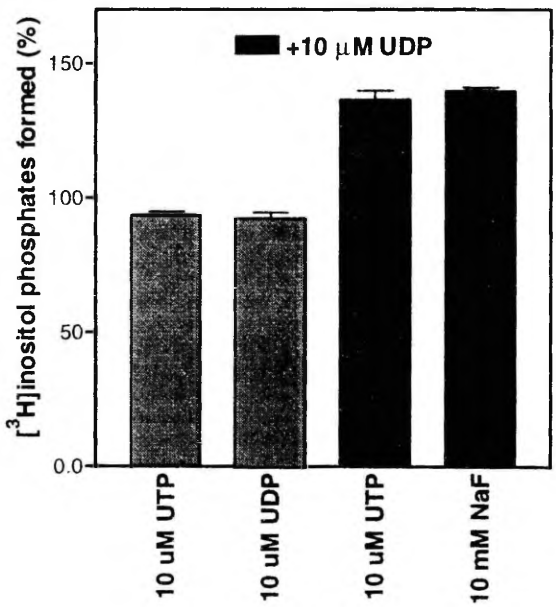


**Figure 2A.** Additivity of agonist induced inositol phosphates formation: UTP (■), UDP (●) and activity of UTP in the presence of 10 μM UDP (◆). **2B.** Effect of simultaneous addition of 10 μM UDP with 10 μM UTP and 10 mM NaF with 10 μM UDP on the synthesis of inositol phosphates in NG108-15 cells. The basal effect is subtracted.

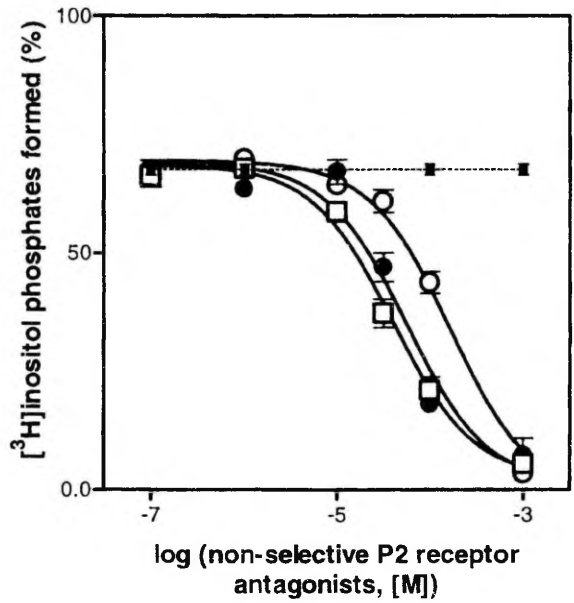


**Figure 3**. Effect of the P2 receptor antagonists suramin (□), reactive blue 2 (●) and PPADS (○) on 1 μM UTP (■) induced hydrolysis of inositol phospholipids.

**Table 1.** Effect of adenosine and its derivatives on the synthesis of inositol phosphates initiated by UTP in NG108-15 cells. The pKi values were calculated by the Gaddum equation (Lazareno and Birdsall, 1993).

| Compound | pKi |
|---|---|
| Adenosine | 3.73 ± 0.07* |
| CGS-21680 | 3.24 ± 0.03* |
| IPAdo | 4.26 ± 0.08* |
| AdoC | N.E.[1] |
| AMP (A5'P) | N.E. |
| A2'P | N.E. |
| A3'P | N.E. |
| A2'3'cP | N.E. |
| A3'5'cP | 4.32 ± 0.14* |

* Significant effect ($P<0.05$). [1]N.E. No significant effect at 300 μM

# VIII

Sak, K., Kelve, M., Uri, A., Järv, J. (1998)
Pyrimidinoceptor potentiation by ATP in NG108-15 cells.
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# Pyrimidinoceptor potentiation by ATP in NG108-15 cells

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**Abstract** **Regulation of inositol phospholipid hydrolysis by UTP and UDP in neuroblastoma×glioma hybrid cell line NG108-15 was potentiated in the presence of ATP. The effect of ATP was dose dependent and shifted the $EC_{50}$ value for these uracil nucleotides up to three powers of magnitude, having no influence on the maximal value of the response. Adenine nucleotides (ADP, AMP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), β,γ-methyleneadenosine 5′-triphosphate (βγMeATP), 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP) and 3′-deoxyadenosine 5′-*O*-(1-thio)triphosphate (dATPαS)) as well as adenosine, had no influence on the pyrimidinoceptor response. The potentiation effect was abolished by excess of EDTA. The results were in agreement with the hypothesis of pyrimidinoceptor affinity regulation via extracellular phosphorylation of the receptor protein, initiated by ATP. This mechanism may have physiological implication for functioning of uracil nucleotides as endogenous signaling molecules.**

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*Key words:* UTP; UDP; ATP; ATP analog; Pyrimidinoceptor; Inositol phospholipid hydrolysis; Extracellular receptor phosphorylation

## 1. Introduction

Extracellular nucleotides regulate physiological responses in various tissues by activating appropriate receptors [1]. Several of these receptors possess clear selectivity against uracil nucleotides, pointing to the possibility that these compounds may function as endogenous signaling molecules [2–5]. The pyrimidinoceptor concept can be supported by the possibilities for recycling of the uracil nucleotides and formation of their pools in cells [2]. However, the amount of the stored UTP in granules of chromaffin cells and platelets forms only about 10% of the appropriate ATP level [2]. Therefore the question whether the uracil nucleotides can be released in quantities sufficient to stimulate pyrimidinoceptors, has still no answer.

Here we present evidence that the effectiveness of UTP as well as UDP to activate phosphoinositol lipid breakdown in neuroblastoma×glioma hybrid cell line NG108-15 can be dramatically increased in the presence of ATP, probably initiating phosphorylation of the receptor by ectokinases. As the shift of the effective concentration interval for UTP and UDP exceeded several powers of magnitude, far enough to compensate for the low abundance of uracil nucleotides in tissues, the effect discovered may have a clear physiological implication in cell regulation by these compounds.

## 2. Materials and methods

### 2.1. Cell culture

Neuroblastoma×glioma hybrid NG108-15 cells were obtained from the European Collection of Cell Cultures and were grown in high-glucose Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) of fetal calf serum, 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 dishes.

### 2.2. Chemicals

UTP, UDP, UMP, ATP, ADP, AMP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), β,γ-methyleneadenosine 5′-triphosphate (βγMeATP), 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP) and 3′-deoxyadenosine 5′-*O*-(1-thio)triphosphate (dATPαS) (Boehringer Mannheim, Sigma, Amersham) were analyzed and purified by HPLC (Gilson) on an anion exchange column Mono Q HR5/5 (Pharmacia Biotech) by monitoring 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. Adenosine and other chemicals were of the highest available grade of purity.

### 2.3. Assay of inositol phosphates

NG108-15 cells were seeded in a 24-well culture plate at density ~$5\times10^4$ cells/well, and used for assay after 3 days when grown to subconfluence. Inositol lipids were radiolabeled by overnight incubation of cells with *myo*-[2-$^3$H]inositol (2 μCi/ml, Amersham) in 200 μl of inositol-free DMEM. The medium was not changed after [$^3$H]inositol addition. Nucleotide effects were initiated by addition of 50 μl of ligand solution in 50 mM LiCl and the assay mixture was incubated 10 min at 37°C. The reaction was stopped by aspirating the medium and adding 500 μl of ice-cold 5% TCA. The TCA-containing supernatant was extracted three times with 500 μl of diethyl ether and inositol phosphates were isolated by using Dowex AG1-X8 gel (Bio-Rad, 100–200 mesh, formate form). The columns (bed volume 0.8 ml) were washed with water (2×5 ml) and 50 mM ammonium formate (10 ml) and inositol phosphates were eluted with 1 M ammonium formate in 0.1 mM formic acid (2×5 ml). The amount of [$^3$H]inositol phosphates formed was determined by counting radioactivity of the eluate (scintillation cocktail EcoLume, ICN). Under the experimental conditions used the basal radioactivity of samples remained between 800 and 1000 cpm/sample and the maximal radioactivity achieved was 6000–7000 cpm/sample. The results were calculated as means ± S.E.M. from three independent experiments and normalized versus the results of the control experiments at 10 μM UTP concentration. The dose-response curves were processed by a non-linear regression analysis program Prism (Version 2.00).

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*Abbreviations:* ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); βγMeATP, β,γ-methyleneadenosine 5′-triphosphate; BzATP, 3′-*O*-(4-benzoyl)benzoyl ATP; dATPαS, 3′-deoxyadenosine 5′-*O*-(1-thio)triphosphate; EDTA, ethylenediamine tetraacetate; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid; HPLC, high performance liquid chromatography

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## 3. Results

### 3.1. Activation of phospholipase C by nucleotides in NG108-15 cells

The synthesis of inositol phosphates in NG108-15 cells was activated by UTP and UDP in a dose-dependent manner, yielding the $EC_{50}$ values $(3.9 \pm 0.6) \times 10^{-7}$ M and $(1.3 \pm 0.6) \times 10^{-6}$ M, respectively (Fig. 1). The potency of UMP was considerably weaker ($EC_{50} = (7.5 \pm 0.6) \times 10^{-4}$ M). The same biochemical response was also activated at high concentrations of ATP, ADP, ATPγS and dATPαS, characterized by the $EC_{50}$ values $(3.0 \pm 0.7) \times 10^{-4}$ M, $(2.4 \pm 0.7) \times 10^{-4}$ M, $(7.0 \pm 0.8) \times 10^{-4}$ M and $(4.4 \pm 0.9) \times 10^{-4}$ M, respectively. In the presence of βγMeATP, AMP and adenosine, there was no statistically significant increase in formation of inositol phosphates if compared with the basal level. Thus, the receptors revealed clear specificity for UTP and UDP.

At nucleotide concentrations above 1 mM a decrease of the ligand-induced effect was observed in the case of UTP and UDP, as well as in the case of adenine nucleotides, resulting in a bell-shaped form of the dose-response curves (data not shown). However, as not relevant to the present study, these effects of high ligand concentration will be discussed elsewhere.

### 3.2. Influence of ATP on UTP and UDP induced synthesis of inositol phosphates

Simultaneous application of ATP and UTP or ATP and UDP led to a non-additive effect, i.e. the total amount of inositol phosphates formed did not exceed the level observed at optimal concentration of UTP alone (Fig. 2). At the same time the dose-response curves for UTP and UDP, measured in the presence of ATP, revealed a significant potentiation of the effect of the uracil nucleotides. This shift of the dose-response curves was clearly dependent on ATP concentration and in the presence of 1 mM ATP the effective UTP and UDP concentrations were lowered into the picomolar range, yielding the $EC_{50}$ values $(1.1 \pm 0.2) \times 10^{-12}$ M and $(1.2 \pm 0.2) \times 10^{-11}$ M, respectively (Fig. 2).

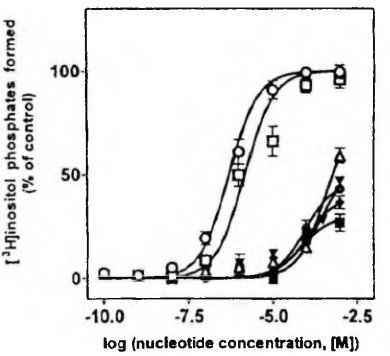


Fig. 1. Dose-dependent stimulation of synthesis of inositol phosphates in neuroblastoma×glioma hybrid NG108-15 cells by uracil and adenine nucleotides (○, UTP; □, UDP; △, UMP; ●, ATP; ■, ADP; ▼, ATPγS; ◆, dATPαS).

### 3.3. Influence of other nucleotides on UTP induced synthesis of inositol phosphates

The experiments described above were repeated in the presence of ADP, AMP and adenosine (10 μM–1 mM), as well as βγMeATP and ATPγS (0.1 mM). It was found that none of these ligands potentiated the UTP effect on synthesis of inositol phosphates. Moreover, these ligands had no influence on the maximal level of the UTP response.

### 3.4. Effect of EDTA on UTP induced synthesis of inositol phosphates

In separate experiments 4 mM EDTA was added into the assay medium before nucleotides to bind the divalent cations present. It was found that the excess of this chelating ligand had no effect on the $EC_{50}$ value for UTP, equal to $(2.0 \pm 0.5) \times 10^{-7}$ M in the $Mg^{2+}$ and $Ca^{2+}$ free medium, as

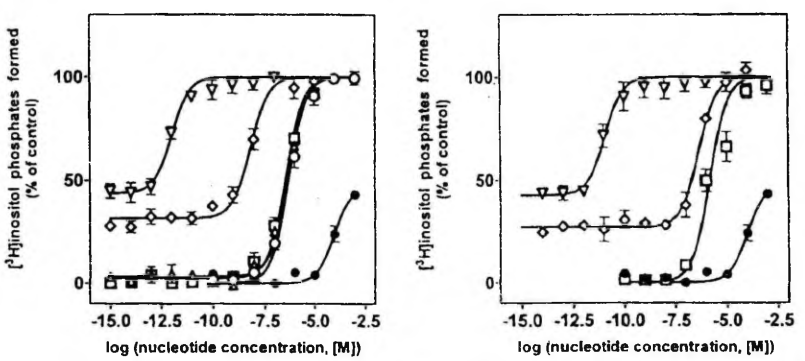


Fig. 2. Potentiation of the effect of UTP (left) and UDP (right) on pyrimidinoceptor regulated synthesis of inositol phosphates in the presence of various concentrations of ATP (△, 1 μM; □, 10 μM; ○, 100 μM; ▽, 1 mM). The dose-response curves for UTP (○), UDP (□) and ATP (●) alone were shown for comparison.

well as on the range of the response of cells observed. At the same time the potentiating effect of ATP on the UTP induced inositol phosphate formation was completely abolished in the presence of EDTA.

## 4. Discussion

Synthesis of inositol phosphates in NG108-15 cells was regulated by nucleotide receptors, which revealed clear preference for UTP and UDP over ATP and its analogs. This result agreed well with the rank order of the effect of uracil and adenine nucleotides, published by Lin for the same cell line [6]. Therefore the uracil nucleotides may well act as endogenous signaling molecules for these cells and the appropriate receptors may belong to the $P2Y_4$ and/or $P2Y_6$ subtypes [7–9].

Although the cell responses were evoked by UTP and ATP at very different concentrations, Lin has suggested that both these nucleotides interact with the same receptor on NG108-15 cells [6]. This suggestion was based on the absence of additivity between the effects of UTP and ATP, as well as on the possibility of desensitization of the cells to the effects of both these nucleotides by their extended pretreatment with UTP. Although the same phenomena were observed in the present study, a more significant difference between the behavior of uracil and adenine nucleotides pointed to the involvement of distinct regulatory mechanisms for uracil and adenine nucleotides.

Firstly, in the presence of ATP a dramatic dose-dependent increase in potency of uracil nucleotides was observed, while no increase in potency of ATP analogs was found under the same conditions. This means that most probably the uracil and adenine nucleotides act at distinct target sites.

Secondly, among all the investigated adenine nucleotides only ATP was able to enhance potency of UTP (and UDP), although the synthesis of inositol phosphates was similarly initiated by several adenine-containing ligands. Thus, the activation of phospholipase C and potentiation of the effect of uracil nucleotides should occur via different mechanisms.

Although the former phenomenon may be related to some still unspecified influence of the externally applied adenine nucleotides on synthesis of inositol phosphates, the ligand specificity of this effect seems to support the idea of involvement of some receptors. These receptors may well belong to the channel-coupled subtypes, also governed by ATP and its analogs. For example, the $P2X_7$ receptor subtype activated by micromolar ATP concentrations and operating a non-selective ion channel was found in this cell line [10]. Therefore, it can be supposed that ATP may cause 'leaking' of intracellular compounds, including uracil nucleotides, into the extracellular medium. Although the amount of uracil nucleotides released through these 'pores' should be small, this may still be sufficient to activate the pyrimidinoceptors simultaneously potentiated by ATP. Following this hypothetical scheme the non-additive nature of the maximal response of UTP and ATP, as well as the desensitization phenomena observed by Lin [6], can be easily understood.

The results of the present study point to the possibility that the observed potentiation of pyrimidinoceptors on NG108-15 cells, occurring in the presence of ATP, can be related to extracellular phosphorylation of these proteins. As the γ-phosphate of ATP is transferred to the target protein in this reaction, the ATP analogs cannot participate in this process and therefore were unable to potentiate pyrimidinoceptors. Moreover, as the actual substrate for protein kinases is MgATP, the abolishment of the potentiation effect in the presence of EDTA also agrees with the phosphorylation hypothesis.

If the intracellular reversible phosphorylation of proteins has been recognized as a key regulatory mechanism in numerous cellular functions, much less is known about the regulatory role of the extracellular protein phosphorylation [11]. The results of the present study point to the possibility that the latter process may be implied in regulation of the responsiveness of NG108-15 cells to extracellular UTP. Until recently the possibilities of cell regulation by uracil nucleotides have been thought to be limited by low physiological concentration of these compounds, not corresponding to the experimentally detectable effectiveness of binding of these ligands on the appropriate receptors. Therefore the possibility of significant increase in affinity of pyrimidinoceptors through extracellular phosphorylation of these proteins may have a clear physiological significance for intracellular communication by means of uracil nucleotides.

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