DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

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KINETIC ASPECTS OF dATPαS INTERACTION WITH $P2Y_1$ RECEPTOR

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III. Järv J, Oras A. Similar dynamics of G-protein coupled receptors molecules in response to antagonist binding. Neurosci Lett., 2005 Jan 10; 373 (2): 150–2.

ABBREVIATIONS

β,γ-MeATP	β,γ-Methylene-adenosine-5'-triphosphate
2-ClATP	2-Chloro adenosine 5'-triphosphate
2-MeSADP	2-Methylthioadenosine-5´-diphosphate
2-MeSATP	2-Methylthioadenosine-5´-triphosphate
A3P5PS	Adenosine 3´-phosphate 5´-phosphosulphate
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
ATPαS	Adenosine 5'-O-(1-thiotriphosphate)
dATPαS	2'-Deoxyadenosine 5'-O-(1-thiotriphosphate)
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N.,N.-tetraacetic acid
GPCR	G-protein coupled receptor
MRS 2179	2'-deoxy-N^6-methyladenosine-3',5'-bisphosphate
MRS 2269	anhydrohexitol derivative of N^6-methyl-2'-deoxyadenosine 3',5'-bisphosphate
MRS 2279	(*N*)-methanocarba-N^6-methyl-2-chloro-2'-deoxyadenosine-3',5'-bisphosphate
MRS 2286	2-[2-(2-chloro-6-methylamino-purin-9-yl)-ethyl]-propane-1,3-bisoxy(diammoniumphosphate)
MRS 2365	(N)-methanocarba-2MeSADP,
PAPET-ATP	2-[2-(4-Aminophenyl)ethylthio]adenosine 5´-triphosphate
PEI	Polyetyleneimine
PPADS	4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid
QNB	Quinuclidinyl benzilate
TLC	Thin layer chromatography
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate

INTRODUCTION

Metabotropic nucleotide receptors belong to the class of the G-protein coupled receptors (IUPHAR Receptor Database) and their structure is assumed to fit into the classical 7TM model, initially proposed for rhodopsin (Kroese et al, 2003, Becker et al, 2003). These receptors form the largest protein family at all, and they can be found in vertebrates, where this family contains between 1000 and 2000 members and forms more than 1% of the proteins encoded by the genome, but also in plants as well as in protozoa and earliest diploblastic metazoa (Bockaert and Pin, 1999). These receptors recognize intercellular (hormones, neurotransmitters) and extracellular messages (light, odours, etc), and it might well be that they are the oldest molecules responsible for cellular signaling (Madabushi, 2004).

The P2Y receptors interact with nucleotides and their subtype $P2Y_1$ reveals specificity for adenine nucleotides (IUPHAR Receptor Database). Today it is generally accepted that ADP and its analogues can activate the $P2Y_1$ subtype, while ATP and other triphosphate derivatives and analogues, like 2-MeSATP, 2-ClATP, ATPαS and β,γ-MeATP are acting as antagonists (Léon et al, 1997; Hechler et al, 1998;) or at least partial agonists (Palmer et al, 1998) at this subtype.

Among various ATP analogues many investigators have selected dATPαS (2'-deoxyadenosine-5'-O-(1-thiotriphosphate)) as a tool for radiolabelling of the $P2Y_1$ subtype (Simon et al, 1995). This triphosphate selectively inhibited the 2-MeSADP-stimulated response on this receptor subtype (Schachter and Harden, 1997) and it is chemically more stable than ATP, which hydrolysis under the assay conditions has become a generally recognized problem (Yegutkin and Burnstock 1998). This ligand is also listed in IUPHAR Receptor Database as the only radioligand for $P2Y_1$ receptor.

The attempts to use this ligand, however, have lead to contradictory results. From one side, this ATP analogue has revealed remarkable selectivity for this receptor subtype, especially if used in pharmacological assays. On the other hand, however, although the non-specific binding of this radioligand with the cellular membranes was low, a more thorough analysis has suggested that a lot of the binding sites traced by [^{35}S]dATPαS may belong to various endogenous ATP-binding proteins, distinct from the $P2Y_1$ receptor sites (Schachter and Harden, 1997). The presence of such extensive additional binding should certainly interfere with radioligand binding assay of this nucleotide receptor.

This complication seems to have a rather general background for binding assay of nucleotide receptors, until the ligands in use remain structurally related to naturally occurring nucleotides, which should possess a large number of various binding sites on cellular membranes. Therefore it was interesting to investigate into details of the interaction of this radioligand with biomembrane variants, where the $P2Y_1$ receptor was present or absent, and test the possi-

bilities of distinction of the receptor sites from other nucleotide binding sites. For this purpose the kinetic method of receptor-ligand interaction analysis was used instead of the conventional equilibrium binding study. The human $P2Y_1$ receptors, expressed in astrocytoma 1321N1 cells, and the analogous binding sites on rat brain membranes were involved in the analysis and in both cases the slow isomerization of the radioligand-receptor complex was discovered, and the slowness of the receptor-ligand complex formation and dissociation was used for distinction of the receptor sites from other specific but kinetically different binding sites on biomembrane. Thus the kinetic analysis provided a unique possibility for determination of a distinct class of receptor binding sites in the presence of an extensive radioligand binding at some other binding sites.

Moreover, the kinetic data obtained provided possibility for discussion of the dynamic properties of the G-protein coupled receptor molecules in general.

1. $P2Y_1$ NUCLEOTIDE RECEPTOR

1.1. P2Y receptor family

Cell-surface P2-receptors mediate the action of extracellular nucleotides in cell-to-cell signaling, and are divided into two major groups. The P2X-receptors are ligand-gated ion channels, whereas P2Y-receptors belong to the superfamily of the metabotropic G-protein-coupled receptors (Burnstock, 2004). Until today, the P2Y family is composed of nine subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$ and one orphan receptor, all encoded by human genome. More detailed pharmacological characterization can be found for five of these subtypes, which can be identified on the basis of their specificity against principal physiological agonists. For $P2Y_1$ receptor this specific agonist is ADP. All the cloned and functionally expressed P2Y-receptors are coupled to phospholipase C and therefore the phosphoinositole-assay can be used to follow the functional activity of these subtypes (Lee and O'Grady, 2003; von Kugelgen and Wetter, 2000). The $P2Y_{11}$ receptor stimulates in addition also adenylate cyclase (von Kugelgen and Wetter, 2000).

The present study is focused on investigation into kinetic properties of human $P2Y_1$ subtype, which is the most well described member of this receptor family and has great pharmacological impact. This impact is caused by the fact that the distribution of $P2Y_1$ is very wide (Guerra et al, 2003; Moore et al 2001). For example, the receptor plays crucial role in blood platelet aggregation (Maayani et al, 2003) and mediates the adenine nucleotide-induced release of the endothelium-derived relaxing factor nitric oxide (Kittner et al, 2003). $P2Y_1$ receptors may also be involved in the modulation of neuro-neural signaling transmission (von Kugelgen and Wetter, 2000).

1.2. Structure of human $P2Y_1$ receptor

The primary structure of the $P2Y_1$ receptor of human origin, consisting of 373 amino acids, has been established in 1996 (Ayyanathan et al, 1996; UniProt), and is listed in Table 1. The understanding of spatial structure of this receptor has been developed on the basis of analogy with rhodopsin structure (Schertler and Hargrave, 1995, Meng and Bourne, 2001). This protein has seven trans-membrane α helices (TM), which bundle together and cross the biomembrane, as shown in Fig. 1.

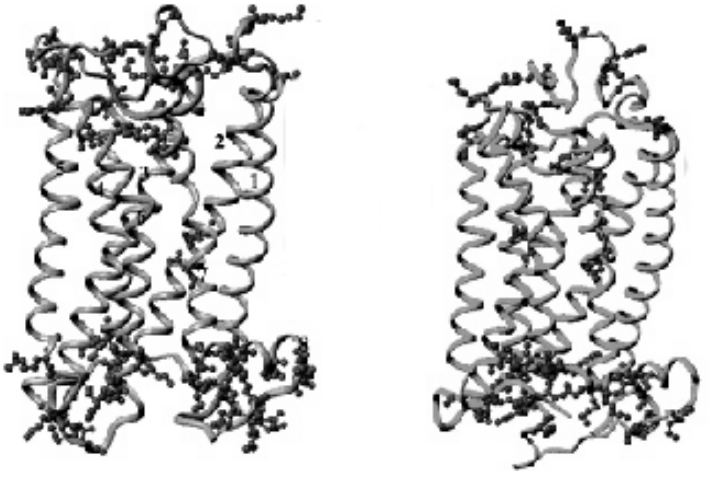

Figure 1. Comparison of the spatial structure of human $P2Y_1$ receptor (left) and rhodopsin (right). The top of the molecules is located in the extracellular space and the bottom is located in the intracellular space. The illustration is taken from recent paper by Major and Fischer (2004).

The seven TM helices of all these proteins are located clockwise in bundle, if viewed from the cytoplasm. Proceeding from this general understanding of the spatial structure of the 7TM receptors and taking into consideration the chemical nature of the amino acids in the human $P2Y_1$ receptor primary structure, the distribution of amino acids between the transmembrane regions (TM1 – TM7) and intracellular (IC) and extracellular (EC) domains was predicted (UniProt). The results of such analysis are shown in Table 2. For illustration, the TM regions are marked by bold in II this Table. These data illustrate the fact that the TM fragments of rather similar length are separated by peptide loops of different length.

As in most of the GPCR-s of this subfamily, there are the following conserved groups in this sequence (Schulz and Schoneberg, 2003). Firstly, the Glu in the TM2 and Arg at the N-terminal part of IL2, which are involved in receptor activation and coupling to G-proteins. Secondly, Cys residues in EL1 and EL2 for formation of a disulfide bond. Finally, several hydrophobic residues in TM6 and TM7 are recognized to be important for functionally active conformation of the protein.

Finally, there are several posttranslational modification sites in human $P2Y_1$ receptor (see in: UniProt). The potential N-glycosylation sites are at Asn residues at positions 11, 27, 113 and 197. The disulfide bond formation can be predicted between amino acids 124 and 202. Finally the potential phosphorylation sites can be at Ser residues 258 and 336 (protein kinase A) and

additionally at 343 (protein kinase C and calmodulin-dependent protein kinase), but also at Thr residues 330 and 339 (protein kinase C).

Table 1. The primary structure of $P2Y_1$ receptor of human origin (Ayyanathan et al, 1996; UniProt). Amino acids of the predicted transmembrane helices are shown in bold.

MTEVLWPAVP	NGTDAAFLAG	PGSSWGNSTV	ASTAAVSSSF	KCALTKTGFQ	50
FYY**LPAVYIL**	**VFIIGFLGNS**	**VAIW**MFVFHM	KPWSGISV**YM**	**FNLALADFLY**	100
VLTLPALIFY	YFNKTDWIFG	DAMCKLQ**RFI**	**FHVNLYGSIL**	**FLTCISA**HRY	150
SGVVYPLKSL	GRLKKK**NAIC**	**ISVLVWLIVV**	**VAISPILF**YS	GTGVRKNKTI	200
TCYDTTSDEY	LRSYFIYS**MC**	**TTVAMFCVPL**	**VLILGCYG**LI	VRALIYKDLD	250
NSPLRRKSIY	LVIIV**LTVFA**	**VSYIPFHVMK**	**TMNLR**ARLDF	QTPAMCAFND	300
RVY**ATYQVTR**	**GLASLNSCVD**	**PILYFLAG**DT	FRRRLSRATR	KASRRSEANL	350
QSKSEDMTLN	ILPEFKQNGD	TSL			373

Table 2. The predicted transmembrane, extracellular and cytoplasmic domains of $P2Y_1$ receptor of human origin (Ayyanathan et al, 1996; UniProt)

Predicted domains	Begins at position	Ends at position	Length
Extracellular (N-terminal)	1	52	52
TM1	53	74	22
Cytoplasmic (IL1)	75	87	13
TM2	88	109	22
Extracellular(EL1)	110	126	17
TM3	127	147	21
Cytoplasmic (IL2)	148	166	19
TM4	167	188	22
Extracellular(EL2)	189	218	30
TM5	219	238	20
Cytoplasmic (IL3)	239	265	27
TM6	266	285	20
Extracellular(EL3)	286	303	18
TM7	304	328	25
Cytoplasmic (C-terminal)	329	373	45

It can be assumed that the helices forming the TM bundle in $P2Y_1$ are tilted relative to one another in a manner, similar to rhodopsin structure. However, the latest computational refinements of the $P2Y_1$ structure have revealed rather specific and unique topology of the human $P2Y_1$ receptor, specifically influenced by its amino acid sequence (Major and Fischer, 2004). In this sequence 14 Pro residues can be found and seven of these residues are located in TM helical regions. These Pro residues stabilize kinks in most of the TM helices, as illustrated in Figure 2. The greatest kinks can be predicted for TM

helices 6 and 7, while TM3 is entirely straight. It is interesting that the Pro residues in TM4 to TM7 are highly conserved in the GPCR family. As these conserved Pro residues are located in the helices that form the binding site in rhodopsin (Palczewski et al, 2000), it can be suggested that the possible ligand binding site in $P2Y_1$ is located in the same region (Moro et al 1998). On the other hand, the Pro residues in TM1 and TM2 are specific to the human $P2Y_1$.

Secondly, eight of 19 Gly residues, which introduce conformational flexibility into the protein structure, can be found in TM regions of the human $P2Y_1$ receptor. Two residues are located in each of TM1, TM3, and TM5 and one in each of TM2 and TM7. Other Gly residues are located close to the ends of the TM helices and probably add flexibility to the helix-loop connections (Major and Fischer, 2004).

Following the spatial structure of rhodopsin, it may also be suggested that the TM domains, formed by alpha helices, bundle together clockwise if viewed from the cytoplasm (Figure 2). Four helices (TM1, TM2, TM3 and TM5) probably span as they cross the membrane, three helices (TM4, TM6 and TM7) are oriented more perpendicularly to the plane of the membrane. In addition to the seven TM helices also a small intracellular helix (H8) of amphiphilic character can be described at the C terminal part of the receptor (Figure 2). It has been suggested that this extracellular helix may be involved in interaction with G-proteins (Hamm, 2001).

The bundle structure of the GPCR-s is stabilized by sum of intermolecular and intramolecular interactions. In general, as the TM helices are located in the membrane in antiparallel way, and their dipole moments are relatively large, the bundle structure is stabilized electrostatically. In addition, several clusters of polar and hydrophobic interactions stabilize the receptor structure from inside of the bundle. In these interactions amino acids of different TM helices are participating. Most often these amino acids are conserved within the GPCR family that explains the similarity of all of these structures (Major and Fischer, 2004). For example, the conserved residues Asn69 (TM1), Asp97 (TM2), and Asp320 (TM7) are involved in one cluster of interactions, located approximately in the middle of the receptor molecule. Other inter-helical interactions include Tyr324 (TM7) and Ser87 (TM2) interacting through a bridging water molecule and the conserved Asn92 (TM2) and Trp176 (TM4) residues interacting through H-bonds. The presence of these interactions is vitally important to retain the active receptor structure.

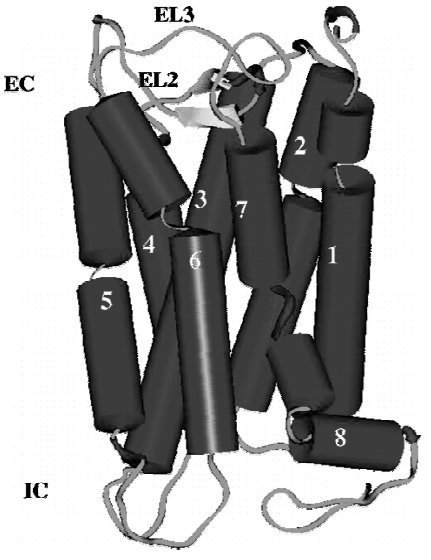

Figure 2. Model of human $P2Y_1$ receptor as proposed by Major and Fischer (2004).

Today the classical GPCR model, based on rhodopsin structure, has been refined by different computational methods, taking into account the influence of the hydrated lipid bilayer of biomembrane (Major and Fischer, 2004). Therefore several details were added into the list of the factors stabilizing the $P2Y_1$ receptor structure. Firstly, the human $P2Y_1$ receptor has many polar and charged residues exposed to the surface of the biomembrane. These residues interact with the polar groups of the lipids on the biomembrane-water interface and anchor the receptor molecule within the membrane via electrostatic and polar interactions. Amino acids involved in these interactions include Trp, Tyr, Lys, and Arg residues. All these strong interactions enhance the stability of the helical bundle in the membrane environment. Secondly, the TM bundle is stabilized by numerous hydrophobic interactions between hydrophobic amino acids, which residues are oriented towards the hydrophobic phase of the lipid membrane.

The extracellular and intracellular loops as well as the C-terminal polypeptide of $P2Y_1$ receptor contain many charged amino acid residues. However, the multitude of positively charged residues localized in the extracellular part of human $P2Y_1$ receptor seems to be its specific feature, and these positively charged residues in extracellular loops may play directing role in the process of binding of negatively charged nucleotide into the receptor binding site (Moro et al, 1999).

1.3. Specific ligands of $P2Y_1$ receptor

The $P2Y_1$ receptor responds to adenine nucleotides and ADP is recognized as its endogenous agonist. Activity of this ligand was characterized by EC_{50} value 0.06 μM at human $P2Y_1$ receptor (Sak et al, 2000b). Besides this diphosphate several ADP analogs are also in use to activate this receptor. But the list of these ligands, published by IUPHAR Receptor Database (IUPHAR Receptor Database), is rather short and involves the compounds listed in Table 3. In this table also the EC_{50} values for these agonists for human $P2Y_1$ receptor are given, and structures of some of these compounds are illustrated in Figure 3. It is clear that all of these ligands are rather closely related to the structure of ADP. Certainly, more ligands have been investigated on this receptor, and significant part of these data were collected and systematized in the P2Y-Receptor-Ligand Database (Sak et al, 2000), which upgrading has, however, stopped some time ago.

It can be observed in Table 3 that the agonists of $P2Y_1$ receptor listed are mostly not very potent. Modification of 2-MeSATP has yielded one of the most potent agonists, PAPET, which activates $P2Y_1$ receptor with EC_{50} value around 1 nM (Fisher et al, 1993). However, this ligand is not selective against the $P2Y_1$ receptor subtype. This is important because besides $P2Y_1$ ADP is also the cognate agonist of $P2Y_{12}$ and $P2Y_{13}$ receptors. Therefore the problem of finding of a high potency agonist that selectively activates the $P2Y_1$ receptor has been investigated by several groups, but solution was found only recently, when the conformationally constrained non-nucleotide analogue (N)-methanocarba-2MeSADP, abbreviated as MRS 2365, was proposed. In this type of compounds the fused cyclopropane and cyclopentane rings replace the ribose moiety (Kim et al, 2002). Although 2-MeSADP is a potent agonist at all three receptors, $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$, MRS 2365 was an extremely potent agonist at the $P2Y_1$ receptor, characterized by the EC_{50} value 1.2 nM. (Chhatriwala et al, 2004). This non-nucleotide analogue exhibited no agonist activity at the $P2Y_{12}$ receptor and very low activity at the $P2Y_{13}$ receptor. MRS 2365 was also unable to block the action of 2-MeSADP at the $P2Y_{12}$ and $P2Y_{13}$ receptors. Thus, MRS 2365 is the first high affinity agonist that discriminates among the three ADP-activated P2Y receptors, and therefore, may introduce new pharmacological tool for investigation into the biological action of these three signaling proteins.

Search for effective and selective antagonists for P2Y receptors has been even more intensive, keeping in mind the potential application of these compounds as drugs. For example, it can be suggested that a selective $P2Y_1$ antagonist may be useful as an antithrombotic agent. Therefore, besides the traditional drug-design approaches, where systematic modification of all structural elements of nucleotide structure, including the adenine ring (base), sugar moiety and polyphosphate group, were tested, the more contemporary site-directed mutagenesis and protein modeling approaches have been used to design better antagonists for $P2Y_1$ receptor. Besides the pharmacological significance also the practical need for reliable tools for receptor research has

driven these studies. As a result of this work, some selective $P2Y_1$ antagonists were proposed.

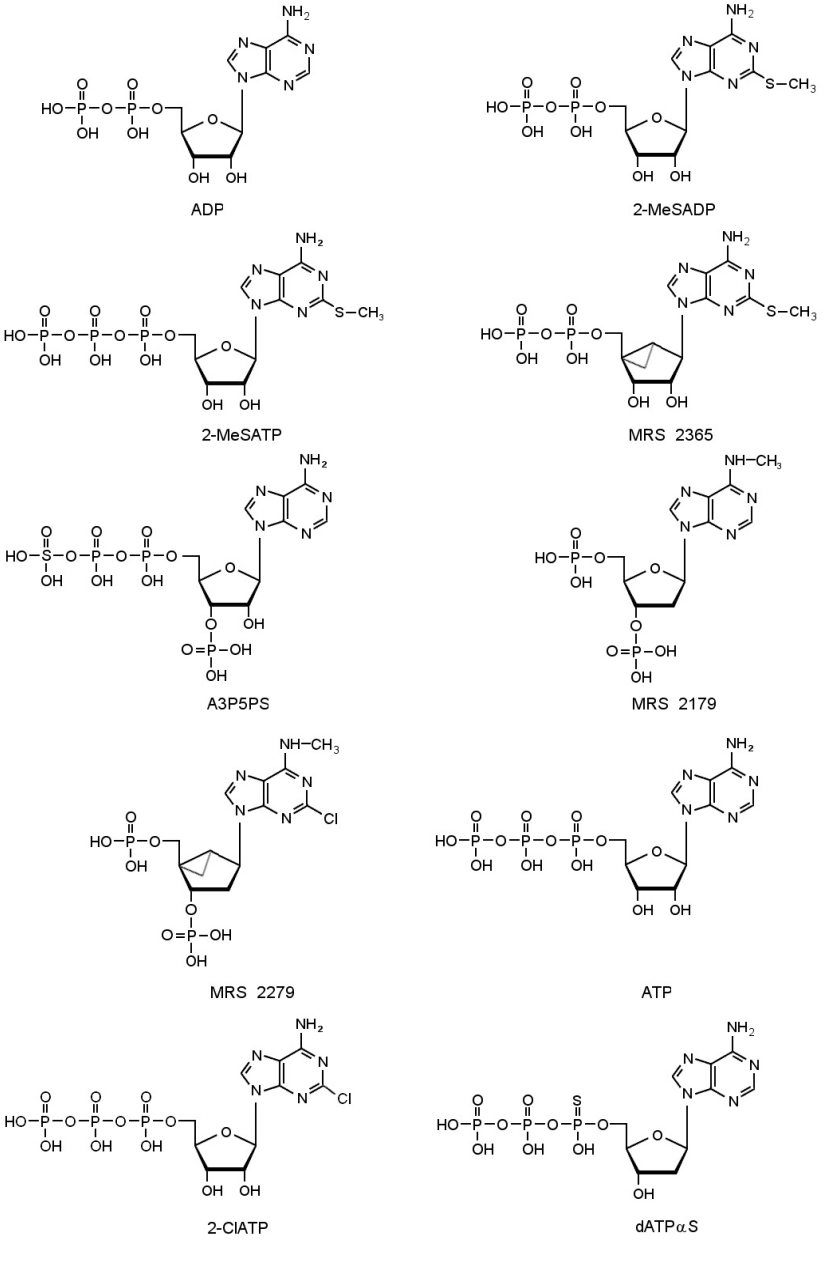

Figure 3. Structures of some ligands, interacting with $P2Y_1$ receptor. Activity of these ligands is characterized in Table 3.

Table 3. Some ligands, interacting with human $P2Y_1$ receptor.

Agonists	**EC_{50}**	**Reference**	**Selectivity against human $P2Y_1$ receptor**
ADP	60 nM	Sak et al, 2000b	No
2-MeSADP	15 nM	Sak et al, 2000b	Yes
2-MeSATP	0.1 μM	Palmer et al, 1998	No
PAPET	1.2 nM	Fisher et al, 1993	No
MRS 2365	1.2 nM	Chhatriwala et al, 2004	Yes
Antagonists	**IC_{50}**		
A3P5PS	0.35 μM	Boyer et al, 1996	No
MRS 2179	0.1 μM	Baurand et al, 2001	No
MRS 2269	1.64 μM	Brown et al, 2000	Yes
MRS 2279	8 nM	Boyer et al, 2002	Yes
MRS 2286	0.84 μM	Brown et al, 2000	Yes
ATP	14 μM	Hechler et al, 1998	No
2-MeSATP	5.7 μM	Hechler et al, 1998	No
2-ClATP	2.3 μM	Hechler et al, 1998	No
dATPαS	7 nM	Simon et al, 1995	Yes

Initially it was found that adenosine 3',5'-bisphosphate and 2',5'-bisphosphate and their derivatives, like A3P5PS, antagonize $P2Y_1$ receptor (Boyer et al, 1996). For example, A3P5PS is a competitive $P2Y_1$ receptor antagonist with the IC_{50} value 0.35 μM. Later, series of deoxyadenosine 3',5'-bisphosphate derivatives (MRS series) were designed and synthesized. Among these compounds $P2Y_1$ is antagonized by MRS 2179 and MRS 2279 and MRS 2216, while another member of the same group, MRS 2365, was found to be an agonist (Jacobson et al, 2004). From this series of compounds MRS 2179 is now recognized as selective $P2Y_1$ antagonist and is available commercially.

Recently an attempt was made to use one of these compounds, [^{3}H]MRS 2279, for radioligand binding experiments with the $P2Y_1$ receptor (Waldo et al, 2002). This antagonist is selective against this subtype and has low non-specific binding on cell membranes. However, this assay has to be carried out in ice-water bath, and even then the off-rate of this ligand from its complex with the receptor was relatively fast (half-life 0.95 min). This means that application of ice-cold buffer could not be used to avoid the loss of the specifically bound radioligand during the dilution, filtration and washing steps of the binding assay and should lead to underestimated receptor content. Therefore, any other radioligands, which have slower rate of dissociation from the receptor complex, may still have preference in this kind of assay. In this dissertation we have studied the applicability of [^{35}S]dATPαS, which is still the only radioligand for $P2Y_1$ receptor assay, mentioned in the IUPHAR Receptor Database (IUPHAR Receptor Database).

[^{35}S]dATPαS was indeed used in several attempts to radiolabel the native $P2Y_1$ receptors in brain (Simon et al, 1995) and recombinant receptors expressed in cultured cells (Schachter and Harden, 1997). Although the non-specific binding of this radioligand with the cellular membranes was low, a more thorough analysis has suggested that most of the detected binding sites belonged to various endogenous ATP-binding proteins distinct from the receptor sites (Schachter and Harden, 1997). The presence of such extensive additional binding interfered with radioligand binding assay of this nucleotide receptor.

This complication seems to have a rather general background for binding assay of nucleotide receptors, until the ligands in use remain structurally related to naturally occurring nucleotides, which should possess a large number of various binding sites on cellular membranes. Therefore it was interesting to test the possibilities of distinction of the nucleotide receptors from other binding sites by using kinetic approach instead of the equilibrium binding study. This analysis is based on special feature of interaction of antagonists with GPCR, the phenomenon of receptor-ligand complex isomerization, which is most probably related to some specific dynamic properties of the receptor structure.

2. DYNAMICS OF GPCR STRUCTURE

The characteristic feature of GPCR structure is the presence of the "central core", which is formed by the bundle of the TM helices. This part of the receptor molecule most probably accommodates ligand-binding site, by analogy with the localization of the retinal residue in rhodopsin structure. Therefore it is generally recognized that activation of the receptor is accompanied by change in conformation of the "core domain" and this change affects thereafter the conformation of the intracellular loops, used for G protein recognition and activation. In the receptors for small ligands (group 1a of GPCR-s) the specificity of interaction with G-proteins is governed mainly by the IL3, but other cytoplasmic loops may be involved in the case of other receptor types. To ensure the possibility of cascade of these necessary conformational changes the structure of the "core" domain seems to be very dynamic, as conformational fluctuations on the micro- to millisecond time scale can be observed for the amide groups of the peptide backbone of the TM helices (Major et al, 2004a; Klein-Seetharaman et al, 2004). This dynamic plasticity seems to be supported by the protein structure, which contains several Gly residues. At the same time the movement of the side chains of several other amino acid is rather restricted, pointing to the fact that some parts of the structure is rigidly fixed.

It is interesting to mention that in peptide receptors (group 1b) or GPCRs for glycoprotein hormones (group 1c) the ligand-binding site includes also the N-terminal domain, the extracellular loops and the superior parts of TM helices. Although the ligand-binding site of these receptors has mostly extracellular location, the appropriate loops have still contact with the TM fragments and probably govern through these structures the conformation of the cytoplasmic loops interacting with G-proteins. This means that the mechanism of the signal transduction could be very similar even in different types of GPCR.

In fact, the idea of common molecular mechanism of the receptor activation by agonists has been initiated by the observation that the core structure of these transmembrane proteins is significantly conserved (Karnik et al, 2003; and other refs to the text above), and was further supported by the results of the evolutionary trace analysis (Madabushi et al, 2004), as well as by similar structure — function relationships for the constitutively activated receptor mutants (Parnot et al, 2002).

Today the movement of the TM helices in response to receptor activation has been documented in the case of rhodopsin by using the site-directed spin labeling experiments (Gouldson et al, 2004; Hubbell et al, 2003; Hubbell et al 2000), were the spin markers were linked with the receptor molecule. These experiments indicated that rhodopsin activation results in a 30° clockwise rotation of TM6, which moves away from TM3. Similarly, the movement of the TM3 and TM7 fragments was observed. Thus TM2, TM3, TM6 and TM7 move apart, whereas TM1, TM4 and TM5 have more fixed positions.

Agonist-induced conformational changes were also studied in the case of other GPCR-s, but most often with beta2-adrenergic receptor, modified with selectively planted spin labels (Gouldson et al, 2004) or fluorophores (Gether et al, 2002; Dunham and Farrens, 1999). This analysis has revealed that agonist binding, indeed, induced disruption of intramolecular interactions between TM3 and TM6 and leads to a conformational change (rotating and/or tilting) of TM6 relative to the rest of the receptor molecule. It was suggested that this could be an evolutionary conserved activation mechanism, characteristic for agonist interaction with various GPCR-s (Gether et al, 2002).

The same spectroscopic methods, including real-time monitoring of receptor conformation by fluorescence-based analysis, were used to follow the time course of the receptor activation process. It has been found that the protein conformation may change passing different states, and these processes can be characterized by different half-lives. It has been suggested that agonists initiate in different receptors similar structural transition with half-life approx 2 s (Swaminath et al, 2004; Vilardaga et al, 2003). In parallel, another kinetically distinguishable conformational change can be observed with half-life of 70 s (Swaminath et al, 2004). The main conclusion from these studies seems to be the possibility that agonists may trigger off similar movement of the transmembrane helices in diverse GPCR-s, altering conformation of the second and the third intracellular loops, the key sites for activating G-proteins.

Although the classical view on GPCR assumes functioning of a single receptor, many studies have revealed that they can form dimers or even higher order complexes (Moepps and Fagni, 2003). For example, dimerization and formation of even larger oligomeric complexes, organized in paracrystalline arrays, was found in the case of the prototypical GPCR rhodopsin in its native membrane by electron and atomic force microscopy (Fotiadis et al, 2004). This means that properties of all proteins belonging to the superfamily of GPCR, including the $P2Y_1$ receptors, should also be analyzed proceeding from this possibility. Increasing evidence suggests that a dimer may be the minimal functional structure, but considerable variation exists between reports on the effects of agonist ligands on quaternary structure of GPCR-s. Recent analysis has revealed that agonist binding with one metabotropic glutamate (mGlu) receptor in dimeric structure of this receptor is sufficient to activate the receptor, but the agonist binding with both receptors is required for its full activity (Kniazeff et al, 2004). This finding is in agreement with another conclusion that subunits of GPCR in complex can be activated by agonist independently rather than cooperatively, but the concerted action of subunits is important to promote G protein activation, possibly by contacting different subunits or regions of the G protein heterotrimer (Chinault et al, 2004). This means that ligand-binding properties of receptor subunit may be less affected by dimerization than the receptor responses initiated through this binding. But even in this case it can be concluded that the dynamics properties of GPCR, especially in relation to ligand binding and receptor activation phenomena, may be of crucial role for understanding the molecular mechanism of signal transduction.

3. MATERIALS AND METHODS

3.1. Chemicals

[^{35}S]dATPαS was purchased from Amersham and its purity was analyzed on PEI TLC plates by using 1 M acetic acid — 4 M LiCl (8:2, v/v) as eluent (Randerath and Randerath, 1964). dATPαS was purchased from Amersham, other non-radioactive nucleotides were obtained from Boehringer Mannheim. The non-radioactive nucleotides were analyzed and purified before their use by HPLC (Gilson) on an anion exchange column Mono Q (Pharmacia Biotech). Absorbance was monitored at 258 nm. Linear gradients from 0 to 1 M NaCl in 40 mM phosphate buffer (pH=7.0) and from 0.1 to 1.2 M ammonium carbonate in water at flow rate 1 ml/min were used. The latter gradient was used for preparative purification of nucleotides. The purity of nucleotides used in the assays was never less 99.5%. Other chemicals were of analytical grade or higher and were used without additional purification.

3.2. Chromatographic analysis of [^{35}S]dATPαS decomposition products

The same chromatographic system, using PEI TLC plates and 1 M acetic acid - 4 M LiCl (8:2, v/v) as eluent was used for investigation into kinetics of decomposition of [^{35}S]dATPαS in the presence of membrane fragments of hP2Y$_1$-1321N1 cells and rat brain, by analyzing aliquots of the assay mixture, taken at appropriate time interval. The di- and monophosphates formed from [^{35}S]dATPαS were detected by radioactivity.

3.3. 1321N 1cell membrane fragments

The transfected astrocytoma hP2Y$_1$-1321N1 cells were kindly donated by Professor Kunapuli (Department of Physiology, Temple University Medical School, Philadelphia, USA). The cells were grown in Dulbecco`s Modified Eagle`s Medium (Gibco Ltd.) supplemented with 10% (v/v) of fetal calf serum, tylosine (8 μg/ml) and geneticin (400 μg/ml) (Gibco BRL). Cultures were kept at 37^{0}C in a humidified atmosphere (95% air and 5% CO_2) in 75-cm^2 tissue culture dishes. For preparation of the membrane fragments the cells were washed with 50 mM Tris-HCl buffer, containing 1 mM EDTA and 1 mM EGTA, pH 7,4 (buffer A). The cells were harvested in the same buffer, containing also 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride, 0.01%(w/v) bacitracin, 0.001% soybean trypsin inhibitor, 40 unit of kallikrein

inhibitor. The freeze-thaw method for cell lysis was used in combination with homogenization (Ultra-Turrax J-25, 20 s, maximum speed). This procedure was repeated two times and membrane fragments were centrifuged for 30 min at 25000g and 4^{o}C. Pellets were washed twice by gentle re-suspension in buffer A and centrifuged (25000g, 30 min). Finally the membranes were rapidly frozen and stored at –80^{o}C.

3.4. Membrane fragments of rat brain

Brains of 7 days old rats were homogenized in ice-cold 50 mM Tris HCl buffer (pH 7.4) and centrifuged for 10 min at 1500g and 4^{o}C. The supernatant was decanted and centrifuged (27000g, 40 min) and the pellet was re-homogenized in the same buffer (1.5 ml per brain) and stored at –80^{o}C.

3.5. Determination of protein concentration

Dye binding method (Peterson, 1983) was used for protein determination throughout the study.

3.6. Equilibrium binding of [^{35}S]dATPαS with membrane fragments

Radioligand binding with the membrane fragments was studied at concentration interval from 2 to 250 nM. The assays were initiated by addition of the radioligand stock solution to membrane suspension (final volume 0.5 ml, 50 mM Tris-HCl buffer, pH 7.4, 0.09–0.1 mg protein/ml). The samples were gently agitated at 25^{0}C (20 min) and the assay was terminated by addition of 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and immediate filtering of the samples over the glass-fiber filters (GF/B, Whatman), presoaked with the washing buffer. Thereafter the filters were washed twice with 5 ml of the ice-cold buffer and the filter-bound radioactivity was counted (scintillation cocktail Ecoscint A™ ICN) on a Beckman LS 1801 liquid scintillation counter at counting efficiency 95%. Before counting the filters were kept in the scintillation cocktail at least 12 h. Separate experiments were made with different amount of the washing buffer according to the protocol described below. The non-specific binding of [^{35}S]dATPαS was determined in the presence of 1 mM or 2.5 mM ATP, added to the membrane fragments 5 min before the radioligand.

3.7. Kinetics of [^{35}S]dATPαS association

The reaction was started by addition of [^{35}S]dATPαS into the membrane suspension (final volume 7 ml, 50 mM Tris-HCl buffer, pH 7.4, 25°C, protein concentration 0.09–0.1 mg/ml). At the appropriate time moments 0.5 ml aliquots of the reaction mixture were rapidly diluted in 5 ml of ice-cold washing buffer and assayed for bound radioactivity as described above.

3.8. Kinetics of dissociation of the receptor-ligand complex

The receptor-radioligand complex was prepared by pre-incubation of membranes with 50 nM [^{35}S]dATPαS (10 min, under the conditions of the binding assay). Thereafter the dissociation reaction was initiated by addition of excess of ATP (final concentration 1 mM in routine assay protocol). Aliquots were taken from this reaction mixture at the appropriate time moments, diluted with 5 ml of the ice-cold washing buffer and the membrane-bound radioactivity was determined by the filtration assay as described above.

3.9. Data processing

The binding experiments were carried out in triplicate and the data were expressed as mean ± SEM. In kinetic runs the number of experiments corresponded to the number of points shown in figures. A non-linear regression analysis program Prism™ (Version 2.00) was used for data processing. The results of calculations are given together with their standard errors (SE).

4. MAIN RESULTS AND DISCUSSION

4.1. Interaction of [^{35}S]dATPαS with cell membrane fragments

Under the equilibrium conditions a rather high level of [^{35}S]dATPαS binding above its non-specific binding level was detected with all membrane preparations used, including the wild type 1321N1 cells which do not express any nucleotide receptors (Figure 4). Moreover, it was found that a significant amount of this radioligand was bound to wild type Sf9 cell membranes, also lacking nucleotide receptors (our unpublished data). And in all cases the radioligand was very largely displaced from these binding sites by excess of the unlabelled ligand or by ATP, to give low level of non-specific binding in each case. These results are in a good agreement with the suggestion made by Schachter and Harden (1997) that biomembranes contains variety of binding sites, which are selective for nucleotides and nucleotide-like ligands, but do not belong to P2Y receptors. Therefore differentiation of these receptors from other selective binding sites is the most crucial point in development of reliable radioligand assay of these receptors.

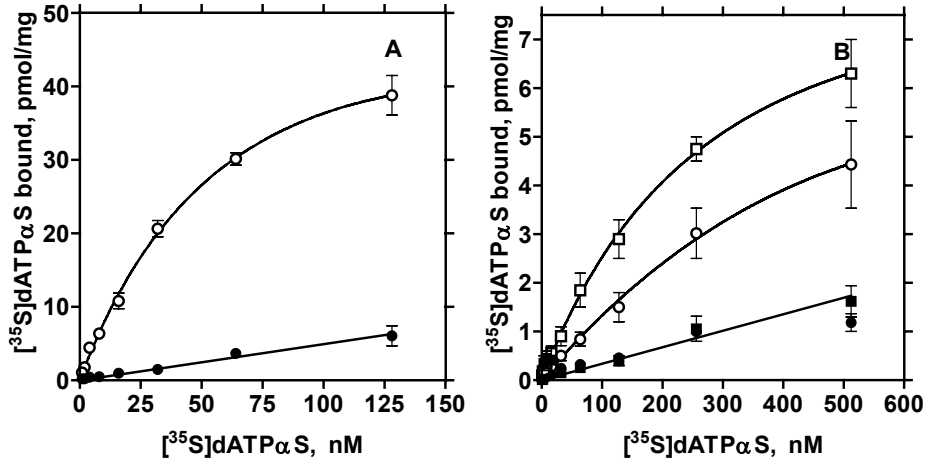

Figure 4. Binding of [^{35}S]dATPαS to membrane fragments of rat brain (left, A) and 1321N1 cells (right, B), expressing human $P2Y_1$ receptors (□) and the same wild type cells (○). Non-specific binding of the radioligand (● – brain and wild type 1321N1 cells; ■ – $hP2Y_1$-transfected 1321N1 cells) was measured in the presence of 1 mM ATP.

The orthodox way of solving this puzzle is application of radioligands, which do not resemble the nucleotide structure and are highly specific for $P2Y_1$ receptor subtype and do not interact with other ATP recognizing sites. However, all ligands proposed for this receptor until today still resemble, at least to some extent, nucleotides, even if methanocarba derivatives of MRS series are taken into consideration. Therefore the complication caused by the "wrong specific binding" seems to have a rather general background and we decided to test an alternative way, namely to distinct the nucleotide receptor sites from other nucleotide binding sites by using kinetic approach instead of the equilibrium binding study.

4.2. Kinetics of [^{35}S]dATPαS association with membrane fragments

This kinetic approach is based on discovery that association of antagonists with various GPCR-s occurs at least in two kinetically distinguishable steps. The first step is fast formation of the receptor-ligand complex RA, and the second step is slow isomerization of this complex into another complex denoted as (RA):

$$R \overset{K_A}{\rightleftharpoons} RA \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} (RA) \qquad (1)$$

The presence of this slow isomerization step can be considered as a specific feature of interaction of potent antagonists with GPCR-s (Järv and Rinken, 1993). Moreover, this slow isomerization process seems to be necessary condition for application of these compounds for relevant radioligand assay of membrane-bound receptors (Järv and Eller, 1988), and its presence has been observed besides the muscarinic receptor (Järv et al, 1979; Järv and Eller, 1988) also in the case of D2 dopaminergic (Lepiku et al, 1996) and adrenergic (Schiliebs and Bigl, 1984) receptors. In the present study the kinetic approach was extended to $P2Y_1$ subtype of nucleotide receptors by using [^{35}S]dATPαS as radioligand.

Indeed, a slow time-dependent increase in filter-attached radioactivity was observed, if [^{35}S]dATPαS was added to the membrane fragments of rat brain (Figure 5A) and the transfected astrocytoma $hP2Y_1$-1321N1 cells (Figure 5B). But in the case of wild-type 1321N1 cell membranes a constant level of the filter-bound radioactivity was reached extremely rapidly, before the first sample of the kinetic assay was filtered (approx. 10 s) (Figure 5B). Thus at least two types of binding sites, characterized by fast and slow radioligand on-rates, can be differentiated by kinetic analysis on these membranes.

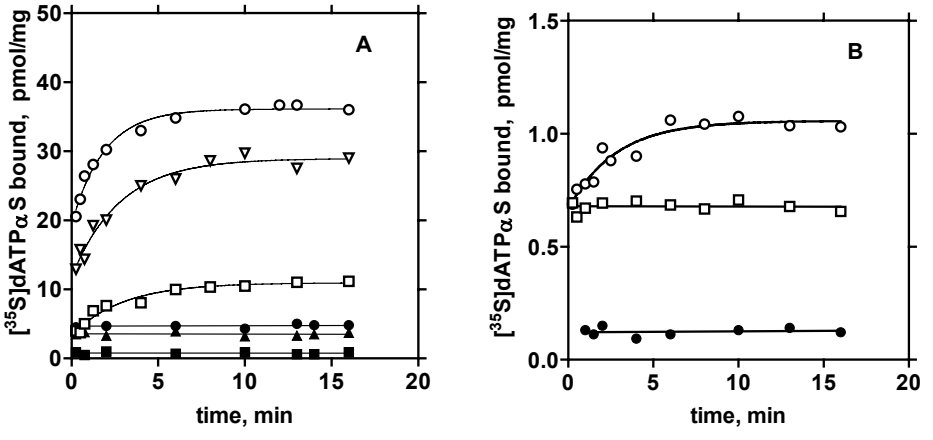

Figure 5. Time-course of [^{35}S]dATPαS association with membrane fragments of rat brain (left, A; □ — 10 nM, ∇ – 60 nM, ○ – 100 nM) and 1321N1 cells, expressing human $P2Y_1$ receptors (right, B; ○ – 25 nM) and the same wild type cells (right, B; □ – 25 nM). Data for non-specific radioligand binding are given by filled symbols.

The slow phase of [^{35}S]dATPαS binding was followed during 15 to 20 min, although the major change of the complex concentration occurred during 3–7 min. During this time interval the radioligand decoposition did not influence the kinetic experiments. Kinetics of degradation of [^{35}S]dATPαS into other phosphates in the standard assay mixtures was characterized by the first order rate constants (3.5 ± 0.3) 10^{-4} s^{-1} and (3.3 ± 0.2) 10^{-4} s^{-1} in the case of membrane fragments of $hP2Y_1$-1321N1 cells and brain, respectively. These results were independent on radioligand concentration in the reaction medium (20 or 100 nM), pointing to the fact that the ligand degradation occurred at least 10 times slower than its binding with the membrane sites.

The time-course of the [^{35}S]dATPαS association with the "slow" binding sites on $hP2Y_1$-1321N1 cell membranes was monitored until constant level of radioligand binding was confirmed (Figure 5). The kinetic data were analyzed by the first-order rate equation:

$$B_t = B_0 + B_{sp}\left[1 - \exp(-k_{obs}t)\right] \qquad (2)$$

where B_t is the amount of bound radioligand per mg membrane protein at time t. The rate constant k_{obs}, as well as the plateau and intercept values B_{sp} and B_0, referring to the slow phase only, were calculated by fitting to this part of the B_t vs. t plot. No systematic deviation of the experimental data from the calculated curve was observed, pointing to the fact that a kinetically homogeneous

population of the "slow" sites of specific binding was quantified by the B_{sp} value.

Although the kinetic properties of the "fast" sites cannot be analyzed by the filtration assay, the difference between B_0 and the radioligand non-specific binding should represent the amount of those sites trapped in the filter. This comparison revealed that the amount of the "fast" sites, calculated relative to the protein content, was essentially the same on the transfected and the wild-type 1321N1 cells, as can be seen at the initial time point in Figure 5B.

4.3. Kinetics of receptor-radioligand complex dissociation

Radioligand dissociation from its complex with membrane-bound binding sites was initiated by addition of excess of unlabelled ATP into the assay mixture. This resulted in a fast drop of the membrane-bound radioactivity, followed by a slow phase of the complex dissociation (Figure 6). Thus at least two distinct fractions of the binding sites can also be observed in the time-course of radioligand dissociation from its complex with membranes. The half-life of the fast "phase" was too short for meaningful kinetic analysis by the filtration assay, while the following part of the process was well described by the rate equation:

$$B_t = B_{ns} + B_{sp} \exp(-k_{off} t) \qquad (3)$$

where B_t is the amount of the membrane-bound ligand at time t, B_∞ is the final level of the radioligand binding, extrapolated to the end of the dissociation reaction, and B_0 is the amount of the complex which dissociation was characterized by the rate constant k_{off}. It has been found that this dissociation kinetics was not affected by increase in ATP concentration from 1 to 2.5 mM.

The same radioligand displacement experiment was done with the wild-type cell membranes. In this case the membrane-bound radioactivity dropped immediately to the level of the non-specific binding, and therefore the time-course of this process could not be monitored by the filtration assay.

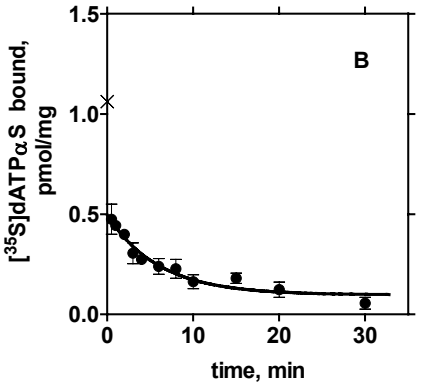

Figure 6. Time-course of dissociation of [^{35}S]dATPαS complex with receptor sites on hP2Y$_1$-1321N1 cell membrane fragments. The dissociation reaction was initiated by excess of ATP (1 mM).

4.4. Kinetic mechanism of [^{35}S]dATPαS binding with the "slow" binding sites

The plot of k_{obs} *vs* [^{35}S]dATPαS concentration was hyperbolic for both membrane preparations, obtained from transfected hP2Y$_1$-1321N1 cells (Figure 7, left) and rat brain (Figure 7, right). This hyperbolic plot gives evidence for the two-step mechanism of ligand association reaction, including the slow isomerization of the receptor-ligand complex (see Eq. 1). This isomerization mechanism was previously analyzed in detail by Stricland et al (1975) and can be described by the following rate equation:

$$k_{obs} = \frac{k_i[A]}{K_A + [A]} + k_{-i} \qquad (4)$$

The kinetic constants k_i, K_A and k_{-i} for the transfected cells and rat brain are listed in Table 4.

The rate constant k_{-i} determined from the intercept of the k_{obs} *vs* [A] plot can be compared with the rate constant of radioligand displacement (k_{off}), as de-isomerization of the complex is the rate-limiting step of the overall dissociation process. It can be seen in Table 4 that k_{-i} and k_{off} have indeed close values.

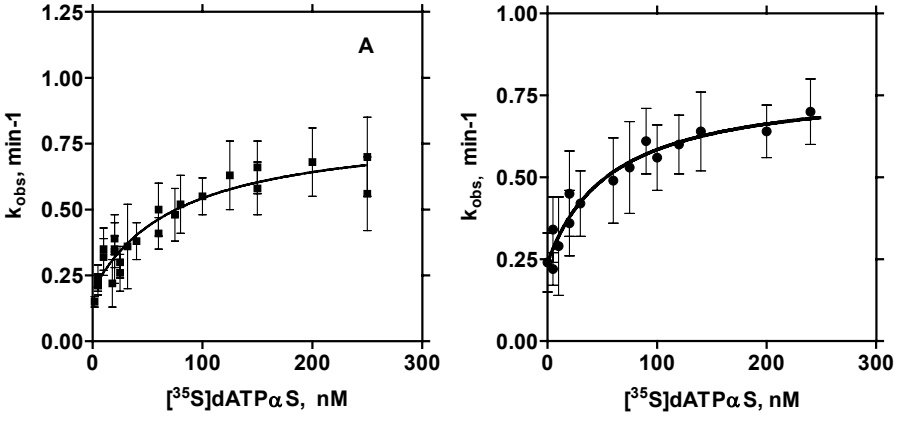

Figure 7. Dependence of the observed rate constant k_{obs} for [^{35}S]dATPαS association with membrane fragments of rat brain (A) and hP2Y1-1321N1 cells (right) on radioligand concentration.

TABLE 4. Results of kinetic analysis of [^{35}S]dATPαS interaction with specific binding sites on hP2Y$_1$-1321N1 and rat brain membrane fragments.

Parameters	hP2Y$_1$-1321N1 cell membrane fragments	Rat brain membrane fragments
k_i	$(9.0\pm0.8)10^{-3}$ s^{-1}	$(1.1\pm 0.1)10^{-2}$ s^{-1}
K_{-i}	$(3.9\pm0.7)10^{-3}$ s^{-1}	$(3.4\pm0.5)10^{-3}$ s^{-1}
K_A	59±16 nM	99±29 nM
k_{off}	$(2.8\pm 0.7)10^{-3}$ s^{-1}	$(3.2\pm 0.6)10^{-3}$ s^{-1}
K_d (from kinetic experiments)	31±8 nM	12±5 nM
B_{max} (from kinetic experiments)	2.5±0.2 pmol/mg protein	20±2 pmol/mg protein

The radioligand binding capacities B_0 and B_{sp} were calculated from kinetic curves of radioligand association with both hP2Y$_1$-1321N1 and rat brain membranes, monitored at different [^{35}S]dATPαS concentrations. The former parameter corresponded to the "fast" phase of the binding process, including the contribution of the non-specific binding. As the B_o values were significantly depending on conditions of the filtration assay, their detailed discussion was meaningless.

4.5. Affinity of the "slow" binding sites for [^{35}S]dATPαS

The B_{sp} values were used for quantification of the "slow" fraction of the binding sites. As these parameters characterize the radioligand binding with the "slow" sites at the end of the association reaction, i.e. under conditions where the equilibrium of the binding process was achieved, the plot of B_{sp} *vs* [^{35}S]dATPαS concentration was analyzed according to a binding isotherm (Figure 8):

$$B_{sp} = \frac{B_{max}[A]}{K_d + [A]} \quad (5)$$

where B_{max} was the maximal amount of the "slow" binding sites detected at ligand concentration [A] and K_d was the appropriate dissociation constant. The K_d and B_{max} values calculated from kinetic data were listed in Table 4.

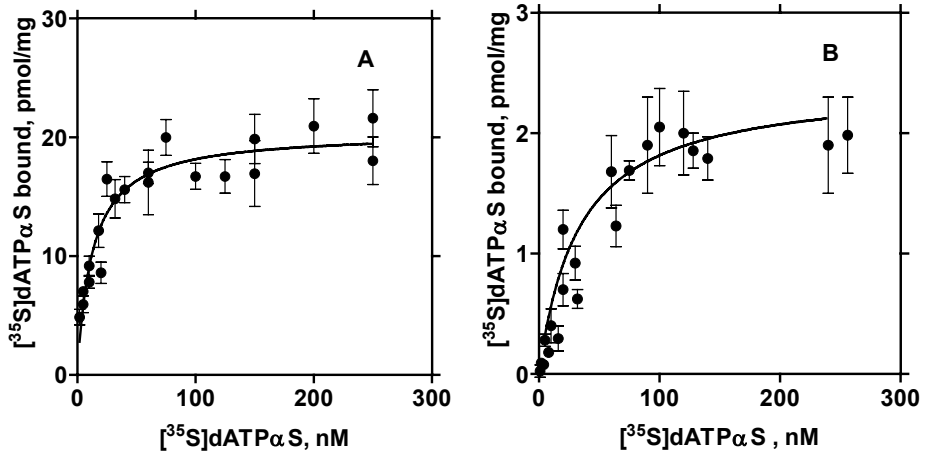

Figure 8. Binding isotherms for specific interaction of [^{35}S]dATPαS with the "slow" binding sites on membrane fragments of rat brain (left, A) and hP2Y$_1$-1321N1 cells (right, B), calculated from kinetic experiments.

In summary, in the present study we were able to distinguish and quantify a part of [^{35}S]dATPαS binding sites, localized on membranes of 1321N1 cells expressing P2Y$_1$ receptors, but also on brain membranes, by means of kinetic analysis, as both on-rate and off-rate of the radioligand were slow enough to be followed by means of the filtration assay. In both cases the slowness of on-rate and off-rate was related to the two-step receptor-ligand interaction mechanism

(1), where the fast receptor-ligand complex formation was followed by a slow isomerization step.

As this two-step binding mechanism has been found to be characteristic for GPCR-s (Järv et al, 1979; Schiliebs and Bigl, 1984; Lepiku et al, 1996), it was suggested that the "slow" binding sites, traced by [^{35}S]dATPαS, also belong to receptors of the same superfamily, particularly to $P2Y_1$ subtype, expressed in the cell-line used.

This assumption was supported by the fact that the "slow" binding sites were observed specifically in the case of the h$P2Y_1$-1321N1 cell line and they behaved as a homogeneous population in all kinetic experiments. In contrary, the membranes of the wild type cells revealed only the "fast" component of the radioligand binding.

Secondly, it was important that the radioligand used was rather specific for $P2Y_1$ receptor subtype if compared with other P2Y receptor subtypes. This drug had neither agonistic nor antagonistic effects at the $P2Y_4$ and $P2Y_6$ subtypes and was a weak agonist at $P2Y_2$ subtype with the EC_{50} value 21 μM (Schachter and Harden, 1997). In the case of $P2Y_1$ receptors dATPαS behaved as competitive antagonist (Schachter and Harden, 1997), interacting with the receptor sites at nanomolar concentration range.

The "slow" binding of [^{35}S]dATPαS on rat brain membranes was kinetically very similar to the putative receptor sites on the transfected cells. Moreover, these "slow" binding sites behaved as a homogeneous population, pointing to the possibility that the same $P2Y_1$ receptor sites were traced by kinetic experiments on brain membranes. At the same time the maximal radioligand binding capacity B_{max} was rather different for these two preparations (Table 4). Following the two-step drug-receptor interaction mechanism the equilibrium between the isomerized and non-isomerized complexes is quantified by the dissociation constant K_i ($K_i = k_{-i}/k_i$). The kinetic data listed in Table 4 revealed that the K_i values for the "slow" binding sites on h$P2Y_1$-1321N1 cell membranes and brain membranes were rather close, 0.5 and 0.3, respectively. As the non-isomerized complexes most probably escape detection by the filtration assay (Järv and Eller, 1988), the relationship between the observed B_{max} value and the genuine amount of the binding sites on membranes is determined by the equilibrium constant K_i (Järv et al, 1979). In the present case it can be estimated that approx. half of the putative receptor sites on h$P2Y_1$-1321N1 cell membranes and 2/3 of the same sites on brain membranes were quantified by [^{35}S]dATPαS. Thus the membrane preparations investigated have indeed different density of the "slow" nucleotide binding sites.

The isomerization equilibrium determines also the effectiveness of receptor-drug interaction, characterized by the dissociation constant K_d. Following the two-step reaction scheme (1) this parameter can be presented as a combination of the constants K_A and K_i: $K_d = K_A K_i$ (Strickland et al, 1975). Proceeding from the results listed in Table 4 the K_d values 19 and 22 nM were calculated

from the kinetic data for the hP2Y$_1$-1321N1 cell membranes and brain, respectively. These results were rather close with the appropriate K_d values 31 and 12 nM, evaluated from the B_{sp} *vs* ligand concentration plots (Figure 8).

The apparent affinity of [^{35}S]dATPαS against the putative P2Y$_1$ receptor sites, estimated from the kinetic data of radioligand binding, was somewhat lower if compared with affinities of other typical radioligands, used for assay of GPCR-s. On the other hand, not affinity alone, but primarily the kinetic aspects of the drug-receptor interaction determine the applicability of any compound in receptor-radioligand analysis. Proceeding from the results of the present study it can be concluded that [^{35}S]dATPαS satisfied well these kinetic criteria until its interaction with the receptor sites is concerned and kinetic analysis is used for distinction of these sites from other nucleotide binding sites of non-receptor origin.

4.6. Similar dynamics of GPCR molecules in response to antagonist binding

GPCR-s as the evolutionary "oldest" transmembrane proteins devoted to transmission of "outside" signals to "inside" of the living cell demonstrate significant structural similarity concerning the "central core" of the seven transmembrane helical domains. This similarity, in turn, points to the possibility that the mechanism of the receptor protein responses on the external signal might also be somewhat conserved. This means that the signal transmission might consist of similar structural rearrangements of the transmembrane helixes and the connecting loops. Indeed, for several receptors and for rhodopsin in particular, similar structural rearrangements of the "central core" accompanying the receptor activation have been documented by different physical methods, as described above. All these experiments have mostly pointed to mutual movement of the transmembrane helices TM3 and TM6 that affects the conformation of the IL2 and IL3 intracellular loops, serving as the key sites for G-protein recognition and activation. This conclusion is also supported by analysis of the structure of the constitutively activated receptor mutants (Parnot et al, 2002), the evolutionary trace analysis (Madabushi et al, 2004) and also by similar structure — function relationships for these proteins (Karnik et al, 2003).

Moreover, the recent achievements in real-time monitoring of the conformational transition in GPCR molecules by fluorescence based methods has revealed the presence of a fast transition with half-life of approx 2 seconds, which can be observed in the case of different receptors and remains in agreement with the hypothesis about the universal signal transmission mechanism by GPCR (Vilardaga et al, 2003, Swaminath et al, 2004).

Much less attention has been paid on the mechanism of antagonist interaction with GPCR. This is quite understandable from the point of view of the physiological meaning of these ligands. On the other hand, application of

antagonists as radioligands in practical receptor research, but also their pharmacological significance, have focused researcher also on these studies. So, since late seventies, methods of chemical kinetics were applied for investigation into the mechanism of antagonist binding with GPCR and this aspect was systematically investigated in the case of several receptors. In this work we would like to draw attention on surprisingly similar transition, observed in these kinetic studies for different receptors of Class 1.

The main finding from all the kinetic investigations into antagonist interaction with various GPCRs can be summarized by discovery of the two-step reaction mechanism (1), which involves fast bimolecular binding step followed by slow monomolecular "isomerization" of the complex. This slow "isomerization" step has been identified also in the case of [^{35}S]dATPαS interaction with $P2Y_1$ receptor in this study, and the conformational transition was characterized by the rate constants k_i and k_{-i} (Table 4). In Table 5 these results are compared with similar kinetic constants for other receptors and their antagonists.

Summary of the kinetic data, presented in Table 5, reveals that the "isomerization" rate constant k_i is surprisingly similar for all of the four receptors under consideration, while the affinity of the appropriate ligands differs more that 100 times. Also, the de-isomerization rate constant k_{-i} has different values. These data point to the fact that the activation barrier, separating the complex RA from the "isomerized" state (RA), is similar for all of these receptors and does not depend much on the receptor type. On the other hand, the variation in the K_A and k_{-i} values represents the specific features of the antagonist-receptor interaction and depends specifically on structure of the ligand and its binding site.

It is noteworthy that the half-life of the "isomerization" step, remaining between 80–50 seconds, is very close to the half-life 70 s, characteristic for the slow phase of the conformational change, observed by direct fluorescence assay of conformational dynamics for beta-adrenergic receptor interaction with some agonists (Swaminath et al, 2004). As mentioned in this paper, the slow phase can be hardly related to physiological phenomenon of the receptor activation and signal transmission, but may well characterize some dynamic properties of the "core domain" of the receptor molecule. In the light of the present compilation the same conformational movements may accompany interaction of the receptor protein with antagonists.

This slow phase of receptor-antagonist complex "isomerization" reflects properties of the receptor protein, but not biomembrane, as the "isomerization" step can be observed also after receptor solubilization. So, solubilization of muscarinic receptor did not change the reaction scheme, but only slowed down the rate of the "isomerization" process, yielding for [^{3}H]-L-QNB interaction with the muscarinic receptor from rat cerebral cortex the k_i value $(2.3 \pm 0.2)\ 10^{-3}\ s^{-1}$. At the same time the affinity of the ligand, characterized by the K_A value 3.8 ± 0.8 nM, was mush less affected, if compared with the appropriate value in Table 5.

Table 5. Summary of the kinetic data for interaction of antagonists with some GPCR-s

GPCR	Antagonist	10^2 k_i , s^{-1}	10^4 k_{-i} , s^{-1}	K_A , nM	Conditions	Ref
β-Adrenergic receptor	[^{3}H]dihydroalprenolol	1.0 ± 0.6	4 ± 3	0.48 ± 0.08	rat cerebral cortex, 25^{0}C	Schliebs and Bigl, 1984
Muscarinic acetylcholine receptor	L- [^{3}H]quinuclidinyl benzilate	1.2 ± 0.3	0.20 ± 0.04	1.3 ± 0.5	rat brain cortex, 25^{0}C	Sillard et al, 1987
		0.9±0.2	0.53±0.02	1.2±0.6	rat heart, 25^{0}C	Järv and Sillard, 1987
	[^{3}H]-4-N-methyl-piperidinyl benzilate	1.4 ± 0.3	4.6 ± 0.3	5.5 ± 3.1	rat brain cortex, 25^{0}C	Järv et al, 1987a
		0.9 ± 0.1	12 ± 3	6.7 ± 2.5	rat small intestine, 25^{0}C	Järv et al, 1979
Dopamine D_2 receptor	[^{3}H]raclopride	1.7 ± 0.3	18 ± 7	12 ± 3	rat brain *corpus striatum*, 25^{0}C	Lepiku et al, 1996
$P2Y_1$ nucleotide receptor	[^{35}S]dATPαS	0.9 ± 0.1	39 ± 7	59 ± 19	h$P2Y_1$-1321N1 cells, 25^{0}C	This study

In summary, the kinetic approach, used for investigation into [^{35}S]dATPαS interaction with $P2Y_1$ receptor has provided a good possibility for selective determination of the receptor sites in the presence of other, non-receptor sites, which are also specific binding sites for nucleotides. This possibility is based on unique and probably rather uniform dynamic properties of GPCR molecules, which may have analogy with the presence of the conserved elements in their tertiary or even quaternary structure.

CONCLUSIONS

In this study we have focused on kinetics of interaction of 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) ([^{35}S]dATPαS) with specific binding sites on three types of membrane fragments. Firstly, we used the membranes of transfected astrocytoma 1321N1 cells, expressing this receptor. Secondly, the membrane fragments of the same wild-type cells, not expressing $P2Y_1$, were used. Finally, binding of the radioligand with brain membranes was investigated.

Significant binding of [^{35}S]dATPαS was observed in the case of membrane fragments of both types of astrocytoma 1321N1 cells: the transfected cells expressing human $P2Y_1$ receptors, and the same wild-type cells, not expressing $P2Y_1$ receptor (Paper I). As in both cases the bound radioligand was displaced by excess of ATP, all these binding sites can be defined as specific sites. But only in the case of the transfected cell membranes a fraction of these binding sites had slow radioligand on-rate. These "slow" binding sites behaved as a kinetically homogeneous population and their interaction with the radioligand was shown to occur in two steps, including isomerization of the complex RA into (RA):

$$R \underset{}{\overset{K_A}{\rightleftharpoons}} RA \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} (RA)$$

These “slow” sites were characterized by the kinetic parameters K_A=59±19 nM, k_i= (9.0±0.8) 10^{-3} s^{-1} and k_{-i}=(3.9±0.7)10^{-3} s^{-1} (25^{o}C). As this two-step ligand association mechanism is typical for interaction of G-protein coupled receptors (GPCR-s) with their antagonists, it was concluded that the “slow” binding sites on cell membranes correspond to the $P2Y_1$ receptor subtype.

Kinetics of interaction of the same radioligand with rat brain membrane fragments was studied (Paper II), and at least two different ways of binding of [^{35}S]dATPαS with these membranes were distinguished on the basis of the kinetic analysis. Firstly, the binding sites characterized by fast on-rate were observed. Secondly, the “slow” binding sites were identified and quantified by means of the kinetic approach. In the “slow” binding sites isomerization of the receptor-ligand complex was observed, as this is typical for interaction of antagonists with GPCR-s, and the kinetic parameters for this process were determined: K_A=99±29 nM, k_i= (11±1) 10^{-3} s^{-1}, k_{-i} =(3.4±0.5)10^{-3} s^{-1} (25^{o}C). As these data are close to the results obtained with the transfected $P2Y_1$ receptor, the “slow” binding sites on brain membranes could be assigned to the same receptors. Conclusion was drawn that kinetic criteria can be used for differentiation of the receptor sites from non-receptor sites, providing possibility of radioligand assay of $P2Y_1$ receptors with [^{35}S]dATPαS as radioligand.

The kinetic parameters for interaction of [^{35}S]dATPαS with $P2Y_1$ receptor were compared with analogical data for other GPCR-s (Paper III). This analysis revealed surprisingly similar rate of the receptor-antagonist complex isomerization in the case of distinct receptors. Hypothesis was proposed that antagonists might induce similar conformational transition in the case of different GPCR-s, reflecting some uniform principles of the spatial structure of these receptor proteins.

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SUMMARY IN ESTONIAN

dATPαS ja $P2Y_1$ retseptori vahelise toime kineetilised aspektid

Käesolevas töös uuriti radioligandi [^{35}S]dATPαS sidumise kineetikat kolme tüüpi rakumembraanide fragmentidele. Esiteks kasutati membraanfragmente astrotsütoomi 1321N1 rakkudest, kus eelnevalt ekspresseeriti $P2Y_1$ retseptorit. Teiseks kasutati samade looduslike rakkude membraanifragmente, kus see retseptor puudub. Viimaks teostati katsed roti ajust valmistatud membraanipreparaadiga.

Leiti, et [^{35}S]dATPαS seostub mõlemat tüüpi 1321N1 rakkude membraanifragmentidel — nii neil, kus on ekspresseeritud $P2Y_1$ retseptor, kui ka membraanidel, mis saadi rakkudest, kus selle retseptori ekspressiooni ei toimu (Artikkel I). Kuna mõlemal juhul oli võimalik seostunud radioligandi välja tõrjuda ATP liiaga, siis võib kõiki selle radioligandi sidumiskohti iseloomustada kui "spetsiifilise" sidumise tsentreid. Erinevalt looduslike rakkude membraanidest ilmnes aga $P2Y_1$ retseptorit ekspresseerivate rakkude membraanifragmentide korral radioligandi sidumise kineetikas nn "aeglane faas". Need "aeglased" sidumiskohad moodustasid kineetiliselt homogeense populatsiooni ning radioligandi seostumine nendel sidumistsentritel kulges kaheastmelise protsessina, kus esmase retseptor-ligand kompleksi RA moodustumisele järgnes selle kompleksi aeglane isomerisatsioon kompleksiks (RA):

$$R \overset{K_A}{\rightleftharpoons} RA \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} (RA)$$

Selle reaktsiooniskeemi kohaselt iseloomustati "aeglasi" sidumiskohti järgmiste kineetiliste parameetritega: K_A=59±19 nM, k_i=(9.0±0.8) 10^{-3} s^{-1} ja k_{-i}=(3.9±0.7)10^{-3} s^{-1} (25^{o}C). Et selline kaheastmeline ligandi sidumise mehhanism on isloomulik G-valguga seotud retseptoritele, siis järeldati, et ka sidumiskineetikas ilmnevad "aeglased sidumiskohad" on tõenäoliselt $P2Y_1$ retseptorid.

Sama radioligandi sidumise kineetikat uuriti ka roti aju membraanfragmentide korral (Artikkel II). Ka siin võimaldas radioligandi sidumise kineetiline analüüs eristada kahte tüüpi sidumiskohti. Esiteks leiti sidumistsentrid, kuhu [^{35}S]dATPαS seostus kiirelt. Teiseks leiti ka aju membranfragmentide korral "aeglased" sidumistsentrid, mida iselomustab retseptori ja antagonist kompleksi isomerisatsioon. Neid "aeglasi" sidumistsentreid iseloomustavad järgmised kineetilised parameetrid: K_A=99±29 nM, k_i= (11±1) 10^{-3} s^{-1}, k_{-i} =(3.4±0.5)10^{-3} s^{-1} (25^{o}C). Kuna need tulemused on sarnased $P2Y_1$ retseptorit sisaldavate transfekteeritud 1321N1 rakkude membraanifragmentide korral leidud kineetiliste parameetritega, siis järeldati, et ka aju "aeglased" sidumistsentrid kuuluvad sama tüüpi retseptorile. Tehti järeldus, et radioligandi sidumise kineetikat

analüüsides on võimalik eristada selle ligandi seostumist G-valguga seotud retseptorile ning mitteretseptoorset päritolu sidumistsentritele.

[^{35}S]dATPαS ja $P2Y_1$ retseptori vahelise toime kineetilisi parameetreid võrreldi analoogiliste andmetega, mis on saadud hoopis teist tüüpi antagonistide ning G-valguga seotud retseptorite korral (Artikkel III). Võrdlus tõi välja üllatava sarnasuse erinevate retseptor-ligand komplekside isomeriseerumise kiiruse osas. Selle võrdluse põhjal püstitati hüpotees, et retseptor-ligand kompleksi isomerisatsioonistaadiumiga kaasnev retseptori konformatsiooniline muutus võib olla ühesugune paljude (kui mitte kõikide) G-valguga seotud retseptorite korral, peegeldades seda tüüpi retseptorite ruumilise ehituse teatud universaalseid külgi.

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PUBLICATIONS

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