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Effects of the adenosine A_1 receptor allosteric modulators PD 81,723 and LUF 5484 on the striatal acetylcholine release

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Abstract

The objective of the present study was to characterize the adenosine A_1 receptor allosteric enhancing and antagonistic actions of (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(3,4-dichlorophenyl)methanone (LUF 5484) and (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD 81,723) on striatal acetylcholine release. Upon local administration in conscious rats, LUF 5484 or PD 81,723 caused a concentration-dependent increase of extracellular acetylcholine levels of approximately 40%, which was similar to that obtained by the selective adenosine A_1 receptor antagonists 8-cyclopentyl-1,3-dimethylxanthine (8CPT) and N^6-cyclopentyl-9-methyladenine (N0840). In interaction experiments, LUF 5484 or PD 81,723 did not change the inhibition of acetylcholine release by the adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA), whereas 8CPT caused an eightfold rightward shift. Acetylcholine concentrations were diminished with 62 ± 3%, 48 ± 11% and 56 ± 9% by CPA, CPA + LUF 5484 and CPA + PD 81,723, respectively. In conclusion, the antagonistic action of LUF 5484 and PD 81,723 seems to counteract the putative allosteric actions with respect to the reduction of striatal acetylcholine release.

Keywords: Allosteric modulation; PD 81,723; Acetylcholine release; Adenosine A_1 receptor; Microdialysis; Striatum

1. Introduction

Extracellular adenosine produces numerous physiological effects via four identified G protein-coupled receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. Adenosine A_1 receptor agonists inhibit the release of various neurotransmitters in the brain, including glutamate, aspartate, norepinephrine, dopamine and acetylcholine (Dunwiddie and Masino, 2001). Therefore, the selective activation of the adenosine A_1 receptor constitutes an attractive approach to treat disorders that are associated with neuroexcitability (Dunwiddie and Masino, 2001). Specifically, the inhibition of central acetylcholine release may be advantageous in the treatment of an organophosphate intoxication (Van Helden and Bueters, 1999) and sleep regulation (Materi et al., 2000). However, the development of adenosine A_1 receptor agonists as centrally active drugs has been hampered by serious cardiovascular side effects (Mathot et al., 1994) and by poor blood–brain barrier transport properties (Pardridge et al., 1994). One approach to enhance selectivity of action of adenosine A_1 receptor agonists in vivo has been the design of partial agonists (Van der Graaf et al., 1999; Bueters et al., 2000). However, these compounds still have difficulties penetrating the blood–brain barrier, as a result of which, very high doses are required to obtain a significant effect in the central nervous system (CNS). Alternatively, CNS-selective actions may be obtained through allosteric modulators that modify the adenosine A_1 receptor into an agonist-preferring state (Soudijn et al., 2001; Van der Klein et al., 1999), thereby enhancing binding of endogenous adenosine and functional activation of the adenosine A_1

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receptor (Bruns and Fergus, 1990; Janusz et al., 1991; Janusz and Berman, 1993; Jarvis et al., 1999). Under pathological conditions, local extracellular adenosine levels are elevated (Berman et al., 2000; Dunwiddie and Masino, 2001), from which the allosteric modulators derive their site specificity.

A potential problem with the currently available allosteric enhancers is that in addition to their modulating effects, they also possess an antagonistic effect for the adenosine A_1 receptor. In binding assays and in functional in vitro assays of neuronal origin, the allosteric modulator (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD 81,723) demonstrated signs of antagonism at the adenosine A_1 receptor, besides enhanced ligand binding and receptor coupling (Bruns and Fergus, 1990; Jarvis et al., 1999; Janusz et al., 1991; Janusz and Berman, 1993; Kourounakis et al., 2000; Van der Klein et al., 1999). Moreover, PD 81,723 failed to attenuate the development of cerebral ischemic injury in gerbils in vivo, which was attributed to the antagonistic actions of the compound (Cao and Phillis, 1995). In contrast, PD 81,723 appeared neuroprotective against hypoxia in pup rats (Halle et al., 1997), albeit that the immature brain differs from the adult brain in many aspects (Geiger et al., 1984; Marangos et al., 1982).

Recently, we have evaluated the actions of a series of partial adenosine A_1 receptor agonists on the release of acetylcholine in freely moving rats through local perfusion of the compounds in the intact striatum (Bueters et al., 2000). This way, restricted blood–brain barrier transport was circumvented as a confounding factor. We have used a similar approach to characterize the adenosine A_1 receptor allosteric modulatory and antagonistic effects of (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(3,4-dichlorophenyl)methanone (LUF 5484) and PD 81,723 on the inhibition of acetylcholine release in order to determine whether in vivo selectivity of action could be achieved by these compounds. LUF 5484 is a newly developed allosteric enhancer, which proved to be more potent relative to PD 81,723 in a rat cortex preparation (Van der Klein et al., 1999). This may improve discrimination between allosteric enhancement and competitive antagonism on adenosine A_1 receptors.

2. Materials and methods

2.1. Chemicals

N^6-cyclopentyladenosine (CPA), N^6-cyclopentyl-9-methyladenine (N0840) and 8-cyclopentyl-1,3-dimethylxanthine (8CPT) were purchased from Research Biochemicals (Zwijndrecht, The Netherlands). Acetylcholinesterase, choline oxidase, neostigmine bromide and atropine sulfate were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Kathon CG® was provided by Rohm and Haas (Croyden, UK). (2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD 81,723) and (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(3,4-dichlorophenyl)methanone (LUF 5484) were synthesized at the Department of Medicinal Chemistry of the Leiden/Amsterdam Center for Drug Research (Van der Klein et al., 1999). The other chemicals used were of standard purity. For high performance liquid chromatography (HPLC) analysis, solvents of the highest purity grade were used.

All solutions were prepared with water tapped from a Milli-Q system (Millipore, Molsheim, France) except for the stock solutions of CPA, PD 81,723, LUF 5484, N0840 and 8CPT, which were made in dimethyl sulfoxide.

2.2. Animal experiments

Male Wistar Rats (280–350 g; Harlan, Horst, The Netherlands) were kept under standard conditions with free access to rodent chow and acidified water. The Ethical Committee on Animal Experimentation of TNO has approved all experiments described.

Rats were anaesthetized with 2.7 ml/kg FFM mix (1.25 mg/ml midazolam, 2.5 mg/ml fluanisone, 0.079 mg/ml fentanyl citrate) and a concentric microdialysis probe was stereotactically implanted in the striatum (A 0.5, L 3.0, V 6.8 mm relative to bregma and the dura mater). A heating pad was used to maintain body temperature. The microdialysis probes used were self-constructed and made of a polyacrylonitryl/sodiummethyl sulfonate copolymer dialysis membrane (Filtral 12, Hospal, Breda, The Netherlands), of which 3.5 mm was exposed.

Microdialysis experiments in conscious rats were initiated between 9:0 and 10:00 a.m. 14–38 h after the surgical procedure. The microdialysis probe was perfused at 2 μl/min with a Ringer solution to which 0.1 μM of the acetylcholinesterase inhibitor neostigmine and 10 μM of the muscarinic antagonist atropine were added. Each rat was connected directly to the injection valve that was automatically activated every 10 min, allowing on-line analysis of the microdialysates. Once a stable acetylcholine response had been reached (usually after 90 min), drugs were co-administered in increasing concentrations through the probe and the inhibition of acetylcholine release was determined for each concentration by averaging four consecutive samples at plateau level (Fig. 1). This allowed the construction of concentration–response relationships to characterize the effects of the different compounds on the acetylcholine release. The concentration ranges of the various drugs were as follows: CPA 0.01–10 μM; PD 81,723 0.1–100 μM; LUF 5848 0.01–100 μM; 8CPT 0.1–10 μM; and N0840 0.1–100 μM, respectively.

2.3. Acetylcholine assay

Microdialysate samples were assayed for acetylcholine using an HPLC system previously described by Damsma et al. (1987) with some modifications. The system was

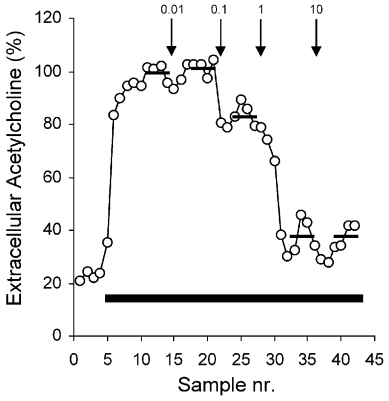

Fig. 1. Typical example of a time-effect profile of the acetylcholine release at increasing concentrations of CPA. After basal release of acetylcholine is established, 10 μM atropine is added, represented by the solid bar at the bottom. This led to an augmentation of acetylcholine release in each rat. Subsequently, increasing concentrations of CPA (0.01–10 μM) were added to the perfusion fluid, indicated with the arrows, which resulted in a concentration-dependent decrease of the acetylcholine release. At each CPA concentration, the inhibitory effect on the acetylcholine release was determined by averaging four consecutive samples at plateau, reflected by the small black bars. These responses were used subsequently to construct the concentration–response curves as shown in Figs. 2 and 3.

equipped with an electrochemical detector containing a VT03 flow cell with a platinum work electrode operated at 450 mV (Antec Leyden, Hazerswoude, The Netherlands), an EC 125/3 Nucleosil 100-5 C18 AB analytical column that was preloaded with 0.5% sodium lauryl sulfate and a post-column enzyme reactor that contained immobilized acetylcholinesterase (80 U) and choline oxidase (40 U). The mobile phase consisted of a 166 mM potassium phosphate buffer (pH 8.5) containing 1 mM tetramethylammonium chloride, 0.79 mM ethylenediaminetetraacetic acid (EDTA) and 250 μl/l Kathon CG ®. The system was run at 0.5 ml/min and the temperature was maintained at 30 °C. Limit of quantification was 1 pmol/ml. Linear calibration curves were obtained in the range from 5 to 1000 pmol/ml (r>0.990). The intra-assay coefficients of variation for 10 and 100 pmol/ml were 2.9% and 4.1%, respectively. Inter-assay variability could not be determined due to variable enzyme activities in the post-column reactor between days.

2.4. Data analysis

The effects of the various drugs on the release of acetylcholine were expressed relative to the baseline release in the presence of 0.1 μM neostigmine and 10 μM atropine, which was set at 100% (Fig. 1).

The antagonistic actions of the allosteric enhancers and adenosine A_1 receptor antagonists could not be completely determined due to induced convulsive activities at higher concentrations. Therefore, the effects were described on the basis of a power function

$$E = E_0 + bC^n, \quad (1)$$

in which E is the effect on the acetylcholine release at concentration C, E_0, the effect with no drug present, n, the Hill coefficient and b, a constant that reflects the slope of the polynomial.

The responses on the inhibition of acetylcholine release obtained with interaction studies of 10 μM of LUF 5848 or PD 81,723 combined with increasing concentrations of CPA were described on the basis of the I_{max} model

$$I = I_0 - \frac{I_{max} C^n}{IC_{50}^n + C^n}, \quad (2)$$

in which I represents the inhibitory effect of the acetylcholine release at CPA concentration C, I_0, the effect with no drug present, I_{max}, the maximal inhibitory effect, IC_{50}, the concentration at 50% of the maximal effect and n, the Hill coefficient. All fitting procedures were performed using the NONMEM software package (NONMEM project group, UCSF, USA). Goodness-of-fit was analyzed using visual inspection, objective function and assessment of parameter correlation. Statistical analysis was performed using analysis of variance (ANOVA) followed by the Dunnett's post hoc test, whenever appropriate. All data are reported as the mean ± S.E.M. and differences were considered significant for P-values < 0.05.

3. Results

Fig. 1 shows an example of an experiment in which increasing concentrations of CPA have been locally deliv-

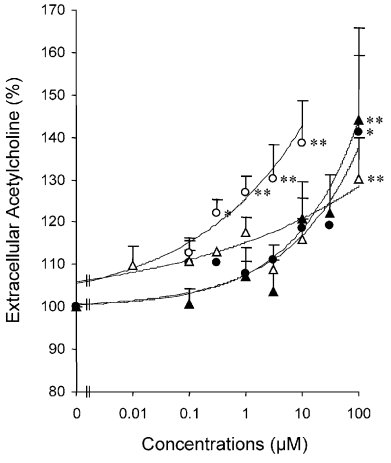

Fig. 2. Concentration-dependent elevation of the striatal acetylcholine release by the adenosine A_1 receptor allosteric enhancers LUF 5484 (open triangles) and PD 81,723 (closed triangles), and the adenosine A_1 receptor antagonists 8CPT (open circles) and N0840 (closed circles). The solid lines represent the fitted data based on a power equation. Effect is expressed as the percentage of the basal acetylcholine release (mean ± S.E.M.; n = 6–7). *P < 0.05; **P < 0.01, significantly different from the basal acetylcholine release, according to ANOVA followed by Dunnett's post hoc test. The significance symbols are on the right side of the corresponding data points for clarity reasons.

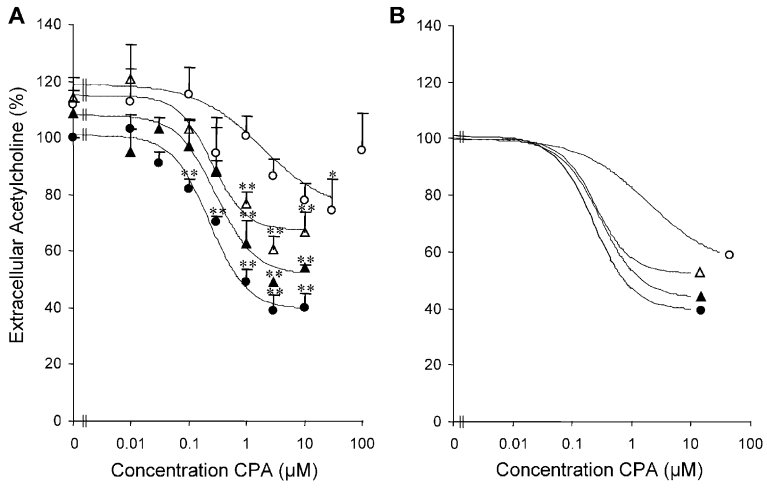

Fig. 3. (A) Illustrates the concentration-dependent inhibition of the striatal acetylcholine release by the selective adenosine A_1 receptor agonist CPA (0.01–10 μM; closed dots), and CPA combined with 10 μM of the adenosine A_1 receptor allosteric enhancers LUF 5484 (closed triangles), and PD 81,723 (open triangles), or 10 μM of the adenosine A_1 receptor antagonist 8CPT (open dots). The solid lines represent the fitted data based on the sigmoidal I_{max} equation (see Table 1 for the pharmacodynamic parameter estimates). In (B) the fitted profiles are normalized to the basal acetylcholine release with no drugs present. In both panels, the effect is expressed as the percentage of the basal acetylcholine release (mean ± S.E.M.; $n = 6–11$). *$P < 0.05$; **$P < 0.01$, significantly different from the basal acetylcholine release, according to ANOVA followed by Dunnett's post hoc test.

ered in the striatum of conscious rats by retrograde microdialysis. The average basal release of acetylcholine was 244.7 ± 40.7 fmol/min ($n = 55$). Administration of 10 μM atropine increased the acetylcholine release to 691 ± 55.0 fmol/min, which was reversible upon omission of the drug. Control experiments with only neostigmine, and with both neostigmine and atropine present, demonstrated a stable signal during the entire experiment. Furthermore, the observed acetylcholine release was almost completely tetrodotoxin-dependent, reflecting its neuronal origin (data not shown).

Infusion of LUF 5484 or PD 81,723 increased the striatal acetylcholine release concentration-dependently up to 40%, which was also reached by the selective adenosine A_1 receptor antagonists 8CPT and N0840 (Fig. 2; $n = 6–7$). Infusions of higher concentrations into the striatum led to convulsive activity, which precluded the determination of the maximal effect of these compounds on the acetylcholine release, and quantitative estimations of drug potencies.

The selective adenosine A_1 receptor agonist CPA reduced the acetylcholine release in a concentration-dependent manner to a maximal extent of 38 ± 2% of control values (Fig. 3; Table 1; $n = 6$). Administration of increasing CPA concentrations combined with 10 μM LUF 5484 or PD 81,723 led to a maximal inhibition of 52 ± 12% and 44 ± 7% of control values, respectively (Fig. 3; Table 1; $n = 7$). The IC_{50} values were unaffected by the allosteric enhancers, whereas co-administration with 1 μM 8CPT resulted in an eightfold rightward shift (Fig. 3; Table 1; $n = 11$). Moreover, a steep increase of acetylcholine release was apparent at 100 μM CPA in the presence of 1 μM 8CPT.

4. Discussion

With retrograde microdialysis in conscious rats, the actions of the adenosine A_1 receptor allosteric modulators on acetylcholine release were investigated to evaluate

Table 1
Pharmacodynamic parameter estimates[a] for the reduction of acetylcholine release in the striatum by the selective adenosine A_1 receptor agonist CPA (0.01–100 μM) alone and in combination with 10 μM PD 81,723, 10 μM LUF 5484 or 1 μM 8CPT (mean ± S.E.M.; see also Fig. 3)

Compound	I_0 (%)	I_{max} (%)	IC_{50} (μM)	Hill factor
CPA	101 ± 1 (1%)	62 ± 3 (6%)	0.23 ± 0.01 (7%)	1.3 ± 0.08 (64%)
CPA + PD 81,723	108 ± 5 (17%)	56.2 ± 8.8 (23%)	0.30 ± 0.04 (112%)	1.3 ± 0.3 (0%)
CPA + LUF 5484	115 ± 4 (6%)	48 ± 11 (29%)	0.25 ± 0.09 (83%)	1.5 ± 0.4 (0%)
CPA + 8CPT	119 ± 3 (10%)	45[b]	1.9 ± 0.4 (117%)	0.8 ± 0.2 (11%)

The coefficient of variation is expressed between parentheses.

[a] The acetylcholine reducing effects were fitted on the basis of the I_{max} model:

$$I = I_0 - \frac{I_{max} C^n}{IC_{50}^n + C^n}.$$

[b] This value was fixed to be able to fit the I_{max} model to the inhibitory part of the curve.

whether these compounds could be useful in obtaining selectivity of action in vivo. Moreover, we have examined if the novel 2-amino-3-benzoylthiophene derivative LUF 5484 would be an improvement relative to the prototypic enhancer PD 81,723. Our microdialysis approach differed from the more usual method in that 10 μM atropine was added to the perfusion fluid, an approach that has also been employed by Materi et al. (2000) to study the inhibition of cortical acetylcholine release by adenosine. The reason for the addition of atropine was to block the presynaptically localized cholinergic autoreceptors. The resulting 2.8-fold increase of acetylcholine levels demonstrated that under basal conditions, i.e. in the presence of 0.1 μM neostigmine alone, the acetylcholine release is partially inhibited by these autoreceptors (Moor et al., 1998). Previous work using synaptosomes has suggested that presynaptic adenosine receptors located on cholinergic terminals and cholinergic autoreceptors modulate the acetylcholine release via a common mechanism, since the inhibitory effects of adenosine were reduced by the simultaneous addition of a cholinergic agonist (Pedata et al., 1986). Thus, without atropine present, these autoreceptors may confound the adenosine A_1 receptor-mediated modulatory effects.

The present results demonstrate that increasing concentrations of LUF 5484 or PD 81,723 affect the striatal acetylcholine release similarly. This is consistent with the results obtained by Van der Klein et al. (1999) in that LUF 5484 and PD 81,723 possess an equal antagonistic effect on the adenosine A_1 receptor. However, the differences in K_i values of the enhancers, both within the μM range (Kourounakis et al., 2000), and the antagonists, 0.011 ± 0.003 and 0.18 ± 0.05 μM for 8CPT and N0840 (De Ligt, personal communication), respectively, raise questions about the underlying mechanism of the observed increase of the acetylcholine release. Based on classic receptor–ligand interactions, a weaker effect of the allosteric enhancers on the acetylcholine release was expected relative to 8CPT and N0840. Adenosine A_1 receptor-independent actions of PD 81,723 have been described and might be involved here as well (Kollias-Baker et al., 1997; Musser et al., 1999).

The elevation of the acetylcholine levels by adenosine A_1 receptor antagonists reveals the presence of a tonic inhibition by endogenous adenosine in the rat brain, which has been demonstrated previously (Carter et al., 1995; Dunwiddie and Masino, 2001). The fact that the allosteric modulators failed to strengthen this tonic inhibition may be indicative for the poor potentiating actions in our experimental set-up. This was confirmed by the results obtained via simultaneous administration of the enhancers and increasing concentrations of the adenosine A_1 receptor agonist CPA. LUF 5484 and PD 81,723 did not affect the CPA-mediated reduction of the acetylcholine release appreciably, i.e. the potencies remained the same, which is illustrated in Fig. 3B. These curves, however, presumably represent a mixed effect by the modulators. A rightward shift would be expected based on the antagonistic properties that the modulators exhibited in Fig. 2, and based on the results obtained with 8CPT in combination with CPA, which resulted in an eightfold rightward shift. The absence of this rightward shift in the presence of CPA suggests that allosteric modulation of the A_1 receptor compensated for this antagonism, yielding a net result equal to the effect of CPA without a modulator present.

The steep increase in acetylcholine release seen at 100 μM CPA in the presence of 1 μM 8CPT is presumably caused by putative binding of CPA to the adenosine A_{2A} receptors, which are well known to augment striatal acetylcholine release (Fig. 3; Kirk and Richardson, 1994).

In conclusion, the present results demonstrate that the principle of allosteric modulation also works in vivo. However, the antagonistic actions of the allosteric enhancers PD 81,723 and LUF 5484 on the adenosine A_1 receptor canceled out the allosteric actions, which makes them unsuitable candidates to improve in vivo selectivity of action in the brain at this time. In order to exploit favorable actions from adenosine A_1 receptor allosteric modulators in the brain, potent enhancers need to be developed lacking antagonistic action.

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