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Modulation of adenylate cyclase activity in rat striatal membranes by adenosine A_{2A} receptors

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Abstract. Possibilities of characterizing adenosine A_{2A} receptor dependent signal transduction in rat striatal membranes by activation of adenylate cyclase were studied. By optimization of membrane preparation methods and the composition of the incubation buffer, an up to 10-fold increase in cyclic AMP (cAMP) accumulation was achieved in response to the binding of A_{2A}-specific agonist CGS 21680. The best response was achieved in the crude striatal homogenate in the buffer where Na^+ and K^+ were omitted and then the potency of CGS 21680 was characterized by $EC_{50} = 0.5 \pm 0.2$ μM. The presence of at least 1 mM Mg^{2+} was required to achieve the maximal specific AC activation, but at higher concentrations magnesium increased the non-specific cAMP accumulation and decreased the receptor-mediated effects. The antagonist of A_{2A} receptors, ZM 241385, had no effect on the basal activity of adenylate cyclase in striatal homogenate, but inhibited the CGS 21680-dependent activation with affinity that was in agreement with the binding affinity of this antagonist to A_{2A} receptors.

Key words: adenosine A_{2A} receptor, CGS 21680, ZM 241385, adenylate cyclase, rat striatum.

INTRODUCTION

Adenosine receptors comprise a large receptor subfamily of purinergic receptors. All adenosine receptors are G protein coupled receptors (GPCRs), and like other receptors of this superfamily have seven transmembrane domains with extracellular amino terminus and intracellular carboxy terminus. To date four adenosine receptors are known and marked as A_1, A_{2A}, A_{2B}, and A_3 receptors [1]. Historically adenosine receptors were pharmacologically divided into two subtypes: adenosine A1 receptors, which decreased the adenylyl cyclase activity, and A2 receptors, which increased the adenylyl cyclase activity [2, 3]. However,

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at the end of the 1980s the existence of four different adenosine receptor subtypes was shown by using the methods of molecular cloning [4].

All four adenosine receptors have been found in the central nervous system, but high expression levels of A_1 receptors were shown also in the spinal cord, eye, adrenal gland, and atria [5]. The distribution of adenosine A_{2A} receptors includes lymphocytes, platelets, nerve cells, vascular smooth muscle, and endothelium [6]. In the brain the A_{2A} receptors are highly expressed in the neostriatum, nucleus accumbens, and olfactory tubercle [7]. Striatal A_{2A} receptors are colocated with dopamine D_2 receptors [8] and there is an antagonistic cross-regulation between these two receptors [9, 10]. High expression of A_{2B} receptor mRNA has been found in cecum, colon, and bladder and high expression of A_3 receptors in testis and mast cell [5]. However, these data have not yet been confirmed pharmacologically because of lack of highly specific radioligands for these receptors.

Activation of adenosine receptors modulates the activity of several effectors, including adenylyl cyclase, phospholipase C, ion channels, etc., depending on the particular conditions in the cell. Generally, the A_1 and A_3 receptors are coupled to $G_{i/o}$ proteins, which are directed to inhibition of adenylate cyclase [11, 12], while the A_{2A} and A_{2B} receptors activate this enzyme via G_s protein subtypes [13]. However, it is shown that also other G protein subtypes can couple to these receptors [14, 15], and the coupling specificity depends on the tissue where the receptors are expressed [16].

As mentioned earlier, all adenosine receptors modulate the activity of adenylyl cyclase (AC, E.C 4.6.1.1), an enzyme that catalyses the formation of an important second messenger, adenosine 3',5'-cyclic monophosphate (cAMP). To date at least 10 mammalian ACs have been identified [17, 18], and most of the isozymes contain two hydrophobic regions, each comprising six transmembrane helixes and three large cytoplasmic domains: N, C1a/b, and C2 [19]. The C1a and C2 domains comprise the cytoplasmic catalytic unit of the enzyme, while the N terminus domains are highly variable between AC isozymes and are proposed to play a regulatory role [20, 21].

The activity of intracellular adenylate cyclase is usually estimated by measuring the accumulation of cAMP in the presence of inhibitors of phosphodiesterases [22]. This method is widely used to characterize physiological effects of various compounds in intact cells, but pharmacological studies require characterization of the signal transduction mechanism also in membrane preparations of tissues. As all components of the AC activation pathway (GPCR-s, G-proteins, and AC) are membrane-bound proteins, the signal transduction has to function also in cell membrane preparations. However, it has been shown that the AC activation sensitivity is much lower in membrane preparations and affinities of several receptor ligands are lower as well [23].

In the present study we searched for the optimal conditions for the measuring of the adenosine A_{2A} receptor dependent modulation of the AC activity in rat striatal membranes. We showed that the activation of the second messenger system is in good agreement with the binding of ligands to the receptors.

MATERIALS AND METHODS

Chemicals

[2-^{3}H]-4-(2-(7-[Amino-2(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]-triazin-5-ylamino]-ethyl)phenol) ([^{3}H]ZM 241385, 21 Ci/mmol) was purchased from Tocris Cookson Ltd., [5',8'-^{3}H] adenosine-3',5'-cyclic monophosphate [^{3}H]cAMP (48 Ci/mmol) was obtained from Amersham Biosciences. 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS 21680), 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]-triazin-5-ylamino]-ethyl)phenol (ZM 241385), and forskolin were obtained from Tocris Cookson Ltd. Adenosine deaminase (ADA, EC 3.5.4.4, 5 mg/mL) and guanosine-5'-(3-thio)-triphosphate (GTPγS) were purchased from Roche Diagnostics. Guanosine-5'-diphosphate (GDP), cyclic adenosine-3',5'-monophosphate (cAMP), and 4-(3-butoxy-4-methoxybenzyl)-imidazolidin-2-one (Ro 20-1724) were from Sigma Chemical Co. and 3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3) was from Pharmaceutical Institute of the University of Bonn. All other reagents were of analytical grade from regular suppliers.

Membrane preparations from rat striatum

Rat striatal membranes were prepared as described previously [24] with slight modifications. The washed membranes were prepared by sonication of striatum tissue in 60 volumes (v/ww) of Tris-HCl buffer (50 mM, pH = 7.4). The homogenate was centrifuged at 20.000×*g* for 40 min at 4 °C, the resulting membrane pellet was resuspended in the same amount of Tris-HCl buffer (50 mM, pH = 7.4) and centrifuged. The homogenization and centrifugation step was repeated once more and the final membrane pellet was resuspended in assay buffer (A.B.) containing 30 mM Tris-HCl (pH = 7.4), 8.25 mM $MgCl_2$, 0.1 mM Ro 20-1724, 0.75 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 7.5 mM KCl, and 0.1 M NaCl. The washed membranes were divided into 1 mL aliquots and stored at −80 °C until use.

The crude homogenate was prepared by homogenization of striatum tissue by sonication in 65 volumes (v/ww) of A.B., divided into 1 mL aliquots, and stored at −80 °C until use. The crude homogenate without sodium/potassium salts was prepared by homogenization of striatum in 50 volumes (v/ww) of 2.5 mM Tris-HCl buffer (pH = 7.4) containing 2 mM EGTA. The homogenate was diluted with the same volume of 50 mM Tris-HCl buffer (pH = 7.4) containing 2 mM EGTA, divided into 1 mL aliquots and stored at −80 °C until use.

The concentration of total protein in samples was determined by the modified method of Lowry [25], using bovine serum albumin (BSA) as standard.

Adenylyl cyclase assay

For the determination of AC activity, the membranes (2–9 μg protein/mL) were incubated in the reaction medium containing 30 mM Tris-HCl (pH = 7.4), 8.25 mM $MgCl_2$, 0.75 mM EGTA, 7.5 mM KCl, 0.1 M NaCl, 0.1 mM Ro 20-1724, 150 μg/mL bacitracin, 0.05% BSA, and an ATP regenerating system (10 mM phosphoenolpyruvate (PEP) and 45 μg/mL pyruvate kinase). For the crude homogenate without sodium/potassium salts the concentration of $MgCl_2$ was 10 mM, while KCl and NaCl were omitted.

The reaction was started by addition of ATP (final 1 mM) and GTP (final 10 μM) (if not stated otherwise) and terminated by addition of 50 μL EDTA (final 25 mM) to the samples and boiling the sample tubes for 3 min. The content of cAMP in the samples was measured by competition binding with [^{3}H]cAMP to cAMP binding protein [26]. Shortly, cAMP standards or samples were mixed with [^{3}H]cAMP (10 000 cpm per sample) and incubated with cAMP binding protein [27] at least for 2 h at 4 °C. The bound radioactivity was determined by rapid filtration through GF/B glass-fibre filters (Whatman Int. Ltd., Madistone, UK) using a Brandell cell harvester and three washes of 5 mL of ice-cold washing buffer containing 100 mM NaCl and 20 mM phosphate buffer (pH = 7.5) as described in [28]. The nonspecific [^{3}H]cAMP binding was determined in the absence of the binding protein. The filters were kept overnight with 5 mL of scintillation cocktail OptiPhase HiSafe®3 (Wallac Perkin Elmer Life Sciences), and the radioactivity content was measured using a Beckman LS 1800 scintillation counter.

[^{3}H]ZM 241385 displacement experiments

Equilibrium binding assays were performed by incubating membranes (100 μg protein/500 μL) in incubation buffer (IB, 20 mM Tris HCl, 120 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, pH = 7.5) with appropriate concentrations of [^{3}H]ZM 241385 (0.04 to 4 nM) for 45 min at 25 °C [29]. The reaction was terminated by rapid filtration and the radioactivity content was measured as described above. Non-specific binding was determined in the presence of 0.5 mM dimethylpropargylxantine (DMPX). Displacement experiments were performed by incubating membranes (70 μg protein/mL) in incubation buffer (30 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.75 mM EGTA, pH = 7.4) with 2.3 nM [^{3}H]ZM 241385 and non-labelled ligands CGS 21680 (concentration range 1 nM to 100 μM), ZM 241385 (concentration range 10 pM to 10 μM), or MSX-3 (concentration range 100 pM to 30 μM), with and without the presence of 30 μM GTPγS for 60 min at 30 °C. The reaction was terminated by rapid filtration, and the radioactivity content was measured as described above.

Data analysis

All data were analysed by means of a non-linear least squares regression method using the commercial program GraphPad PRISM™ (GraphPad Software, Inc.). Data are presented as mean ± SEM of at least two independent determinations. The K_i values of competition experiments were calculated according to the equation of Cheng–Prusoff [30]: $K_i = IC_{50}/(1+[L]/K_d)$, where $[L]$ is the concentration of the radioligand and K_d is the radioligand dissociation constant. The statistical significance of differences was determined by the Student–Newman–Keuls test, where $P < 0.05$ was taken as the criterion of significance.

RESULTS AND DISCUSSION

CGS 21680-specific increase in cAMP formation

Biochemical and molecular pharmacological characterization of G protein-coupled receptors has been usually carried out in intact cells or in partially purified membrane preparations. This has also been a standard for characterization of A_{2A} receptor-coupled events, including the activation of AC. However, increasingly more information has been accumulated indicating that other proteins besides GPCRs, G proteins, and AC may have an important role in this signal transduction mechanism [31] and it has been suggested that some of them may be discarded during the membrane preparation. Comparison of the influence of adenosine A_{2A}-specific agonist CGS 21680 (10 μM) on the initiation of the accumulation of cAMP revealed that the biggest effect, 0.33 ± 0.05 pmol/min/μg, was achieved in the crude homogenate without sodium and potassium salts in comparison with values of 0.08 ± 0.01 and 0.15 ± 0.03 pmol/min/μg for washed membranes and crude homogenate with salts, respectively. Forskolin, which is reported to be a direct activator of all AC subtypes [18], did not clearly distinguish the preparations, causing an increase of cAMP concentration by 940%, 1030%, and 710% above the basal level in the preparations of washed membranes and crude homogenate with and without sodium and potassium ions, respectively. This means that the biggest absolute as well as relative activation of AC by CGS 21680 was achieved in crude homogenate without Na^+ and K^+ and therefore all the following experiments were carried out with these preparations.

Effect of guanosine nucleotides and adenosine deaminase on CGS 21680 activation effect

The ratio of guanosine nucleotides GTP and GDP is a very important parameter in the signal transduction systems of G protein-coupled receptors [32]. The rate of GDP/GTP exchange on G proteins determines the efficiency of signal transduction and the rate of GTP/GDP hydrolysis the signal's latency. To find possibilities for further optimization of conditions for the receptor-specific

activation of AC activity in membrane preparation, we substituted GTP in the reaction medium with 1 μM GTPγS. This caused an overall increase of cAMP formation, but did not affect the activation effect of CGS 21680. GTPγS is a nonhydrolysable analogue of GTP, which could not be degraded by the activated G proteins and the system could not be regenerated. This means that GTPγS forces the activation of all G proteins available with a single turn and so decreases the part of receptor-dependent effect, which in normal activation causes multiple turns. This was supported also by the finding that the addition of 1 μM GDP into the reaction medium decreased the activation effect of CGS 21680 by 30% (data not shown). The G proteins in their idle state are usually coupled with GDP and therefore this nucleotide is usually an inhibitor of signal transduction. However, in very effective systems GDP is used to suppress receptor independent activation of G proteins, as it has been found to be useful for the design of the [^{35}S]GTPγS binding activation assay [24]. The inhibition of A_{2A} receptor-specific AC activation by GDP indicates a low level of intrinsic activity of the system and it can be assumed that here the rate limiting step is the exchange of GDP to GTP on the receptor-coupled G proteins as it is also in the intact signal transduction system.

It is known that adenosine and its derivatives are abundant in brain, and it is expected that they remain bound to the A_{2A} receptors in high affinity state also during the homogenization and membrane preparation. The treatment of the homogenate with adenosine deaminase (ADA, 10 U/mL) to remove the endogenous adenosine had no significant influence on the CGS 21680 dependent accumulation of cAMP. It has been previously reported that ADA is highly expressed in striatum [33] and tightly linked to the adenosine receptors [34, 35]. This seems to be sufficient to eliminate the endogenous adenosine in the membrane preparations of striatum and therefore in all the following experiments ADA was omitted from the reaction medium.

Effect of Mg^{2+} on CGS 21680 dependent cAMP accumulation

The presence of Mg^{2+} is required for an effective coupling of receptors with G proteins [36] and in the generation of second messengers. However, depending on the steps of the signal transduction of interest, the required concentration of Mg^{2+} may vary greatly. For the optimization of the AC assay of adenosine A_{2A} receptors in striatal membranes we have varied the concentration of Mg^{2+} in the assay medium from 0 to 30 mM. In the absence of Mg^{2+}, the formation of cAMP was very low at both basal and CGS 21680 activated state (Fig. 1). Substantial activation of AC by CGS 21680 could be determined already at 1 mM Mg^{2+}, while the basal activity remained very low. Additional increase in Mg^{2+} did not cause a rise of the A_{2A}-specific cAMP accumulation, while at concentrations above 5 mM its decrease occurred approaching the level of 50% at 30 mM $MgCl_2$ compared to the effect at 1 mM $MgCl_2$ (Fig. 1 streaked columns). On the other hand, the basal level of the cAMP formation increased with the increase of

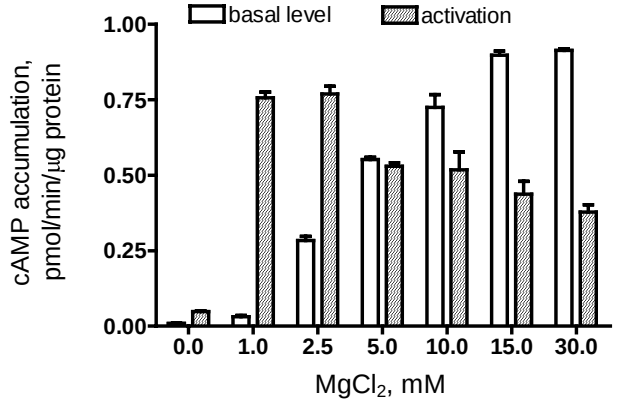

Fig. 1. Influence of the concentration of $MgCl_2$ on the formation of cAMP in rat striatal crude homogenate. Homogenate (5 μg/mL of protein) was incubated with the indicated concentration of $MgCl_2$ in the absence (basal) and presence of 10 μM CGS 21680 (activated) as described in Materials and Methods. Basal level (open columns) and CGS 21680-dependent cAMP formation, which was defined as the difference between the total and the basal level of cAMP, are presented as a mean of three independent determinations ± SEM as error bars.

Mg^{2+} concentration at all ion concentrations studied (Fig. 1 open columns). Already at 5 mM Mg^{2+} the basal cAMP accumulation reached the level of specific cAMP accumulation and at higher Mg^{2+} concentrations the specific effect remained below 50% of the total binding (Fig. 1). It has also to be pointed out that at Mg^{2+} concentrations above 2.5 mM the total amount of cAMP formed remained constant, but with an increase in the basal level caused by the increase of the magnesium concentration the adenosine receptor-coupled amount started to decrease. The obtained results clearly indicate that for the determination of A_{2A}-specific cAMP accumulation 1 mM Mg^{2+} has to be preferred, as then the maximal receptor-specific effect could be achieved and the basal cAMP formation is suppressed.

Parameters of adenosine A_{2A} receptor specific regulation of AC activity

CGS 21680, a highly specific adenosine A_{2A} receptor agonist, caused a concentration-dependent increase of cAMP formation in the crude homogenate of rat striatal membranes (Fig. 2). The potency of CGS 21680 was characterized by an $EC_{50} = 0.5 \pm 0.2$ μM, and the Hill coefficient was not different from unity ($n_H = 1.1 \pm 0.2$). The obtained potency was considerably lower than $EC_{50} = 15 \pm 3$ nM found in the intact CHO cells expressing adenosine A_{2A} receptors [29], and the $K_i = 45 \pm 8$ nM found in competition with [^{3}H]ZM 241385 binding. It is proposed that the decrease of potency of CGS 21680 is caused by the perturbation of the receptor – G protein – AC signalling pathway during the preparation of the membranes. A similar decrease in the potencies of agonists in

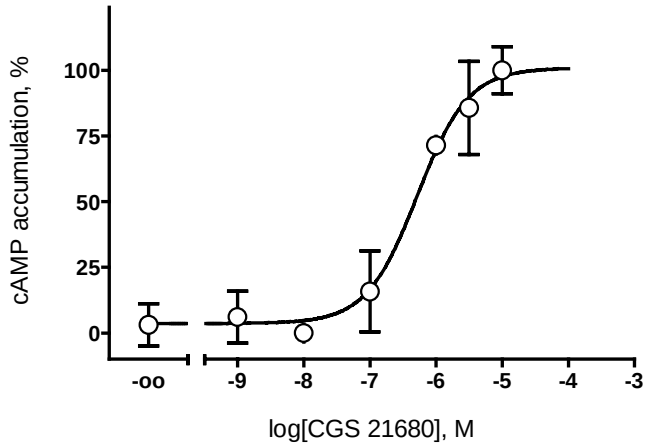

Fig. 2. Activation of the formation of cAMP by A_{2A}-specific agonist CGS 21680. Crude homogenate of rat striatum (5 μg/mL of protein) was incubated with the indicated concentrations of CGS 21680 in the AB at 30 °C for 15 min and the formation of cAMP was determined as described in Materials and Methods. Data presented as percentage of maximal activation are representative of three independent experiments carried out in triplicate (mean ± SEM as error bars).

the activation of AC in membrane preparation has been found for A_3 adenosine receptors, where a higher than 200-fold discrepancy was observed [23]. Of course, also the relatively high concentration of nucleotides in the reaction medium may lead the receptor into a low affinity state and decrease the potency of agonists [32].

ZM 241385, a highly specific adenosine A_{2A} receptor antagonist, had no effect on the basal level of cAMP formation in the homogenate of striatal membranes, but inhibited the CGS 21680 induced activation with an IC_{50} = 32 ± 12 nM in the presence of 10 μM CGS 21680. The inhibition by ZM 241385 was competitive as it caused a concentration-dependent rightward shift of AC activation curves without affecting the level of maximal response (data not shown). Similar inhibition of AC activity was achieved also by the antagonist MSX-3 with IC_{50} = 63 ± 30 nM (n_H = 0.6 ± 0.3). Using a mechanistic approach for the activation of receptors, the inhibition constants of the antagonists could be estimated as K_i = 1.2 nM for ZM 241385 and K_i = 2.4 nM for MSX-3.

Characteristics of [^{3}H]ZM 241385 binding to striatal membranes and competition binding with other A_{2A} ligands

[^{3}H]ZM 241385 is the first commercially available radiolabelled A_{2A} antagonist and it has been found to be useful for pharmacological experiments [37]. We have found that the binding of [^{3}H]ZM 241385 to rat striatal membranes was saturable and described with a K_d = 0.14 ± 0.01 nM and a B_{max} = 1620 ± 40 fmol/mg protein. All the studied adenosine receptor ligands were able to compete with [^{3}H]ZM 241385 binding in a concentration dependent

manner (Fig. 3) with estimated K_i values 45 ± 8 nM for CGS 21680, 0.8 ± 0.1 nM for ZM 241385, and 50 ± 1 nM for MSX-3. The difference in MSX-3 K_i values from displacement and AC inhibition experiments may be caused by the shallowness ($n_H = 0.6 \pm 0.3$) of the AC inhibition curve. Activation of G proteins with 30 μM GTPγS had no significant influence on the affinities of antagonists, but slightly lowered the affinity of the agonist (K_i = 55 nM). The slightly lower Hill coefficient of the CGS 21680 displacement curve in the absence of GTPγS ($n = 0.8 \pm 0.1$) and F test in comparison with binding models proposed the presence of two binding sites for the agonist with affinities K_{iH} = 1.3 nM and K_{iL} = 61 nM. The fraction of high affinity sites was $\alpha_H = 0.15 \pm 0.05$, which was completely lost in the presence of 30 μM GTPγS.

In summary, the optimization of experimental conditions revealed a clear adenosine A_{2A} receptor-specific activation of adenylate cyclase activity in the crude homogenate of rat striatum. The presence of at least 1 mM Mg^{2+} was required to achieve the specific AC activation, but higher Mg^{2+} concentrations increased the nonspecific AC activity and decreased the receptor-mediated effects. However, the obtained agonist potencies in the activation of AC in membrane preparations were lower than in assays using intact cells and in radioligand binding displacement assays. It could be proposed that the decrease in the agonist potency is caused by the perturbation of the receptor – G protein – AC signalling pathway during the preparation of the membranes. Thus, the properties of adenosine A_{2A} receptors in rat striatal membranes can be characterized by the activation of AC, but the decrease in sensitivity has to be taken into account for the interpretation of the obtained data.

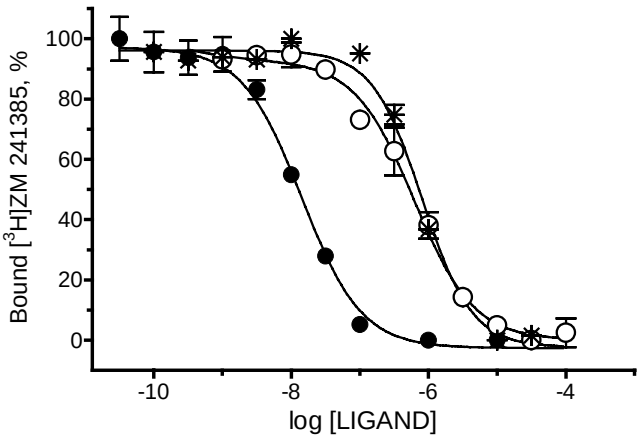

Fig. 3. Inhibition of specific binding of [^{3}H]ZM 241385 by ligands of adenosine receptors. The crude homogenate of rat striatum was incubated for 60 min at 30 °C at indicated concentrations of ZM 241385 (●), CGS 21680 (o), or MSX-3 (∗) and 2.3 nM [^{3}H]ZM 241385. Binding of [^{3}H]ZM 241385 is presented as the percentage of specific binding in the absence of ligands (± SEM as error bars) and is from two independent experiments carried out in duplicate.

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Adenülaat-tsüklaasi aktiivsuse moduleerimine roti aju juttkeha membraanides A_{2A} retseptorite abil

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Käesolevas töös on uuritud võimalusi roti aju juttkeha membraanides adenosiini A_{2A} retseptorite poolt edastatava signaali mõõtmiseks, lähtudes adenülaat-tsüklaasi aktiivsusest. Varieerides membraanide valmistamise metoodikat ja inkubatsioonipuhvri koostist, saavutati retseptorite aktiveerimisel A_{2A}-spetsiifilise agonisti CGS 21680-ga ligi 10-kordne cAMP akumulatsiooni taseme tõus üle baastaseme ja selle ligandi afiinsust membraanpreparaadis iseloomustas $pEC_{50} = 6{,}3 \pm 0{,}2$. Parim aktivatsioon saavutati puhastamata juttkeha suspensioonis, millele polnud lisatud Na^+ ja K^+ soolasid. Vähemalt 1 mM Mg^{2+} juuresolek oli vajalik maksimaalse spetsiifilise adenülaat-tsüklaasi aktivatsiooni saavutamiseks, kusjuures kõrgematel kontsentratsioonidel vähendas Mg^{2+} cAMP retseptor-spetsiifilist akumulatsiooni ja tõstis baasaktiivsuse taset. A_{2A}-spetsiifiline antagonist ZM 241385 ei mõjutanud adenülaat-tsüklaasi baasaktiivsust, kuid inhibeeris CGS 21680-sõltuvat aktivatsiooni afiinsustega, mis oli kooskõlas sidumisafiinsusega A_{2A} retseptoritele.