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Adenülaadi tsüklaasi aktiivsuse moduleerimine adenosiin-A_{2A} retseptorite poolt

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Sisukord

Kasutatud lühendid

AC	adenülaadi tsüklaas
ADA	adenosiindeaminaas
AMP	adenosiin-5'-monofosfaat
ATP	adenosiin-5'-trifosfaat
BSA	veise seerumi albumiin
cAMP	tsükliline adenosiin-3',5'-monofosfaat
CGS21680	2-p-(2-karboksüetüül)-fenetüülamino-5'-N-etüülkarboksamido-adenosiin
EDTA	etüüldiaminotetraetaanhape
EGTA	etüleenglükool-bis(β-aminoetüül eeter)-N, N, N', N'-tetraetaanhape
GDP	guanosiin-5'-difosfaat
GTP	guanosiin-5'-trifosfaat
GTPγS	guanosiin-5'-0-(3 tiotrifosfaat)
MSX-3	3-(3-hüdroksüpropüül)-8-(m-metoksüstürüül)-7-metüül-1-propargüülksantiin
PBS	Fosfaat-puhverdatud saliin
PEP	fosfoenüülpüruvaat
PK	püruvaat kinaas
Ro 20-1724	4-(3-butoksü-4-metoksübensüül)-imidasolidiin-2-oon
Tris-HCl	trihüdroksümetüül-aminoetaan kloriid
ZM241385	4-(2-(7-amino-2-(2-furüül)[1,2,4]-triasolo-[2,3-a][1,3,5]-triasiin-5-üülamino)-etüül)-fenool

Sissejuhatus

Tänapäeval on üks levinum retseptorite ligandide füsioloogiliste efektide iseloomustamise meetodeid retseptoriga heterotrimeerse G valgu kaudu seotud efektori aktiivsuse mõõtmine. Üheks selliseks efektoriks on adenülaadi tsüklaas (AC), ensüüm mis katalüüsib ühe tähtsama sekundaarse virgatsaine-tsüklilise adenosiin-3',5'-monofosfaadi (cAMP) moodustumist. Seetõttu on cAMP-i akumulatsiooni mõõtmise kaudu on võimalik iseloomustada AC-ga seotud retseptori aktiivsusi.

Rakusisese AC-i aktiivsust määratakse cAMP-i akumulatsiooni mõõtmisega fosfodiesteraasi (PDE) inhibiitorite juuresolekul [1]. Seda meetodit kasutatakse laialt erinevate ühendite füsioloogiliste efektide iseloomustamisel tervetes rakkudes, kuid farmakoloogilisi uuringuid on enamasti vajalik teostada ka koepreparaatides, kus rakkude terviklikkust ei ole võimalik säilitada. Kuna retseptor/G valk/AC signaaltee toimimiseks vajalikud valgud on membraaniga seotud valgud siis peaks võimalik olema retseptorite ligandide füsioloogiliste efektide mõõtmine ka membraanpreparaatides, kuid siis tuleb lisada substraadi regenereerimise süsteem. Vaatamata sellele pole selle meetodi efektiivsus saavutanud radioligandi sidumise ja tervetes rakkudes AC-i aktiivsuse mõõtmise taset. Seetõttu on uurimistähelepanu all uute mõõtmismeetodite leidmine retseptorite indutseeritud biokeemiliste reaktsioonide mõõtmiseks. Käesoleva töö eesmärgiks on leida optimaalsed tingmused adenosiin-A_{2A} retseptorite vahendatud AC-i aktiivsuse määramiseks ning võrrelda saadud tulemusi ligandide sidumisega retseptorile ning AC-i aktivatsiooniga tervetes rakkudes.

Kirjanduse ülevaade

Adenosiini metabolism

Adenosiin on üks tähtsamaid neuromodulaatoreid kesk kui ka perifeerses närvisüsteemis. Normaaltingimustel tekib adenosiin nii rakusiseses kui ka rakuvälises keskkonnas. Rakusiseselt toimub adenosiini tootmine 5'-nukleotidaasi poolt, mis defosforüleerib adenosiin-5'-monofosfaati (AMP) ja S-adenosüülhomotsüsteiini hüdrolüüsil S-adenosüülhomotsüsteiin hüdrolaasi poolt, rakuväliselt aga toimub adenosiini tootmine ekto-5'-nukleotidaasi poolt, mis defosforüleerib samuti AMP-i [2]. Raku sees toodetud adenosiini transporditakse vajadusel rakuvälisesse keskkonda spetsiifiliste kahesuunaliste transporterite abil, mis efektiivselt hoiavad rakusisese ning –välise adenosiini taseme võrdsena. Adenosiini kasutavad substraadina ensüümid adenosiin kinaas ja adenosiin deaminaas (ADA). Esimesed kaks nimetatud ensüümi toodavad adenosiini baasil vastavalt AMP-i ja S-adenosüülhomotsüsteiini kuid ADA-i produktiks on inosiini [3].

Adenosiini retseptorid

Adenosiini poolt vahendatavaid närvisignaale edastatakse aktiveerides adenosiini retseptoreid, mis moodustavad ühe purinergiliste retseptorite alamperekonna. Adenosiini retseptorid on G valkudega seotud retseptorid ja omavad G valkudega seotud retseptoritele omaseid struktuurielemente nagu seitset transmembraanset domeeni ja neid ühendavaid kolme rakusisest ja kolme rakuvälist silmust, rakuvälist aminoterminali ja rakusisest karboksüterminali. Tänapäeval tuntakse nelja erinevat adenosiini retseptorit, mida nimetatakse A_1, A_{2A}, A_{2B} ja A_3 retseptoriteks [4]. A_1 ja A_{2A} retseptorid on laialt levinud kesknärvisüsteemis, A_1 retseptorite kõrget ekspressiooni on leitud ajukoores, väikeajus, hipokampuses, seljakeelikus, silmas, arteris ja mujal [5]. A_{2A} retseptorid on kõrgelt ekspresseeritud põrnas, harkelundis, leukotsüütides, vereliistakus, aju juttkehas, nucleus accumbens'is [5]. Aju juttkehas on adenosiin A_{2A} retseptor koos leitud olevat tihedalt dopamiini D_2 retseptoriga [6] ning nende

vahel on täheldatud antagonistlike interaktsioone [7, 8]. Seetõttu eeldame, et A_{2A} retseptorite antagonistid võivad osutuda Parkinsoni tõve ravimiteks [9].

A_{2B} ja A_3 retseptorite farmakoloogilist uurimist kudedes on seni takistanud spetsiifilise radioligandi puudumine ning seetõttu on nende paiknemise kohta hinnanguid tehtud vastava retseptori vastava mRNA ekspressiooni kaudu. A_{2B} retseptori ekspressiooni on täheldatud käärsooles, põies ja pimesooles ning A_3 retseptorite olulist ekspressiooni (rottidel) aga testises ja mastotsüütides [5].

A_{2A} retseptori agonistid ja antagonistid

A_{2A} retseptori looduslik ligand on adenosiin, mis on kõigi adenosiini retseptorite agonistiks. Kuna erinevate adenosiini retseptorite uurimiseks on vaja ligande, mis oleksid antud retseptorile spetsiifilised siis adenosiin selleks ei kõlba, kuna aktiveerib kõiki adenosiini retseptoreid võrreldaval tasemel ja laguneb ADA toimel. 5'-N-etüülkarboksüamidoadenosiin (NECA) oli esimene agonist, mida kasutati spetsiifiliselt A_{2A} retseptorite uurimiseks, kuid hiljem leiti, et see seostub ka teistele adenosiini retseptoritele [10]. 2-p-(2-karboksüetüül)-fenetüülamino-5'-N-etüülkarboksamido-adenosiin (CGS21680) on selektiivsem A_{2A} retseptori agonist kui NECA, kuid tema puuduseks on A_{2A} retseptoriga võrreldav seostumine ka A_3 retseptoritele [11].

Spetsiifilistest A_{2A} retseptorite antagonistidest tasub ära märkida 3-(3-hüdroksüpropüül)-7-methüül-8-(m-methoksüstürüül)-1-propargüülksantiini (MSX-2), 5-amino-2-(2-furüül)-7-fenüületüül-pürasolo[4,3-e]-1,2,4-triasolo[1,5-c]pürimidiin (SCH58261) ja 4-(2-(7-amino-2-(2-furüül)[1,2,4]-triasolo-[2,3-a][1,3,5]-triasiin-5-üülamino)-etüül)-fenool (ZM241385), mis kõik omavad suuremat afiinsust A_{2A} retseptori suhtes võrreldes vähemalt A_1 ja A_3 retseptoritega [5].

G valgud

Adenosiini retseptorid on seotud mitmesuguste efektorite aktiivsuse modulatsiooniga, olenevalt G valgu alatüübist, mida retseptor aktiveerib. Guanosiin nukleotiidi (GTP ja GDP) siduvad regulatoorvalgud (G valgud)

jaotatakse kahte rühma: monomeersed (väiksesed) G valgud ja heterotrimeersed (suured) G valgud. Monomeersed G valgud on väiksema molekulmassiga ning nad on seotud mitmete rakuprotsesside kontrolliga. Nende aktivatsioon ja deaktivatsioon on reguleeritud mitmete teiste valkude poolt. Heterotrimeersed G valgud koosnevad kolmest alaühikust (α, β ja γ alaühikud), mis G valgu puhkeolekus moodustavad heterotrimeerse kompleksi. Praegu on teada üle 20 erineva α-alaühiku, millede molekulmass varieerub vahemikus 38,000-52,000 kDa. Samuti on teada vähemalt viite erinevat β-alaühikut, millede molekulmassid jäävad vahemikku 35,000-36,000 kDa ning seitset γ-alaühikut, millede molekulmassid jäävad vahemikku 6,000-9,000 kDa. Kuna α-alaühiku tüüp on määrav G valgu interaktsioonil retseptoritega ning ka efektorite aktiveerimisel, jagatakse heterotrimeerseid G valke perekondadeks just α-alaühiku tüübi alusel, milledeks on G_s, G_i, G_q ja G_{12} perekonnad (tabel 1). β ja γ alaühikud moodustavad dimeeri, mida on võimalik lahutada ainult pärast kompleksi denatureerimist. [3]

G valgu signaaliülekande tsükkel (joonis 1) algab α-alaühikuga seostunud guanosiin-5'-difosfaadi (GDP) dissotsiatsiooniga ja guanosiin-5'-trifosfaadi (GTP) assotsiatsiooniga α-alaühikule, mida initsieerib agonistiga seotud aktiveeritud retseptor. Pärast GTP-i assotsiatsiooni α-alaühikule toimuvad selles konformatsioonilised muutused, mille tulemusena heterotrimeerne G valgu kompleks laguneb α- ja βγ-alaühikuteks. Vabanenud α-alaühik millega on seotud GTP on efektori (ensüümi) suhtes aktiivses olekus ja reguleerib selle aktiivsust. Signaali ülekandmisel G valgu α-alaühiku sisemise GTP-aasse aktiivsuse tõttu lagundatakse seotud GTP-i GDP-ks ja sellega saab võimalikuks α- ja βγ-alaühikute taasühinemine inaktiivseks heterotrimeerseks kompleksiks . Inaktiivset G valku on võimalik uuesti aktiveerida, initsieerides GDP-i vahetust GTP-i vastu α-alaühikus aktiveeritud retseptori poolt. [3]

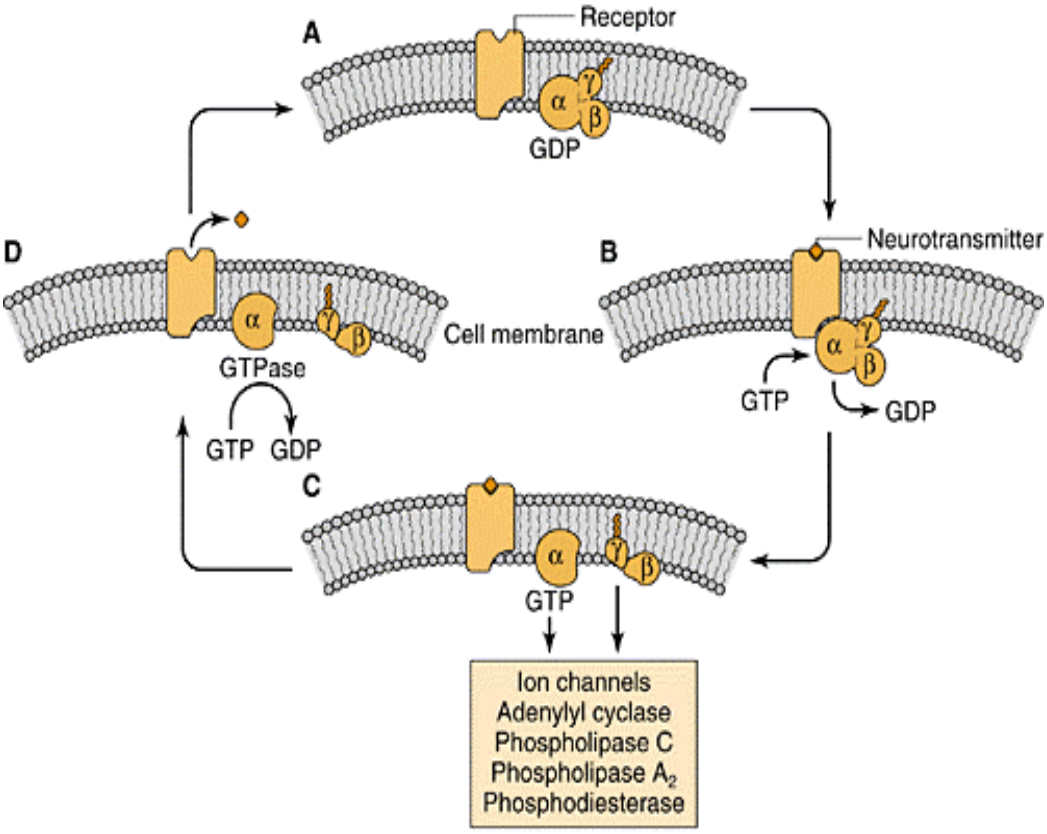
Adenosiini retseptorid on võimelised aktiveerima erinevaid G valgu alatüüpe. A_1 retseptorid on seotud peamiselt G_i perekonda kuuluvate G valkude alatüüpide aktiveerimisega [12, 13]. A_{2A} retseptorid on võimelised aktiveerima mõningaid G_s perekonda kuuluvaid valke [14-16], kuid on näidatud ka G_q valkude aktiveerimist [17]. Ka A_{2B} retseptorid on seotud G_s ja G_q perekonda

kuuluvate G valkude aktiveerimisega [18, 19], kuid A_3 retseptorite poolt on avastatud G_i ja G_q perekonda kuuluvate G valkude aktiveerimist [20].

Tabel 1. Heterotrimeersete G valkude perekonnad ning nendega seotud efektorid

Perekond	Alatüüp	M_r	Efektorvalk(ud)
G_s			
(aktiveerimine)	$G\alpha_{s1}$	52,000	Adennülaadi tsüklaas
	$G\alpha_{s2}$	52,000	
	$G\alpha_{s3}$	45,000	
	$G\alpha_{s4}$	45,000	
	$G\alpha_{olf}$	45,000	
G_i			
	$G\alpha_{i1}$	41,000	Adenülaadi tsüklaas (inhibeerimine)
	$G\alpha_{i2}$	40,000	K^+ kanal (aktiveerimine)[a]
	$G\alpha_{i3}$	41,000	Ca^{2+} kanal (inhibeerimine)[a]
			Fosfolipaas C (aktiveerimine)[a]
			Fosfolipaas A2[a]
	$G\alpha_{o1}$	39,000	K^+ kanal (aktiveerimine)
	$G\alpha_{o2}$	39,000	Ca^{2+} kanal (inhibeerimine)
	$G\alpha_{t1}$	39,000	Fosfodiesteraas (aktiveerimine)
	$G\alpha_{t2}$	40,000	
	$G\alpha_{gust}$	41,000	Fosfodiesteraas (aktiveerimine)
	$G\alpha_z$	41,000	Adenülaadi tsüklaas(inhibeerimine)[a]
G_q		41,000-43,000	
	$G\alpha_q$		Fosfolipaas C (aktiveerimine)
	$G\alpha_{11}$		
	$G\alpha_{14}$		
	$G\alpha_{15}$		
	$G\alpha_{16}$		
G_{12}		44,000	Teadmata
	$G\alpha_{12}$		
	$G\alpha_{13}$		

[a] – G valgu α-alaühiku tüübi otsene seotus antud efektoriga pole lõplikult tõestatud



Joonis 1. G-valgu signaaliülekande tsükkel.

Adenülaadi tüklaas

Kõigi adenosiini retseptorite puhul on täheldatud seotust G valkudega, mis on võimelised moduleerima adenülaadi tsüklaasi (AC) aktiivsust [5]. AC (E. C. 4.6.1.1) on ensüüm, mis katalüüsib ATP-i transformatsiooni tsükliliseks adensoiin-3', 5'-monofosfaadiks (cAMP-ks). Praeguseks on identifitseeritud vähemalt 10 erinevat imetajate AC-i isosüümi [21, 22]. Enamik AC-i isosüüme omab kahte hüdrofoobset regiooni, millest mõlemad moodustavad kuus korda membraani läbivad transmembraansed heeliksid, ja kolme tsütoplasmaatilist domeeni, mida nimetatakse N, C1a/b ja C2 domeenideks [23]. C1a ja C2 domeenid moodustavad AC-i ensüümi tsütoplasmaatilise katalüütilise tsentri. N terminali doomenid on erinevatel isosüümidel väga erinevad ja oletatakse, et neil on ensüümi aktiivsust reguleeriv roll [24, 25].

Kõiki imetajate membraanseotud AC-i isosüüme aktiveerivad forskoliin, mis on taimse päritoluga diterpeen, ja $G\alpha_s$ perekonna G-valkud. $G\alpha_i$ perekonna valgud on üldiselt tuntud AC-i inhibeerijatena, aga nad on võimeline inhibeerima

vaid I, III, V, VI ja VIII AC-i isosüüme. $G\alpha_o$ valgud inhibeerivad ainult isosüüme I, III ja VIII. Lisaks mõjutavad osade isosüümide aktiivsust veel G valgu βγ-alaühikud. Isosüümide I, III ja VIII puhul on βγ-alaühikute kompleksid AC-i inhibiitorid, kuid isosüümide II ja IV puhul põhjustavad βγ-kompleksid koos $G\alpha_s$-ga sünergistilist aktivatsiooni. Samuti Ca^{2+}/kalmoduliin kompleks põhjustab koos $G\alpha_s$-ga sünergilist aktivatsiooni isosüümide I, III ja VIII puhul. Proteiin kinaas A (PKA), proteiin kinaas C (PKC) ning Ca^{2+} inhibeerivad isosüümide V ja VI aktiivsust. [3]

AC aktiivsuse määramise võimalused

AC-i aktiivsust on võimalik määrata AC-i poolt sünteesitud cAMP-i koguse järgi. Kuna sünteesitav cAMP-i kogus katsetes on väga väike siis on tarvis tundlikke meetodeid cAMP-i koguse määramiseks. Üheks võimaluseks on cAMP-i kogus määrata radioaktiivsuse kaudu. Märgistades AC-i substraadi radioaktiivse isotoobiga, võime sünteesitud cAMP-i radioaktiivsuse põhjal leida AC-i aktiivsuse. Et pääseda radioaktiivselt märgistatud AC-i substraadi kasutamisest, on võimalik määrata AC-i aktiivsust kasutades spetsiifiliselt cAMP-i siduvat valku ja radioaktiivselt märgistatud ja märgistamata cAMP-i konkureerivat seostumise metoodikat. Teiseks võimaluseks on cAMP-i koguse määramine spektroskoopiliselt valguse emissiooni või neeldumise intensiivsuse kaudu, mis sõltub cAMP-i kogusest.

Tervete rakkude puhul toimub cAMP-i süntees füsioloogilistes tingimustes, kusjuures substraadi [^{3}H]ATP-i sünteesib rakk ise söötmisel lisatud [^{3}H]adeniinist. Sel juhul on tarvilik rakkude purustamine homogeniseerimisega ja membraanide eraldamine tsentrifuugimisega, et oleks võimalik mõõta rakkude sees sünteesitud radioaktiivset cAMP-i.

Tänapäeval enamkasutatavad radioaktiivsed meetodid AC-i aktiivsuse leidmiseks võib jaotada kahte gruppi: kromatograafilised ja cAMP-i spetsiifiliselt siduva valgu kasutamise meetodid. Neid meetodeid võib vaadelda ka kui otsest ja kaudset, konkureerivat meetodit.

Kromatograafilised meetodid põhinevad enamasti radioaktiivselt märgistatud ATP-st AC-i poolt sünteesitud radiaktiivse cAMP-i eraldamisel

teistest radioaktiivsetest fosfaatidest, peale mida mõõdetakse tema radioaktiivsus. Kui AC-i aktiivsust mõõdetakse tervetes rakkudes, on tarvis määrata ka märgistatud ATP-i radioaktiivsus, kuna antud [^{3}H]adeniiniga inkubeerides pole teada rakkudesse siseneva radioaktiivsuse kogus. Tavaliselt kasutatakse AC-i substraadina [^{3}H]ATP-i. Enamlevinumat kasutust [^{3}H]cAMP-i eraldamisel teistest radioaktiivsetest adenosiinfosfaatidest on leidnud Al_2O_3 täidisega kolonnid, kus cAMP lahutub üpris hästi teistest fosfaatidest [26]. On välja töötatud ka meetodeid, kus kasutatakse ka pöördfaas ning ioon-vahetus kolonne [27,28].

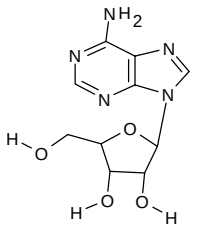
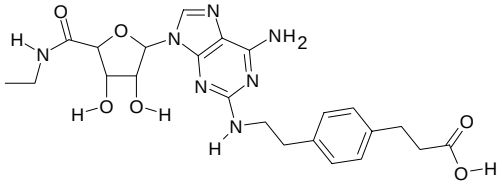
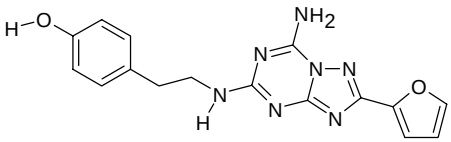
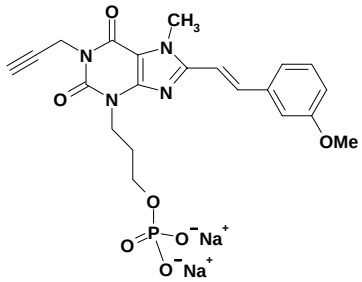
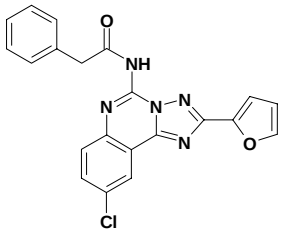
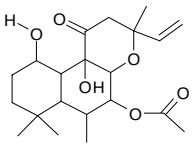
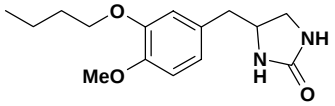
cAMP-i spetsiifiliselt siduva antikeha kasutamise meetodi põhimõte seisneb AC-i poolt sünteesitud cAMP-i ja märgistatud cAMP-i konkureerival seostumisel spetsiifilisele valgule. Meetodi võib jagada kaheks, radioaktiivsed ning mitteradioaktiivsed meetodid. Radioaktiivsete meetodite puhul kasutatakse AC-i poolt sünteesitud cAMP-i konkureeriva ühendina radioaktiivse isotoobiga (enamasti triitium) märgistatud cAMP-i. Mida rohkem on sünteesitud cAMP-i, seda vähem seostub antikehaga [^{3}H]cAMP-i. Antikehaga seostunud [^{3}H]cAMP eraldatakse seostumata [^{3}H]cAMP-st enamasti filtreerimis- või söesadestusmeetodil [29, 30]. AC-i poolt sünteesitud cAMP-i kontsentratsioon määratakse antikehaga seostunud [^{3}H]cAMP-i radioaktiivsuse kaudu.

Mitteradioaktiivse meetodite puhul AC-i poolt sünteesitud cAMP-i konkureeriva ühendina kasutatakse modifitseeritud cAMP-i, mille kontsentratsiooni on võimalik spektroskoopiliselt määrata. Ühe võimalusena kasutatakse cAMP–le kovalentselt seotud aluselist fosfaataasi. Aluseline fosfaataas on ensüüm, mis eraldab fosfaatrühmasid molekulidest. Pärast antikehaga seostumata cAMP-aluselise fosfaatasi eraldamist lisatakse paranitrofenüülfosfaati, mis aluselise fosfaataasi poolt lagundatakse paranitrofenooliks ja fosfaadiks. Paranitrofenooli kollase värvuse intensiivsus on võrdeline aluselise fosfataasi konts.-ga ja sellega seotud cAMP-ga. Tekkiva kollase värvuse intensiivsuse järgi tehakse kindlaks cAMP-i kontsentratsioon. [31]

Üheks eelnevatest erinevateks meetodiks on cAMP-i kontsentratsiooni määramine tema lagundamisel tekkiva anorgaanilise fosfaadi kaudu. Kõigepealt cAMP hüdrolüüsitakse PDE (fosfodiesteraas) poolt AMP-ks ning seejärel hüdrolüüsib 5’-nukleotitaas AMP-i adenosiiniks ja fosfaadiks. Süsteemile lisatud

malahhiitroheline moodustab fosfaadiga kompleksi ning sellega väheneb malahhiitrohelise valguse neeldumisvõime 620 nm juures. Valmistades kaliibrimisgraafiku saab arvutada cAMP-i kontsentratsiooni lähtudes malahhiitrohelise neeldumisvõimest. [31]

Tabel 2. Mõningate A_{2A} retseptorite ligandide struktuurid, adenülaadi tsüklaasi otsese aktivaatori forskoliini struktuur ning fosfodiesteraasi (PDE) inhibiitori Ro 20-1724 struktuur.

Ühend	Struktuur
Adenosiin	
CGS 21680	
ZM241385	
MSX-3	
MRS 1220	
Forskoliin	
Ro 20-1724	

Materjalid ja metoodika

Materjalid

[5',8'- ^{3}H] adenosiin-3', 5'-monofosfaat ([^{3}H]cAMP, 48Ci/mmol) oli fimalt Amersham Pharmacia Biotech, adenosiindeaminaas (ADA, EC 3.5.4.4), adenosiin-5'-trifosfaat (ATP), guanosiin-5'-trifosfaat (GTP), guanosiin-5'-0-(3tiotrifosfaat) (GTPγS), fosfoenüülpüruvaat (PEP) ja püruvaat kinaas (PK) firmalt Roche Diagnostics GmbH., bacitracin, tsükliline adenosiin-3',5'-monofosfaat (cAMP), etüleenglükool-bis(β-aminoetüül eeter)-N, N, N', N'-tetraetaanhape (EGTA), guanosiin-5'-difosfaat (GDP) ja 4-(3-butoksü-4-metoksübensüül)-imidasolidiin-2-oon (Ro 20-1724) firmalt Sigma-Aldrich Fine Chemicals, 2-p-(2-karboksüetüül)-fenetüülamino-5'-N-etüülkarboksamido-adenosiin (CGS21680), 4-(2-(7-amino-2-(2-furüül)[1,2,4]-triasolo-[2,3-a][1,3,5]-triasiin-5-üülamino)-etüül)-fenool (ZM241385), 9-kloro-2-(2-furüül)-5-fenüülatsetüülamino-[1,2,4]-triasolo[1,5-c]guinasoliin (MRS 1220) ja forskoliin osteti firmalt Tocris Cookson Ltd., etüüldiaminotetraetaanhape (EDTA) ja trihüdroksümetüül-aminoetaan kloriid (Trizma base) firmalt Merck & Co., Inc., naatriumkloriid ja dimetüülsulfoksiid (DMSO) firmalt OÜ Naxo , veise seerumi albumiin (BSA) firmalt Boehringer Mannheim ja magneesiumkloriid pärines firmalt Acros Organics.

Roti aju juttkeha membraanpreparaatide valmistamine

Rotiaju juttkehad eraldati Wistari liini rottidest prof. J. Harro poolt ja külmutati kuival jääl ning hoiti –80°C juures kuni kasutamiseni.

Membraanide valmistamisel kasutati kolme erinevat metoodikat. Pestud membraanide valmistamisel homogeniseeriti kude ultraheli-sonikeerimisega (10 sek.) 50 mM Tris-HCl puhvris (pH=7.4) (60 ml/g koe kohta). Järgnes tsentrifuugimine 20,000×g juures 40 min 4°C. Saadud sade resuspendeeriti samas koguses Tris-HCl puhvris, millele järgnes tsentrifuugimine eelpool toodud tingimustel. Sademe homogeniseerimist ja tsentrifuugimist korrati veel üks kord ning lõplik membraansade resuspendeeriti P.B. puhvris (30 mM Tris-HCl

(pH=7.4), 0.1 mM Ro 27-1470, 8.25 mM $MgCl_2$, 0.75 mM EGTA, 7.5 mM KCl, 0.1 M NaCl) ja jagati alikvootideks ning hoiti -80°C juures kuni kasutamiseni.

Üldise juttkeha suspensioon valmistamisel homogeniseeriti kude ultraheli-sonikeerimisega (/10 sek.) P.B. puhvris (30 mM Tris-HCl (pH=7.4), 0.1 mM Ro 27-1470, 8.25 mM $MgCl_2$, 0.75 mM EGTA, 7.5 mM KCl, 0.1 M NaCl) 65 ml/g koe kohta, jaotati alikvootideks ja hoiti -80°C juures kuni kasutamiseni.

Na/K sooladeta üldise juttkeha suspensiooni valmistamisel homogeniseeriti kude Potteri klaas/teflon homogenisaatoriga 2.5 mM Tris-HCl puhvris (pH=7.4), mis sisaldas 2 mM EGTA-d (50 ml/g koe kohta). Saadud suspensioonile lisati täiendavalt 50 mM Tris-HCl puhvrit (pH=7.4), mis sisaldas 2 mM EGTA-d (50 ml/g koe kohta) Saadud lahus segati ja jagati alikvootideks ning hoiti -80°C juures kuni kasutamiseni.

Adenülaadi tsüklaasi aktiivsuse määramine rakkudes

Hiina hamstri munarakkude (CHO) liini, milles on ekspresseeritakse koera A_{2A} retseptoreid, valmistas Dr. M. Torvinen Karolinska Instituudis (Rootsi) [32]. Rakud kasvatati 10 % veiseloote seerumit, 100 U/ml streptomütsiini ja penitsiliini ning 300 µl/ml hügromütsiini sisaldavas rakukultuuri meediumis MEM α (Gibco) ilma nukleosiiditeta Petri tassidel 37°C juures 5 %-lise CO_2 atmosfääris Raili Remmeli poolt.

Ööpäev enne eksperimenti pandi rakud kasvama multivell plaatidele eelpooltoodud tingimustel, kusjuures söötmesse oli lisatud ADA (3 U/ml). Enne eksperimenti pesti rakke seerumivaba α-MEM meediumiga ja inkubeeriti rakke 400 µl seerumivabas α-MEM keskkonnas, mis sisaldas ADA (1.5 U/ml), 30 min 37°C juures 5 %-lise CO_2 atmosfääris. Pärast ADA-ga töötlemise lõppu lisati rakkudele PBS puhvris lahustatuna erineva konts.-ga ligandid koos fosfodiesteraasi (PDE) inhibiitori Ro 20-1724 (lõplik konts. 0.1 mM). Järgnes 15 minutiline inkubatsiooniperiood 37 °C ja 5% CO_2 juures ja cAMP akumulatsiooni reaktsiooni peatamiseks lisati rakkudele $HClO_4$ (lõplik 0.4 M) ning seejärel reaktsioonisegu inkubeeriti 1 h jääl. Enne cAMP koguse määramist neutraliseeriti proovid KOH lahusega.

Akumuleerunud cAMP hulga määramiseks kasutati cAMP-i spetsiifiliselt siduva veise neerupealse ekstrakti valku ja metoodikat [30]. Lühidalt, cAMP standardeid ning proove inkubeeriti koos [^{3}H]cAMP lahuse (10000 cpm/punktis) ja cAMP-i spetsiifiliselt siduva valgu lahusega vähemalt 2 h 4°C juures.Seostunud radioaktiivsus eraldati filtreerimisel läbi GF/B klaaskiudfiltrite (Whatman Int. Ltd., Madistone, UK) kasutades firma Brandell filtreerimissüsteemi ja pestes filtreid 4x4 ml jääkülma 20 mM fosfaatpuhvriga (pH=7.4), mis sisaldas 100 mM NaCl. Filtreid hoiti ööpäev 5 ml stsintilatsioonikokteilis OptiPhase HiSafe®3 (Wallac Perkin Elmer Life Sciences) ja radioaktiivsus loendati Beckman LS 1800 stsintilatsiooni loendaja abil.

[^{3}H]cAMP mittespetsiifiline sidumine määrati tühiprooviga, kuhu sidumisvalgu lahust ei lisatud ning mõõdeti sidumisvalguga seostumisest sõltumatu radioaktiivsus.

Adenülaadi tsüklaasi aktiivsuse määramine aju juttkeha membraanides

Aju juttkeha membraanpreparaati (2-9 μg/ml valku katsepunktis) inkubeeriti eri konts.-ni ligandidega reaktsioonipuhvris, mis sisaldas 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, ATP regenereerivat süsteemi (10 mM PEP ja 45 μg/ml PK). Na/K sooladeta suspensiooni puhul oli reaktsioonikeskkonnas $MgCl_2$ konts. 10 mM ning NaCl ja KCl ei kasutatud.

cAMP akumulatsiooni reaktsiooni käivitamiseks lisati proovidele ATP (lõplik konts. 1 mM) ja GTP (lõplik konts. 10 μM) lahus ning reaktsioon peatati 50 μl EDTA lisamisega (lõplik konts 25 mM), misjärel proove keedeti 3 min. Tekkinud cAMP hulka määrati [^{3}H]cAMP konkureerimise järgi nagu on kirjeldatud ülalpool.

cAMP spetsiifiliselt siduva valgu lahuse valmistamine

Sidumisvalk puhastati koostöös TÜ Arstiteaduskonna Biokeemia instituudiga veise neerupealsetest eelnevalt kirjeldatud metoodika järgi [29].

Värskelt eraldatud veiste neerupealsed (Vastse-Kuuste Lihatööstus) homogeniseeriti 1:1.5 ruumala suhtes 50 mM Tris-HCl puhvris (pH=7.4), mis sisaldas 0.25 M glükoosi ja 5 mM $MgCl_2$. Homogenaat tsentrifuugiti 2000×g juures 5 minutit ja saadud sade eemaldati ning tsentrifuugiti 5000×g juures 15 minutit. Saadud supernantant lahus jaotati alikvootideks ning säilitati -20°C juures kuni eksperimendini.

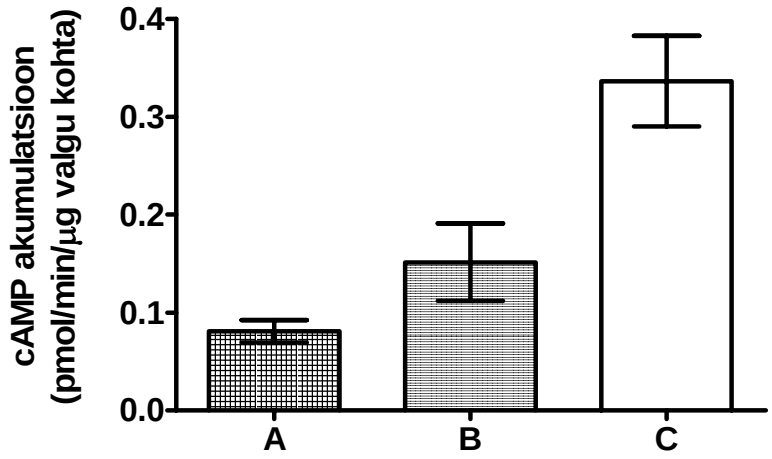
Tulemuste töötlus

Kõik tulemused analüüsiti programmi GraphPad Prism™ 4.0 abil. Kalibreerimisgraafikute puhul kasutati lineaarset vähimruutude töötlust, teiste tulemuste töötluseks kasutati mittelineaarset vähimruutude meetodit.

Tulemused ja arutelu

CGS21680 spetsiifiline cAMP akumulatsiooni kasv

G valguga seotud retseptorite farmakoloogilisi uuringuid on tavaliselt teostatud tervetes rakkudes või osaliselt pestud membraanpreparaatides, mida rakendati esialgu ka A_{2A} retseptori iseloomustamiseks roti aju juttkehas. Kuid üha enam koguneb informatsiooni retseptor/G valk/efektor signaalülekandes osalevate teiste valkude olulisest rollist [33], mis tsentrifuugimise käigus võidakse välja pesta membraanpreparaadist. Seetõttu on oluline kasutada optimaalsemat membraanpreparaadi valmistamise metoodikat ning samuti reaktsioonikeskkonna optimaalseimaid tingimusi, et saavutada suuremat A_{2A} retseptor spetsiifilist signaali. Optimaalsemate tingimuste leidmiseks võrreldi kolmes erinevalt valmistatud membraanpreparaadis A_{2A}-spetsiifilise agonisti CGS21680 (10 μM) poolt initsieeritud cAMP akumulatsiooni taset. Suurim CGS21680 spetsiifiline cAMP akumulatsiooni kasvu efekt (0.33±0.05 pmol/min/μg valgu kohta) saavutati Na/K sooladeta üldises suspensioonis võrreldes pestud membraanides (0.08±0.01 pmol/min/μg valgu kohta) ja üldises suspensioonis (0.15±0.03 pmol/min/μg valgu kohta) saadud efektiga (Joonis 2). Forskoliin põhjustatud cAMP akumulatsiooni kasvu efektid erinevates juttkeha membraanpreparaatides ei erinenud oluliselt, mis olid vastavalt basaalsest tasemest 940, 1030 ja 710 %-ti pestud membraanides ja Na/K soolade ja sooladeta üldises juttkeha suspensioonis. Seega suurib absoluutne ja samuti suhteline AC aktivatsioon saavutati sooladeta üldises juttkeha suspensioonis ning edaspidistes eksperimentides kasutati just seda roti aju juttkeha membraanpreparaati.



Joonis 2. A_{2A} retseptori agonisti CGS21680 põhjustatud cAMP akumulatsioon. Aju juttkeha membraanpreparaadid: A -pestud membraanid, B - üldine suspensioon ja C - Na/K sooladeta üldine suspensioon. Preparaate (2-9 μg valku/ml) inkubeeriti ilma ja 10 μM CGS21680 juuresolekul nagu on kirjeldatud peatükis materjalid ja metoodika. Tulemused on esitatud kui triplikaatide keskmised ± SEM.

Guanosiin nukleotiidide ja ADA toime CGS21680 sõltuva aktivatsiooni efektile

GTP/GDP vahekord on oluline parameeter retseptor/G valk/efektor signaali ülekande mehhanismis [34]. GDP vahetuse kiirus GTP vastu G valgu α alaühikul määrab signaali ülekande efektiivsuse ja GTP-st GDP-ks hüdrolüüsi kiirus määrab signaali kestvuse. Et uurida GTP (10 μM) juuresolekul esinevat G valkude aktiveerimise taset A_{2A} retseptori poolt AC aktiivsuse kaudu, vahetati reaktsioonikeskkonnas GTP tema mittehüdrolüüsuva analoogi GTPγS vastu. 1 μM GTPγS kasutamisel A_{2A} retseptoritega seotud G valkude aktiveerimise taseme suurenemist ei toimu võrreldes 10 μM GTP juures esineva tasemega, kuna CGS21680 (10 μM) aktivatsiooni efekt jääb samaks GTP vahetamisel GTPγS vastu reaktsioonikeskkonnas. Küll aga üleüldise cAMP akumulatsiooni taseme kasv GTPγS kasutamisel näitab A_{2A} retseptoriga mitteseotud G valkude aktiveerimise taseme kasvu. GDP (1 μM) juurdelisamine GTP sisaldavasse inkubeerimiskeskkonda vähendas CGS21680 aktivatsiooni 30 %-di võrra ja

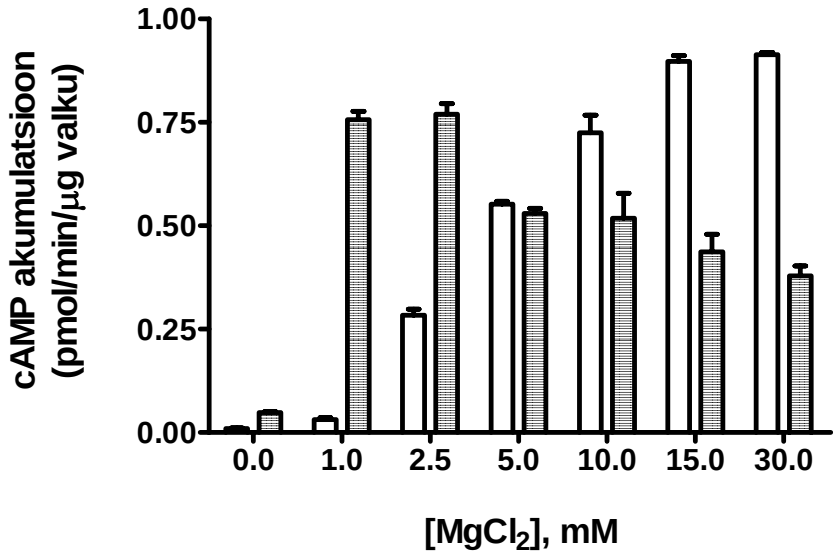
samuti üleüldist cAMP akumulatsiooni taset, mis näitab G valgu aktiveerimise inhibeerimist GDP poolt.

Adenosiin, mida esineb ajus küllaldases koguses, et aktiveerida adenosiini retseptoreid, võib retseptoriga seotuks jääda ka pärast homogeniseerimist või tsentrifuugimist membraanpreparaadi valmistamisel. ADA ensüümiga membraanpreparaadi töötlemisel on võimalik sisemine adenosiin eemaldada. Aju juttkeha membraanpreparaadi töötlus ADA-ga aga ei mõjutanud CGS21680 (10 μM) aktivatsiooni efekti ega basaalset AC aktiivsust, mis näitab aju juttkeha membraanpreparaadis sisemine adenosiini puudumist või madalat kontsentratsiooni. On leitud, et aju juttkehas on ADA kõrgelt ekspresseeritud [35] ja tihedalt seotud adenosiini retseptoritega. Lisaks näitavad radioligandiga tehtud eksperimendid aju juttkeha membraanpreparaatides , et ligandide afiinsused ei muutu ADA-ga töötlemisel [36]. CHO rakkudes, kus on ekspresseeritud A_{2A} retseptor, on aga radioligandide afiinsus sõltuv ADA-ga töötlemisest, suurenedes viimase rakendamisel [36], mis näitab sisemise adenosiini mõju nendes. Seega aju juttkeha membraanpreparaadi kasutamisel katsetes ADA-ga töötlust ei rakendatud, CHO rakkude puhul aga teostati alati eelnev ADA-ga töötluse etapp.

Mg^{2+} kontsentratsioon-sõltuv toime basaalsele ja CGS21680 sõltuva aktivatsiooni tasemele

Mg^{2+} on vajalik efektiivseks retseptor initsieeritud G valgu aktivatsiooniks [37] ja ATP-st cAMP-i tootmise reaktsiooni katalüüsimisel AC ensüümi poolt. Kuid sõltuvalt signaaliülekande mehhanismi astmetest võib vajalik Mg^{2+} konts.-n olla erinev. Parima A_{2A} retseptor spetsiifilise efekti leidmise eesmärgil varieeriti Mg^{2+} kontsentratsiooni roti aju juttkeha membraanpreparaadi reaktsioonipuhvris 0 – 30 mM vahemikus. Mg^{2+} puudumisel olid nii basaalne kui CGS21680 aktiveeritud cAMP akumulatsiooni tasemed väga madalad (Joonis 3). 1 mM Mg^{2+} kontsentratsiooni juures basaalne cAMP akumulatsioon oluliselt ei kasvanud, kuid CGS21680 juuresolekul tõusis cAMP akumulatsiooni tase oluliselt. 2.5 mM Mg^{2+} konts.-st kõrgematel väärtustel CGS21680-spetsiifiline efekt cAMP akumulatsioonile vähenes järjest, jõudes 30 mM Mg^{2+} konts.-ni juures 50 %-di tasemeni võrreldes 1 mM Mg^{2+}

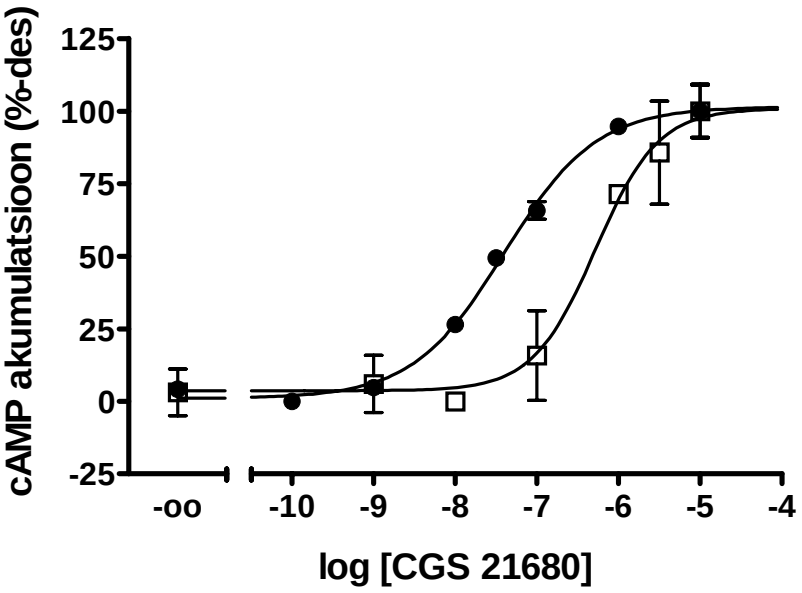
konts.-ni juures esinenud aktivatsiooniga (joonis 3, viirutatud tulbad). Basaalne cAMP akumulatsiooni tase kasvas koos Mg^{2+} konts.-ga ning juba 5 mM Mg^{2+} konts.-ni juures basaalne cAMP akumulatsioon oli jõudnud CGS21680-spetsiifilise efekti tasemeni (Joonis 3, tühjad tulbad). Basaalse ja CGS21680-spetsiifilise cAMP akumulatsiooni võrdlemine näitab, et kõrgemal kui 2.5 mM Mg^{2+} konts.-nil maksimaalne totaalne cAMP akumulatsioon jääb konstantseks ning Mg^{2+} konts.-ni suurenemisega väheneb retseptor spetsiifilise cAMP akumulatsiooni osa. Saadud andmed näitavad, et A_{2A} retseptor spetsiifiline efekt cAMP akumulatsioonile on kõige suurem 1 mM Mg^{2+} kontsentratsiooni juures ja seega on kõige optimaalsem A_{2A} retseptor spetsiifilise AC aktiivsuse mõõtmisel.



Joonis 3. Mg^{2+} mõju cAMP akumulatsioonile roti juttkeha suspensioonis. Mg^{2+} kontsentratsioon-sõltuv mõju basaalsele cAMP akumulatsiooni tasemel (tühi tulp) ja CGS21680-spetsiifilise cAMP akumulatsiooni efektile (viirutatud tulp). Na/K sooladeta üldist juttkeha suspensiooni (5 μg valku/ml) inkubeeriti erinevatel Mg^{2+} kontsentratsioonidel koos ja ilma 10 μM CGS21680 nagu on kirjeldatud peatükis materjalid ja metoodika. Tulemused on esitatud kui triplikaatide keskmised ± SEM.

CGS21680 sõltuva aktivatsiooni efektid CHO rakkudes ja aju juttkeha membraanpreparaadis

A_{2A} retseptori agonist CGS21680 suurendas kontsentratsioonist sõltuvalt cAMP akumulatsiooni taset CHO rakkudes, kus on ekspresseeritud A_{2A} retseptor, ja aju juttkeha membraanpreparaadis (Joonis 4). 10 μM CGS21680 mõjul kasvas cAMP akumulatsiooni tase võrreldes basaalse tasemega ligikaudu 6 korda CHO rakkudes ning aju juttkeha membraanpreparaadis tingimuste varieerumise tulemusena ligi 10 korda. CGS21680 kontsentratsioon-sõltuvat cAMP akumulatsiooni aktiveerimist iseloomustas CHO rakkudes pEC_{50} = 7.4±0.1 ja aju juttkeha membraanpreparaadis pEC_{50} = 6.3±0.2. Nende väärtuste võrdlemisel ilmneb, et CHO rakkudes on CGS21680 afiinsus märgatavalt suurem A_{2A} retseptori suhtes kui aju juttkeha membraanpreparaadis. Radioligandiga tehtud sidumiskatsed ei näita, et [^{3}H]CGS21680 afiinsus antud retseptori suhtes oluliselt erineks [36]. CGS21680 afiinsuse suur vahe A_{2A} retseptorile aju juttkeha membraanpreparaadis ja CHO rakkudes võib olla põhjustatud aju juttkeha koe homogeniseerimisel funktsionaalse retseptor/G valk/AC signaaltee efektiivsuse vähenemisest. Sellist ligandi afiinsuse vahet on täheldatud ka adenosiin-A_3 retseptori puhul, kus tervetes rakkudes ning rakkude membraanpreparaadis erines A_3 retseptori agonisti afiinsus kuni 200 korda [38].



Joonis 4. CGS21680 mõju cAMP akumulatsioonile CHO rakkudes ja roti aju juttkeha suspensioonis. CGS21680 kontsentratsioon-sõltuv cAMP akumulatsiooni aktivatsioon CHO rakkudes (●) ja Na/K sooladeta üldises suspensioonis (□). CHO rakke ja Na/K sooladeta üldist suspensiooni inkubeeriti erinevatel CGS21680 kontsentratsioonidel nagu on kirjeldatud peatükis materjalid ja metoodika. Tulemused on esitatud kui triplikaatide keskmised ± SEM.

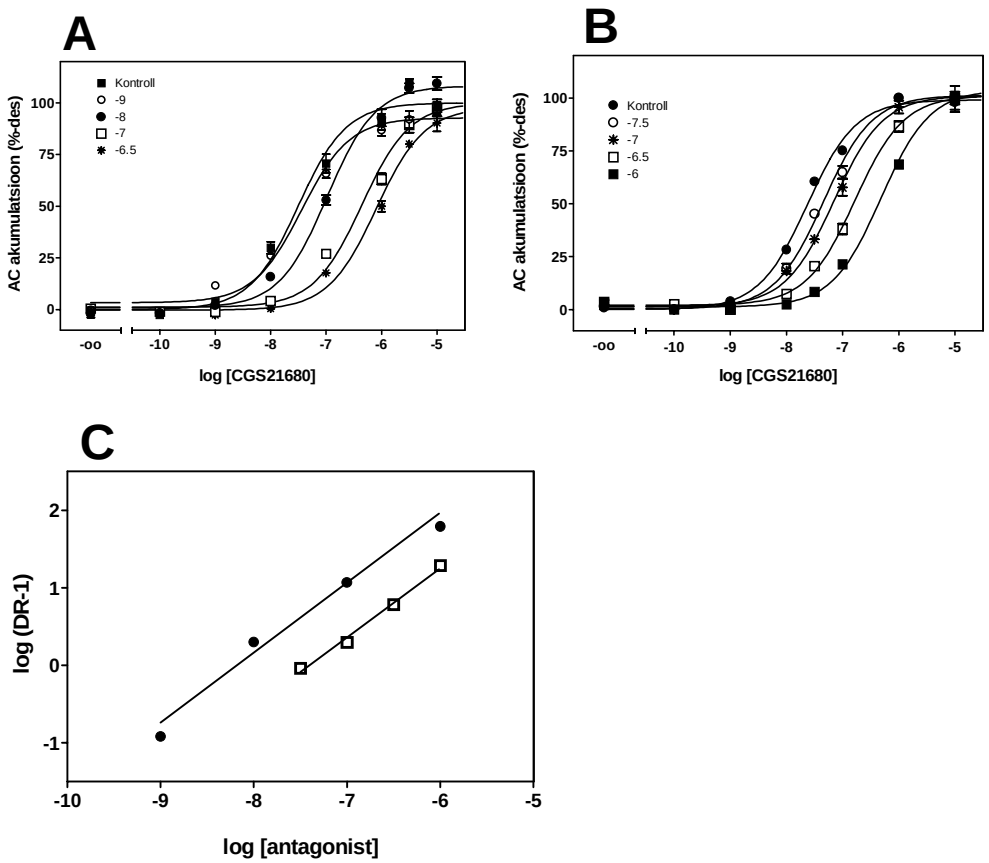
Antagonistide mõju CGS21680 sõltuvale aktivatsioonile CHO rakkudes ja aju juttkeha membraanpreparaadis

A_{2A} retseptori antagonistid ZM241385 ja MSX-3 ei omanud mõju basaalsele cAMP-i akumulatsioonile kuid inhibeerisid CGS21680 sõltuvat aktivatsiooni nii CHO rakkudes kui ka roti aju juttkeha membraanpreparaadis. Antagonistide toime puudumine basaalsele cAMP-i akumulatsioonile viitab sisemise adenosiini mõju puudumisele nii CHO rakkudes kui ka juttkeha membraanpreparaadis cAMP akumulatsioonile.

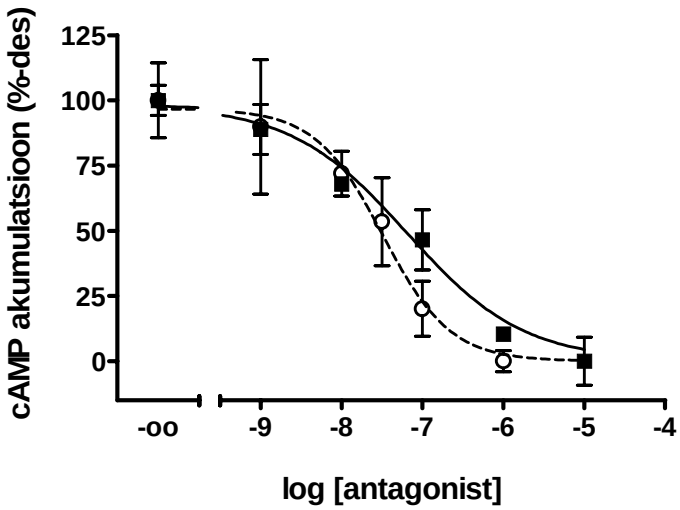
Mõlemate antagonistide konts.-ni suurendamine reaktsioonikeskkonnas põhjustas CGS21680 sõltuva aktivatsiooni EC_{50} väärtuse kasvu CHO rakkudes ning seejuures maksimaalset cAMP akumulatsiooni taset oluliselt mõjutamata (Joonis 5 A ja B). Antagonistide konts.-sõltuva toime põhjal CGS21680 sõltuva

aktivatsiooni EC_{50} väärtusele koostati Schildi graafikud (Joonis 5 C) ning leiti antagonistide K_i väärtused (6.6 nM ja 39.7 nM vastavalt ZM241385 ja MSX-3 jaoks) ja konts.-sõltuvuste tõusu väärtused (0.89±0.05 ja 0.89±0.06 vastavalt ZM241385 ja MSX-3 jaoks). K_i väärtuste võrdlemisel ilmneb, et ZM241385 on kõrgema asiinsusega ligand kui MSX-3 ja mõlemad antagonistid seostuvad konkureerivalt agonist CGS21680-ga samale sidumiskohale A_{2A} retseptoril, mida näitab antagonistide kontsentratsioon-sõltuvuse tõusu 1 lähedased väärtused Schild graafikul.

Roti aju juttkeha membraanpreparaadis antagonistide konts.-sõltuv CGS21680 põhjustatud aktivatsiooni inhibeerimise IC_{50} väärtused (32±15 ja 63±30 nM vastavalt ZM241385 ja MSX-3 jaoks) näitasid samuti ZM241385 suuremat afiinsust A_{2A} retseptori suhtes kui MSX-3 (Joonis 6) ja ka inhibeerimist iseloomustavad Hill'i koefitsiendid olid antagonistidel erinevad (1.0±0.4 ja 0.6±0.3 vastavalt ZM241385 ja MSX-3 jaoks). Radioligandi väljatõrjumise katsest konkureeriva ligandi K_i väärtuse arvutamiseks kasutatavat Cheng-Prusoffi valemi ($K_i = IC_{50} / (1 + [L]/K_d)$) analoogiat kasutati antagonistide ZM241385 ja MSX-3 K_i väärtuste hindamisel membraanpreparaadis iseloomuliku inhibeerimise põhjal. Arvutamisel kasutati antagonistide konts. -sõltuva inhibeerimise efektist saadud IC_{50} väärtusi ja CGS21680 K_d väärtuse asemel membraanpreparaadis saadud EC_{50} väärtust. Võrreldes saadud tulemusi [^{3}H]ZM241385 väljatõrjumise eksperimendist saadud väärtustega näeme, et ZM241385 puhul erinevalt saadud K_i väärtused on üsna hästi kooskõlas (K_i = 1 nM ja K_i = 1.2 nM vastavalt radioligandi väljatõrjumise ja CGS21680 aktivatsiooni inhibeerimise eksperimendist). MSX-3 puhul aga sellist kokkulangevust ei saavutatud (K_i = 50 nM ja K_i = 2.4 nM vastavalt radioligandi väljatõrjumise ja CGS21680 aktivatsiooni inhibeerimise eksperimendist).



Joonis 5. **A** - A_{2A} retseptori antagonisti ZM241385 toime CGS21680 sõltuva cAMP-i akumulatsiooni aktivatsioonile CHO rakkudes. CGS21680 konts. - sõltuvat cAMP-i akumulatsiooni kasvu mõõdeti erinevate ZM241385 konts.-nide (graafikul A näidatud logaritmiliste ühikutena) juuresolekul nagu on kirjeldatud peatükis materjalid ja metoodika. **B** – A_{2A} retseptori antagonisti MSX-3 toime CGS21680 sõltuva cAMP-i akumulatsiooni aktivatsioonile CHO rakkudes. Mõõtmised teostati sarnaselt ZM241385-ga tehtud katsetes kuid ZM241385 asemel kasutati MSX-3 erinevaid konts.-ne (graafikul B näidatud logaritmiliste ühikutena). **C** – A_{2A} antagonistide ZM241385 ja MSX-3 Schild graafik. Erinevatel antagonistide ZM241385(●) ja MSX(□) juuresolekutel saadud CGS21680 sõltuva aktivatsiooni EC_{50} väärtuste põhjal arvutati doos-suhted ning konstrueeriti Schild graafik telgedega doos-suhe – 1 ja antagonisti konts. logaritmilise skaalas.



Joonis 6. A_{2A} retseptori antagonistide mõju 10µM CGS21680-initsieeritud cAMP akumulatsioonile roti aju juttkeha suspensioonis. Na/K sooladeta üldist suspensiooni inkubeeriti koos 10 µM CGS21680 ja erinevate antagonisti ZM241385 (○) või MSX-3 (■) kontsentratsioonil nagu kirjeldatud peatükis materjalid ja metoodika. Tulemused on esitatud kui triplikaatide keskmised ± SEM.

Kokkuvõte

Kasutades erinevaid metoodikaid roti aju juttkehast membraanpreparaadi valmistamiseks leiti, et suurim adenosiini A_{2A} retseptori aktivatsioonist tingitud cAMP-i akumulatsioon saavutati üldises koe suspensioonis, millele ei olnud lisatud ka Na^+ ja K^+ ioone. Vähemalt 1 mM Mg^{2+} on vajalik maksimaalse CGS21680-spetsiifilise cAMP-i akumulatsiooni saavutamiseks. Kuna ka üldine, A_{2A}-sõltumatu cAMP-i akumulatsioon sõltus otseselt Mg^{2+} kontsentratsioonist siis hakkas see üle 5 mM Mg^{2+} juuresolekul retseptor-sõltuvat efekti inhibeerima ja varjutama. CGS21680 mõju cAMP-i akumulatsioonile roti aju juttkeha üldises suspensioonis iseloomustas pEC_{50} = 6.3±0.2. Samas aga CHO rakkudes, kuhu oli ekspresseeritud A_{2A} retseptor, oli CGS21680 spetsiifilise AC-i aktivatsiooni pEC_{50} väärtus 7.4±0.1.

Antagonistid ZM241385 ja MSX-3 ei mõjutanud cAMP-i akumulatsiooni baastaset, kuid inhibeerisid CGS21680 initsieeritud cAMP-i akumulatsiooni tõusu nii aju membraanide suspensioonis kui ka CHO rakkudes. Aju juttkeha üldises suspensioonis antagonistide CGS21680 initsieeritud cAMP-i akumulatsiooni inhibeerimisest saadud K_i väärtused olid 1.2 ja 2.4 nM vastavalt ZM241385 ja MSX-3 jaoks. ZM241385 ligandi puhul oli see väärtus heas kooskõlas radioligandi väljatõrjumise eksperimendist saadud K_i väärtusega (K_i = 1 nM), kuid CHO rakkudes cAMP-i akumulatsiooni inhibeerimise põhjal saadud K_i väärtusest madalam (K_i = 6.6 nM). MSX-3 ligandi puhul oli eelpool saadud K_i väärtus madalam nii radioligandi väljatõrjumises kui CHO rakkudes cAMP-i akumulatsiooni inhibeerimise eksperimendist saadud K_i väärtustest (K_i = 50 nM ja K_i = 39.7 nM vastavalt radioligandi väljatõrjumise ja CHO rakkudes cAMP-I akumulatsiooni inhibeerimise eksperimendis). Antagonistide jaoks leitud afiinsuste erinevuste põhjusteks võivad olla kineetilised limitatsioonid ja saadud konstantide erinev sisu, kuid agonisti afiinsuse muutus võib olla seotud retseptor/G valk/AC signaaltee efektiivsuse vähenemisega koe homogeniseerimisel.

Seega A_{2A} retseptori spetsiifilist efektorsüsteemi aktivatsiooni on võimalik mõõta ka aju juttkeha membraanpreparaatides, kuid andmete töötlemisel tuleb arvestada ka süsteemi tundlikkuse vähenemisega.

Summary

Using different methods for the preparation of membranes from rat striatum, the highest adenosine A_{2A} receptor-specific cAMP accumulation was achieved in crude tissue homogenate without added Na^+ and K^+ ions. The presence of at least 1 mM Mg^{2+} was required for a maximal CGS-21680-dependent cAMP accumulation in this preparation. The increase of Mg^{2+} caused also increase of the A_{2A} receptor independent cAMP accumulation and at concentrations above 5 mM it the receptor-specific effect was decreased and also shaded by the nonspecific accumulation. The potency of CGS21680 to activate AC in the homogenate was characterized with pEC_{50} = 6.3±0.2, but in intact CHO cells expressing A_{2A} receptors the potency was pEC_{50} = 7.4±0.1.

Antagonist ZM241385 and MSX-3 had no effect on basal level of cAMP formation, but inhibited competitively CGS21680 stimulated cAMP accumulation in the homogenate as well in the CHO cells. The potencies of the antagonists to inhibit the effects of CGS21680 in the striatal homogenate were K_i= 1.2 nM and K_i= 2.4 nM for ZM241385 and MSX-3, respectively. The constant for ZM241385 was in good agreement with K_i= 1 nM obtained in radioligand displacement experiment, but lower than K_i= 6.6 nM obtained from Schild plot of cAMP accumulation in the CHO cells. The constant of MSX-3 was considerably lower than both constants 50 nM and 39.6 nM, obtained from radioligand binding and from cAMP inhibition in the intact cells, respectively. Obtained discrepancies in antagonist potencies are connected with kinetic limitations and different meanings of obtained constants, while for agonists the efficacy has decreased probably by the disruption of the receptor / G protein / AC signal transduction pathway during the preparation of the homogenate.

Thus, the activation of adenosine A_{2A} receptors can be measured in the rat striatal membranes, but the decrease in the sensitivity and kinetic limitations has to be taken into account during the interpretation of the obtained data.

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Kinetic and functional properties of [^{3}H]ZM241385, a high affinity antagonist for adenosine A_{2A} receptors.

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Abstract

We have characterized the binding of [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]ZM241385) to adenosine A_{2A} receptors in membranes of rat striatum and transfected CHO cells. Saturation experiments showed that [^{3}H]ZM241385 binds to a single class of binding sites with high affinity (K_d = 0.23 nM and 0.14 nM in CHO cell and striatal membranes, respectively). The membranes of CHO cells required pretreatment with adenosine deaminase (ADA) to achieve high-affinity binding, while ADA had no influence on the ligand binding properties in striatal membranes. The binding of [^{3}H]ZM241385 was fast and reversible, achieving equilibrium within 20 minutes at all radioligand concentrations. The kinetic analysis of the [^{3}H]ZM241385 interaction with A_{2A} receptors indicated that the reaction had at least two subsequent steps. The first step corresponds to a fast equilibrium, which also determines the antagonist potency to competitively inhibit CGS21680-induced accumulation of cAMP (first equilibrium constant K_A = 6.6 nM). The second step corresponds to a slow process of conformational isomerization (equilibrium constant K_i = 0.03). The combination of the two steps gives the dissociation constant K_d = 0.20 nM based on the kinetic data, which is in good agreement with the directly measured value. The data obtained shed light on the mechanism of the [^{3}H]ZM241385 interaction with adenosine A_{2A} receptors from different sources in vitro. The isomerization step of the A_{2A} antagonist radioligand binding has to be taken into account for the interpretation of the binding parameters obtained from the various competition assays and explain the discrepancy between antagonist affinity in saturation experiments versus its potency in functional assays.

Keywords: [^{3}H]ZM241385; adenosine A_{2A} receptors; adenylate cyclase; CGS 21680; kinetic mechanism; isomerization

Introduction

Adenosine is an endogenous modulator of a wide range of biological functions, which acts via at least four G-protein-coupled receptor subtypes classified as A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2000). The A_{2A} and A_{2B} receptors are positively coupled to adenylyl cyclase, while A_1 and A_3 adenosine receptors cause inhibition of cAMP formation (Fredholm et al., 1994). Adenosine A_1 receptors are abundant in the brain with high levels being expressed in many regions such as the cerebral cortex, hippocampus, cerebellum, thalamus, brain stem, and spinal cord (Ralevic and Burnstock, 1998). The distribution of adenosine A_{2A} receptors includes lymphocytes, platelets, nerve cells, vascular smooth muscle and endothelium (Ralevic and Burnstock, 1998). In the brain the A_{2A} receptors are highly expressed in the neostriatum, nucleus accumbens and olfactory tubercle (Ongini and Fredholm, 1996). Striatal A_{2A} receptors are colocated with dopamine D_2 receptors (Fink et al., 1992) and an antagonistic cross-regulation between these receptors exists in the striatum (Ferré et al., 1997; Agnati et al., 2003), but synergistic interactions have been observed in PC12 cells (Kudlacek et al., 2003). There exist a number of findings, suggesting that A_{2A} receptor antagonists may have a role in the treatment of Parkinson's disease, whereas A_{2A} receptor agonists show an atypical antipsychotic profile (Ferré et al., 1991; Ongini and Fredholm, 1996; Rimondini et al., 1997; Ferre, 1997; Kase et al., 2003). The pharmacological characterization of the A_{2A} receptors has been hampered by the lack of selective, high affinity A_{2A}-specific radioligands. For a long time the agonists [^{3}H]CGS21680 and [^{3}H]NECA have been the only radioligands used for the characterization of A_{2A} receptors (Fredholm et al., 1994). [^{3}H]SCH58261 was the first reported radiolabelled A_{2A}-specific adenosine antagonist (Dionisotti et al., 1997) but is not yet generally available. [^{3}H]ZM241385, ([2-^{3}H]-4-

(2-[7-Amino-2-(2-furyl)-[1,2,4]-triazolo-[2,3-α]-[1,3,5]triazin-5-ylamino]ethyl)phenol) was the first commercially available radiolabelled A2A antagonist and has been found to be useful for pharmacological experiments (DeMet and Chicz-DeMet, 2002; Rebola et al., 2003). ZM241385 was introduced as a very selective A_{2A} receptor antagonist in 1995 (Poucher et al., 1995) which has been confirmed in numerous experiments in vivo and in vitro (Cunha et al., 1997; Ohkubo et al., 2000). Nevertheless, there is only limited information available on the A_{2A} binding properties of [^{3}H]ZM241385 (Alexander and Millns, 2001).

In the present study we have used a kinetic approach for unraveling the mechanism of interactions of [^{3}H]ZM241385 with the adenosine A_{2A} receptors in rat neostriatum and transfected CHO cell membranes. The results indicate the presence of two kinetically distinguishable steps for the A_{2A} antagonist radioligand receptor interaction, where the fast binding equilibrium (characterized with equilibrium constant K_A) is followed by a relatively slow isomerization step (with equilibrium constant K_i, for comparison see (Lepiku et al., 1996)). The physiological potency of the A_{2A} antagonist is determined by its equilibrium constant for fast binding, while affinity in binding experiments is a product of both equilibrium constants (K_A * K_i).

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]ZM241385, 17 Ci/mmol) was purchased from Tocris Cookson Ltd., [carboxyethyl-^{3}H(N)]-4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid

hydrochloride ([^{3}H]CGS21680, 30.0 and 42.5 Ci/mmol) from Perkin Elmer Life Sciences and [5',8-^{3}H]adenosine 3', 5'-cyclic phosphate, ammonium salt ([^{3}H]-cyclicAMP, 48 Ci/mmol) was purchased from Amersham Biosciences. Forskolin, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone (Ro 20-1724), N-[9-chloro-2-(2-furanyl)[1,2,4)-triazolo[1,5-c]quinazolin-5-benzeneacetamide (MRS 1220) and ZM241385 were obtained from Tocris Cookson Ltd., 2-chloroadenosine, 3,7-dimethyl-1-porpargylxanthine (DMPX) from Sigma-Aldrich Fine Chemicals, 3-(3-hydroxypropyl)-8-(m-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3) from Pharmaceutical Institute, University of Bonn (Germany) and cell culture media and reagents from GIBCO^{TM} and all other reagents were of analytical grade from regular suppliers.

Membrane preparations from CHO cells expressing A_{2A} adenosine receptors

Chinese hamster ovary cell line (CHO-K1) stably expressing double hemagglutinin-tagged dog adenosine A_{2A} receptors were described and characterized previously (Torvinen, 2002; Torvinen et al., 2004). The cells were grown to adherence and maintained in α-MEM without nucleosides, containing 10% foetal bovine serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and geneticin (G-418, 500 µg/ml) at 37°C in a 5% CO_2-95% air atmosphere with saturated humidity on plastic Petri dishes. For radioligand binding experiments the cells were collected, washed with PBS, and homogenized by sonication in homogenization buffer (HB, 20 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH=7.5) as described earlier (Rinken et al., 1999a). The crude homogenate was centrifuged at 40,000 g for 20 min at 4°C and the pellet homogenized in HB. The resulting membrane suspension, unless otherwise indicated, was incubated with adenosine deaminase (ADA, EC 3.5.4.4, Roche

Diagnostics GmbH, 5 U/ml) for 30 min at 37°C to remove endogenous adenosine. The membranes were centrifuged and homogenized two more times as above but without ADA treatment. The final pellet was resuspended in HB (0.2 mg protein /ml) and was used directly for binding experiments.

Membrane preparations from rat striatum.

Rat striatal membranes were prepared as described previously (Rinken et al., 1999b) with slight modifications. Rat striata were homogenized in ice-cold homogenization buffer HB and centrifuged at 40,000 g for 20 min at 4°C. The membrane pellet was washed by homogenization in HB and centrifuged two more times. The final suspension was divided into aliquots and stored at -80°C until use.

Radioligand binding assays

Equilibrium binding assays were (unless otherwise stated) performed by incubating membranes (100 µg protein/500 µl) in incubation buffer (IB, 20 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA pH=7.5) with appropriate concentrations of [^{3}H]ZM241385 (0.04 to 4 nM) for 45 min at 25°C and were terminated 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 20 mM Tris-HCl and 100 mM NaCl, (pH=7.5). Non-specific binding was determined in the presence of 0.5 mM dimethylpropargylxantine (DMPX). Displacement experiments were performed in the same way including 1.4 nM [^{3}H]ZM241385 and appropriate concentrations of non-labelled compounds (from 1 pM to 100µM, depending on ligand studied) after the incubation for 60 min at 25°C. The filters were kept overnight with 5 ml of scintillation cocktail OptiPhase HiSafe®3

(Wallac Perkin Elmer Life Sciences) and radioactivity content was measured using a Beckman LS 1800 scintillation counter.

Kinetic association experiments were started by addition of [^{3}H]ZM241385 (final concentration from 0.1-12 nM) at a time moment 0 to a membrane suspension in the IB (2 ml). At timed intervals aliquots (200 μl) were taken and filtered on GF/B as described above. Parallel incubations with corresponding concentration of [^{3}H]ZM241385 and 0.5 mM DMPX were used to estimate the non-specific binding of the radioligand.

Dissociation kinetics were measured after preincubation of membranes with 1 nM [^{3}H]ZM241385 for 45 min at 25°C. Dissociation was then initiated by addition of unlabelled CGS 21680 (100 μM final concentration), unlabelled ZM241385 (10 μM) or 20 times dilution of reaction medium. At timed intervals aliquots (500 μl) were filtered on GF/B and the bound radioactivity was determined as described above.

Measurement of cyclic AMP accumulation in CHO cells.

The cells were sown onto 24-well Petri dishes 24 h before experiments and the cell medium was supplemented with adenosine deaminase (ADA, 3 U/ml). The cells were washed with serum-free α-MEM medium and incubated in 400 μl serum-free α-MEM medium containing 1.5 U/ml ADA for 30 min at 37°C. The ligand dilutions and the phosphodiesterase inhibitor Ro 20-1724 (final concentration 100μM) in 100 μl PBS per well were added and the incubation carried out for 15 minutes at 37^{o}C. The reaction was terminated with addition of ice-cold $HClO_4$ (final 0.4 M). After 1h incubation on ice, the cell lysates were neutralized with KOH, centrifuged at 16,000 g for 2 min and the cAMP content of the supernatant fractions were determined by the modified protein-binding method (Nordstedt and Fredholm, 1990).

Data analysis

Equilibrium binding data were analysed by computer modelling, by fitting the data to appropriate equations, assuming that ligands bound to one or two independent sites, using non-linear regression using commercial program GraphPad PRISM™ (GraphPad, San Diego, CA, U.S.A.).

The pseudo-first-order rate constants of association k_{obs} were calculated from the equation:

$$B_t = B_{nonsp} + B(1 - e^{-k_{obs}t}) \qquad (1),$$

where B_t is the binding at time t, B_{nonsp} the non-specific binding, B the equilibrium binding and k_{obs} the observed association rate constant. Dissociation kinetic data were fitted into equation, which assumes that dissociation occurs in exponential fashion:

$$B_t = B_{nonsp} + B(e^{-k_{-1}t}) \qquad (2),$$

where B_t, B_{nonsp} and B have the same meaning as in Eq.(1).

For the interpretation of kinetic and equilibrium binding data first the simple bimolecular reversible reaction scheme:

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \qquad (3),$$

where the dissociation constant of the receptor·antagonist complex is characterized by the equation:

$$K_d = k_{-1} / k_1 = \frac{[R][L]}{[RL]} \qquad (4)$$

having k_1 and k_{-1} as corresponding association and dissociation rate constants. Solving scheme (3) in the case of pseudomonomolecular conditions (total [L]>>total [R], hence [L] does not change with time) reveals linear dependence of association rate constants on the radioligand concentration: $k_{obs}=k_1[L] + k_{-1}$.

As it was found in the case of several G protein coupled receptors, the ligand receptor interaction is more complex than scheme (3) (Järv et al., 1979; Lepiku et al., 1996; Chappell et al., 1992). Thus, the simplest reaction scheme that describes the ligand receptor interaction involves two consecutive steps, ligand association and isomerization of receptor ligand complex:

$$R + L \overset{K_A}{\rightleftharpoons} RL \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} RL^* \qquad (5),$$

where K_A stands for the fast first equilibrium, while k_i and k_{-i} describe the slower isomerization step of the receptor-ligand complex. For this scheme in pseudomonomolecular conditions ([L] does not change in time) the association rate constants k_{obs} depend on the radioligand concentration according to the hyperbolic function:

$$k_{obs} = \frac{k_i[L]}{K_A + [L]} + k_{-i} \qquad (6)$$

Dissociation constant of ligand according to scheme (5) is a product of dissociation constants for each step: $K_d = K_A \cdot K_i = K_A \cdot (k_{-i}/k_i)$.

All data are presented as mean ± SEM of at least two independent determinations carried out in duplicates. Statistical significance of differences was determined by Student-Newman-Keuls test, where $P<0.05$ was taken as a criterion of significance.

Results

[^{3}H]ZM241385 binding characteristics in CHO and striatal membranes

The binding of [^{3}H]ZM241385 to membranes of CHO cells expressing A_{2A} adenosine receptors was saturable and characterized by an affinity with a K_d= 0.23±0.03 nM and a density by B_{max} = 360±15 fmol/mg protein. The binding of the radioligand to striatal membranes was described with a K_d=0.14±0.01 nM and a B_{max}=1620±40 fmol/mg protein (Figure 1). The studies with [^{3}H]ZM241385 were carried out in an incubation buffer containing 50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA (pH=7.4), which was found to be optimal for the high-affinity binding of the radioligand. The presence of at least 30 mM Na^+ was required for the high-affinity binding of [^{3}H]ZM241385, while Mg^{2+} and Ca^{2+} at concentrations up to 10 mM had no significant influence on the binding properties of the radioligand (data not shown). Dithiothreitol (DTT), which is often used as an antioxidant in radioligand binding assays, decreased the affinity of [^{3}H]ZM241385 binding to striatal membranes (K_d=0.63±0.13 nM in the presence of 3 mM DTT). The binding was also sensitive towards the presence of dimethylsulfoxide (DMSO), a solvent that is often used to dissolve ligands. The affinity of [^{3}H]ZM241385 was decreased more than three times in the presence of 3% DMSO in the reaction buffer.

Role of G proteins and ADA

Activation of G proteins with the nonhydrolyzable GTP analogue GTPγS (150 μM) had no influence on [^{3}H]ZM241385 binding properties to membranes of rat striatum (K_d=0.14±0.03 nM, B_{max}=1690±90 fmol/mg protein, P>0.05 vs. control). The treatment of membranes with adenosine deaminase (ADA) to remove the endogenous

adenosine in the preparations, had no significant influence on the binding properties of [^{3}H]ZM241385 to striatal membranes (K_d=0.14±0.02 nM and K_d=0.17±0.03 nM for the treated and untreated membranes, respectively), but considerably increased the binding affinity to CHO cell membranes (K_d=0.23±0.03 nM and K_d=0.49±0.07 for the treated and untreated membranes, respectively $P<0.05$). This indicates that endogenous adenosine plays an essential role in membranes of CHO cells expressing A_{2A} receptors and all following experiments with these membranes were carried out after the ADA treatment.

[^{3}H]CGS21680 binding charateristics in CHO and striatal membranes

The A_{2A}-specific agonist [^{3}H]CGS21680 binds to CHO membranes with considerably lower affinity (K_d=78±3 nM), but the number of binding sites B_{max}=400±30 fmol/mg protein did not differ from the number of the binding sites of [^{3}H]ZM241385 in these membranes (P=0.24). The situation was the same in the case of striatal membranes, where the binding of [^{3}H]CGS21680 was characterized by a K_d=68±3 nM and a B_{max}=1650±60 fmol/mg protein (P=0.68).

Dissociation kinetics of [^{3}H]ZM241385

Dissociation of [^{3}H]ZM241385 from the the A_{2A} receptor complex in membranes was monophasic having a half-life of about 3 min (k_{-1}= 0.25±0.04 min^{-1} and 0.20±0.01 min^{-1} for CHO and striatal membranes, respectively) and was completed within 15-20 min (Figure 2). The dissociation rate did not depend on whether the dissociation reaction was initiated by addition of excess of antagonist 10 μM ZM241385, agonist 100 μM CGS21680 or by 20 times dilution.

Association kinetics of [^{3}H]ZM241385

The association of [^{3}H]ZM241385 to A_{2A} receptors was fast and reached steady-state at 0.8 nM of the radioligand within six minutes (Figure 3). Increasing the concentration of the radioligand caused a proportional increase of the association rate, reaching the value k_{obs} = 3.8 ± 0.8 min^{-1} at 5.9 nM of [^{3}H]ZM241385, which indicates that the amount of bound radioligand reached 50% of equilibrium values in 11 ± 2 sec. ($\tau_{1/2}$) (Fig. 4). The analysis of the dependence of the observed association rate constants according to the simple bimolecular reversible ligand binding model revealed the second order (true association) rate constant k_1 = 0.42 ± 0.01 $min^{-1}nM^{-1}$. The dissociation constant of the complex calculated from these kinetic constants K_d = k_{-1}/k_1 = 0.48 ± 0.04 nM is higher, than obtained with equilibrium binding.

This discrepancy and high dispersion of rate constants at higher radioligand concentration (Fig. 4) proposed the usage of a more complex model of the ligand binding, where the simple bimolecular reaction is followed by the isomerization of the receptor-ligand complex (Eq. 5). Fitting of the observed association rate constants to Eq. 6 revealed a rough estimation of the constants k_i= 6.9 ± 1.2 min^{-1} and K_A= 8.5 ± 2.2 nM that reveal the apparent dissociation constant K_d = $K_A(k_{-i}/k_i)$ = 0.24 nM, which is in better agreement with the equilibrium binding experiment (Fig.1)

Displacement of [^{3}H]ZM241385 binding by adenosine agonists and antagonists

All studied adenosine receptor ligands were able to compete with [^{3}H]ZM241385 binding in a concentration dependent manner and with K_i values which are in agreement with earlier reported data for A_{2A} receptors (Table 1). Activation of G proteins with 100 μM GTPγS had no significant influence on the affinities of neither the agonists nor the antagonists. Only the Hill coefficient of the

A_{2A}-specific agonist CGS 21680 was increased by GTPγS, indicating preferential affinity of the agonist for the G protein-receptor complex.

Modulation of accumulation of cAMP in CHO cells

CGS 21680 caused a concentration–dependent increase of cAMP accumulation in CHO cells expressing A_{2A} adenosine receptors. The activation level was 600% of the basal level and the potency of CGS21680 was characterized by an EC_{50} = 15±3 nM. Other adenosine receptor agonists adenosine and N-cyclopentyladenosine caused similar activation of adenylate cyclase with EC_{50} values of 51±10 nM and 250±70 nM, respectively, which are in agreement with the potencies of these ligands for A_{2A} receptors (Klotz, 2000). ZM241385 had no effect on the basal level of cAMP formation in these cells, but inhibited the CGS21680 induced activation with an IC_{50}= 54 ± 28 nM in the presence of 100 nM of the agonist. The inhibition by ZM241385 was competitive as it caused a concentration-dependent rightward shift of AC activation curves without affecting the level of maximal response (Fig. 5) and the obtained Schild plot had a slope value close to unity (Fig. 5 insert). Similar inhibition curves and Schild plots were obtained also for the two other A_{2A}-specific antagonists namely MRS1220 and MSX-3 (Table 2).

Discussion

The results show that [^{3}H]ZM241385 is a valuable tool for the characterization of A_{2A} adenosine receptors, for which it has a high affinity with a fast developing equilibrium. The ligand binding to the A_{2A} receptor is reversible with a dissociation half-life of about 3 min and the equilibrium is achieved within 15-20 minutes at all radioligand concentrations. These results therefore support the view that [^{3}H]ZM241385 is a highly suitable ligand for A_{2A} receptor binding analysis. Comparison of [^{3}H]ZM241385 binding characteristics in striatal (rat A_{2A}) and CHO (dog A_{2A}) cell membranes did not reveal significant differences under the present experimental conditions indicating no substantial differences in the A_{2A} receptor antagonist binding characteristics of rat and dog A_{2A} receptors even in spite of the fact that they are from different origins (brain vs. CHO cell membranes). However, treatment of membranes with adenosine deaminase had an influence on the binding of [^{3}H]ZM241385 in CHO cell membranes but not in striatal membranes. Nevertheless it is known that adenosine and its derivatives are abundant in brain, and it is expected that they remain bound to the to the A_{2A} receptors in high affinity state also during the membrane preparation. On the other hand, it is shown that adenosine deaminase is highly expressed in striatum (Yamamoto et al., 1988), and tightly linked to the adenosine receptors (Preston et al., 2000; Herrera et al., 2001; Torvinen et al., 2002). CHO cells are transfected with adenosine receptors but not with adenosine deaminase and thus treatment of cells with the enzyme was required to eliminate the receptor-bound endogenous adenosine (Shryock et al., 1998). Therefore, it is important to take into account the influence of endogenous adenosine when working with CHO cells.

Reliable kinetic data could be obtained at [^{3}H]ZM241385 concentrations of up to 6 nM. These data could be fitted according to simple one step bimolecular reaction

model (Eq. 3) with a linear relationship between the pseudo-first order constant k_{obs} and ligand concentration $k_{obs}=k_1[L] + k_{-1}$. However, at higher concentrations, where the the association of [^{3}H]ZM241385 is too fast to be exactly determined (Fig. 4), a second isomerization step may appear (Eq. 5) that significantly alters the meaning of the dissociation constant K_d. According to this two-step binding model the apparent dissociation constant of the ligand becomes a combination of two equilibrium constants: $K_d = K_A \cdot K_i = K_A \cdot (k_{-i}/k_i)$. The obtained data (Fig. 4) did not allow the exact determination of these constants, but a rough estimation revealed constants $K_A = 8.5 \pm 2.2$ nM and $K_i \approx 0.029$. According to the inverse agonist theory, the ligand behaves in the first step of the two-step binding model as a competitive antagonist, while isomerization reveals its inverse agonist properties (Kenakin, 2004). In its inhibition of CGS 21680- induced activation of adenylate cyclase the potency of ZM241385 (K_A = 6.6 nM) was considerably lower than its dissociation constant from the binding analysis but not significantly different from the estimated constant obtained from the fitting of the radioligand binding data to Eq. 6, which corresponds to the first step of the A_{2A} binding according to the two-step binding model (Eq. 5). Taking into account the K_A value from adenylate cyclase assays reveals the kinetic parameters of isomerization step as $k_i = 6.5 \pm 0.2$ min^{-1} and $K_i = 0.031$. Thus, the apparent dissociation constant of ZM241385 according to this model is $K_d = K_A \cdot K_i =$ 0.20 nM, which is in very good agreement with values obtained with radioligand binding curves under equilibrium conditions (Fig. 1). Thus, the present results can explain the discrepancy between dissociation constant measured in saturation (equilibrium) experiments and the inhibition constant obtained in adenylate cyclase experiments, based on the theory of Kenakin (2004).

The physiological potency of antagonists is determined under fast equilibrium conditions where agonists and antagonists compete for the binding sites, but the dissociation of the ligands from these complexes is too fast to be detected by filtration assay. Only the isomerized receptor-antagonist complex RL* can be measured by the radioligand binding. As the formation of the isomerized complex RL* occurs faster than its decomposition ($K_i = k_{-i}/k_i = 0.031$), most of the receptors (97%) are isomerized and can be determined by the radioligand binding. Therefore [^{3}H]ZM241385 is a valuable tool for the determination of the binding characteristics of A_{2A} adenosine receptors, and the directly measured dissociation constant (K_d) is a product of K_A and the isomerization equilibrium constant. The latter should be taken into consideration when interpreting ligand displacement data where [^{3}H]ZM241385 has been used as a reporter ligand.

The binding of [^{3}H]ZM241385 was sensitive to the composition of buffer and the presence of Na^+ was required for the high-affinity binding of the radioligand. It is shown in several studies that sodium ions enhance antagonist binding and inhibit agonist binding to G protein-coupled receptors (Lepiku et al., 1997; Lallement et al., 1994; Rinken, 1996). These effects are usually connected with a conserved aspartate residue in the second transmembrane domain of the receptors (Ceresa and Limbird, 1994). It is proposed that conformational changes caused by Na^+ shifts the receptor towards the isomerized complex, which according to the inverse agonist theory corresponds to the inactive receptor state (Milligan et al., 1995). It is also reported that a glutamic acid residue in the first transmembrane domain (Glu13) and a histidine residue in the seventh transmembrane domain (His278) play a crucial role in sodium-dependent modulation of [^{3}H]ZM241385 binding to A_{2A} adenosine receptors (Gao et al., 2000). We have also found that DMSO, which is widely used as an aprotonic

solvent for solubilization of different drugs, decreased the [^{3}H]ZM241385 binding affinity. It might be speculated that under these conditions DMSO reverses the influence of Na^+, but for the true understanding of its mechanism of action additional studies would be required.

In summary, [^{3}H]ZM241385 is a valuable tool for the determination and characterization of the binding properties of adenosine A_{2A} receptors from different sources in vitro. Its interaction with the A_{2A} receptor is complex and likely involve two kinetically distinguishable steps, where apparently the equilibrium constant of the first step determines the potency of the antagonist in functional assays, but the isomerization step of the radioligand binding has to be taken into account for the interpretation of constants obtained from displacement curves.

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Legends for figures

Figure 1. Specific binding of [3H]ZM241385 to rat striatal membranes. The membranes in the incubation buffer (20 mM Tris HCl, 120 mM NaCl, 5 mM KCl, 10 mM MgCl2, 1 mM CaCl2, 1 mM EDTA, pH 7.5) were incubated with different concentrations of [3H]ZM241385 in the absence (total binding) and presence (nonspecific binding) of 130 μM of DMPX for 60 min at 25°C. The specific binding was defined as the difference between total and nonspecific binding. Non-linear least squares fit of these data gave K_d=0.14±0.01 nM and B_{max}=1620±40 fmol/mg protein. **Inset:** Scatchard plot of the corresponding data.

Figure 2. Dissociation of [^{3}H]ZM241385 from the complex with A_{2A} adenosine receptors in rat striatal membranes. The complex was formed by incubation of striatal membranes (4 mg protein/ml) with 0.87 nM [^{3}H]ZM241385 for 45 min at 25°C. Dissociation was initiated by addition of large excess (10 μM) of non-radioactive ZM241385. **Inset:** Data in semilogarithmic coordinates.

Figure 3. Time course of total (○) and non-specific binding of [^{3}H]ZM241385 to rat striatal membranes. The Receptor preparation (3.7 mg protein/ml) was incubated with 0.81 nM [^{3}H]ZM241385, and at various time intervals the bound radioactivity in aliquots were determined as described in *Methods*. The non-specific binding was determined in the presence of 1 mM of DMPX.

Figure 4. Dependence of the pseudo-first-order rate constants for [^{3}H]ZM241385 binding to A_{2A} adenosine receptors of rat striatal membranes (k_{obs}) on the radioligand concentration. The kinetics of association were determined as described in the legend of Fig. 3 and in the Material and Methods. The standard errors of separate determinations are shown by the bars. The dotted line corresponds to the simple one step model and the solid line corresponds to the two-step kinetic model.

Figure 5. Inhibition of CGS 21680 stimulation of formation of cAMP by ZM241385. Activation of adenylate cyclase in CHO cells was measured at the indicated concentrations of CGS 21680 in the absence (∗) and in the presence of 1 nM (○), 10 nM (▼), 100 nM (△) and 300 nM (●) of ZM241385 as described in Materials and Methods. Data are presented as percent of maximal activation and are representatives of 4 independent experiments carried out in triplicate. **Inset:** Schild plot of ZM241385 antagonism. Basal values are not given

Table 1. Affinities of adenosine ligands obtained in competition experiments with [^{3}H]ZM241385 in rat striatal membranes.

Ligand	K_i (nM) and n_H values[a]	
	control	+GTPγS
ZM 241385	0.29±0.05 (1.2±0.2)	0.31±0.04 (1.1±0.2)
CGS 21680	310±48 (0.6±0.1)[b]	367±53 (0.8±0.2)
SCH 58261	26±11 (0.9±0.1)	32±13 (0.9±0.1)
DMPX	1120±165 (1.0±0.2)	1650±245 (1.1±0.3)
2-Cl-adenosine	1430±215 (1.0±0.1)	1465±215 (1.1±0.2)

[a] Ki values were calculated from displacement curves against 1.4 nM [^{3}H]ZM241385 with corrections by equations of Chen-Prusoff (Cheng and Prusoff, 1973). Hill coefficients (nH) are given in parentheses.

[b] the Hill coefficient is significantly lower from unity ($P < 0.05$)

Table 2. Potencies of adenosine antagonists to inhibit CGS 21680–dependent accumulation of cAMP in CHO cells expressing A_{2A} receptors.

Ligand	IC_{50} (nM) [a]	Schild plot	
		K_i (nM)	slope
ZM241385	54 ± 28	6.6	0.89±0.05
MRS1220	81 ± 26	16.8	0.80±0.07
MSX-3	175 ± 89	39.7	0.89±0.06

[a] Concentration of the antagonist causing 50% inhibition of the cAMP accumulation activated by 100 nM CGS21680

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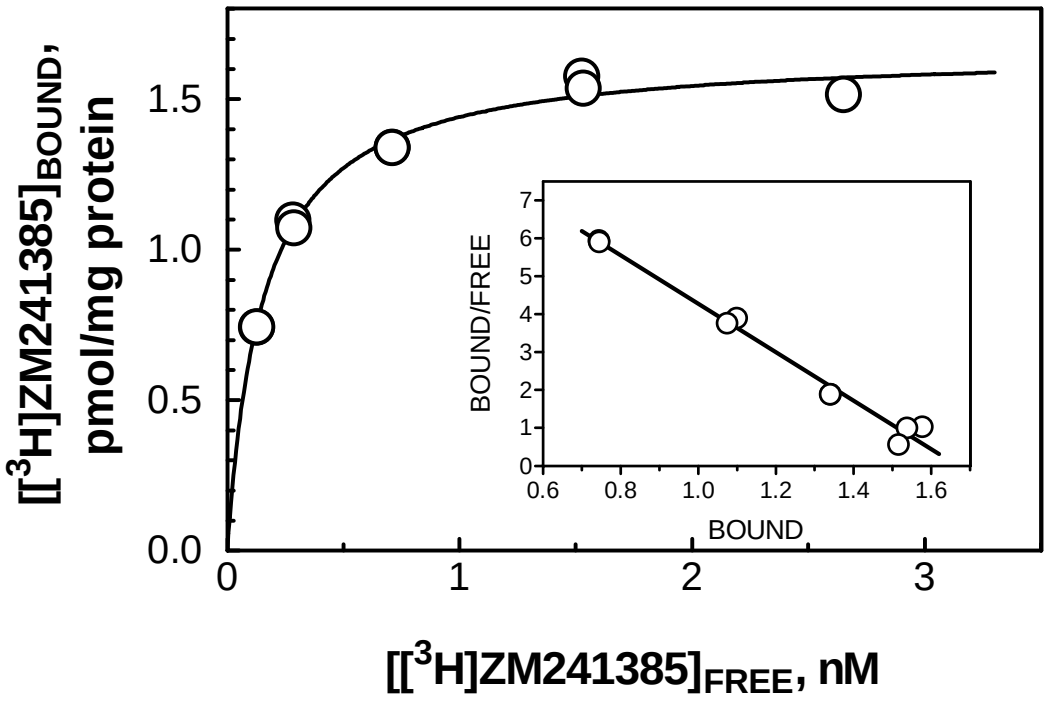
Figure 1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6
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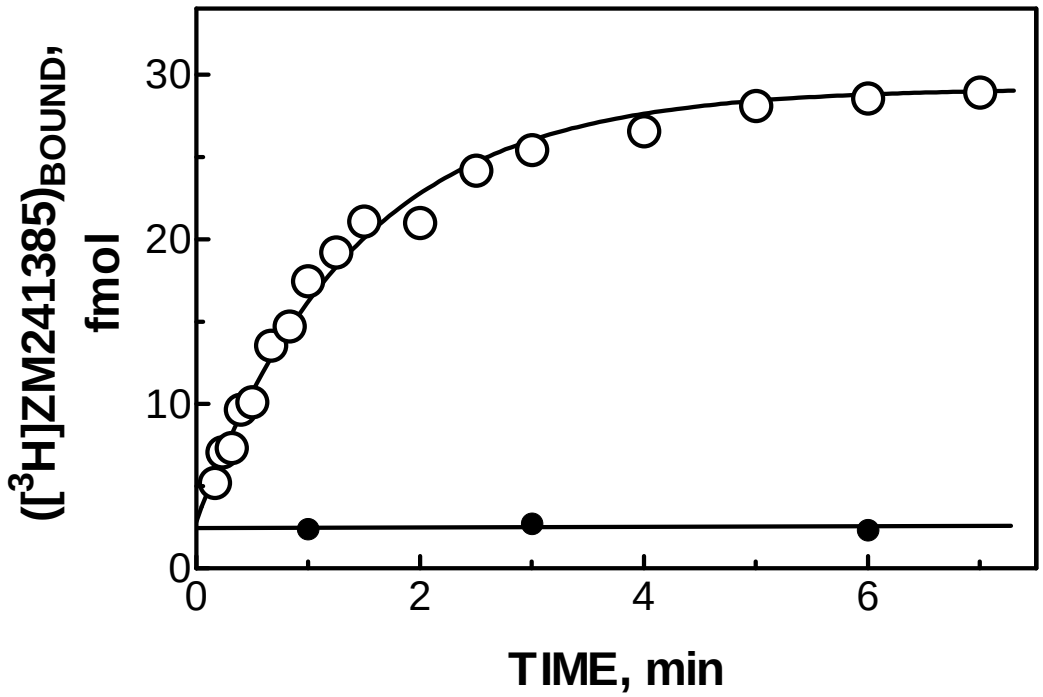
Figure 2,



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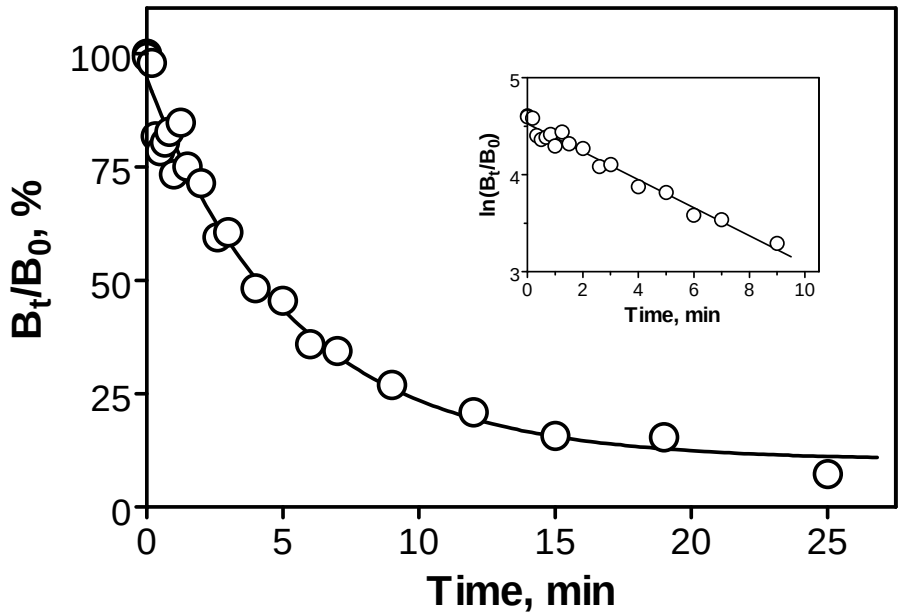
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Time, min
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Figure 4.

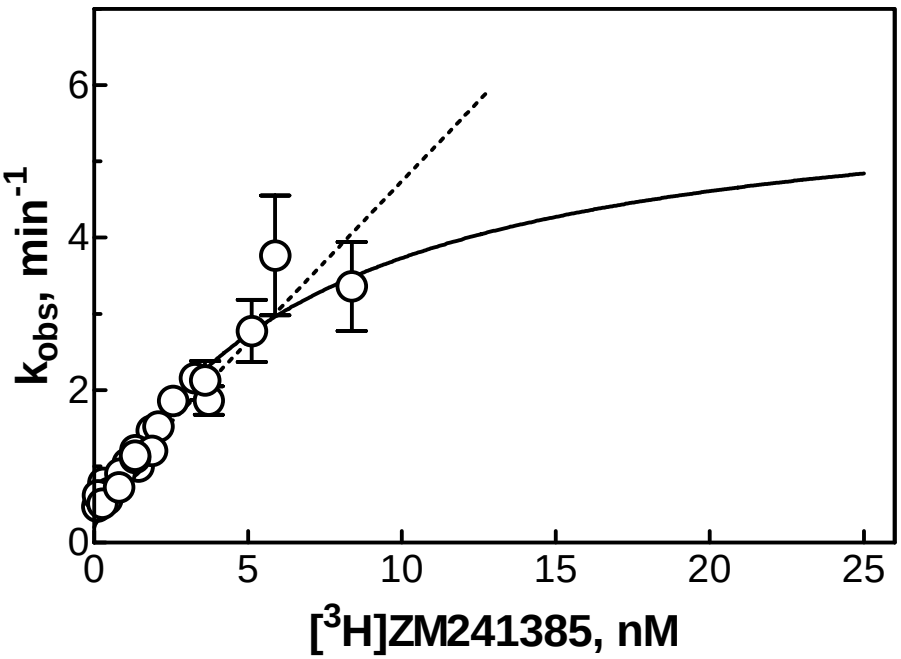
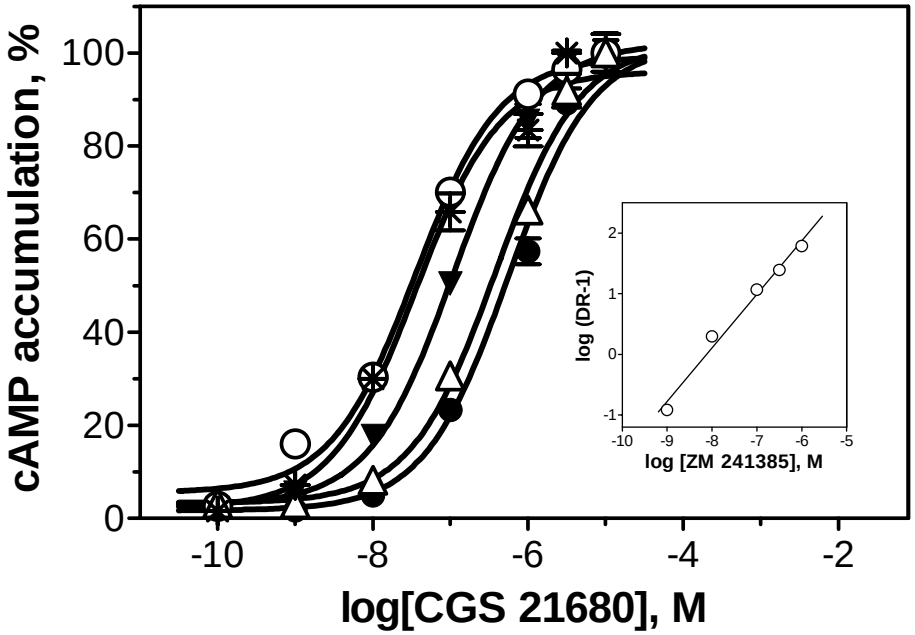


Figure. 5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60
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-10
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-6
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log[CGS 21680], M
log (DR-1)
2
1
0
-1
-10
-9
-8
-7
-6
-5
log [ZM 241385], M

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

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Abstract

The possibilities to characterize of 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 composition of incubation buffer, up to 10-fold increase in cyclic AMP (cAMP) accumulation was achieved in response of 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 ± 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 antagonists of A_{2A} receptors, ZM241385 and MSX-3 had no effect on the basal activity of adenylate cyclase in striatal homogenate, but inhibited the CGS21680-dependent activation with affinities that were in good agreement with the binding affinities of those antagonists to A_{2A} receptors.

Keywords: Adenosine A_{2A} receptor, CGS 21680, ZM241385, adenylate cyclase, rat striatum

Kokkuvõte

Käesoleva töös uuritakse võimalusi roti aju juttkeha membraanides adenosiini A_{2A} retseptorite poolt edastatava signaali mõõtmiseks lähtudes adenülaadi tsüklaasi aktiivsusest. Varieerides membraanide valmistamise metoodikat ja inkubatsioonipuhvri koostist saavutati retseptorite aktiveerimisel A_{2A}-spetsiifilise agonisti CGS21680-ga ligi 10-kordne cAMP akumulatsiooni taseme tõus üle baastaseme ja selle ligandi afiinsust membraanpreparaadis iseloomustas pEC_{50}=6.3±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 adenülaadi tsüklaasi aktivatsiooni saavutamiseks, kusjuures kõrgematel kontsentratsioonidel vähendas Mg^{2+} cAMP retseptor-spetsiifilist akumulatsiooni ja tõstis baasaktiivsuse taset. A_{2A}-spetsiifilised antagonistid ZM241385 ja MSX-3 ei mõjutanud adenülaadi tsüklaasi baasaktiivsust, kuid inhibeerisid CGS21680- sõltuvat aktivatsiooni afiinsustega, mis olid kooskõlas nende antagonistide sidumisafiinsusega A_{2A} retseptoritele.

Introduction

Adenosine receptors comprise a large receptor subfamily of purinergic receptors. All adenosine receptors are G protein coupled receptors (GPCR-s), 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], but in the end of eighties, using the methods of molecular cloning, existence of four different adenosine receptor subtypes was shown [4].

All four adenosine receptors have been found in central nervous system, but high expression levels of A_1 receptors is show also in 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 a 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 was in testis and mast cell [5], but these data has not yet confirmed pharmacologically due to 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 and 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 has been identified [17, 18] and most of the isozymes contain two hydrophobic regions, each comprising of six transmembrane helixes and three large cytoplasmic domains: N, C1a/b and C2 [19]. The domains 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 the characterization of signal transduction mechanism also in membrane preparations of tissues. Since 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 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 have searched for the optimal conditions for measuring of the adenosine A_{2A} receptor dependent modulation of AC activity in

rat striatal membranes and shown that the activation of 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]ZM241385, 21 Ci/mmol) was purchased from Tocris Cookson Ltd., [5',8'- ^{3}H] adenosine-3',5'-cyclic monophosphate [^{3}H]cAMP (48 Ci/mmol) were 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 (ZM241385) 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, 3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3) was from Pharmaceutical Institute of University of Bonn and 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 vols. (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 resulted 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 vols. (v/ww) of A.B. and 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 vols. (v/w) 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 and 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 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 1mM) and GTP (final 10 μM) (if otherwise not stated) 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 (10000 cpm per sample) and incubated with cAMP binding protein [27] for at least for 2h 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 [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 radioactivity content was measured using a Beckman LS 1800 scintillation counter.

[^{3}H]ZM241385 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]ZM241385 (0.04 to 4 nM) for 45 min at 25°C [29]. The reaction was terminated by rapid filtration and 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]ZM241385 and non-labeled ligands CGS 21680 (concentration range 1 nM – 100 μM), ZM241385 (concentration range 10 pM – 10 μM) or MSX-3 (concentration range 100 pM – 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 radioactivity content was measured as described above.

Data analysis

All data were analyzed 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. 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 radioligand and K_d is the radioligand dissociation constant. Statistical significance of differences was determined by Student-Newman-Keuls test, where $P<0.05$ was taken as a criterion of significance.

Results & 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 the partially purified membrane preparations. This has also been a standard for characterization of A_{2A} receptor-coupled events, including the activation of AC. However more and more information has been accumulated indicating that other proteins in addition to GPCR-s, 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 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/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 direct activator of all AC subtypes [18], did not clearly distinguish the preparations, causing 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/potassium ions, respectively. This means that the biggest absolute as well as relative activation of AC by CGS21680 was achieved in crude homogenate without Na^+/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 the 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 overall increase of cAMP formation, but had no effect on the activation effect of CGS 21680. GTPγS is a nonhydrolyzable 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 data, where the addition of 1 μM GDP into the reaction medium decreased the activation effect of CGS 21680 by 30 %. The G proteins in their idle state are usually coupled with GDP and therefore this nucleotide is usually as inhibitor of signal transduction. However, in very effective systems the 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 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 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 the 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 the effective coupling of receptors with G proteins [36] and in generation of second messengers. However, depending on the steps of the signal transduction of interest, the required concentration of Mg^{2+} may be very different. 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 raise of the A_{2A}-specific cAMP accumulation, while at concentrations above 5 mM caused its decrease, approaching the level of 50% at 30 mM $MgCl_2$ compared to the effect at 1 mM $MgCl_2$ (Fig. 1 streaked columns). From the other side, the basal level of the cAMP formation increased with the increase of 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 point that at Mg^{2+} concentrations above 2.5 mM, the total amount of cAMP formed remained constant, but with the increase of basal level caused by the increase of magnesium concentration, the adenosine receptor-coupled amount started to decrease. Obtained results clearly indicate that for the determination of A_{2A}-specific cAMP accumulation the 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, 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 ± 0.2 μM, and the Hill coefficient was not different from the unity (n_H = 1.1±0.2). The obtained potency was considerably lower than EC_{50} = 15 ± 3 nM found in the intact CHO cells expressing adenosine A_{2A} receptors [29], and the K_i = 45 ± 8 nM found in competition with [^{3}H]ZM241385 binding. It is proposed, that the decrease of potency of CGS 21680 is caused by the perturbation of the receptor – G protein – AC signaling pathway during the preparation of the membranes. Similar decrease in potencies of agonists in activation of AC in membrane preparation has been found for A_3 adenosine receptors, where the discrepancy exceeded 200 fold [23]. Of course, also the relatively high concentration of nucleotides in the reaction

medium may lead the receptor into low affinity state and decrease the potency of agonists [32].

ZM241385, a highly specific adenosine A_{2A} receptor antagonist had no effect on the basal level of cAMP formation in 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 ZM241385 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 antagonist MSX-3 with IC_{50}=63±30 nM (n_H = 0.6 ± 0.3). Using mechanistic approach for the activation of receptors, the inhibition constants of the antagonists could be estimated as K_i = 1.2 nM for ZM241385 and K_i = 2.4 nM for MSX-3.

Characteristics of [^{3}H]ZM241385 binding to striatal membranes and competition binding with other A_{2A} ligands

[^{3}H]ZM241385 is the first commercially available radiolabelled A_{2A} antagonist and has been found to be useful for pharmacological experiments [37]. We have found that the binding of the [^{3}H]ZM241385 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 studied adenosine receptor ligands were able to complete with [^{3}H]ZM241385 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 ZM241385 and 7 ± 1 nM for MSX-3. 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 CGS 21680 displacement curve in the absence of GTPγS (n = 0.8±0.1) and F test in comparison of 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 α_H=0.15±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 non-specific AC activity and decreased the receptor-mediated effects. However, obtained agonist potencies in activation of AC in membrane preparations were lower than in assays using intact cells and in radioligand binding displacement assays. It would be proposed that the decrease in agonist potency is caused by the perturbation of the receptor – G protein – AC signaling 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 obtained data.

Acknowledgements

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Legends for figures

Figure 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 difference between total and basal level of cAMP, are presented as a mean of three independent determinations ± SEM as error bars.

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 percent of maximal activation are representative of 3 independent experiments carried out in triplicate (Mean ± SEM as error bars).

Fig 3. Inhibition of specific binding of [^{3}H]ZM241385 by ligands of adenosine receptors. The crude homogenate of rat striatum was incubated for 60 min at 30°C at indicated concentrations of ZM241385 (●), CGS 21680 (O) or MSX-3 (∗) and 2.3 nM [^{3}H]ZM241385. Binding of [^{3}H]ZM241385 is presented as the percentage of specific binding in the absence of ligands (± SEM as error bars) and are from two independent experiments carried out in duplicate.

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Fig 1.

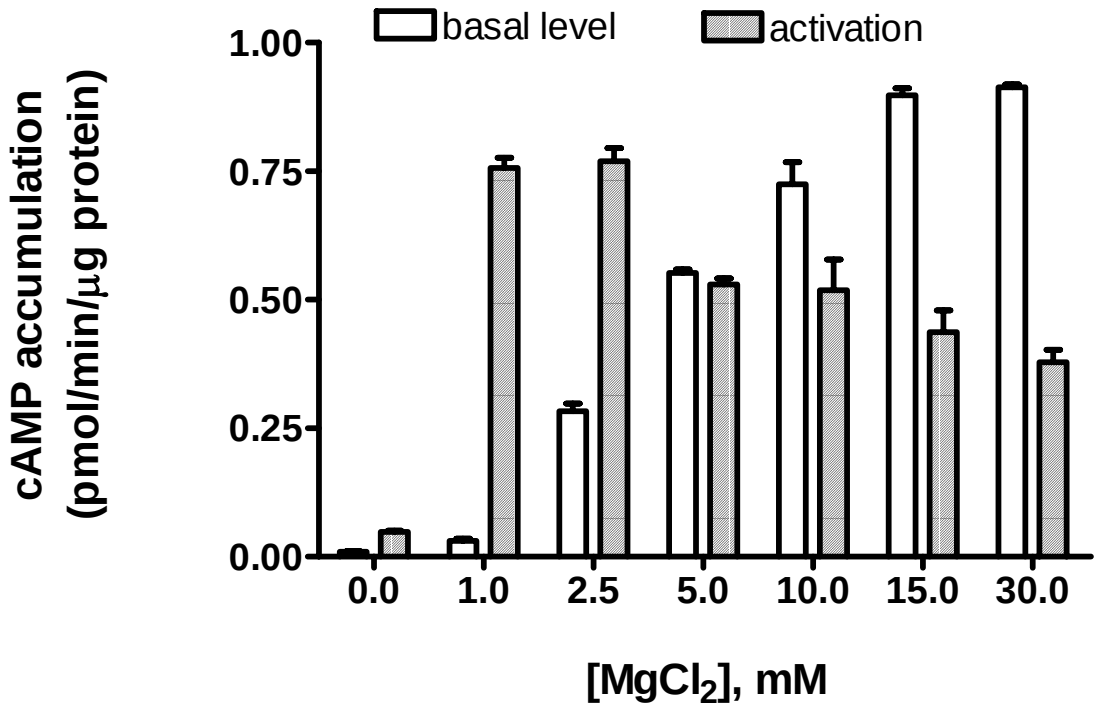
basal level
activation
cAMP accumulation (pmol/min/μg protein)
1.00
0.75
0.50
0.25
0.00
0.0
1.0
2.5
5.0
10.0
15.0
30.0
[MgCl2], mM

Fig 2

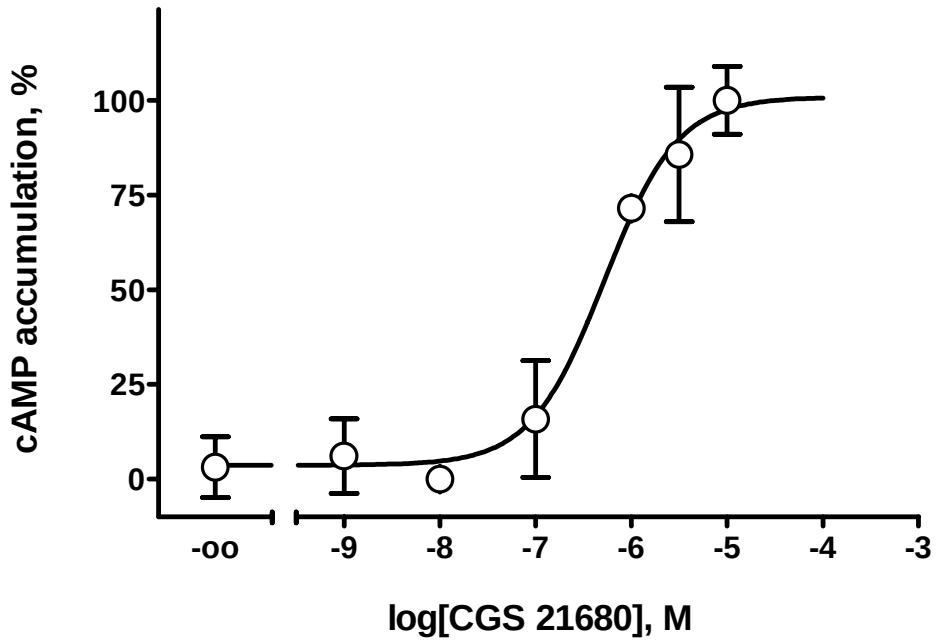

Fig. 3